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SYNTHESIS AND EVALUATION OF CYCLIC CATIONIC PEPTIDES AS ANTIMICROBIAL AGENTS FOR USE IN PLANT PROTECTION

Sylvie Martine Colette MONROC

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Synthesis and evaluation of cyclic cationic peptides as antimicrobial agents for use in plant protection

Sylvie Monroc

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Synthesis and evaluation of cyclic cationic peptides as antimicrobial agents for use in plant protection

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Sylvie Monroc

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Dra. Marta Planas Grabuleda Dra. Lídia Feliu Soley Dr. Eduard Bardají Rodríguez

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To Guillem, Laura and Joan

If I am mad about the Chemistry,

You are my reasons for living.

Never forget it.

Love

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Abbreviations

Ac-	acetyl
Acm-	acetamidomethyl
AcOH	acetic acid
Aib	α-aminoisobutyric acid
Al-	allyl
Alloc-	allyloxycarbonyl
AMPs	antimicrobial peptides
BHA	benzhydrylamine
Boc-	*tert*-butyloxycarbonyl
Cbz-	benzyloxycarbonyl
CD	circular dichroism
DCD	dermcidin
DIC	*N*,*N*'-diisopropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DNA	deoxyribonucleic acid
DOE	design of experiments
EDCI	1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide
ESI-MS	electrospray ionization mass spectrometry
FAB-MS	fast atom bombardment mass spectrometry
Fm-	9-fluorenylmethyl
FMDV	foot-and-mouth disease virus
Fmoc-	9-fluorenylmethyloxycarbonyl
G^+	Gram-positive
G^-	Gram-negative

GlcNAc	*N*-acetylglucosamine
HATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMFA	9-(hydroxymethyl)-2-fluoreneacetic acid
HOAt	7-aza-1-hydroxybenzotriazole
HOBt	1-hydroxy-1,2,3-benzotriazole
HPLC	high performance liquid chromatography
LPS	lipopolysaccharide
MALDI-MS	matrix-assisted laser desorption ionization mass spectrometry
MBHA	*p*-methylbenzhydrylamine
MIC	minimal inhibitory concentration
MurNAc	*N*-acetylmuramic acid
MWI	microwave irradiation
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
NMM	*N*-methyl morpholine
NMP	*N*-methylpyrrolidinone
NMR	nuclear magnetic resonance
Nonb	3-nitro-4-aminomethylbenzoic acid
non-RAMPs	non-ribosomally synthesized antimicrobial peptides
OBOC	one-bead one-compound
ONb	3-nitro-4-hydroxymethylbenzoic acid
Pbf-	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PEG	polyethylene glycol
PEGA	polyethylene glycol-polyacrylamide
PE-g-AA	poly(acrylic acid) grafted onto polyethylene

PPTS	pyridinium *p*-toluenesulfonate
PS	polystyrene
PS-SCL	positional scanning synthetic combinatorial library
PyAOP	7-aza-benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
PyBOP	benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
PyBrOP	bromo-tris(pyrrolidino)phosphonium hexafluorophosphate
QSAR	quantitative structure-activity relationships
RAMPs	ribosomally synthesized antimicrobial peptides
RNA	ribonucleic acid
RP-HPLC	reverse phase high performance liquid chromatography
SAR	systemic acquired resistance
SCAL	safety-catch amide linker
S_NAr	nucleophilic aromatic substitution
SPS	solid-phase synthesis
SPPS	solid-phase peptide synthesis
TCP-	tetrachlorophthaloyl
TES	triethylsilane
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TG	Tentagel
THP	tetrahydropyranyl
TIS	triisopropylsilane
t_R	retention time
Trt-	trityl
XAL	xanthenylamide linker

Introduction

Plant diseases caused by bacteria and fungi are a major economical problem in cultivated and stored crops due to the devastating damages that they produce resulting in considerable economic losses which affect growers and, indirectly, consumers (Figure 1) (Agrios, 1998; Pinstrup-Andersen, 2001; Vidaver, 2002; Vidaver and Lambrecht, 2004). Furthermore, while the crop losses involve important reduction of the worldwide food production, the world's population should increase by 2.5 billion by 2050 bringing the overall number to 9.2 billion inhabitants (European Parliament H-0228/07).

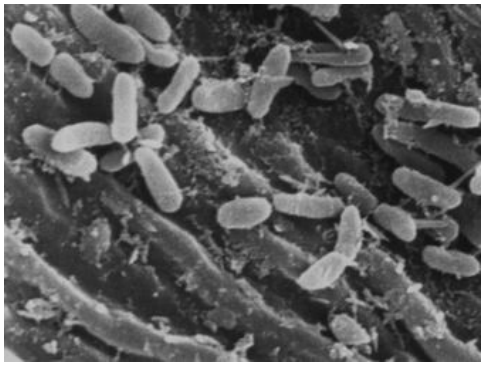


Figure 1. Phytopatogenic bacteria on pear leaf
by electron scanning microscopy (Photo: C. Moragrega)

In the 1840's, potato late blight caused by *Phytophthora infestans* provoked an Irish famine which was responsible for the death of more than a million people. Nowadays, although such dramatic situations do not occur, it remains worrying for some plant diseases. Then, in the area of plant protection, various strategies are been developed with the objective to control these diseases caused by bacteria and fungi, and to increase the food production and the product quality. The selected orientation will depend largely on the involved pathogen, the part of the plant infected, the vector implicated and economic considerations (Riley et al., 2002). Then, the accurate pathogen identification and the knowledge of the plant disease management are essential.

Plant pathogens

The research project of this PhD thesis has been mainly centred on the phytopathogenic bacteria *Erwinia amylovora*, *Pseudomonas syringae*, *Xanthomonas vesicatoria*, *Clavibacter michiganensis*, and the fungi, *Verticillium dahliae*, *Fusarium oxysporum*, *Penicillium expansum*, *Aspergillus niger*. These pathogens are responsible for diseases in plants of great economic importance and for which no effective methods are available. More emphasis has been given to the first three bacteria, *E. amylovora*, *P. syringae* and *X. vesicatoria*, responsible for severe plant diseases.

Erwinia amylovora is a Gram-negative bacterium which causes fire blight, a devastating disease that affects several plant species of the rosaceous family, e. g. fruit trees such as apple and pear trees, and also to ornamental plants (Cabrefiga and Montesinos, 2005; EPPO, 2005). Fire blight has been described worldwide (Figure 2) (Montesinos and López, 1998).

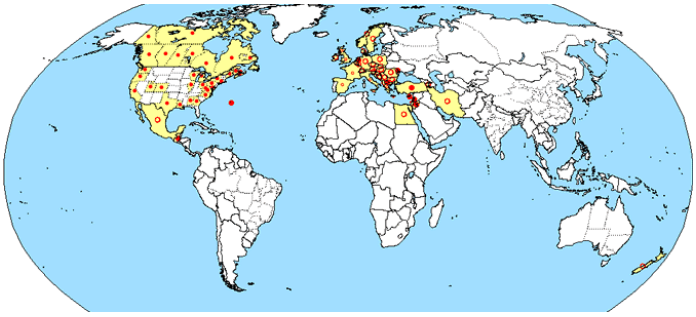


Figure 2. Worldwide distribution of *E. amylovora* reviewed by EPPO in 2005

present, national record
present only in some areas, national record
present, subnational record
present only in some areas, subnational record

It was first detected in 1780 in New York state (USA), and later in bordering areas. In 1919, it was observed in New Zealand, in 1960 in Egypt, and in the early 1980s in Israel and in several Arabian countries. Since its introduction in northern Europe in the 1950s to 1960s, the pathogen slowly spread southwards until it arrived to Spain in 1995 in Guipúzkoa (Euskadi). Later, outbreaks were detected in Vizcaya, Álava,

Navarra, Jaca, La Rioja and Zaragoza. In Catalonia, fire blight was detected in 1998 in Lleida and in 2003 in Puigcerdà (Gerona) (López et al., 2002).

E. amylovora infects the plant through open blossoms, natural openings such as stomata**,** and wounds (Norelli et al., 2003). Then, it multiplies rapidly during warm and moist conditions causing branch cankers and, sometimes, trunk attacks. These cankers provoke the quick death of branches or the whole tree by girdling. The bacterium overwinters in stem cankers and infected tissues. During springtime, it can be disseminated from cankers to flowers by insects, wind and rain. The most common and characteristic symptoms are the wilting and death of flower clusters, the withering and death of shoots and twigs, and the blight of leaves and fruits (Figure 3). Once these symptoms are visible, the fire blight is well established and very difficult to control.

Figure 3. Fire blight symptoms in an infected growing pear shoot (Photo: Dr. Montesinos)

Pseudomonas syringae is a Gram-negative bacterium which causes bacterial canker on stone fruit trees including apple, pear and nectarine trees (EPPO, 2005).

P. syringae infects the plant in autumn and winter, through wounds, natural openings, and frost or winter-injured tissues. In spring, during wet periods, *P. syringae* can multiply and continues its invasion. The bacterium produces a protein that acts as an ice nucleus, increasing frost wounds. It also produces syringomycin, a powerful plant toxin, that destroys plant tissues (Bender et al., 1999). Bacterial canker symptoms are wilt, vascular discoloration, leaf spots, scorching of leaf margins, and lesions on fruits (Figure 4). But the most conspicuous symptom is stem and branch cankers which exude gum during late spring and summer.

Figure 4. Bacterial canker symptoms in an infected growing pear shoot (Photo: Dr. Montesinos)

Xanthomonas vesicatoria is an aerobic Gram-negative bacterium which causes bacterial spot on Solanaceae plants of economic importance, mainly on tomatoes and peppers (EPPO, 2005). This disease occurs worldwide where these crops are grown in warm, moist areas (Figure 5).

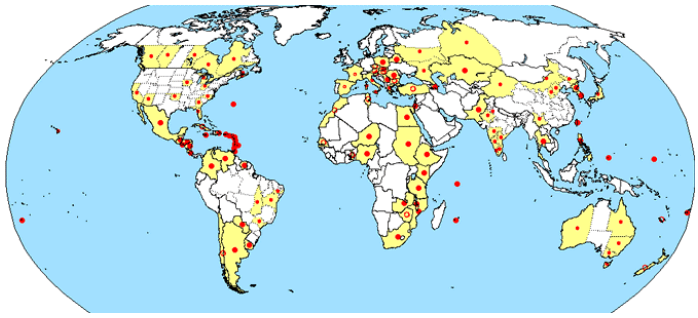


Figure 5. Worldwide distribution of *X. vesicatoria* reviewed by EPPO in 2005

present, national record
present only in some areas, national record
present, subnational record
present only in some areas, subnational record

This bacterium infects leaves through stomata and young fruits through small wound such abrasions and insect punctures. Lesions on leaves are irregular water-soaked areas, at first green and later becoming brown and necrotic (Figure 6). Diseased leaves drop prematurely causing an excessive fruit exposition to the sun and, consequently, increasing sunscald. Although fruit lesions are often only superficial, they reduce quality for sale fresh and also for processing.

Figure 6. Bacterial spot symptoms in infected pepper leafs (Photo: Dr. Montesinos)

Clavibacter michiganensis is a Gram-positive bacterium responsible for the bacterial canker of tomatoes (*C. michiganensis* subsp. *michiganensis*) and the bacterial ring rot of potatoes (*C. michiganensis* subsp. *sepedonicum*) (Rowe et al, 1995; Partridge, 2003; EPPO, 2005; Tisserat, 2006). These diseases are a continuing threat to the tomato and potato industries worldwide because they have the potential to spread quickly throughout a farm and may lead to severe losses. The most diagnostic feature of bacterial canker is the formation of fruit spots bordered by a distinct white halo.

Verticillium dahliae is a common soil-borne fungus which causes Verticillium wilt, a serious disease mostly found in temperate zones (Ash, 1994; Pataky, 1997; Berlanger and Powelson, 2000). It affects over 300 host plants, including many fruits, vegetables, trees, shrubs, and flowers as well as numerous weeds and some field crops. The characteristic symptom of the disease is wilting by vascular system invasion.

Fusarium oxysporum is a fungus widely distributed in soil and organic matter (Gonsalves and Ferreira, 1993). It is one of the most common species of the genus *Fusarium* and has several specialized forms which cause wilt diseases on a broad range of agricultural and ornamental plants. The typical symptoms are yellowing, vascular wilt, corm rot, and root rot.

The fungus ***Penicillium expansum*** causes blue mould which is the most common postharvest decay of stored fruits, mainly apples and pears. This pathogen also produces the carcinogenic mycotoxin patulin, which can rise to unacceptable levels in fruits and may result in off flavours. The decay caused by *P. expansum* is

characterized by the soft, watery consistency of the rotted tissues, by the musty odour and by the formation of conidial tufts.

Aspergillus niger is a common saprophytic fungus of the soil (U.S. Environmental Protection Agency, 1997). It can cause the rotting of numerous fruits and vegetables such as onions, grapes and tomatoes, especially on stored agricultural produce, resulting in substantial economic losses.

Plant disease management

Plant disease control has been traditionally based on single, and often severe, measures such as pesticide application, soil fumigation or burning of infected crops. However, this reactive approach has evolved into plant disease management, a more proactive, multifaceted system that integrates control methods on the basis of ecological and economic principles. Rather than to eliminate diseases completely, the goal is to keep them at acceptable levels, mainly combining two principles, prevention (tactics applied before infection) and therapy (measures applied after infection) (Maloy, 2005). Examples of preventive actions are exclusion measures and transgenic modification. Curative actions include eradication, biological control and chemical treatment (McManus and Stockwell, 2000; Maloy, 2005).

Exclusion measures are used to prevent entrance and establishment of a pathogen into a region where it does not occur (Directive 2000/29/EC). It includes the use of certified seeds or plants, as well as the selection of bulbs or the treatment of seeds, tubers or corns before planting. Unfortunately, these measures usually only delay the entry of a pathogen, but they do provide time to plan how to manage the pathogen when it arrives. In order to prevent the import and spread of plant pathogens, some laws regulate the conditions under which some crops may be grown and distributed between countries. Such control is applied by means of quarantine enforcement. Nine regional plant protection organizations (PPOs) have been established for promulgating and enforcing plant quarantine measures (Maloy, 2005; EPPO, 2006 (a); EPPO, 2006 (b)). Thus, *Erwinia amylovora*, *Pseudomonas syringae* pv. *Persicae*, *Xanthomonas vesicatoria*, *Clavibacter michiganensis*, *Fusarium*

oxysporum f.sp. *albedinis* and *Verticillium dahliae* have been listed as quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO).

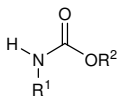
Transgenic modification of crops is used to confer resistance in plants to the diseases. This method of genetic engineering allows to an increasing crop yield and decreasing need for pesticides. It consists in the introduction of new genetic material (DNA) into the plant's genome to confer new characteristics on the plant. It is the most effective preventive action when available. This management tool has been most successfully applied against the more specialized pathogens such as rush fungi, smut fungi, powdery mildew fungi and viruses, but less against general pathogens such as many blight, canker, root rot and leaf spotting pathogens (Maloy, 2005). In addition, while numerous transgenic plants resistant to specific microbial disease are commercially available, their uses are very limited in European Union mainly by the consumer controversy (Montesinos, 2007).

Eradication involves the elimination of a pathogen once it has been introduced on a plant or in a field but before it has become well established or widely spread. Depending on the type of pathogen, its host, and the size of area affected, eradication can be accomplished by different measures such as removal of the disease plants or parts, pruning, crop rotation, soil fumigation, or burning agriculture field (partial or entire) (Maloy, 2005). The authorities must control the last method because it can result in contamination of groundwater or in killing of beneficial organisms.

Biological control of plant diseases involves the use of beneficial organisms that have the capacity to inhibit pathogens and control the disease that they cause (Compant et al., 2005). Biological control is sustained by beneficial interactions resulting from competition, antagonism or hyper parasitism of some microorganisms against plant pathogens, insects and weed (Rocco and Pérez, 2001; Montealegre et al., 2003). Several products based on biological agents are commercially available for the control of many plant diseases and pests (Montesinos et al., 2002; Montesinos, 2003; Elmer et al., 2005, Montesinos, 2007). For example, three biological control agents have been developed and are commercially available to inhibit *Erwinia amylovora*: *Bacillus subtilis* (Biopro®), *Pantoea agglomerans* (Blossom Bless®) and *Pseudomonas fluorescens* (Blightban A506®) (Lindow and

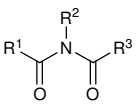
Suslow, 2003). These bacteria confer biological control of fire blight by competitive exclusion of *E. amylovora* or by production of antimicrobial substance(s) which inhibit the pathogen growth. Best control is achieved in combination with antibiotics for managing blossom and shoot infections. However, in spite of they are commercially available, the biological agents are not widely employed because chemical compounds are cheaper, easier to use and with higher efficacy and consistency of control. Therefore, biological control cannot still be viewed as an alternative to chemical treatment, but as a complementary disease management strategy (Francés et al., 2006).

Chemical treatment has been used to control plant diseases for centuries. Most pesticides targeted at plant diseases are applied prior infection. Only few pesticides have the capacity to stop infection once the pathogen has invaded the plant. Although fungicides can be very efficacious against fungal pathogens, bacterial plant pathogens are much more difficult to control. Chemicals used in plant protection include a great variety of structures (Thomson and Ockey, 1998). Sulphur and inorganic copper compounds are some of the most common inorganic fungicides. Unfortunately, most of them are phytotoxic and, in general, they display low persistence and penetration capacity. Carbamates, dicarboximides, benzene derivatives and organic copper compounds play a major role as fungicides because they are generally more effective and less toxic than the inorganic compounds (Figure 7).



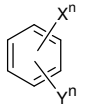
R^1 = alkyl, aryl
R^2 = alkyl, aryl

Carbamates



R^1 = acyl, alkyl, other
R^2 = alkyl, aryl
R^3 = alkyl, alkoxy

Dicarboximides

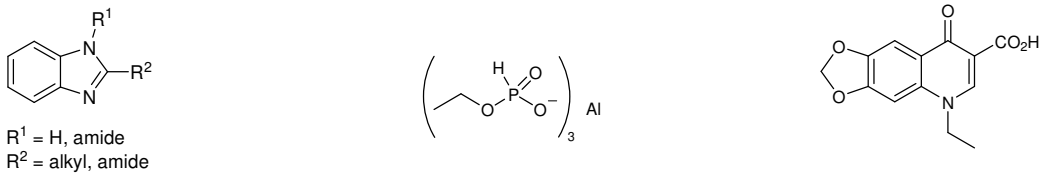


X = Cl
Y = alkoxy, CN, NO_2, alkyl
n = variable number of substituents

Benzene derivatives

Figure 7. Structure of some chemicals used in plant protection

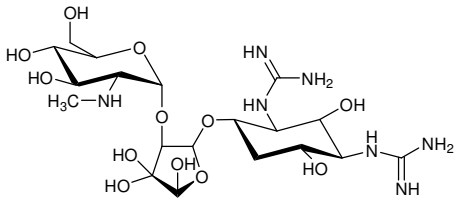
Recently, benzimidazoles, oxathiins, sterol inhibitors or strobilurins have been developed as systemic fungicides. For example, fosetyl-Al and oxolinic acid exhibit activity against *E. amylovora* (Figure 8) (Wolfson and Hooper, 1985; Kleitman et al., 2005). Unlike non-systemic fungicides, which need to be sprayed evenly into the plant surface, systemic ones are absorbed into the leaf tissue and then translocated throughout the plant, giving a better protective effect.



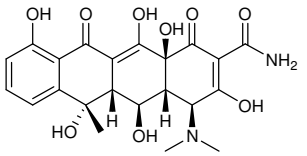
Benzimidazoles **Fosetyl-Al** **Oxolinic acid**

Figure 8. Systemic compounds used to control fire blight

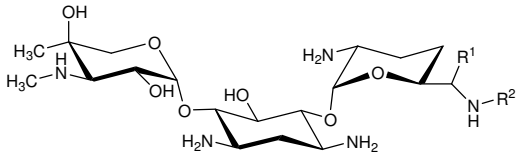
Another group of chemical compounds used in plant protection are antibiotics. They include organic compounds produced by microorganisms which inhibit other microorganisms as well as synthetic and semi-synthetic products (Cordiés Jackson et al., 1998; McManus et al., 2002). While in the European Union the use of antibiotics is not authorized, in USA they are allowed, being streptomycin and oxytetracycline the applied antibiotics on plants (Figure 9) (Chopra et al, 2001; McManus and Stockwell, 2001). In several Latin American countries, gentamycin, another aminoglycoside antibiotic, is also used (Figure 9) (McManus et al., 2002). However, due to its important utilization to treat human diseases, USA has not allowed its registration as an agricultural pesticide (McManus and Stockwell, 2000). Due to their high cost, the antibiotic application is mainly limited to fruit and vegetable crops of economic importance and to ornamental plants (McManus and Stockwell, 2000). In fact, while antibiotics are authorized under strict conditions in infection cases by *Pseudomonas syringae* and *Xanthomonas vesicatoria*, controlling fire blight of apple, pear, and related ornamental plants accounts for the majority of antibiotic use on plant disease control (Vidaver, 2002). However, an increasing emergence of antibiotic-resistant strains has been detected and associated with a loss of antibiotic efficiency (Chiou and Jones, 1991; McManus et al., 2002).



Streptomycin



Oxytetracycline

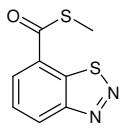


gentamycin C1a $R^1 = R^2 = H$
gentamycin C2 $R^1 = CH_3$ $R^2 = H$
gentamycin C1 $R^1 = R^2 = CH_3$

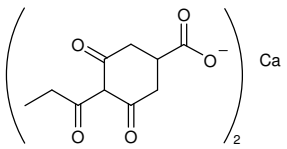
Gentamycin

Figure 9. Structure of antibiotics used in plant protection

As an alternative to traditional pesticides, two systemic acquired resistance (SAR) inducers called Actigard® (USA) or BION® (Europe) and Messenger® are available (Brisset et al., 2000; Hammerschmidt et al., 2001; Tsiantos et al., 2003; Vallad and Goodman, 2004). The active ingredient of Actigard® is a benzothiadiazole derivative (Figure 10), while harpin is the active ingredient of Messenger®. These compounds do not have an direct effect on pathogens but stimulate the natural plant defence mechanisms (salicylic acid pathway). Harpin is a naturally occurring bacterial protein of 403 amino acids in length, rich in glycine without cysteine residue. It is present in a number of species of plant pathogenic bacteria. The first harpin protein was isolated from the bacterium *Erwinia amylovora*. Prohexadione calcium (Phd-Ca), a plant growth regulator and active substance of the bioregulator Regalis®, is an other alternative agent to traditional pesticides (Figure 10) (Byers and Yoder, 1999; Rademacher, 2004). It reduces longitudinal shoot growth by inhibiting gibberellin biosynthesis reducing in this way the plant disease severity (Norelli and Miller, 2004).



Active ingredient of Actigard®



Prohexadione calcium

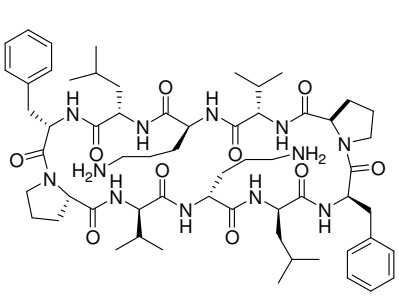
Figure 10. Organic compounds used as alternative to traditional pesticides

In general, the aim of plant disease management is to achieve the best disease control while maintaining the minimum ecological impact. Unfortunately, transgenic plants, pesticides and biological agents available are not enough to cover the real needs. Furthermore, the abusive use of chemicals produces environmental pollution, accumulation of toxic residues in the food chain and in water, and the development of resistance in the pathogens in addition to different human diseases detected mainly for the pesticide applicators (Schnabel and Jones, 1999; Alavanja et al., 2004; Blair et al., 2005; Engel et al., 2005). Consequently, crop protection has been progressively reoriented to a rational use of pesticides and to a reduction of the number of registered active ingredients to those certainly unavoidable, more selective, less toxic and with a lower negative environmental impact (Directive 91/414/EC). Consequently, a great deal of studies is actually devoted to the search for new chemical agents with high activity, low toxicity and low tendency to develop resistance (Mari et al., 2003). For several years, antimicrobial peptides have emerged as good candidates for the development of new anti-infective agents (Reddy et al., 2004; Andrès and Dimarcq, 2005; Mcphee and Hancock, 2005; Hancock and Sahl, 2006). Over the past few years, research efforts have been also performed in the antimicrobial peptides as potential candidates to control plant diseases (Montesinos, 2007).

Antimicrobial peptides

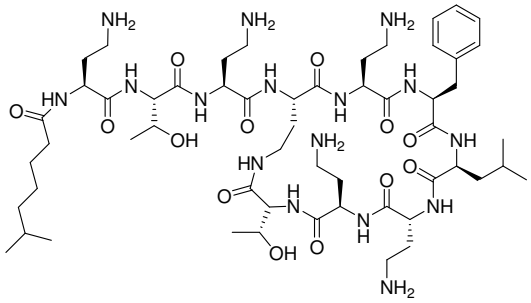
Antimicrobial peptides have been found in a broad variety of many living organisms, including mammals, amphibians, insects, plants and microorganisms (Hancock and Chapple, 1999). These peptides show a great diversity in the number of residues, primary structures, positioning of charged residues and secondary structures (Powers and Hancock, 2003; Patrzykat and Douglas, 2005; Brogden, 2005; Yount and Yeaman, 2005; Yount et al., 2006). They can be divided in non-ribosomally synthesized antimicrobial peptides (non-RAMPs) and ribosomally synthesized antimicrobial peptides (RAMPs) (Andreu and Rivas, 1998; Kohli et al., 2001; Kohli et al., 2002; Ganz, 2003).

Non-ribosomally synthesized antimicrobial peptides are produced by large multidomain enzymes, the so-called non-ribosomal peptide synthetases (NRPS) (Sieber and Marahiel, 2003). The number and order of these domains usually correspond to size and the primary structure of the peptide to construct, respectively. Several domains are able to introduce modifications to amino acids that are incorporated such as racemization of the L-amino acid pool, *N*-methylation of the α-amino group, and ring formation. Also, posttranslational modifications such as *C*-terminal amidation, glycosylation, oxidative cross-linking and halogenation can be introduced by NRPS. The cationic peptides gramicidin S and polymyxin B as well as the lipopeptide daptomycin and the noncationic glycopeptide vancomycin are some prominent examples of non-ribosomally synthesized antimicrobial peptides (Figure 11). A common feature of these peptides is their conformational restriction imposed by heterocyclization or macrocyclization.



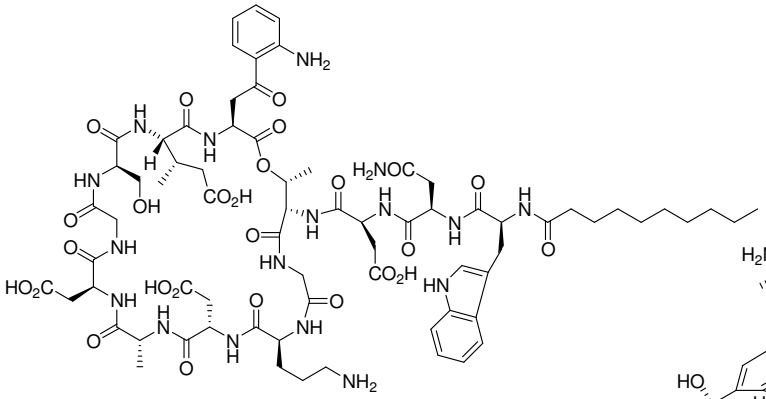
c(Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro)

Gramicidin S

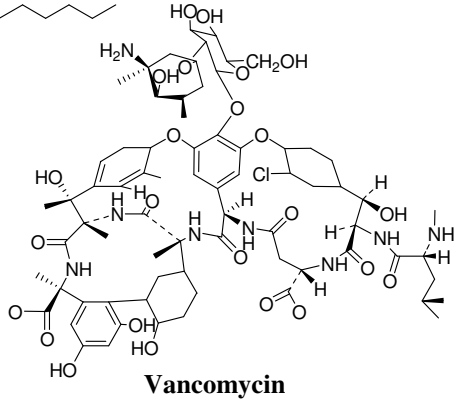


Mha-Dab-Thr-Dab-cDab(Dab-D-Phe-Leu-Dab-Dab-Thr)

Polymyxin B



Daptomycin



Vancomycin

Figure 11. Non-ribosomally synthesized peptide structures
Dab, 2,4-diaminobutyric acid; Mha, 6-methylheptanoic acid.

Unlike the non-RAMPs, the **ribosomally synthesized antimicrobial peptides** are synthesized as proforms which are subsequently processed into mature peptides after one or more proteolytic activation steps. The RAMPs are found to be important components of the innate defence systems in almost all forms of life, plants, a wide variety of invertebrates and vertebrates, and also prokaryotes (Table 1) (Hancock and Chapple, 1999, Bulet et al., 2004; Bulet and Stöcklin, 2005). In vertebrates, the ribosomally synthesized antimicrobial peptides provide a sophisticated first line of host defence, having the capacities to kill microbes directly and stimulate innate and adaptive defence systems (Hancock, 2001; Patrzykat and Douglas, 2005). In plants and insects, the RAMPs are major effector molecules to prevent and combat microbial infections. They show a broad spectrum of antimicrobial activity against a wide range of pathogens, including bacteria, fungi, and enveloped viruses (Zasloff, 2002). Although they differ significantly in their amino acid composition, most of them share the ability to form amphipathic residue distribution in which patches of hydrophobic and hydrophilic amino acids are spatially separated. This ability allows an antimicrobial peptide classification according to their secondary structure into four subgroups (van't Hof et al., 2001).

One subgroup contains linear peptides with an α-helical structure such as magainin and cecropin A (Figure 12). They adopt disordered structures in aqueous solution and fold into an α-helical conformation upon interaction with hydrophobic solvents or lipid surfaces. While the majority of these peptides are cationic, there are also hydrophobic and anionic α-helical peptides such as alamethicin extracted from the fungus *Trichoderma viride* (Figure 12) (Haris et al., 2004).

H-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-OH
Magainin

H-Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Glu-Lys-Val-Gly-Gln-Asn-Ile-Arg-Asp-Gly-Ile-Ile
-Lys-Ala-Gly-Pro-Ala-Val-Ala-Val-Val-Gly-Gln-Ala-Thr-Gln-Ile-Ala-Lys-NH_2
cecropin A

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-PhOl
Alamethicin

Figure 12. Primary α-helical peptide structure
Aib, *α*-methylalanine; PhOl, amino-alcohol derivative of Phe

A second subgroup contains conformationally more restrained peptides consisting of β-strands connected by intramolecular disulfide bridges as in the case of tachyplesins (Figure 13) (Tincu and Taylor, 2004). Usually in a β-sheet conformation in aqueous solution, they may be stabilized upon interactions with lipid surfaces. While studies with linear tachyplesin analogs suggested that the cyclic tachyplesin structure was essential for antimicrobial activity but not crucial for membrane permeabilization, others indicated a maintenance of a certain antimicrobial activity depending of the amino acid residues used to substitute the cysteine residues (Matsuzaki et al., 1997; Tamamura et al., 1998).

Tachyplesin I	**H-Lys-Trp-Cys$_1$-Phe-Arg-Val-Cys$_2$-Tyr-Arg-Gly-Ile-Cys$_2$-Tyr-Arg-Arg-Cys$_1$-Arg-NH$_2$**
Tachyplesin II	**H-Arg-Trp-Cys$_1$-Phe-Arg-Val-Cys$_2$-Tyr-Arg-Gly-Ile-Cys$_2$-Tyr-Arg-Lys-Cys$_1$-Arg-NH$_2$**
Tachyplesin III	**H-Lys-Trp-Cys$_1$-Phe-Arg-Val-Cys$_2$-Tyr-Arg-Gly-Ile-Cys$_2$-Tyr-Arg-Lys-Cys$_1$-Arg-NH$_2$**

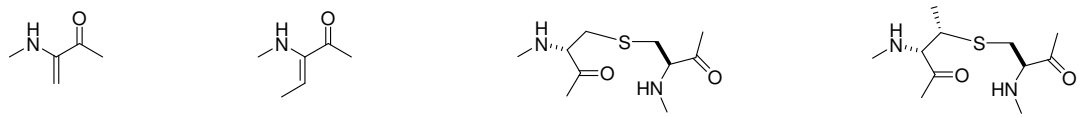
Figure 13. Primary tacheplesin structures. Subscript cysteine numbers represent amino acids that are joined by disulfide bridges.

A third subgroup contains linear peptides with an extended structure and an sequence rich in one or more specific amino acids. Indolicidin rich in tryptophan belongs to this subgroup (Figure 14).

H-Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH$_2$

Figure 14. Primary indolicidin structure

A fourth subgroup contains peptides with a looped structure such as lantibiotics. Lantibiotics are a class of potent antibacterial peptides produce by and primarily act on Gram-positive bacteria, and have been used for a variety of applications including food preservation (Jack et al., 1995; Diep and Nes, 2002; Pag and Sahl, 2002). Their cyclic structures are formed by posttranslational modifications of the side chains of the respecting amino acids mainly serine, threonine and cysteine residues. These modifications occur in the *C*-terminal propeptide while the *N*-terminal sequence remains unchanged. The first modification reaction is a selective dehydration of Ser and Thr in the lantibiotic precursor resulting in the α,β-unsaturated amino acids didehydroalanine (Dha) and didehydrobutyrine (Dhb), respectively (Figure 15). Cys residues then react with the dehydro residues to form the thioether amino acids lanthionine (Lan) and 3- methyllanthionine (MeLan) (Figure 15).



Didehydroalanine **Didehydrobutyrine** **Lanthionine** **Methyllanthionine**

Figure 15. Structures of modified amino acids found in lantibiotics

Nisin produced by *Lactococcus lactis* subsp. *lactis* is the most prominent lantibiotic. It is currently used as food biopreservative in several countries for its strong activity against food pathogens. Furthermore, it is reported to have successfully undergone phase I clinical trials as an oral treatment for *Helicobacter pylori* infection (Hancock and Chapple, 1999). Nisin is an small cationic peptide of 34 residues. Its structure consists of two amphipathic structured domains: an *N*-terminal domain (residues 3-19) containing three intramolecular ring structures formed by a single Lan residue and two MeLan residues (*e.g.* rings A, B and C in Figure 16), and a *C*-terminal domain (residues 22-28) containing two MeLan rings (*e.g.* rings D and E in Figure 16).

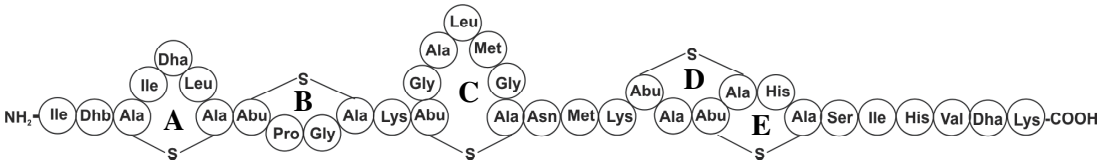


Figure 16. Nisin structure. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllantionine. The five ring are signed with letters A, B, C, D and E

The heterogeneous antimicrobial peptide group allows, also, an antimicrobial peptide classification according to their primary structure into five subgroups (Table 1) (Nissen-Meyer and Nes, 1997; Brogden, 2005):

One subgroup contains anionic peptides such as dermcidin (DCD). They are small, active against Gram-positive and Gram-negative bacteria, and require zinc as a cofactor for antimicrobial activity. Dermcidin is expressed in human sweat glands. Constantly secreted into sweat, DCD is transported to the epidermal surface and takes part in the defence mechanisms of human skin (Schittek et al., 2001). Full-length DCD consists of 110-amino acid residues.

A second subgroup contains linear cationic α-helical peptides (CAMP) such as cecropin A (Figure 12). They have less than 40 amino acid residues, lack cysteine, and are rich in arginine or/and lysine residues. They tend to be highly flexible in

solution, and adopt amphipathic structures only upon contact with membranes and membrane-mimicking environments.

A third subgroup contains cationic peptides enriched with specific amino acids such as indolicidin (Figure 14). These peptides are linear and also lack cysteine residues.

A fourth subgroup contains anionic and cationic peptides rich in cysteine residues such as brevenins. They form disulfide bonds between the thiol groups of cysteine residues and stable β-sheets.

A fifth subgroup contains anionic and cationic peptide fragments from larger proteins such as lactoferricin from lactoferrin.

Table 1. Overview of ribosomally synthesized antimicrobial peptides (RAMPs)

Group		Example	Origin
I	anionic peptides	dermcidin	human
II	linear cationic α-helical peptides	cecropin (A) melittin magainin	insects insects frog
III	cationic peptides enriched for specific amino acids	indolicidine (tryptophan-rich) bactenectins (proline- and arginine-rich) prophenin (proline- and phenylalanine-rich)	cattle cattle, sheep and goats pig
IV	anionic and cationic peptides rich in cysteine residues	brevenins (one disulphide bridge) tachyplesin (two disulphide bridges) defensins (three disulphide bridges)	amphibians horseshoe crab human, rabbit, rat, cattle, mice, pig, goat, poultry, monkey
V	anionic and cationic peptide fragments from larger proteins	casocidin (from casein) lactoferricin (from lactoferrin)	human mammals

The ribosomally synthesized a**ntimicrobial peptides from plants** are expressed after the contact with the pathogen. Thionins, defensins and lipid transfer proteins constitute the main groups of these peptides (García-Olmedo et al., 1998; Castro and Fontes, 2005).

Thionin family is constituted by cationic peptides called α and β thionins rich in arginine, lysine and cysteine residues with low molecular weight (~5 kDa) (Hancock and Lehrer, 1998; Pelegrini and Franco, 2005). They look like L-shaped molecules with their long arm formed by two disulphide-linked α-helices and their short arm containing two antiparallel β sheets. Most of them are toxic against both Gram-positive and Gram-negative bacteria such as *Pseudomonas*, *Xanthomonas* and *Erwinia*, and towards fungi and yeast. Unfortunately, most thionins, also, lyse a wide variety of mammalian and plant cells.

Plant defensins, named also γ thionins in spite of the low degree of similarity with the thionin family, are cationic β-sheet Cys-rich peptides from 45 to 54 amino acids long (Broekaert et al., 1995; Pelegrini and Franco, 2005). This group is characterized by a high antifungal activity.

Lipid transfer proteins (LTPs) are 90–95 amino acid polypeptides rich in disulfide-linked cysteines. Their globular structure is composed of a bundle of four α-helices cross-linked by four disulfide bridges. While, positively charged amino acids are exposed on the surface of the protein, the hydrophobic residues line the internal cavity, which is a characteristic conserved in all LTPs. They inhibit bacterial and fungal pathogens.

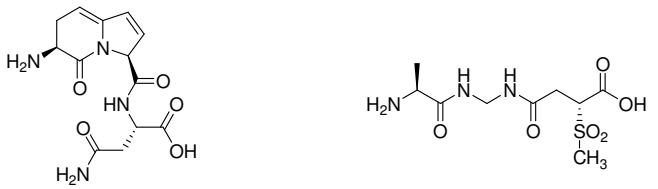
In addition to the antimicrobial peptides expressed from plants, there are other **antimicrobial peptides active against plant pathogenic microorganisms**. They can be classed in two groups, naturally occurring antimicrobial peptides and synthetic peptides (Montesinos, 2007). In the former, many antimicrobial peptides are produced by microorganisms.

In naturally occurring antimicrobial peptides, large bacteriocins and plant defensins-like peptides synthesized by several filamentous fungi have demonstrated an antibacterial activity for the former and an antifungal activity for the latter (Jack et al., 1995).

Some peptaibols also exhibit antimicrobial activity against plant pathogenic Gram-positive bacteria such as *Clavibacter michiganensis* and fungi such as *Fusarium oxysporum*. They are non-ribosomally synthesized linear peptides characterized by the presence non-natural amino acids, an acetylated *N*-terminus and a hydroxylated *C*-terminal amino acid.

Cyclopeptides produced through nonribosomal peptide synthesis by bacteria and fungi, particularly lipidic cyclopeptides (LCPs) display activity against bacteria and fungi such as *Fusarium oxysporum*. Their cyclic backbones which may be linked to a linear lipid or a peptide tail are usually without disulfide bridge and contain D- and L-amino acid residues.

Some pseudopeptides are also effective against plant pathogenic microorganisms. While some of them such as pantocins A and B show antibacterial activity, others such as polyoxins are antifungal agents (Figure 17, Table 2).

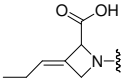
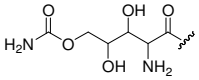
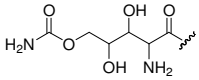


Pantocin A **Pantocin B**

Figure 17. Pantocin structures

The significant phytotoxicity of some naturally occurring antimicrobial peptides, such as peptaibols, explain the restricted use of these peptides in the plant disease control. However, some of them are involved in plant disease control through biological control or transgenic technology.

Table 2. Polyoxin structures

Polyoxin	R_1	R_2	R_3
A			
B			
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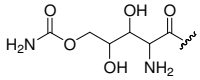
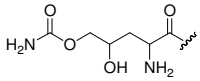
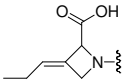
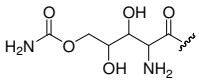
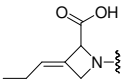
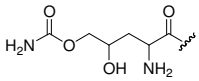
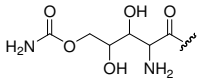
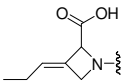
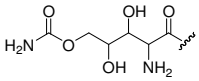
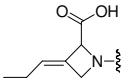
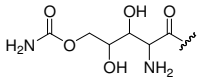
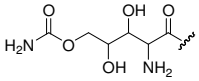
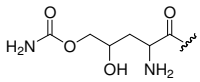
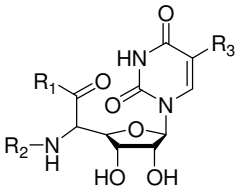
D			
E			
F			
G			
H			
I			
J			
K			
L			
M			



Synthetic antimicrobial peptides such as PEP6 show an antimicrobial activity against plant pathogens (Table 3) (López-García et al, 2002). Some of them such as the cecropin-melittin hybrid PEP3 or $LfcinB_{20-25}$ are peptides based on a naturally occurring peptide(s) (Andreu et al., 1992; Muños and Marcos, 2006). While few of them are peptides open-ended containing disulphide bridges, many are linear (Montesinos, 2007). It is worth noting the lack of sequence homology and standard size of these synthetic antimicrobial peptides (Table 3).

Table 3. Synthetic antimicrobial peptides active against plant pathogens

Peptide	Size	Sequence	Activity
BP76	11	H-Lys-Lys-Leu-Phe-Lys-Lys-Ile-Leu-Lys-Phe-Leu-NH_2	antibacterial
CAMEL	15	H-Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-NH_2	antibacterial
Iseganan	17	H-Arg-Gly-Gly-Leu-Cys-Tyr-Cys-Arg-Gly-Arg-Phe-Cys-Val-Cys-Val-Gly-Arg-NH_2	antibacterial
ESF12	18	H-Met-Ala-Ser-Arg-Ala-Ala-Gly-Leu-Ala-Ala-Arg-Leu-Ala-Arg-Leu-Ala-Leu-Arg-OH	antibacterial
Pexiganan	22	H-Gly-Ile-Gly-Lys-Phe-Leu-Lys-Lys-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Lys-Ile-Leu-Lys-Lys-NH_2	antibacterial
PEP11	11	H-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Leu-Lys-Val-Leu-OH	antibacterial, antifungal
D4E1	17	H-Phe-Lys-Leu-Arg-Ala-Lys-Ile-Lys-Val-Arg-Leu-Arg-Ala-Lys-Ile-Lys-Leu-OH	antibacterial, antifungal
MSI-99	22	H-Gly-Ile-Gly-Lys-Phe-Leu-Lys-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Lys-Ile-Leu-Asn-Ser-OH	antibacterial, antifungal
MB-39	39	H-His-Gln-Pro-Lys-Trp-Lys-Val-Phe-Lys-Lys-Ile-Glu-Val-Val-Gly-Arg-Asn-Ile-Arg-Asn-Gly-Ile-Val-Lys-Ala-Gly-Pro-Ala-Ile-Ala-Val-Leu-Gly-Glu-Ala-Lys-Ala-Leu-Gly-OH	antibacterial, antifungal
Pen4-1	46	H-His-Ser-Ser-Gly-Tyr-Thr-Arg-Pro-Leu-Arg-Lys-Pro-Ser-Arg-Pro-Ile-Phe-Ile-Arg-Pro-Ile-Gly-Cys-Asp-Val-Cys-Tyr-Gly-Ile-Pro-Ser-Ser-Thr-Ala-Arg-Leu-Cys-Cys-Phe-Arg-Tyr-Gly-Asp-Cys-His-Leu-NH_2	antibacterial, antifungal
D32R	47	H-Lys-Ser-Cys-Cys-Arg-Asn-Thr-Trp-Ala-Arg-Asn-Cys-Tyr-Asn-Val-Cys-Arg-Leu-Pro-Gly-Thr-Ile-Ser-Arg-Glu-Ile-Cys-Ala-Lys-Lys-Cys-Arg-Cys-Lys-Ile-Ile-Ser-Gly-Thr-Thr-Cys-Pro-Ser-Asp-Tyr-Pro-Lys-OH	antibacterial, antifungal
PEP6	6	H-Phe-Arg-Leu-Lys-Phe-His-OH	antifungal
PAF26	6	Ac-Arg-Lys-Lys-Trp-Phe-Trp-NH_2	antifungal
$LfcinB_{20-25}$	6	Ac-Arg-Arg-Trp-Gln-Trp-Arg-NH_2	antifungal
PEP3	11	H-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Leu-Lys-Val-Leu-NH_2	antifungal
$LfcinB_{17-31}$	15	Ac-Phe-Lys-Cys-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-NH_2	antifungal
TPY	17	H-Lys-Trp-Val-Phe-Arg-Val-Asn-Tyr-Arg-Gly-Ile-Lys-Tyr-Arg-Arg-Gln-Arg-OH	antifungal
ESF1	20	H-Met-Ala-Ser-Arg-Ala-Ala-Gly-Leu-Ala-Ala-Arg-Leu-Ala-Arg-Leu-Ala-Leu-Arg-Ala-Leu-OH	antifungal

The **mechanism of action** of antimicrobial peptides is not fully established. Furthermore, the mechanism combining by a given antimicrobial peptide is a phenomenon which could be widely used. Nevertheless, although there is evidence that some antimicrobial peptides may interact with bacterial components other than membranes, they work primarily by compromising the cell wall (Reddy et al., 2004; Brogden, 2005).

The bacterial cell wall forms a rigid structure which provides strength, rigidity and shape, and protects the cell from changes in water pressure. It surrounds the cytoplasmic membrane, also called plasma membrane, which is a phospholipid bilayer with peripheral and integral protein. Classification of bacteria into Gram-positive (G^+) and Gram-negative (G^-) groups can be done by the ability of bacterial cell wall to retain a purple dye called crystal violet. While the alcohol/acetone washing step in Gram staining does not decolourise the G^+ cells, the crystal-violet escapes from the G^- cell when the decolouriser is added. The differences in cell wall composition explain these results. While Gram-positive bacteria have a cell wall containing many layers of peptidoglycan, Gram-negative bacteria have a cell wall composed of a thin peptidoglycan layer surrounded with a lipoprotein-lipopolysaccharide-phospholipid outer membrane (Hancock, 1997 (a); van Heijenoort, 2001) (Figure 18). Unlike in G^+ bacteria, the G^- bacterium cell wall has therefore a high lipid content and a low peptidoglycan content. Consequently, the crystal violet escapes from cells at the alcohol/acetone washing step.

In both bacteria, the peptidoglycan is constituted of chains of alternating units of *N*-acetylglucosamine (NAG or GlcNAc) and *N*-acetylmuramic acid (NAM or MurNAc) which are β-1,4-linked. The 2-hydroxypropanoic acid of some NAM are linked to peptides, generally tetrapeptides, consisting of both L- and D-amino acid residues such as D-glutamic acid and D-alanine. Cross-bridges link peptides of NAG-NAM chains. The specific amino acid sequence, the amino acid residues involved in the cross-bridges and the proportion of chains cross-linked vary with the bacterial genera (Schleifer and Kandler, 1972). But there is conservation of architecture throughout the Gram-positive and Gram-negative groups. While in Gram-positive bacteria the peptidoglycan is heavily cross-linked, it is only intermittently cross-linked in Gram-negative bacteria.

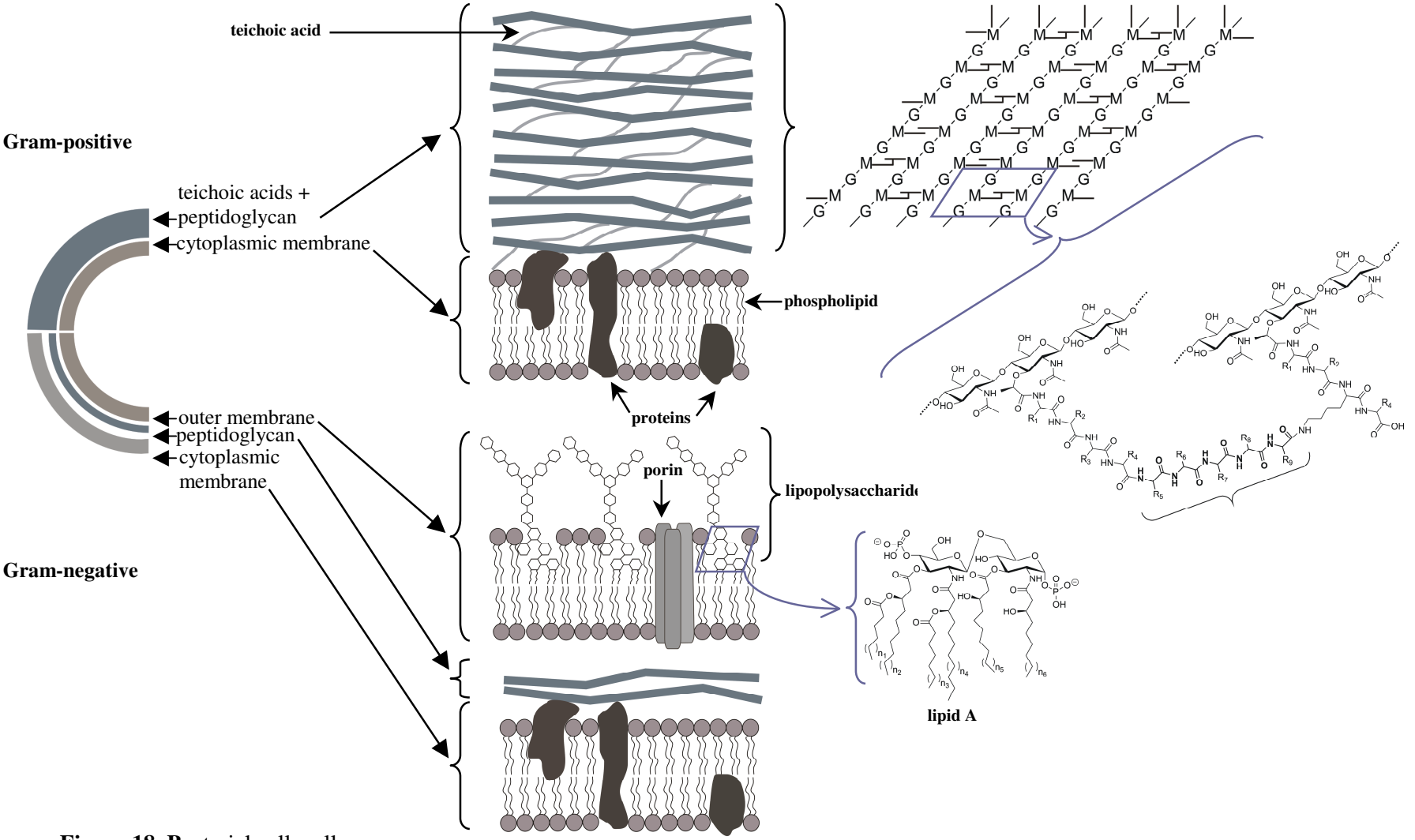


Figure 18. Bacterial cell wall
G, *N*-acetylglucosamine; M, *N*-acetylmuramic acıd

Another structure in the cell wall of Gram-positive bacteria is the teichoic acids (Ward, 1981). Teichoic acids of the G^+ bacteria are polyol phosphate polymers of glycerol or ribitol usually with glucose, *N*-acetylglucosamine, *N*-acetylgalactosamine and/or D-alanine as substituents. These teichoic acids can be covalently linked to the peptidoglycan NAM through phosphodiester bonds and link various layers of the peptidoglycan chains together, or bonded to the cytoplasmic membrane lipids. Therefore, teichoic acids stabilize the G^+ bacteria cell wall and make it stronger. Furthermore, teichoic acids negatively charged contribute to the negative charge of the gram-positive cell wall.

The G^- bacteria outer membrane is another lipid bilayer similar to the cytoplasmic membrane which contains, also, lipoproteins and lipopolysaccharides (LPS). The most abundant outer membrane proteins are the porins which contain a central channel that allows the passage of most small hydrophilic molecules. Very large or hydrophobic molecules cannot penetrate the outer membrane through the porin pores. Covalently bound to the peptidoglycan, the lipoproteins are embedded in the outer membrane by their hydrophobic head that help to tightly bind the peptidoglycan to the outer membrane. LPS, as amphiphilic macromolecules of the outer membrane, contain a hydrophilic heteropolysaccharide portion which is covalently linked to a hydrophobic lipid portion, termed lipid A. The heteropolysaccharide section consists of a conserved core polysaccharide attached to lipid A and distal specific polysaccharide side chains called the O-antigen. Lipid A is a glucosamine disaccharide, usually β-1,6-linked, covalently attached to fatty acid such as hydroxy-myristic acid, and phosphate residues. When released from bacterial cells, free LPS is toxic to mammals and is therefore called endotoxin. The lipid A of LPS is embedded into the the outer leaflet of the outer membrane while the rest of the LPS projects from the surface**.** The lipopolysaccharides confer a negative charge on the outer leaflet of the outer membrane of G^- bacteria

The mode of action of cationic antibacterial peptides is the most well known among all antimicrobial peptide mechanisms known (Hancock, 2001; Brogden, 2005). The cationic peptide portion is proposed to initiate electrostatic interaction with the negatively charged components of the bacterial membrane of microorganisms. Once arrived at the cell surface, the peptides either bind to the lipopolysaccharides or

neutralize the charge over an area of membrane, subsequently distorting the membrane structure. The peptides then can translocate across the outer membrane of the Gram-negative bacteria via self-promoted uptake pathway (Hancock, 1997 (a, b)). Once the outer membrane permeated, cationic peptides interact with the negatively charged phospholipids of the cytoplasmic membrane. The peptides with structural constraints, either by disulfide bonds or by cyclization of the peptide backbone, largely exist in the β-sheet conformation that may be further stabilized upon interactions with lipid bilayer. On the contrary, the α-helical peptides fold into their α-helical amphipathic arrangement upon interaction with lipid surfaces that permits the insertion of a well-defined hydrophobic sector into the lipid bilayer. Once a threshold concentration of CAPs on the lipid bilayer surface, several models including the barrel-stave model, the toroidal-pore model and the carpet model, have been proposed to explain the membrane permeabilization or cell lysis (Papo and Shai, 2003; Yeaman and Yount, 2003; Brogden, 2005).

In the "barrel-stave model" known also as "transmembrane helical bundle model", the individual peptides traverse the membrane and are bundled together forming a highly organized transmembrane pores (Figure 19). The hydrophobic amino acid residues front on the acyl chains of the bilayer phospholipids while the hydrophilic amino acid residues line the central pore hole.

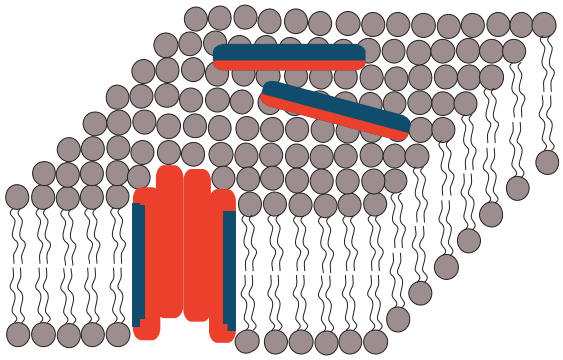


Figure 19. Barrel-stave model proposed for the cationic antibacterial peptides Hydrophobic and hydrophilic peptide faces are illustrated in blue and red, respectively. The grey spheres and the line represent the headgroups and the acyl chains of the bilayer phospholipids, respectively.

In the “toroidal-pore model” or “wormhole model”, the individual peptides insert into the membrane with the negatively charged headgroups of lipids separating the positive peptide charge. Thus, they form supramolecular peptide-lipid complexes with polar peptide side-chains and the lipid head groups lining the central pore hole. (Figure 20).

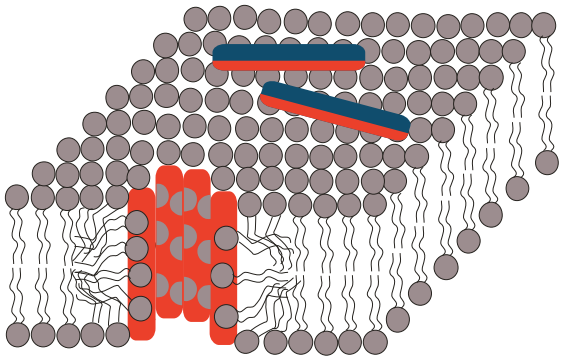


Figure 20. Toroidal-pore model proposed for the cationic antibacterial peptides Hydrophobic and hydrophilic peptide faces are illustrated in blue and red, respectively. The grey spheres and the line represent the headgroups and the acyl chains of the bilayer phospholipids, respectively.

In the “carpet model”, peptides cover the membrane surface in a carpet-like manner. At a critical threshold concentration, surface-oriented peptides disrupt the bilayer in a detergent-like manner eventually leading to the formation of micelles (Brodgen, 2005).

In contrast to conventional antibiotics, that in many cases are bacteriostatic, cationic antibacterial peptides generally cause cell death as quickly as 2 to 3 min after initial exposure (Epand and Vogel, 1999; Hancock and Chapple, 1999; Kondejewski et al., 2002).

It is worth noting that nisin (Figure 16) displays several mechanisms of action known (Peschel and Sahl, 2006). It acts by forming pores in bacterial membranes causing cell death due to loss of essential intracellular substances after target recognition, nisin/lipid II and complex formation and nisin/undecaprenyl pyrophosphate complex formation (Wiedemann at al., 2001; Bonev et al., 2004; Wiedemann at al., 2004). In addition, it inhibits bacterial growth by interfering with cell wall biosynthesis of sensitive bacteria (Hyde et al., 2006). Nisin also activates autolytic enzymes displacing them from their anionic binding sites in the Gram-positive cell wall as

well as inhibits the outgrowth of spores by disrupting some vital functions (Pol et al., 2001).

The mechanism of action of anionic antibacterial peptides is not well known. However, few studies suggest mode of actions of peptides. For instance, investigators have published their work about antimicrobially active dermcidin (DCD) peptides (Steffen et al., 2006). These peptides, such as DCD-1L consisting of 48 amino acids and DCD-1 containing 47 amino acids, are derived from the *C*-terminal region of the 110-amino-acid dermicin. In contrast to most other AMPs which are cationic, dermcidin and some proteolytically processed DCD peptides, such as DCD-1L and DCD-1, have a net negative charge. Studies of dermcidin-derived peptides suggested a model in which individual peptides bound to the bacterial membrane without causing massive permeabilization. The process finished possibly by insertion into the membrane, and finally cell death. It was, also, suggested that DCD-1 bound to the bacterial membrane in the form of clusters through defined membrane targets. Interestingly, time kinetics indicated that dermcidin-derived peptides kill bacteria in a rather slow process, taking at least 2 h *in vitro*.

The fungal cell wall surrounds the cytoplasmic membrane and is a rigid structure essential for the cell survival. Although its chemical composition can vary considerably between and within different groups of fungi, the fungal cell wall usually contains chitin. It is a linear polysaccharide of β-(1,4)-linked *N*-acetylglucosamine (NAG or GlcNAc) cross-linked by highly branched glucans (glucose polymers) (Figure 21) (Debono and Gordee, 1994; Kollár et al, 1997; Lipke and Ovalle, 1998; Thevissen et al., 2003). Proteins represent the third important chemical component of the cell wall. Many of them are modified by glycosylation.

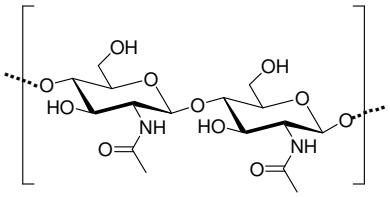


Figure 21. Structure of a unit from a chitin polymer chain

Different mechanisms of action of antifungal peptides are been identified. However in all events, some specific biochemical characteristics such as chitin-binding ability seem to be binded to an antifungal activity. The antifungal peptides may act by lysis or affect synthesis (De Lucca and Walsh, 1999; De Lucca and Walsh, 2000). Once binding to the membrane, the former peptides cause the cell death by disrupting the membrane structure or penetrating it and interacting with specific internal targets or forming pores of variable sizes. The latter peptides interfere in the cell wall synthesis or in the biosynthesis of essential cellular components such as chitin or glucan.

Selectivity of some antimicrobial peptides between bacterial and mammalian cells has been, interestingly, observed. The basis of this selectivity has been attributed to the differences in membrane lipid composition (Huang, 2000; Zasloff, 2002). Indeed, unlike the bacterial cells, the mammalian cells have neither cell wall nor negatively charged lipids on the surface of the cytoplasmic membrane (Verkleij et al., 1973; Papo and Shai, 2003). Furthermore, the cytoplasmic membrane in mammalian cells contains sterols mainly cholesterol (Figure 22). Thus, the absence of negatively charged lipids on the surface and the presence of sterols reduce the susceptibility of eukaryotic cells to lytic peptides.

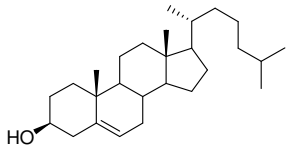
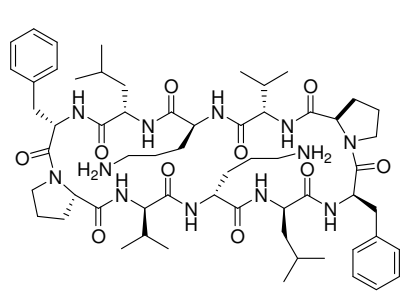


Figure 22. Cholesterol structure

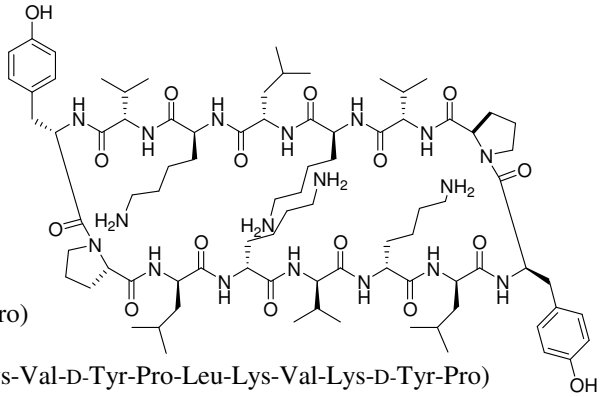
Some **examples of studies on synthetic peptides**, either based on natural occurring structures or synthetic ones, have been made to understand the mode of action and the selectivity of the antimicrobial peptides, and are relevant in the context of this work.

Increasing the ring size of gramicidin S to 14 residues produced a peptide (GS14) with weaker antimicrobial activity but stronger hemolytic activity than its parent peptide (Figure 23) (Kondejewski et al., 1996). Furthermore, the activity profile of GS14 was reversed through a reduction of peptide amphipathicity by substitution of residue for their enantiomer (Kondejewski et al., 1999; McInnes et al., 2000; Lee et al., 2003). Thus, the least amphipathic peptide, produced by substituting a D-Lys at

position 4 of GS14 (GS14K4), displayed the highest degree of specificity for microbial cells over human cells. Moreover, the alteration of the non-polar face hydrophobicity of GS14K4 provided analogues with a range of activities and specificities, where the peptides with the intermediate hydrophobicities showed the best biological profiles (Kondejewski et al., 2002). These studies suggested the possibility to rationally design cyclic antimicrobial peptides with high specificity towards bacterial membranes and minimal toxicity to eukaryotic cells.



(Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro)



c(Val-Lys-Leu-Lys-Val-D-Tyr-Pro-Leu-Lys-Val-Lys-D-Tyr-Pro)

Figure 23. Structure of gramicidin S and GS14

Blazyk and co-workers also observed the influence of amphipathicity on antimicrobial and hemolytic activities in synthetic linear cationic α-helical and β-sheet peptides bearing identical charge and hydrophobicity (Figure 24) (Blazyk et al., 2001; Jin et al., 2005). They showed that an increase of amphipathicity correlated with an increase of antimicrobial and hemolytic activities.

H-(Lys-Ile-Gly-Ala-Lys-Ile)$_3$-NH$_2$ **H-(Lys-Ile-Ala-Gly-Lys-Ile-Ala)$_3$-NH$_2$**

Figure 24. Primary structure of the α-helical and β-sheet peptide families

Rozek and co-workers showed that cyclization of indolicidin analogue CP-11 through disulfide bridge formation significantly improved peptide stability towards protease degradation and, in addition, increased the antimicrobial activity (Figure 25) (Rozek et al., 2003).

CP-11 **H-Ile-Leu-Lys-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-Lys-NH$_2$**
1 5 10

cycloCP-11 **H-Ile-c(Cys-Leu-Lys-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-Cys)-Lys-NH$_2$**
1 5 10 15

Figure 25. Primary structure of CP-11 and its cyclic analogue

Similar results were obtained by Dathe and co-workers using synthetic arginine- and tryptophan-rich hexapeptides (Figure 26) (Dathe et al., 2004; Wessolowski et al., 2004). Peptide cyclization was shown to promote a substantial increase of the selectivity for bacteria versus mammalian cell leading to a decrease in the hemolytic activity.

Ac-Arg-Arg-Trp-Trp-Arg-Phe-NH_2	**c(Arg-Arg-Trp-Trp-Arg-Phe)**
Ac-Arg-Arg-Trp-Trp-Phe-Arg-NH_2	**c(Arg-Arg-Trp-Trp-Phe-Arg)**
Ac-Arg-Arg-Trp-Phe-Trp-Arg-NH_2	**c(Arg-Arg-Trp-Phe-Trp-Arg)**
Ac-Arg-Trp-Arg-Phe-Trp-Arg-NH_2	**c(Arg-Trp-Arg-Phe-Trp-Arg)**
Ac-Phe-Arg-Trp-Trp-Arg-Arg-NH_2	**c(Phe-Arg-Trp-Trp-Arg-Arg)**

Figure 26. Primary structure of the linear and cyclic synthetic arginine- and tryptophan-rich peptides

Synthetic peptides composed solely of Lys and Leu residues maintaining the crucial features of native antibacterial peptides have also been described to display interesting antimicrobial activities (Table 4) (Blondelle and Houghten, 1992; Oren et al., 1997; Papo et al., 2002; Epand et al., 2003; Papo and Shai, 2005). Similarly to the studies described above, peptide size, D-amino acid incorporation and cyclization were shown to influence the biological activity, the selectivity and the stability towards protease degradation.

Table 4. Amino acid sequences of Leu-Lys containing peptides

Amino acid sequence	References
Ac-Leu-Lys-Leu-(Leu-Lys-Lys-Leu-Leu-Lys-Lys)$_2$-Leu-NH_2	Blondelle and Houghten, 1992
H-Lys-Leu-D-Leu-D-Leu-Lys-Leu-(Lys-D-Leu)$_2$-Leu-Lys-NH_2	Oren et al., 1997
H-Lys-(Leu-Lys-Leu-Leu-Lys-Leu)$_2$-Leu-Lys-NH_2	Papo et al., 2002
Ac-(Leu-Lys-Lys-Leu)$_5$-NHEt **Ac-(Lys-Leu)$_{10}$NHEt**	Epand et al., 2003
H-Lys-Leu-D-Leu-D-Leu-Lys-Leu-(Lys-D-Leu)$_2$-Leu-Lys-NH_2	Papo and Shai, 2005

All these studies pointed out that a good balance between the net positive charge, the overall hydrophobicity and the ability to adopt an amphipathic structure are essential to design cyclic peptides with optimal biological properties. They also indicated that

peptide cyclization generally improves their metabolic stability by limiting the accessibility to proteases and increases the selectivity for bacteria versus mammalian cells reducing the hemolytic activity.

Bacterial resistance to antimicrobial peptides has been considered for a long time as impossible or, at least, very difficult to occur. The main argument of that was the primary peptide compromise with bacterial membrane which require significant alteration for bacterial resistance (Reddy et al., 2004; Brogden, 2005). However, important alteration of the anionic bacterial cell-envelope molecules, such as peptidoglycan, lipid A, teichoic acids or phospholipids, can not occur easily. Nevertheless, CAMP-resistance mechanisms used by bacterial pathogens have been, nowadays, found (Peschel and Sahl, 2006).

CAMP-degrading protease production is one obvious bacterial method to inactivate the antimicrobial peptides (Bell and Gouyon, 2003; Brogden, 2005; Yount et al., 2006). However, while linear peptides are relatively susceptible to proteolysis, the cyclic peptides are much more protease resistant. Nevertheless, the presence of proline residues or an amidated *C*-terminal end renders the linear peptides more stable. The protease activity is therefore effective only against specific peptide structures.

CAMP-specific resistance by trapping or extrusion from the bacterial membrane is an other bacterial resistance mechanism which requires first recognition of certain CAMP sequences or structural motifs. Consequently, appropriate residue exchange can circumvent this resistance mechanism without affecting antimicrobial activity.

Reducing net negative surface charge of the bacterial membrane is the least specific resistance mechanism. As above-mentioned, bacteria can not modify their net negative surface charge by changing complex biosynthesis of anionic bacterial cell-envelope molecules, but they can partially neutralize the negative charge by incorporation of D-alanine to Gram-positive cell wall teichoic acids, by modification of anionic phospholipids with L-lysine or by adding aminoarabinose to the lipopolysaccharide (LPS) lipid A moiety of Gram-negative bacteria. However, bacteria with these partial modifications are still susceptible to antimicrobial peptides at high concentrations.

The other resistance mechanisms are changing in hydrophobicity by replacement of the lipid A acyl group myristate with 2-OH myristate, altering the fluidity of the outer membrane by formation of hepta-acylated lipid A with the addition of palmitate (Campos et al., 2004; Brogden, 2005; Yount et al., 2006).

Peptide synthesis and combinatorial chemistry

Combinatorial chemistry embraces a diversity of techniques that, rapidly and efficiently, enable the production of large collection of compounds rapidly tested for desirable properties (Maclean et al., 1999). This large collection of substances and the technique used to evaluate them are known as library and high-throughput screening, respectively. The synthesis of these libraries can be automated by robot controlled by computer. The combinatorial chemistry advantages have made that it has been, rapidly, viewed by the pharmaceutical, agrochemical and biotechnological industries as a means for accelerating the discovery of new bioactive candidates against human, veterinary and plant diseases (Houghten et al., 2000; Golebiowski et al., 2001; Edwards et al., 2006). Its major impact in drug discovery is on the lead finding and optimisation steps (Figure 27) (Lazo and Wipf, 2000). Lead finding is carried out by screening large libraries in order to identify compounds that interact with the target receptor. Once a hit is identified, it is further optimised to produce leads with improved biological properties that can then be converted into drug development candidates (Dolle, 2002; Golebiowski et al., 2001).

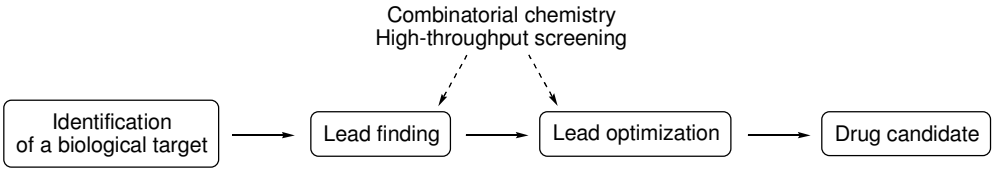


Figure 27. Drug discovery process

In addition to being a powerful tool in drug discovery, its is also a great tool in basic research. Combinatorial chemistry, first applied to peptides, has grown exponentially allowing generate a great variety of compounds including peptidomimetics, oligosaccharides, and small organic molecules can be available (Thompson and

Ellman, 1996; Nefzi et al., 1997; Dolle, 2002). Since then, combinatorial methods has been used in other fields such as semiconductors, supraconductors, catalysts and polymers.

The first report of combinatorial chemistry was published by Geysen and co-workers in 1984 using multi-pin technology to prepare peptides (Geysen et al., 1984). In this work, combinatorial libraries of hexapeptides were synthesized and then evaluated, still coupled to the solid support, as epitopes of the VP1 protein of foot-and-mouth disease virus (FMDV). The first library contained 208-members corresponding to all possible overlapping hexapeptides from the 213-amino acid sequence of the VP1 protein. The second library corresponded to all 120 possible hexapeptides differing by only a single amino acid from relevant parent peptide of the first library AcNH-Gly-Asp-Leu-Gln-Val-Leu-Linker-(PE-g-AA). Since then, other **combinatorial peptide library methods** were introduced. Actually, there are five basic combinatorial peptide library methods: biologic peptide library method, spatially addressable parallel library method, combinatorial library method requiring deconvolution, one-bead one-compound combinatorial library method and synthetic library method using chromatography selection (Liu et al., 2003). All these methods involve library synthesis, screening and structure determination of active compounds. There are advantages and disadvantages of each method. The approach chosen then depends greatly on the library size planned, the peptide sequences and the resources available for investigators.

Biological library method, such as phase displayed and plasmid, can provide up to thousand million peptides. Biological peptide library is generated on the surface of the microorganisms by growing microorganisms. One advantage of this technique is that the peptides are synthesized biologically, and therefore manual synthesis and expensive chemicals are not required. However, unlike in the other approaches, only naturally occurring amino acids can be incorporated in this method.

In spatially addressable parallel library method such as SPOT technology, peptides are synthesized and screened in parallel on solid phase support or in solution phase in a spatially addressable format, typically microtiter plates. Consequently, the libraries generated are relatively small and the structure of each library peptide is well known.

The IRORI's Kan technology and the SynPhase Lantern technology, exclusively used for solid-phase applications, differ a bit from the other approaches. Indeed, they combine the advantages of parallel synthesis yielding discrete compounds in multi-milligram quantities and split-and-pool synthesis. The IRORI's Kans are a more advanced variation of the teabags where resin is placed in porous containers called Kans, which contain glass-encased miniature radiofrequency Rf tag or a ceramic 2D laser-etched bar-coded cap (Figures 28 and 29) (Houghten, 1985). The SynPhase Lanterns are polymer plugs which can be tagged by means of a coloured code or radio frequency tags (www.mimotopes.com; Parsons et al., 2003). Then, the synthetic history of each SynPhase Lantern or Kan can be traced.

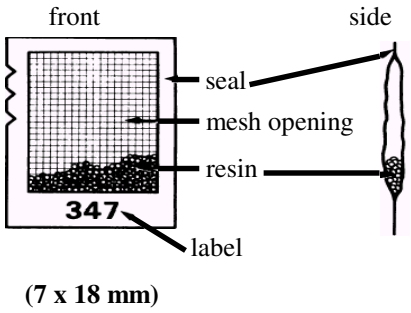


Figure 28. Houghten's tea-bag

Figure 29. Kan family

In these technologies, synthesis of peptide libraries starts by tagging the Kans or the SynPhase Lanterns to identify them in the synthesis (Figure 30) (Xiao et al., 2000). They are then sorted depending on the reagent with which they have to react, and placed into the corresponding reaction flask. When the reaction is complete, all the Kans or the SynPhase Lanterns are combined for the washing step. The sort-reaction-combine-wash-resort cycle is repeated for each reaction step in the combinatorial synthesis. Steps that involve treating all Kans or the SynPhase Lanterns with the same reagent can be carried out in a single large flask. At the completion of the synthesis, resin in each Kan or each SynPhase Lantern, both containing a single compound, is placed in single eppendorf or in a 96-well plate for cleavage to form a spatially addressable compound library.

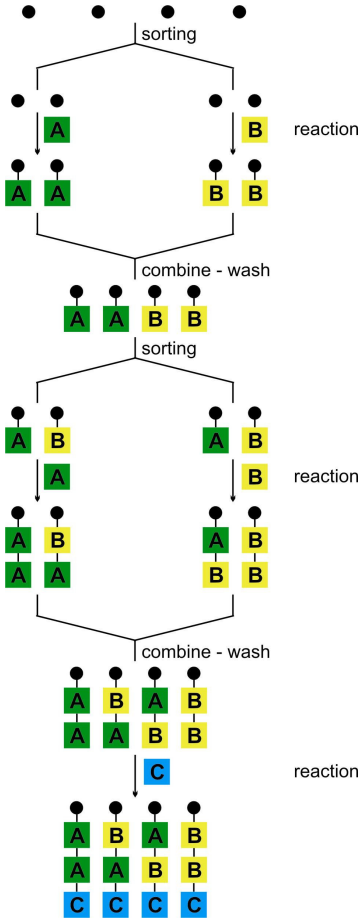


Figure 30. Sort-reaction-combine-wash-resort cycle

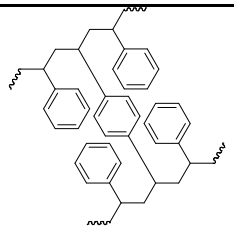
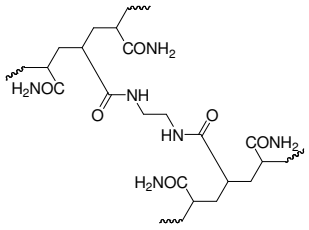
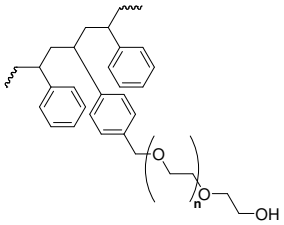
In combinatorial library method requiring deconvolution, mixtures of compounds are obtained. Among several different approaches to identify the active peptide(s) in the screened mixtures, the positional scanning approach avoids the multiple different syntheses and screening of the others (Blondelle et al., 1994; Blondelle et al., 1996, Pinilla et al., 2003). In this method, the library consists of sub-libraries. Each sub-library is composed of peptides with an amino acid residue fixed at one defined position and randomised in the other positions. A single screening assay of each sub-library permits to determine the most important amino acid(s) for every position considering negligible for biological activities the interactions between residues at the different position (Hong et al., 1998). Successful applications of the positional scanning synthetic combinatorial library (PS-SCL) method such as the synthesis of linear peptides effective against phytopathogenic fungi were published (López-García et al., 2002).

One-bead one-compound (OBOC) combinatorial library method provides, after several split and mix operations, millions of beads each expressing only one peptide (Lam et al., 1997). Once synthesized, the random library can be screened in parallel using solid-phase or solution-phase assays. Then, the structure of the individual positive bead peptides can be determined using Edman sequencing method, mass spectrometry method or encoding strategy (Czarnik, 1997; Affleck, 2001).

Synthetic library method using chromatography selection allows to generate, using usually a split-and-pool synthesis approach, mixture libraries with equimolar peptide quantity. After cleaving, peptides are screened loading them onto an affinity column with receptor immobilized to the stationary phase. Then, after thorough washing, the bound peptides can be eluted and microsequenced by Edman degradation.

The **solid support** commonly employed in solid-phase synthesis (SPS) of peptide libraries is resin beads (Sherrington, 1998; Boyle and Janda, 2002). The widely resins used are gel type polymers made from cross-linked polystyrene (PS), polyacrylamide, polyethylene glycol (PEG) grafted onto a cross-linked polystyrene such as Tentagel (TG) (Table 5) (Kates and Albericio, 2000; Vaino and Janda, 2000; Gerritz, 2001).

Table 5. Common solid supports

Resin		Swelling solvents
polystyrene		from low to medium polar solvents (from aliphatic hydrocarbons to CH_2Cl_2)
polyacrylamide		in polar and aprotic solvents (e.g. DMF, or *N*-methyl pyrrolidinone)
polyethylene glycol-polystyrene		in all solvents

SynPhase™ Lanterns are another type of solid support (www.mimotopes.com; Parsons et al., 2003). They are modular and made of a mobile surface polymer grafted onto a rigid unreactive base polymer such as polyethylene (Figure 31). The surface polymer may consist of polystyrene or polyamide. The polystyrene SynPhase Lanterns (SynPhase-PS) are hydrophobic and solvate well in most solvents used in organic synthesis except in low molecular weight alcohol solvents. The polyamide SynPhase Lanterns (SynPhase-PA) are preferable for hydrophilic applications due to their compatibility with solvents such as water, polar alcohols, and typical organic solvents such as methylene chloride and DMF. SynPhase Lanterns are available in different dimensions and loadings: A-, D- and L-series (Figure 32, Table 6). They can be easily tagged by means of coloured code based on spindles and cogs or radio frequency tags (Transponders™) (Figure 33). Furthermore, they can be easily attached to stems and arranged in the standard 96-well plate for parallel synthesis or/and cleavage (Figure 34). This feature makes them highly convenient for the solid-phase synthesis of peptide libraries (Subra et al., 2003).

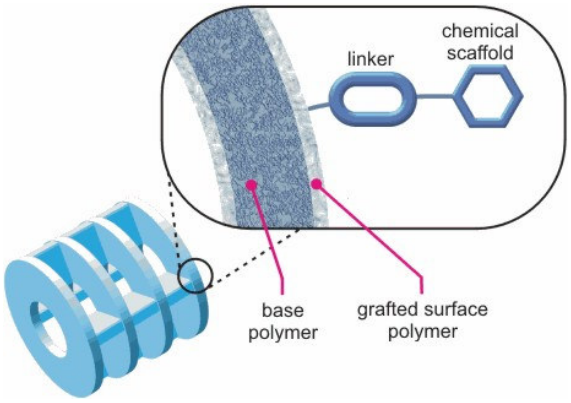


Figure 31. Schematic representation of the SynPhase Lantern surface

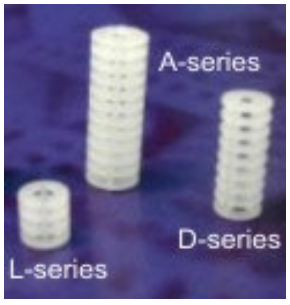


Figure 32. The SynPhase Lantern family

Table 6. Physical properties of SynPhase™ Lanterns

		A-series	D-series	L-series
Height (mm)		17	12.5	5
Diameter (mm)		6	5	5
Loading (μmol)	SynPhase-PS	75	35	15
	SynPhase-PA	N/A	8 and 18	N/A

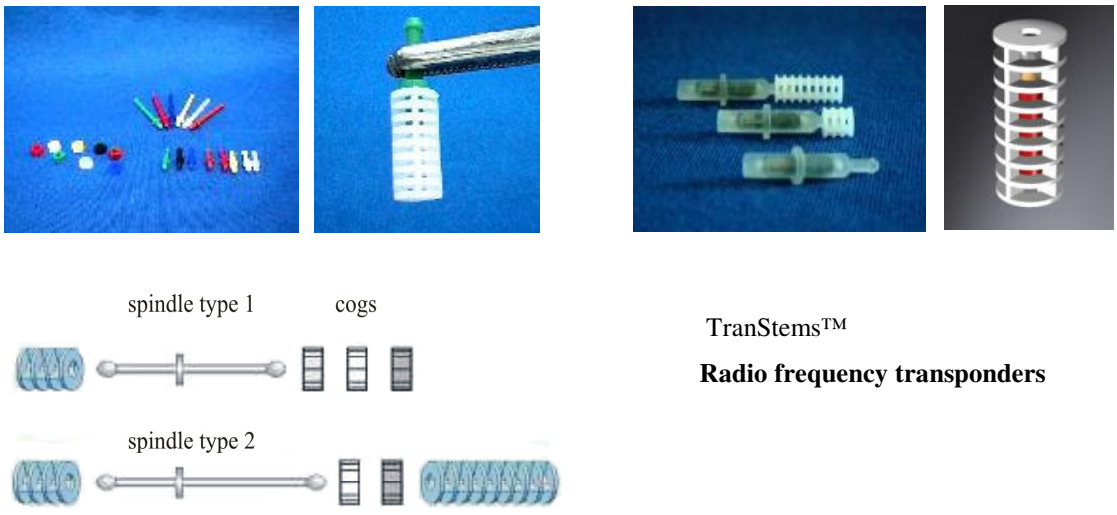


Figure 33. Lantern tags

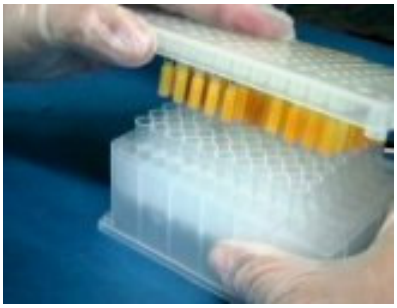
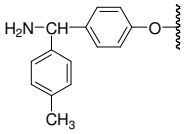
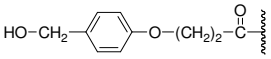
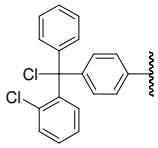
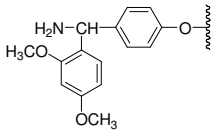
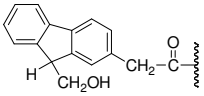
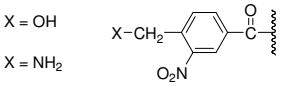
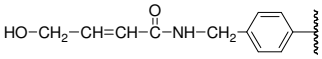
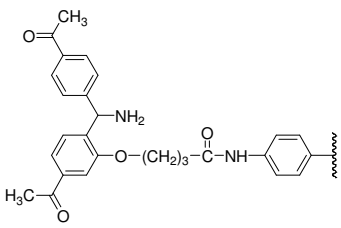
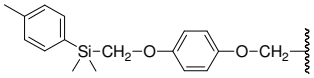


Figure 34. Stem holder

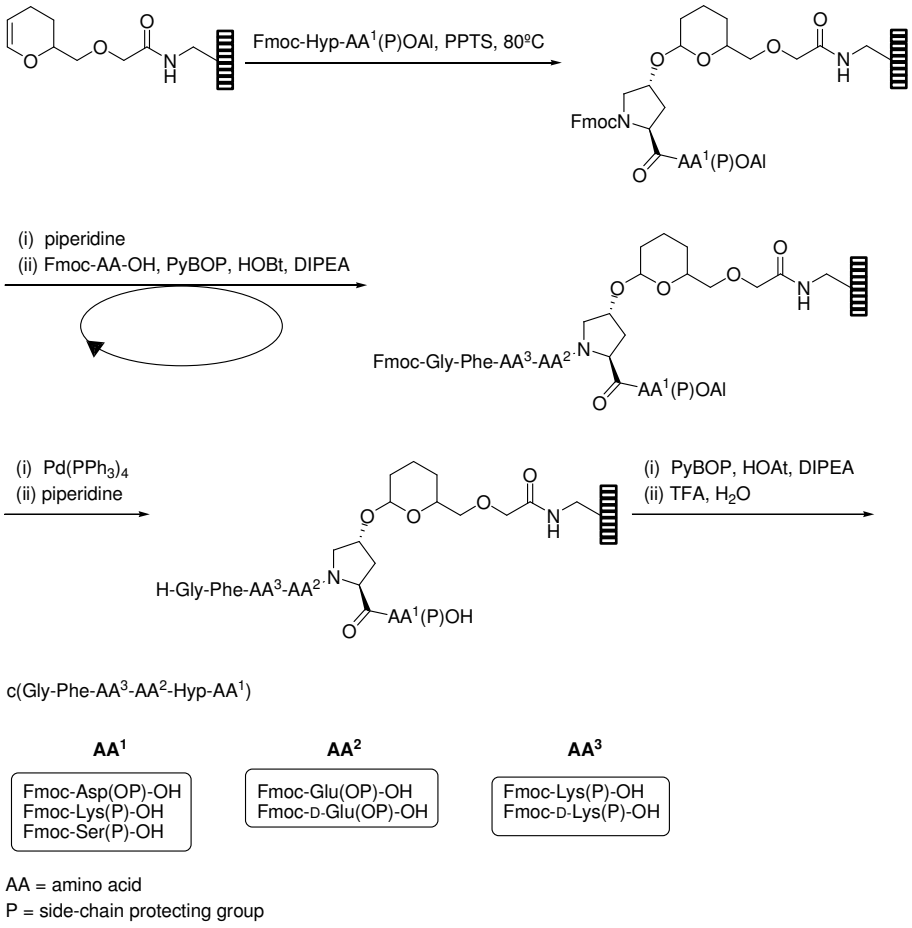
In the solid-phase peptide synthesis (SPPS) approach the insoluble polymer-support is usually covalently attached to the substrate through a linker. A **linker** is a bifunctional molecule which can be bound irreversibly to the solid support, must be stable under the synthesis conditions and also possess protecting group-like stability to allow the cleavage of the products from the support at the end of the synthesis (Yraola et al., 2004). Linkers can be classified based on the cleavage conditions. Table 7 shows some of the most representative linkers used (Guillier et al., 2000).

Table 7. Some representative linkers

Cleavage conditions	Linker	Resulting *C*-terminus	Structure
HF	MBHA	amide	
TFA	Wang	acid	
TFA, HOAc	2-chlorotrityl chloride	acid	
dilute TFA	Rink	amide	
piperidine	HMFA	acid	
hν (350 nm)	ONb	acid	
hν (350 nm)	Nonb	amide	
Pd(0)	HYCRAM	acid	
$(CH_3)_3SiBr$/thioanisole/TFA	SCAL	amide	
strong acid	traceless linker	–	

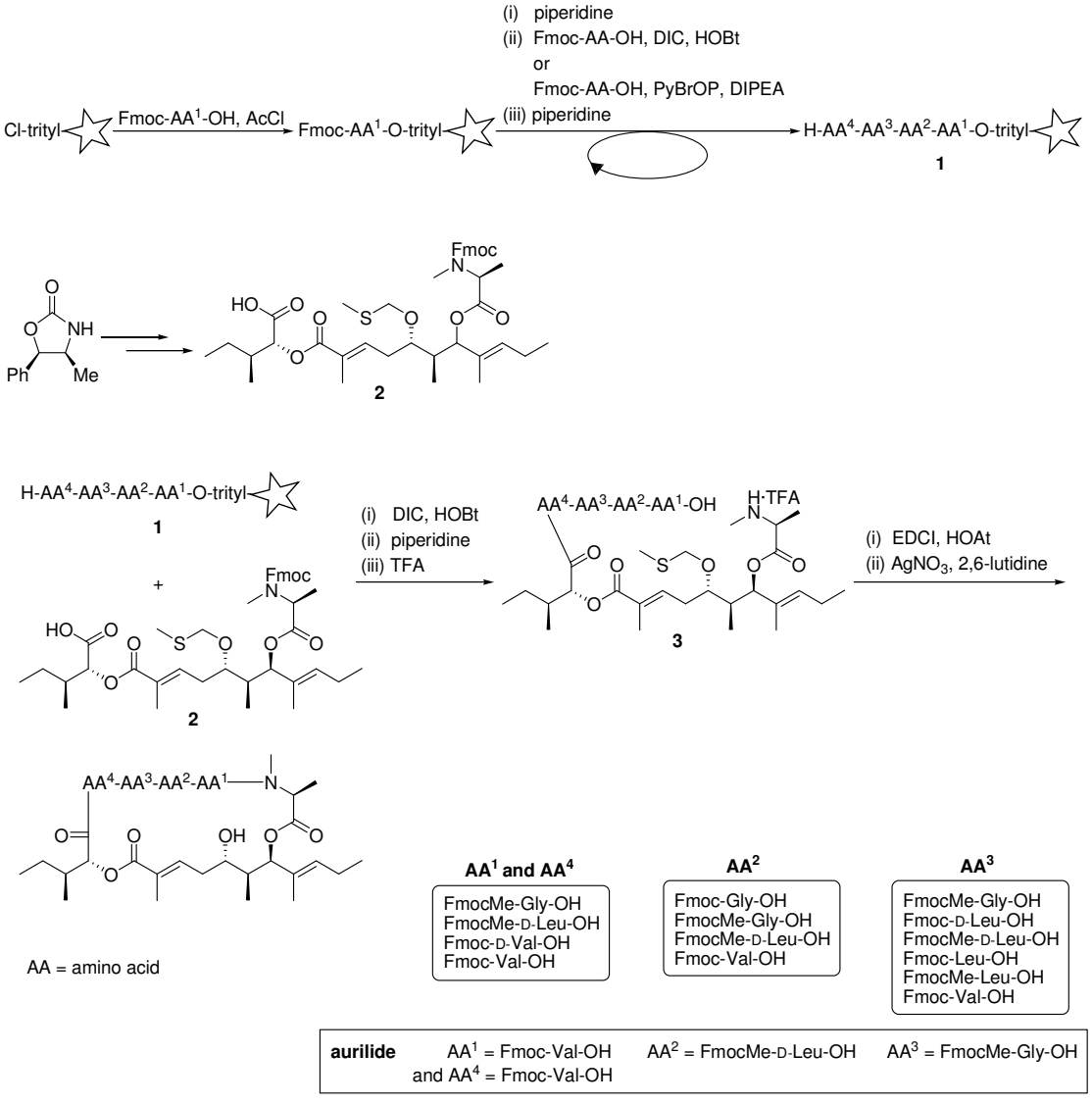
Combinatorial chemistry provides a powerful tool rapidly to synthesize and to identify peptides with improved or new biological properties. Numerous linear peptide libraries have been reported. However, few studies have been centred on the preparation of **cyclic peptide libraries**. Some of the works reported in the literature are reported below.

Basso and Ernst described the synthesis of a 12-member library of cyclic hexapeptides, designed to be selectin antagonists (Basso and Ernst, 2001). The peptides were prepared on SynPhase^{TM} Lanterns, using a radio frequency tagging system. A modified THP linker was attached to the aminomethyl SynPhase Lanterns and it then served to bind the hydroxyproline residue through its hydroxyl group. Linear peptides were synthesized following a standard Fmoc/^{t}Bu/Allyl strategy. After Fmoc and Allyl group removal, cyclization was performed on SynPhase Lanterns (Scheme 1). The HPLC of the crude products performed after the acidolytic cleavage with TFA-H_2O showed purities from 42 to 82%.



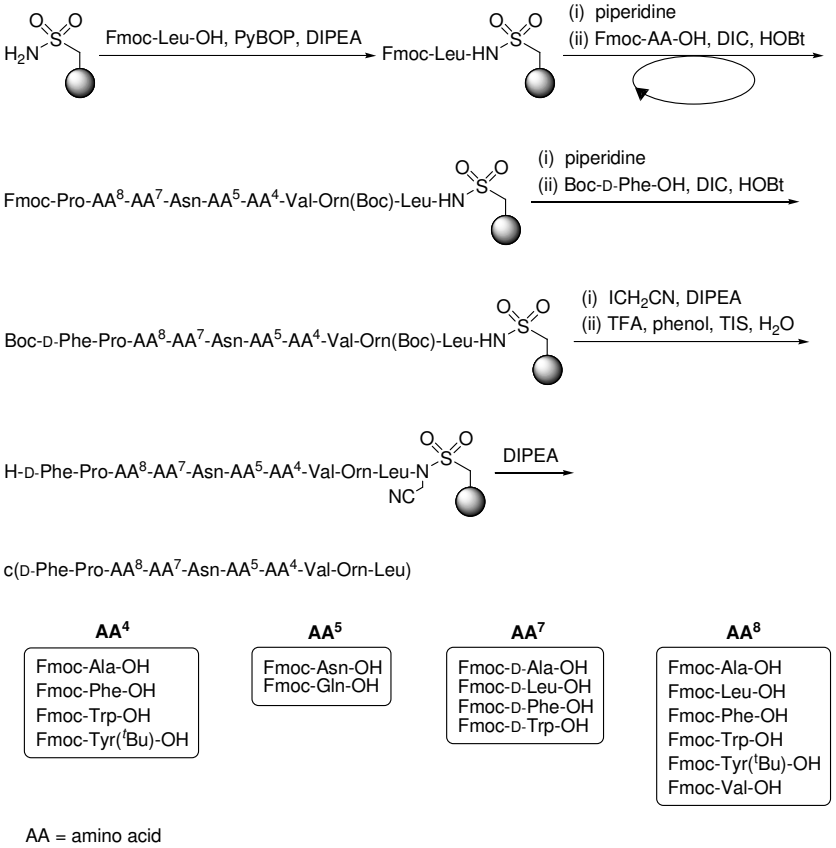
Scheme 1. Solid-phase synthesis of a cyclic hexapeptide library

A solid-phase library based on the natural cyclic depsipeptide aurilide was reported by Takahashi and co-workers (Takahashi et al., 2003). Library of the tetrapeptide moiety **1** was assembled on trityl linker-functionalised SynPhase™ Crowns, other unavailable SynPhase™ products, using a Fmoc strategy (Parsons et al., 2003). The aliphatic moiety **2** was synthesized in solution and then coupled to the solid-supported tetrapeptides **1**. Deprotection and cleavage of the linear precursors **3** were followed by macrocyclization under high dilution conditions and removal of the methylthiomethyl group (Scheme 2). The HPLC of the crude products showed purities from 18 to 58% and the overall yields after purification by reversed-phase HPLC were from 11 to 25%.



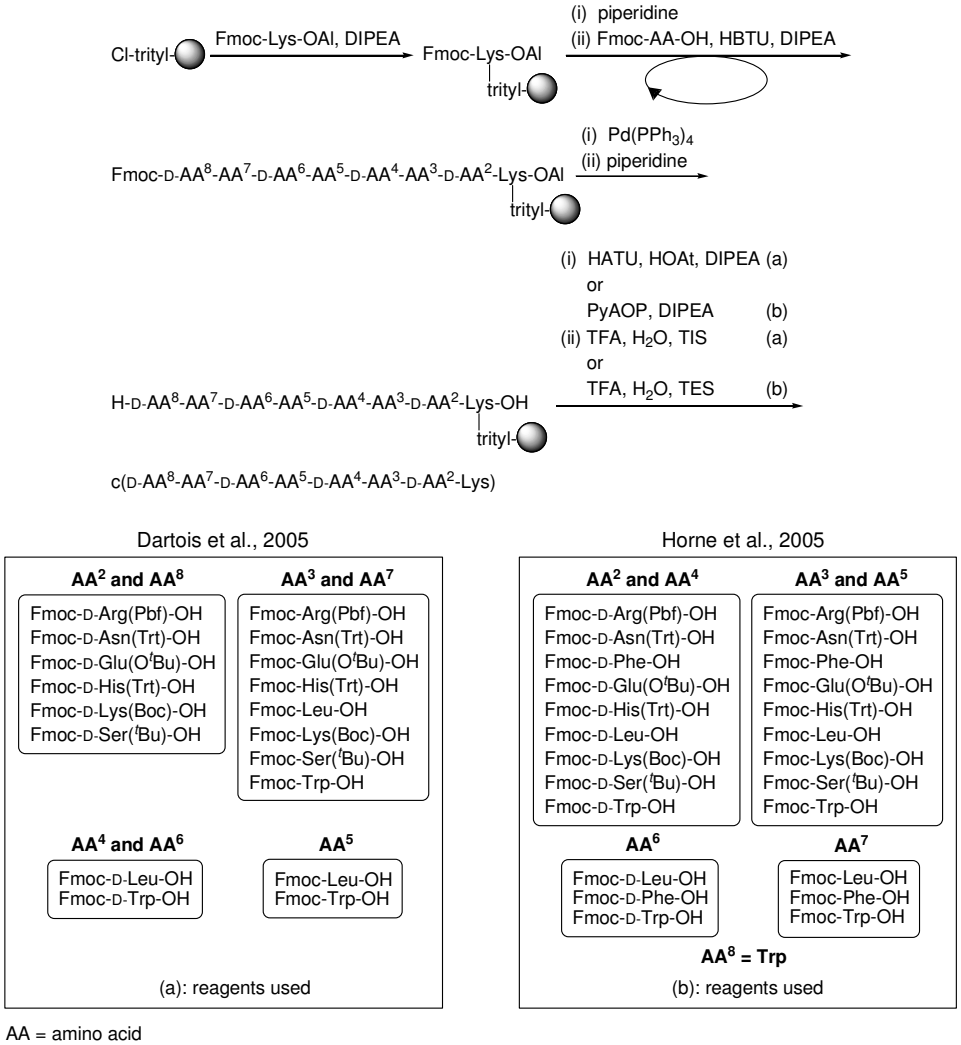
Scheme 2. Library synthesis of aurilide analogues

Qin and co-workers described the synthesis of a 192-member library designed on the basis of the natural products tyrocidines, streptocidins and loloatins to increase the therapeutic indices of these antibacterial peptides (Qin et al., 2004). The library peptides were prepared in MicroKan reactors using 4-sulfamyl butyryl AM resin and following a standard Fmoc/DIC/HOBt chemistry (Scheme 3). The on-solid support cyclization steps were performed after the cyanomethylation activation process and the removal of *tert*-butyloxycarbonyl groups. The HPLC of the crude products showed purities from 80 to 95% and the overall yields after purification by reversed-phase HPLC were from 10 to 21%. Library screening provided nine new analogues whose therapeutic indices were up to 90-fold improved in comparison to the natural parent peptides.



Scheme 3. Solid-phase synthesis of a cyclic decapeptide library

Libraries of cyclo-D-L-α-hexa- and octapeptides were prepared and screened for antimicrobial and antiviral activities (Dartois et al., 2005; Horne et al., 2005). The syntheses were performed on macrobeads of trityl chloride polystyrene-based resin using the one-bead one-compound combinatorial library method. A three-dimensional orthogonal Fmoc/^{t}Bu/Allyl strategy was used and synthesis started by anchoring Fmoc-Lys-OAl onto the resin (Scheme 4). Cyclic peptides were prepared by first carrying out solid-phase synthesis of linear sequences followed by on-resin cyclization and the acidolytic cleavage.



Scheme 4. Solid-phase synthesis of two cyclo-D-L-α-octapeptide libraries

Following this approach, two libraries of 59049 and up to one million cyclic peptides were prepared. The screening results suggested that these peptides hold considerable potential for the treatment of antibiotic-resistant infections and for the discovery of broad-spectrum antiviral agents.

Analysis of combinatorial libraries by design of experiments

Computational chemistry allows the calculation of relationships between structural features and activity by means of mathematical and statistical methods. Then, combining this mathematical approach with combinatorial chemistry can provide designed libraries with relatively a small size but big enough to enable an accurate screening.

Within computational chemistry, design of experiments (DOE) constitutes a well-known statistical methodology which permits to determine the inner rules of a process from relevant experimental data information (Box et al., 1978). Originally, DOE was applied to industrial and engineering fields, but the range of action spread rapidly across other scientific areas. Although its employment in chemistry is still limited, it has been proven to be useful in quantitative structure-activity relationships (QSAR) studies. Unlike other methods, which associate the biological compound activities to compound/receptor interactions using physicochemical properties (Hansch and Fujita, 1964), the DOE approach uses a factorial design to grasp simultaneous synergic and non-linear effects among experimental factors. The magnitude of these effects allows establishing their importance in the biological activities defining the inner rules governing the system's behaviour.

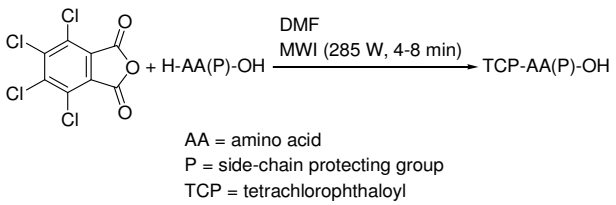
At the University of Gerona, DOE has been successfully applied to activity prediction (Barroso and Besalú, 2005). This study was performed on a 512-member peptide library previously synthesized by Wang et al. based on the sequence H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 and screened against neurokinin 1 receptors (Wang et al., 1993). Barroso and Besalú partitioned the whole set of 512 structures into two sets: a training set and a test set. The training set was selected in order to extract all the possible informations from a minimal number of molecules and accounted for 32 peptides. A two level factorial design (DOE) was applied over this set of peptides in order to obtain a QSAR equation model. This model was then applied to the test set to establish a molecular ranking. Comparison of the results with the experimental data reported by Wang et al. demonstrated the validity of DOE for library design and activity prediction.

Microwave-assisted organic synthesis

The use of microwave irradiation to simplify or improve classic organic reactions has gained increasing popularity in recent years. Primarily used in inorganic chemistry to rapid thermal digestion of samples, microwave technology was introduced in organic chemistry in the mid-1980s with two pioneering independent works reported by Gedye and Giguere in 1986 (Gedye et al., 1986; Giguere et al, 1986). Since the mid-1990s, the amount of publications related to microwave assisted organic reactions has increased quickly due to the significant speed gains and the availability of commercial microwave equipment such as sensors for pressure and temperature, thereby reducing the inherent explosion potential (Santagada et al., 2002; Favretto, 2003; Ferguson, 2003). In addition to these main microwave advantages, microwave irradiation in organic synthesis has been shown to improve yields, to obtain cleaner reactions and higher stereo- or regioselectivity, and to promote reactions that can not be performed under conventional heating (Bose et al., 2002; Lew et al., 2002; Alexandre et al., 2003; Hayes, 2004).

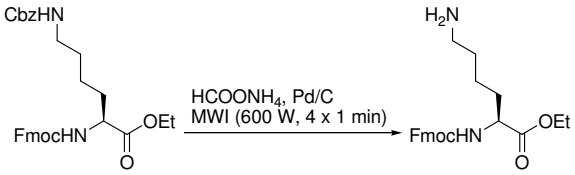
Microwave irradiation was first applied to peptide synthesis in 1992 to improve the coupling of amino acids, especially side-chain-hindered amino acids (Yu et al., 1992). Since then, a large amount of microwave-promoted reactions had been reported in the peptide field. Indeed, microwave irradiation has been applied N^{α}-amino acid protection, protecting group removal, amino acid coupling, and peptide cyclization.

In our research group, a range of **N^{α}-tetrachlorophthaloyl protected amino acids** were synthesized by condensation of the corresponding amino acid and tetrachlorophthaloyl anhydride under microwave irradiation (Cros et al., 2001). After 4-8 minutes of irradiation (285 W) the reactions were complete (Scheme 5). N^{α}-TCP-protected amino acids were obtained with high yields after straightforward work-up.



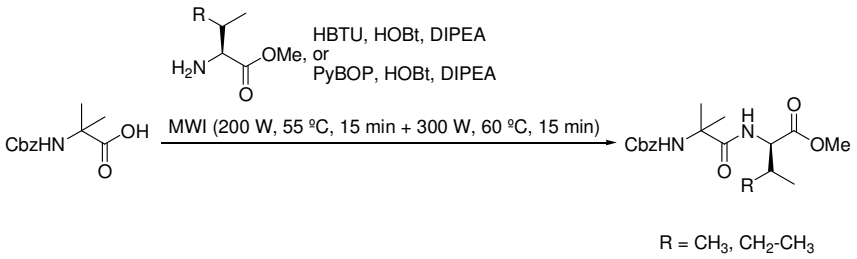
Scheme 5. Synthesis of N^{α}-tetrachlorophthaloyl protected amino under microwave irradiation

Daga and co-workers reported the **benzyloxycarbonyl group (Cbz) removal** by microwave-assisted transfer hydrogenation (Daga et al., 2001). The reactions were performed by treatment of Cbz-protected peptides with ammonium formate and Pd/C in *i*-PrOH (Scheme 6). This methodology allowed the selective deprotection of amino acids in solution and in solid-phase with good yields, and without racemization observed.



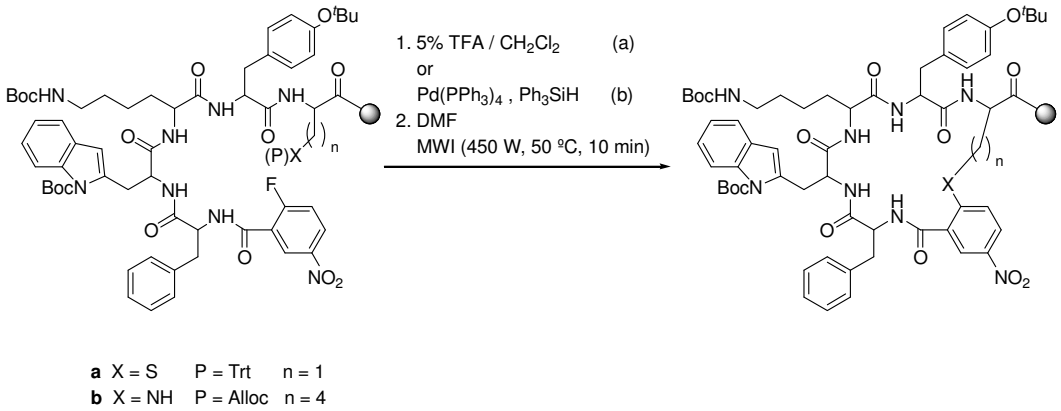
Scheme 6. *N*-Cbz group removal under microwave irradiation

Microwave irradiation was applied to the **coupling in solution of sterically hindered amino acids** such as α-aminoisobutyric acid (Santagada et al., 2001). PyBOP/HOBt or HBTU/HOBt were used as coupling reagents (Scheme 7). Compared to conventional heating, microwave irradiation significantly reduced reaction times and afforded final compounds in higher yields and purities.



Scheme 7. α-Aminoisobutyric acid coupling under microwave irradiation

Grieco and co-workers reported the synthesis of **cyclic peptidomimetics** by a microwave-assisted cyclization via a nucleophilic aromatic substitution (S_NAr) (Grieco et al., 2003). The S_NAr occurred between the thiol group of a cysteine residue or the ε-amino group of a lysine residue and a 2-fluoro-5-nitro benzene ring. Cyclizations were performed by irradiation with 450 W at 50 ºC in DMF for 10 minutes (Scheme 8). Cyclic peptidomimetics were obtained with high yields and without racemization observed.



Scheme 8. Solid-phase cyclization under microwave irradiation

Objectives

The present PhD thesis was centred on the study of the application of *de novo* designed head-to-tail cationic cyclic peptides as inhibitors of the plant pathogenic bacteria *Erwinia amylovora*, *Pseudomonas syringae*, *Xanthomonas vesicatoria*, *Clavibacter michiganensis*, and the plant pathogenic fungi, *Verticillium dahliae*, *Fusarium oxysporum*, *Penicillium expansum*, *Aspergillus niger*. More emphasis has been given to the first three bacteria, *E. amylovora*, *P. syringae* and *X. vesicatoria*, responsible of severe plant diseases.

In particular, this work involved the following steps:

1. Solid-phase synthesis of head-to-tail cyclic peptides of 4 to 10 residues bearing alternating cationic (lysine) and hydrophobic (leucine and phenylalanine) amino acids, and a glutamine residue. The general formula of these cyclic peptides is:

   $c(X_n\text{-}Y\text{-}X_m\text{-}Gln)$ X = Lys or Leu
   Y = L-Phe or D-Phe
   m = n = 1 or m = 3 and n = 0-5

2. Evaluation of the biological activity of the cyclic peptides against the plant pathogenic bacteria *E. amylovora*, *P. syringae*, *X. vesicatoria*, *C. michiganensis*, and the fungi, *V. dahliae*, *F. oxysporum*, *P. expansum*, *A. niger*.

3. Improvement of the biological properties of the most effective cyclic peptide by combinatorial chemistry.

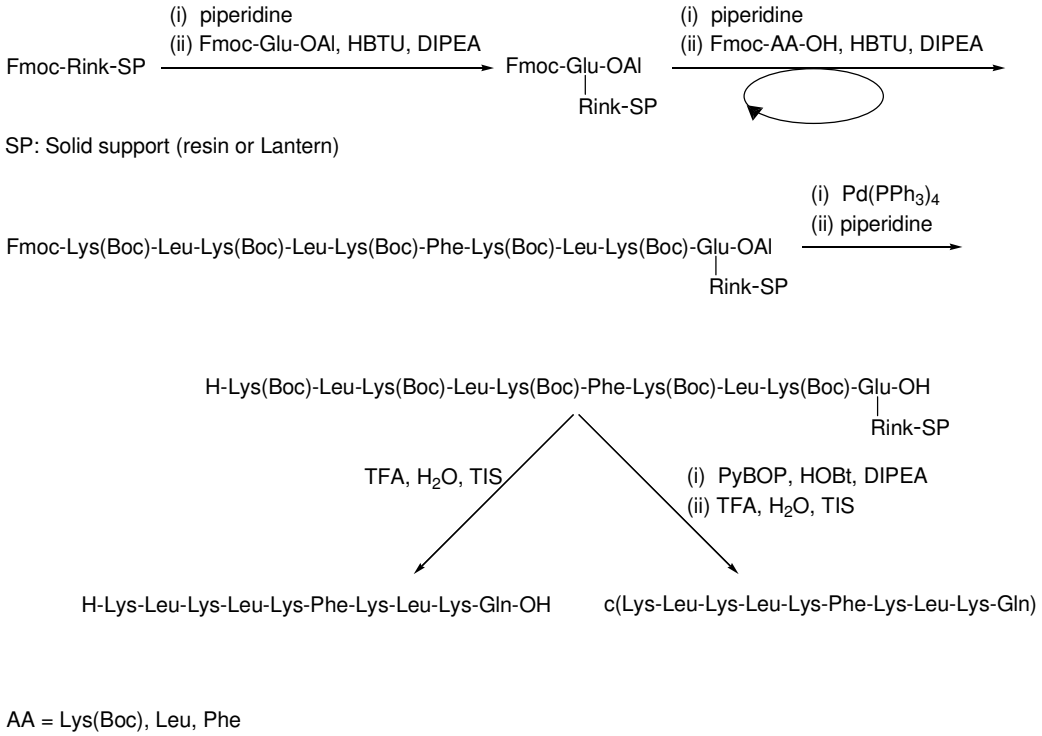
Paper 1 results

Microwave-assisted cyclization of peptides on Synphase™ Lanterns.

Synlett **2006**;9:1311-1314.

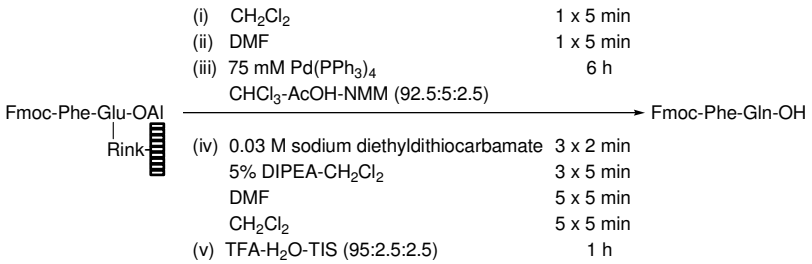
Abstract: SynPhase Lanterns and microwave irradiation have been combined to set up an efficient and rapid cyclization strategy of short peptides.

Cyclic peptides were prepared by first carrying out solid-phase synthesis of the linear sequences, followed by on-solid support cyclization (Scheme 9). Polystyrene Rink amide SynPhase^{TM} Lanterns (35 µmol) and Fmoc-Rink-MBHA resin (0.3 mmol/g) were chosen as solid support (Yraola et al., 2004). A three-dimensional orthogonal Fmoc/^{t}Bu/Allyl strategy was followed (Alsina, et al., 1996; Albericio, 2000; Kates and Albericio, 2000; Farrera-Sinfreu, 2002). This strategy consisted on first anchoring Fmoc-Glu-OAl onto the support. The Fmoc group was then removed followed by coupling of the corresponding Fmoc-protected amino acid. The deprotection-coupling cycle was repeated to get the desired fully protected peptide sequences. After sequentially removing the *C*-terminal allyl ester and the Fmoc group, the linear peptides were either cleaved or subjected to cyclization by treatment with PyBOP in the presence of DIPEA (Ehrlich et al., 1996; Albericio et al., 1997; Albericio et al., 1998; Lambert et al., 2001; Davies, 2003). Finally, cyclic peptides were released from the support by acidolytic cleavage.



Scheme 9. Solid-phase synthesis of cyclic peptides

One of the key steps of the above strategy was the allyl group removal. While this step is well established using resin, it has not been described a general protocol on Lanterns. To study this reaction the protected dipeptide Fmoc-Phe-Glu(Rink-Lantern)OAl was taken as model and prepared following the general strategy described above. It was treated with $Pd(PPh_3)_4$ in $CHCl_3$-AcOH-NMM under N_2, followed by the corresponding washes (Bräse et al., 2003). Several experiments were performed by varying the $Pd(PPh_3)_4$ concentration, the reaction time, under or without stirring, and the washing protocol. After each assay, one stacked disk of the Lantern was cut and subjected to acidolytic cleavage with TFA-H_2O-TIS. The crude product mixture was then analysed by HPLC and ESI-MS. Results showed that the $Pd(PPh_3)_4$ concentration was the most important parameter, stirring is not necessary, and washes with sodium diethyldithiocarbamate and DIPEA are needed but their order was not important. The best conditions for the allyl group removal on Lanterns are summarized in Scheme 10, which yielded Fmoc-Phe-Gln-OH with purity higher than 99%.



Scheme 10. Allyl group removal

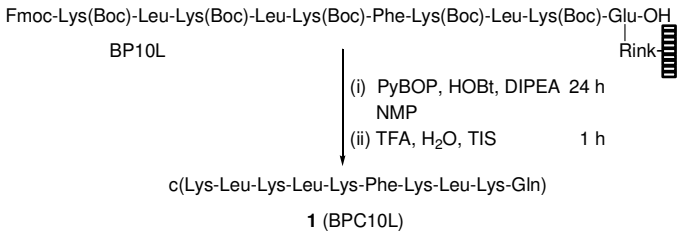
Linear peptides of 4 to 10 residues were then prepared (Table 8). After sequentially removing the allyl ester and the Fmoc group, acidolytic cleavage of a portion of Lantern or of an aliquot of resin afforded the linear peptides with high purities (≥ 98%). Linear peptides were characterized by ESI-MS. It is worth noting that any by-product corresponding to the Rink linker amide attached to the target peptide was detected (Yraola et al., 2004).

Table 8. Sequences, HPLC retention times, molecular weight, and purity obtained for linear peptides of 4-10 residues

Code	Peptide sequence	t_R (min)[a]	$[M + H]^{+b}$	Purity (%)[c]
BP10L	H-Lys-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln-OH	3.72	1273.7	99
BP9L	H-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln-OH	3.87	1145.5	99
BP8L	H-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln-OH	3.55	1032.6	98
BP7L	H-Leu-Lys-Phe-Lys-Leu-Lys-Gln-OH	3.69	904.6	98
BP6L	H-Lys-Phe-Lys-Leu-Lys-Gln-OH	3.35	791.6	99
BP5L	H-Phe-Lys-Leu-Lys-Gln-OH	3.49	663.5	98
BP4L	H-Lys-Phe-Lys-Gln-OH	2.94	550.2	99
BP10D	H-Lys-Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln-OH	3.65	1273.9	98
BP9D	H-Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln-OH	3.68	1145.4	98
BP8D	H-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln-OH	3.44	1033.0[d]	99
BP7D	H-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln-OH	3.50	904.5	98
BP6D	H-Lys-D-Phe-Lys-Leu-Lys-Gln-OH	3.26	791.4	98
BP5D	H-D-Phe-Lys-Leu-Lys-Gln-OH	3.21	663.3	99
BP4D	H-Lys-D-Phe-Lys-Gln-OH	2.90	550.2	98

[a]HPLC retention time
[b]Determined by ESI-MS
[c]Relative amount of linear peptide is based on uncorrected total HPLC values
[d]Determined by MALDI-MS

The other key step for the preparation of cyclic peptides was the ring closure. Since the application of the Lanterns to the synthesis of cyclic peptides is still very limited, an optimisation study was undertaken. Cyclization was first examined at room temperature using the linear decapeptide BP10L as model. The best results were obtained by treatment of five stacked disks of the peptidyl-Lantern with PyBOP (16 equiv), HOBt (16 equiv) and DIPEA (32 equiv) in NMP for 24 h. Acidolytic cleavage afforded the corresponding cyclodecapeptide, coded as **1** or BPC10L (according to papers Monroc et al., 2006a and Monroc et al., 2006b), with 97% purity which was characterized by ESI-MS (Scheme 11).



Scheme 11. Solid-phase cyclization of BP10L

The above-described conditions were applied to the simultaneous cyclization of the linear peptides BP4L-BP10L and BP4D-BP10D. After acidolytic cleavage of each Lantern and analysis of the crude reaction mixtures by ESI-MS, it was observed that cyclization were not complete, being the linear peptides the major products. To investigate this result, some experiments were made varying the decapeptidyl-Lantern and the cyclization reagent stoichometry. They suggested the need to use an increasing excess of reagent when the amount of disks of support was augmented to obtain a substantial conversion of the linear L-decapeptide. Therefore, cyclizations of BP4L-BP10L and BP4D-BP10D were studied separately into individual flasks using five stacked disks of each Lantern and the amount of reagents as outlined for BP10L. Experiments were performed, on the one hand, at room temperature and, on the other hand, at 50 ºC using microwave irradiation. Furthermore, cyclization under conventional heating at 50 ºC was examined using peptides BP10L, BP8L and BP4L. Results obtained are summarized in Table 9.

At room temperature after 24 h, cyclization was only complete for decapeptide BP10L affording the cyclodecapeptide BPC10L (**1**) with high purity (97%).

At 50ºC under conventional heating after 1 h, linear peptides were still observed together with PyBOP decomposition products.

Microwave irradiation led to a clear improvement of the cyclization associated to a reduction of the reaction time. For all the peptides except for BPC10L which only needed two cycles of 15 minutes and BPC7L which did not cyclize, best results were obtained using four cycles of 15 minutes. It is worth noting that poorer results were obtained when one single cycle of 1 hour was applied. In the case of the tetrapeptide (BP4L and BP4D) and pentapeptide (BP5L and BP5D) cyclizations, FAB-MS analysis revealed the formation of the corresponding cyclic peptides but also the presence of cyclodimerisation products. Therefore, except for cycloheptapeptide BPC7L (**4**), this microwave irradiation methodology promoted the cyclization of all the sequences. The cyclodecapeptide **1** (BPC10L) was obtained with high purity, and the cyclohexapeptides **5** (BPC6L) and **12** (BPC6D) and the cyclooctapeptides **3** (BPC8L) and **10** (BPC8D) were synthesized with moderate to good purities.

Table 9. Cyclization at room temperature and at 50 ºC under conventional heating and microwave irradiation

Codes		Peptide sequence	Non-microwave irradiation				Microwave irradiation	
			25 ºC (24 h)		50 ºC (1 h)		50 ºC (4 x 15 min)	
			Product distribution[a]		Product distribution[a]		Product distribution[a]	
			cyclic	linear	cyclic	linear	cyclic	linear
1	**BPC10L**	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	97	0	70	5	90[c]	0
2	**BPC9L**	c(Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	12	55	–[b]	–	62	6
3	**BPC8L**	c(Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	38	41	60	9	91	0
4	**BPC7L**	c(Leu-Lys-Phe-Lys-Leu-Lys-Gln)	0	33	–	–	0	33
5	**BPC6L**	c(Lys-Phe-Lys-Leu-Lys-Gln)	31	36	–	–	84	1
6	**BPC5L**	c(Phe-Lys-Leu-Lys-Gln)	19	56	–	–	79[d]	5
7	**BPC4L**	c(Lys-Phe-Lys-Gln)	26	57	2	8	86[d]	0
8	**BPC10D**	c(Lys-Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	5	50	–	–	12	52
9	**BPC9D**	c(Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	7	55	–	–	48	0
10	**BPC8D**	c(Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	25	26	–	–	78	0
11	**BPC7D**	c(Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	0	43	–	–	20	0
12	**BPC6D**	c(Lys-D-Phe-Lys-Leu-Lys-Gln)	44	12	–	–	60	0
13	**BPC5D**	c(D-Phe-Lys-Leu-Lys-Gln)	20	34	–	–	72[d]	0
14	**BPC4D**	c(Lys-D-Phe-Lys-Gln)	42	34	–	–	81[d]	0

[a]Percentage determined by HPLC. Relative amount of species reported are based on uncorrected total HPLC values.
[b]Not studied
[c]In 2 x 15 min irradiation time
[d]Dimerisation product was detected by FAB-MS.

Results showed that, except for the D-decapeptide, formation of cyclic peptides with an even number of residues was easier than cyclization of peptides with an odd number of amino acids. Furthermore, the cyclization difficulties for linear precursors with even residue numbers increased with the decreasing ring size. This trend was reversed when the L-phenylalanine was substituted for its enantiomer. On the other hand, independently of phenylalanine conformation, cyclization of linear pentapeptides was easier than cyclization of linear nonapeptides.

Cyclic peptides **3** (BPC8L), **5** (BPC6L), **6** (BPC5L), **7** (BPC4L) and **8** (BPC10D) were also prepared using Fmoc-Rink-MBHA resin (0.3 mmol/g) as solid support. Synthesis of the linear sequences was carried out as described previously (Scheme 9). After removal of the allyl and Fmoc groups, on-resin cyclization was performed by treatment of the linear sequence with PyBOP (5 equiv), HOBt (5 equiv) and DIPEA (10 equiv) at room temperature for 12 h. Acidolytic cleavage of the resin afforded the desired cyclic peptides in good to high purity which were characterized by ESI-MS and MALDI-MS (Table 10). No cyclodimerisation product was observed by mass spectrometry.

Table 10. Synthesis of cyclic peptides **3** and **5**-**8** on Fmoc-Rink-MBHA resin

Codes		Peptide sequence	Purity (%)[a]
3	**BPC8L**	c(Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	99
5	**BPC6L**	c(Lys-Phe-Lys-Leu-Lys-Gln)	94
6	**BPC5L**	c(Phe-Lys-Leu-Lys-Gln)	89
7	**BPC4L**	c(Lys-Phe-Lys-Gln)	97
8	**BPC10D**	c(Lys-Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	95

[a]Percentage determined by HPLC. Relative amounts of species are based on uncorrected total HPLC values.

Analogues of the cyclooctapeptide **3** (BPC8L) were also prepared on resin (Table 11). BPC8S1-BPC8S5 include a Leu instead of the Phe residue. Moreover, BPC8S2 contains Glu instead of Gln. BPC8S3 and BPC8S4 bear three or five Lys, respectively, and in BPC8S5 cyclization occurs through the side-chain of the glutamic acid residue. These peptides were characterized by ESI-MS.

Table 11. BPC8L analogues

Codes		Peptide sequence[a]	$[M + H]^{+b}$
3	**BPC8L**	c(Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	1014.5
	BPC8S1	c(Lys-Leu-Lys-**Leu**-Lys-Leu-Lys-Gln)	980.5
	BPC8S2	c(Lys-Leu-Lys-**Leu**-Lys-Leu-Lys-**Glu**)	491.6[c]
	BPC8S3	c(**Leu**-**Lys**-**Leu**-**Lys**-**Leu**-**Lys**-**Leu**-Gln)	483.4[c]
	BPC8S4	c(Lys-**Lys**-**Leu**-**Lys**-**Leu**-**Lys**-Lys-Gln)	995.6
	BPC8S5	c**Glu**(Lys-Leu-Lys-**Leu**-Lys-Leu-Lys)-$\mathbf{NH_2}$	980.3

[a]Bold letters indicate the modifications introduced in the parent sequence **3** (BPC8L)
[b]Determined by ESI-MS
[c]$[M + 2H]^{2+}$

Paper 2 results

De novo designed cyclic cationic peptides as inhibitors of plant pathogenic bacteria.

Peptides **2006**;27:2567-2574.

Abstract: Head-to-tail cyclic peptides of 4–10 residues consisting of alternating hydrophilic (Lys) and hydrophobic (Leu and Phe) amino acids were synthesized and tested against the economically important plant pathogenic bacteria *Erwinia amylovora*, *Xanthomonas vesicatoria* and *Pseudomonas syringae*. The antibacterial activity, evaluated as the minimal inhibitory concentration (MIC), the cytotoxicity against human red blood cells and stability towards protease degradation were determined. The influence of cyclization, ring size, and replacement of L-Phe with D-Phe on antibacterial and hemolytic activities was studied and correlated with the degree of structuring and hydrophobicity. Our results showed that linear peptides were inactive against the three bacteria tested. Cyclic peptides were active only toward *X. vesicatoria* and *P. syringae*, being c(KLKLKFKLKQ) (BPC10L) the most active peptide with MIC values of 6.25 and 12.5 μM, respectively. The improved antibacterial activity of cyclic peptides compared to their linear counterparts was associated to an increase of the hydrophobicity, represented as RP-HPLC retention time (t_R), and secondary structure content which are related to an enhanced amphipathicity. A decrease of antibacterial and hemolytic activities was observed when a D-Phe was introduced into the cyclic sequences, which was attributed to their low amphipathicity as shown by their low secondary structure content and low t_R. The small size, simple structure, bactericidal effect, and stability to protease degradation of the best peptides make them potential candidates for the development of effective antibacterial agents for use in plant protection.

The antimicrobial activity of cyclic peptides was evaluated against *Erwinia amylovora*, *Pseudomonas syringae*, *Xanthomonas vesicatoria*, *Clavibacter michiganensis*, *Verticillium dahliae*, *Fusarium oxysporum*, *Penicillium expansum* and *Aspergillus niger*. Their cytotoxicity against human red blood cells and their secondary structure content were also determined. The antimicrobial activity of the parent linear peptides was also evaluated. From the experimental data obtained, it was studied the influence of different parameters on biological activities. In particular, cyclization, ring size, incorporation of a D-amino acid, substitution of L-phenylalanine by L-leucine, hydrophobicity, amphipathicity and secondary structure content were examined. RP-HPLC was used to determine the overall hydrophobicity and peptide amphipathicity (Blondelle and Houghten, 1992; Lee et al. 2004). Furthermore, the stability towards protease degradation and the bactericidal effect were determined.

Results showed that cyclization significantly increased the antibacterial activity of the peptides. While the linear sequences were not effective against the bacteria tested, the cyclic peptides were active against *P. syringae*, *X. vesicatoria* and *C michiganensis* (Tables 12, 13 and 14). In contrast to bacteria, linear peptides of 8-10 residues as their cyclic counterparts were active against fungi (Tables 13 and 14).

In general, higher antimicrobial activity was displayed by peptides with an even number of amino acids than peptides with an odd number of amino acids. Peptides with L-Phe were, generally, more active than their D-Phe diastereoisomer. Substitution of L-phenylalanine by L-leucine leaded a biological activity decrease. In contrast to bacteria, the antifungal activity depended on the ring size. An increase of the ring size was found to be associated with an increase of the antifungal activity.

X. vesicatoria resulted to be more susceptible pathogen than *P. syringae*.

Therefore, the cyclodecapeptide **1** (BPC10L) was the most active against bacteria and displayed antibacterial activity at 12.5 μM, 6.25 μM and 6.25 μM against *P. syringae*, *X. vesicatoria* and *C michiganensis*, respectively. Its linear counterpart BP10L was the most active peptide against fungi and displayed antifungal activity at 1.0-6.2 μM against *V. dahliae* and *F. oxysporum* and at 6.2-12.5 μM against *A. niger*.

Table 12. Effect of the ring size, introduction of a D-Phe and several modifications in BPC8L on antibacterial (MIC) and hemolytic activities, RP-HPLC retention time and molar ellipticity

Codes		Peptide sequence	t_R (min)	$[\theta]_{208}$[a]	MIC (μM)		Hemolysis (%)[b]
					P. syringae	*X. vesicatoria*	
1	**BPC10L**	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	4.02	–17866	12.5	6.25	81 ± 5.3
2	**BPC9L**	c(Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	4.18	–[c]	25	25	–
3	**BPC8L**	c(Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	4.03	–18395	25	12.5	68 ± 7.5
5	**BPC6L**	c(Lys-Phe-Lys-Leu-Lys-Gln)	3.83	–12318	25	25	11 ± 4.6
6	**BPC5L**	c(Phe-Lys-Leu-Lys-Gln)	4.07	–	75	25	–
7	**BPC4L**	c(Lys-Phe-Lys-Gln)	3.79	–	25	12.5	0
8	**BPC10D**	c(Lys-Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	3.70	–12230	25	12.5	42 ± 6.8
9	**BPC9D**	c(Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	3.90	–	50	12.5	–
10	**BPC8D**	c(Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	3.94	–8119	25	25	37 ± 5.2
12	**BPC6D**	c(Lys-D-Phe-Lys-Leu-Lys-Gln)	3.70	–5655	>100	75	0
13	**BPC5D**	c(D-Phe-Lys-Leu-Lys-Gln)	3.99	–	>100	50	–
14	**BPC4D**	c(Lys-D-Phe-Lys-Gln)	3.30	–	75	25	0
	BPC8S1	c(Lys-Leu-Lys-Leu-Lys-Leu-Lys-Gln)	–	–4800[d]	75	50	1 ± 0.1
	BPC8S2	c(Lys-Leu-Lys-Leu-Lys-Leu-Lys-Glu)	–	–	75	12.5	0
	BPC8S3	c(Leu-Lys-Leu-Lys-Leu-Lys-Leu-Gln)	–	–	75	75	5 ± 0.1
	BPC8S4	c(Lys-Lys-Leu-Lys-Leu-Lys-Lys-Gln)	–	–	>100	>100	–
	BPC8S5	cGlu(Lys-Leu-Lys-Leu-Lys-Leu-Lys)-NH_2	–	–	>100	>100	–

[a]Determined in 50% TFE in 10 mM sodium phosphate buffer (pH = 7.4)
[b]Percent hemolysis at 360 μM plus confidence interval (α = 0.05)
[c]Not determined
[d]Minima observed at 203 nm

Table 13. Antimicrobial and hemolytic activities of linear and cyclic peptides of 4-10 residues incorporating a L-Phe

Codes		Peptide sequence	MIC intervals (μM)					Hemolysis (%)[a]
			Cm[b]	Vd[c]	Fo[d]	Pe[e]	An[f]	
	BP10L	H-Lys-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln-OH	50-100	1.0-6.2	1.0-6.2	25-50	6.2-12.5	–
1	**BPC10L**	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	6.2-12.5	<6.2[g]	<6.2[g]	25-50	12.5-25	81 ± 5.3
	BP9L	H-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln-OH	>100	<50[g]	–[h]	<50[g]	<50[g]	–
2	**BPC9L**	c(Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	<50[g]	<50[g]	25-50	<50[g]	50-75	–
	BP8L	H-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln-OH	>100	25-50	6.2-12.5	>100	>100	–
3	**BPC8L**	c(Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	6.2-12.5	12.5-25	<6.2[g]	>100	>100	68 ± 7.5
	BPC8S2	c(Lys-Leu-Lys-Leu-Lys-Leu-Lys-Glu)	>100	12.5-25	12.5-25	>100	>100	0
	BP7L	H-Leu-Lys-Phe-Lys-Leu-Lys-Gln-OH	>100	>100	–	>100	>100	–
	BP6L	H-Lys-Phe-Lys-Leu-Lys-Gln-OH	>100	>100	–	>100	>100	–
5	**BPC6L**	c(Lys-Phe-Lys-Leu-Lys-Gln)	<50[g]	75-100	<75[g]	>100	>100	11 ± 4.6
	BP5L	H-Phe-Lys-Leu-Lys-Gln-OH	>100	>100	–	>100	>100	–
6	**BPC5L**	c(Phe-Lys-Leu-Lys-Gln)	<50[g]	>100	>100	>100	>100	–
	BP4L	H-Lys-Phe-Lys-Gln-OH	>100	>100	–	>100	>100	–
7	**BPC4L**	c(Lys-Phe-Lys-Gln)	50-75	>100	<75[g]	>100	>100	0

[a]Percent hemolysis at 360 μM plus confidence interval (α = 0.05)
[b]Cm stands for *C. michiganensis*
[c]Vd stands for *V. dahliae*
[d]Fo stands for *F. oxysporum*
[e]Pe stands for *P. expansum*
[f]An stands for *A. niger*
[g]Lowest concentration tested
[h]Not determined

Table 14. Antimicrobial and hemolytic activities of linear and cyclic peptides of 4-10 residues incorporating a D-Phe

Codes		Peptide sequence	MIC intervals (μM)					Hemolysis (%)[a]
			Cm[b]	Vd[c]	Fo[d]	Pe[e]	An[f]	
	BP10D	H-Lys-Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln-OH	>100	25-50	25-50	75-100	25-50	–
8	**BPC10D**	c(Lys-Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	6.2-12.5	12.5-25	12.5-25	>100	>100	42 ± 6.8
	BP9D	H-Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln-OH	>100	75-100	–	75-100	>100	–
9	**BPC9D**	c(Leu-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	<50[g]	<50[g]	<75[g]	>100	>100	–
	BP8D	H-Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln-OH	>100	–[h]	>100	>100	>100	–
10	**BPC8D**	c(Lys-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln)	50-100	–	50-75	>100	>100	37 ± 5.2
	BP7D	H-Leu-Lys-D-Phe-Lys-Leu-Lys-Gln-OH	>100	>100	–	>100	>100	–
	BP6D	H-Lys-D-Phe-Lys-Leu-Lys-Gln-OH	>100	>100	–	>100	>100	–
12	**BPC6D**	c(Lys-D-Phe-Lys-Leu-Lys-Gln)	<50[g]	>100	>100	>100	>100	0
	BP5D	H-D-Phe-Lys-Leu-Lys-Gln-OH	>100	>100	–	>100	>100	–
13	**BPC5D**	c(D-Phe-Lys-Leu-Lys-Gln)	75-100	>100	>100	>100	>100	–
	BP4D	H-Lys-D-Phe-Lys-Gln-OH	>100	>100	–	>100	>100	–
14	**BPC4D**	c(Lys-D-Phe-Lys-Gln)	>100	>100	>100	>100	>100	0

[a]Percent hemolysis at 360 μM plus confidence interval (α = 0.05)
[b]Cm stands for *C. michiganensis*
[c]Vd stands for *V. dahliae*
[d]Fo stands for *F. oxysporum*
[e]Pe stands for *P. expansum*
[f]An stands for *A. niger*
[g]Lowest concentration tested
[h]Not determined

An increase of the ring size was found to be associated with a loss of selectivity between pathogen and erythrocytes. Except for the cyclotetrapeptides **7** and **14** (BPC4L and BPC4D) both non-hemolytic at 360 µM, the D-diastereoisomers were less hemolytic than their L-counterparts. Therefore, the cyclotetrapeptides **7** and **14** (BPC4L and BPC4D) were the less hemolytic, whereas the cyclodecapeptide **1** (BPC10L) was also the most hemolytic (Table 12). Furthermore, the hemolytic activity of **3** (BPC8L) analogues displayed lower eukaryotic cytotoxicity than their parent sequence with 5% hemolysis at the most to 68% of **3** (BPC8L) at 360 µM (Table 12).

In general, cyclic peptides with an odd number of amino acids showed higher t_R values than cyclic peptides with an even number of residues which were higher than their diastereoisomer (Table 12).

Secondary structure content was determined by CD spectrometry in phosphate buffer and a membrane-mimicking solvent such as TFE (Holak et al., 1988; Sönnichsen, et al., 1992; Jayaraman et al., 1996). Linear peptides showed a disordered structure in both solvents, whereas cyclic peptides became structured in TFE. Indeed, the cyclic peptide CD spectra showed a gramicidin S-like CD spectrum with a stronger absorption band at ~ 208 nm and an other minimum at ~ 220 nm (Figures 35 and 36) (Kondejewski et al., 1996; Gibbs et al., 2002). It is worth noting that the cyclooctapeptide BPC8L exhibited a gramicidin S-like CD spectrum with the absorption band at 208 nm stronger that in the decapeptide CD. Incorporation of a D-Phe or a L-Leu provided peptides with lower secondary structure content (Table 12).

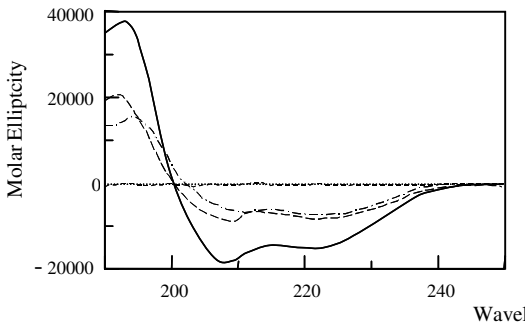


Figure 35. CD spectra of BPC8L and BPC8D at different TFE concentrations in 10 mM sodium phosphate buffer at pH 7.4. BPC8L in 0% TFE (- - -); BPC8L in 50% TFE (– · – · –); BPC8L in 100% TFE (—); BPC8D in100% TFE (– – –).

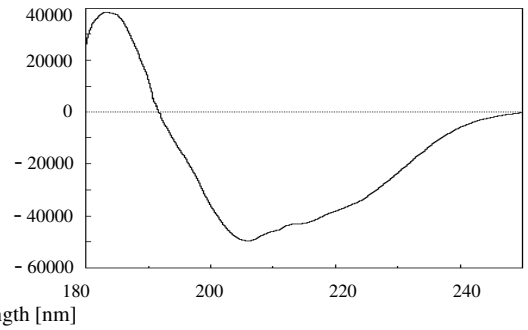


Figure 36. CD spectra of gramicidin S in 50% TFE

Cyclization and D-amino acid substitution afforded peptides less susceptible to protease degradation.

Unlike most of the antibacterial cationic peptides, BPC10L (**1**), BPC10D (**8**), BPC6L (**5**) and BPC6D (**12**) displayed a slow bactericidal effect against *P. syringae* and *X. vesicatoria* at concentrations around their MIC (Figure 37). For a given pathogen, peptides exhibited differences in the time to start bacterial cell killing and in the slope of the survival kinetics, being the L-diastereoisomers more bactericidal than their D-counterparts. It was, also, observed a different effect depending on the pathogen except for BPC10L that showed the same kinetics. All peptides showed a slower bactericidal activity against *P. syringae* than against *X. vesicatoria*.

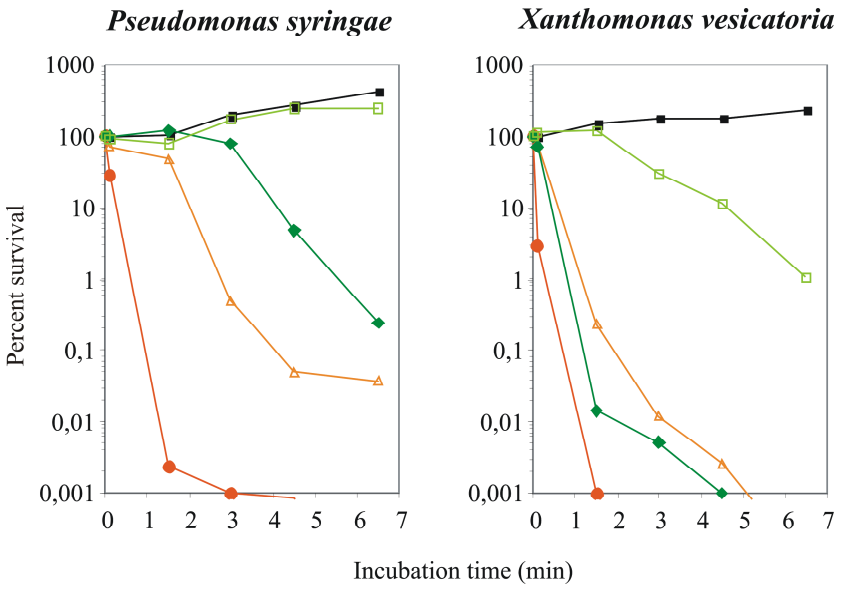


Figure 37. Kinetics of survival of *P. syringae* and *X. vesicatoria* in presence of cyclic peptides. Bacterial suspensions were untreated (■) or treated with MIC concentrations of BPC10L (●), BPC10D (△), BPC6L (◆) and BPC6D (□)

Paper 3 results

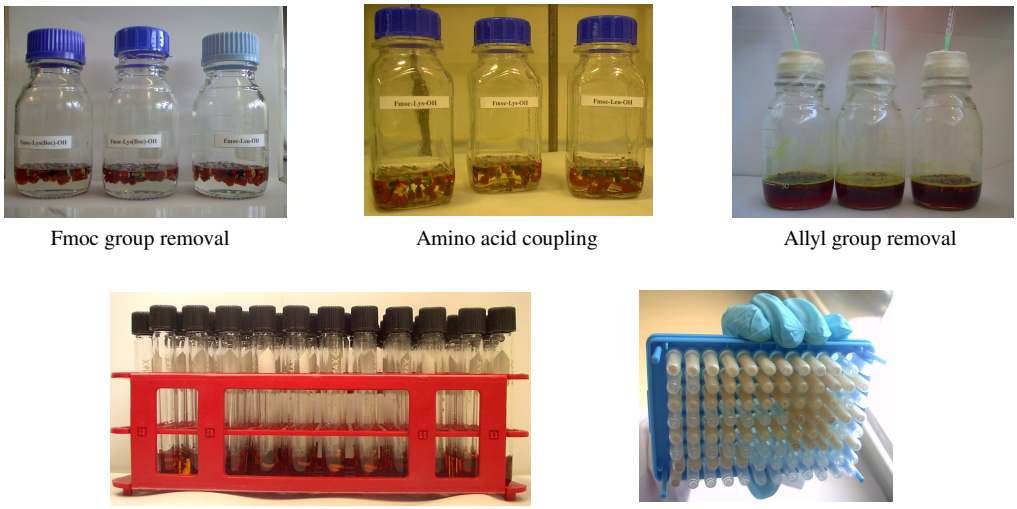
Improvement of cyclic decapeptides against plant pathogenic bacteria using a combinatorial chemistry approach.

Peptides **2006**;27:2575-2584.

Abstract: Cyclic decapeptides were developed based on the previously reported peptide c(LysLeuLysLeuLysPheLysLeuLysGln). These compounds were active against the economically important plant pathogenic bacteria *Erwinia amylovora*, *Pseudomonas syringae* and *Xanthomonas vesicatoria*. A library of 56 cyclic decapeptides was prepared and screened for antibacterial activity and eukaryotic cytotoxicity, and led to the identification of peptides with improved minimum inhibitory concentration (MIC) against *P. syringae* (3.1–6.2 μM) and *X. vesicatoria* (1.6–3.1 μM). Notably, peptides active against *E. amylovora* (MIC of 12.5–25 μM) were found, constituting the first report of cyclic peptides with activity towards this bacteria. A second library based on the structure c($X^1X^2X^3X^4$LysPheLysLysLeuGln) with X being Lys or Leu yielded peptides with optimised activity profiles. The activity against *E. amylovora* was further improved (MIC of 6.2–12.5 μM) and the best peptides displayed a low eukaryotic cytotoxicity at concentrations 30–120 times higher than the MIC values. A design of experiments permitted to define rules for high antibacterial activity and low cytotoxicity, being the main rule $X^2 \neq X^3$, and the secondary rule X^4 = Lys. The best analogs can be considered as good candidates for the development of effective antibacterial agents for use in plant protection.

Improvement of the cyclic decapeptide BPC10L c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln) was performed using a combinatorial chemistry approach. Two combinatorial libraries were prepared and screened for antibacterial activity against *Erwinia amylovora*, *Pseudomonas syringae* and *Xanthomonas vesicatoria*, and for hemolytic activity (library I and library II). A DOE based on the biological activities of library II was performed in order to check for the structural interactions responsible for activity.

Libraries I and II were prepared on SynPhase Lanterns and resin, respectively, by carrying out solid-phase synthesis of linear sequences, followed by on-solid support cyclization as previously described (Scheme 9, Figure 38). Peptides were analysed by RP-HPLC and characterized by mass spectrometry.



Fmoc group removal | Amino acid coupling | Allyl group removal

Cyclization | Cleavage

Figure 38. Solid-phase synthesis of cyclic peptides of library I

Library I was designed based on the sequence of BPC10L. It consisted of 56 cyclic peptides containing a Phe and a Gln at positions 6 and 10, respectively. The rest of positions included all possible combinations of three Leu and five Lys. This library led to a first optimisation of the biological properties of BPC10L (Figure 39, Table 15).

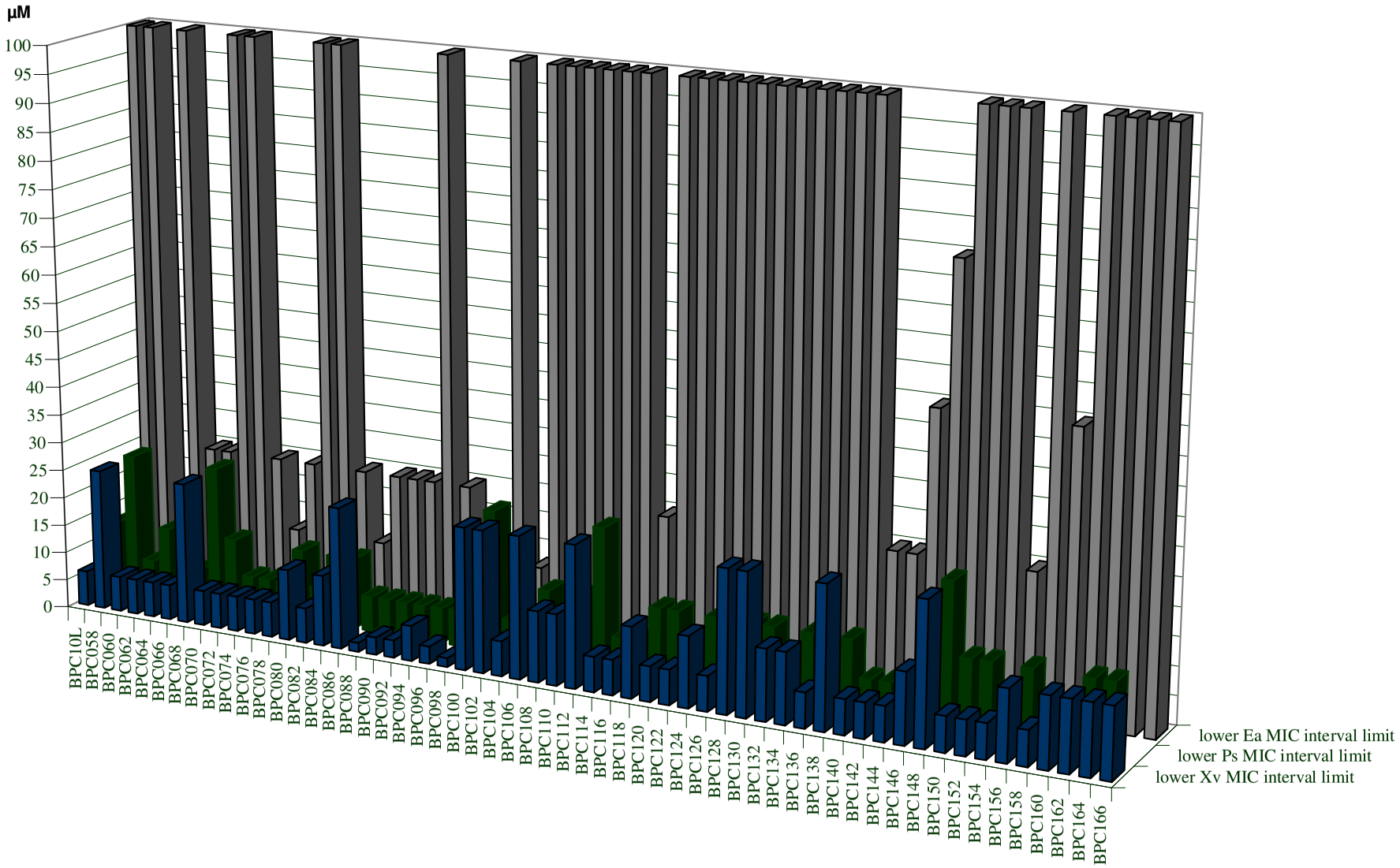


Figure 39. Antibacterial activity (MIC) of cyclic peptides of library I

Peptides with improved antibacterial activity against *P. syringae* (MIC of 3.1-6.2 μM) and *X. vesicatoria* (MIC of 1.6-3.1 μM) were identified (Figure 30, Table 15). Remarkably, peptides active against *E. amylovora* were also found (MIC of 12.5-25 μM), being the first cyclic peptides active against this bacterium described so far (Figure 39, Table 15). The hemolytic activity was also significantly improved (Table 15). All peptides displayed lower eukaryotic cytotoxicity than BPC10L, with 38 peptides exhibiting less than 15% hemolysis at 375 μM.

Table 15. Antibacterial activity and cytotoxicity of the most effective peptides of library I[a]

Code	Peptide sequence	MIC intervals (μM)			Hemolysis
		Ps[c]	Xv[d]	Ea[e]	(%)[b]
BPC10L	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Leu-Lys-Gln)	12.5-25	6.2-12.5	>100	84 ± 6.9
BPC060	c(Lys-Lys-Lys-Lys-Leu-Phe-Lys-Leu-Leu-Gln)	6.2-12.5	6.2-12.5	12.5-25	72 ± 5.5
BPC064	c(Lys-Lys-Leu-Lys-Lys-Phe-Lys-Leu-Leu-Gln)	12.5-25	6.2-12.5	25-50	10 ± 2.0
BPC066	c(Lys-Leu-Lys-Lys-Lys-Phe-Lys-Leu-Leu-Gln)	6.2-12.5	6.2-12.5	25-50	22 ± 4.0
BPC072	c(Lys-Lys-Lys-Leu-Lys-Phe-Leu-Lys-Leu-Gln)	6.2-12.5	6.2-12.5	25-50	7 ± 2.0
BPC074	c(Lys-Lys-Leu-Lys-Lys-Phe-Leu-Lys-Leu-Gln)	6.2-12.5	6.2-12.5	12.5-25	36 ± 1.7
BPC076	c(Lys-Leu-Lys-Lys-Lys-Phe-Leu-Lys-Leu-Gln)	6.2-12.5	6.2-12.5	25-50	13 ± 1.9
BPC080	c(Lys-Lys-Lys-Leu-Leu-Phe-Lys-Lys-Leu-Gln)	6.2-12.5	12.5-25	75-100	19 ± 0.7
BPC082	c(Lys-Lys-Leu-Lys-Leu-Phe-Lys-Lys-Leu-Gln)	6.2-12.5	12.5-25	25-50	36 ± 2.5
BPC084	c(Lys-Leu-Lys-Lys-Leu-Phe-Lys-Lys-Leu-Gln)	12.5-25	12.5-25	12.5-25	45 ± 3.5
BPC086	c(Leu-Lys-Lys-Lys-Leu-Phe-Lys-Lys-Leu-Gln)	6.2-12.5	25-50	25-50	8 ± 0.9
BPC088	c(Lys-Lys-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	6.2-12.5	1.6-3.1	25-50	33 ± 3.5
BPC090	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	6.2-12.5	3.1-6.2	25-50	35 ± 4.7
BPC092	c(Leu-Lys-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	6.2-12.5	3.1-6.2	>100	9 ± 0.9
BPC094	c(Lys-Leu-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	6.2-12.5	6.2-12.5	25-50	73 ± 1.6
BPC096	c(Leu-Lys-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	6.2-12.5	3.1-6.2	12.5-25	32 ± 7.2
BPC098	c(Leu-Leu-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	6.2-12.5	1.6-3.1	12.5-25	36 ± 3.7
BPC102	c(Lys-Lys-Lys-Leu-Lys-Phe-Leu-Leu-Lys-Gln)	6.2-12.5	25-50	12.5-25	26 ± 3.0
BPC104	c(Lys-Lys-Leu-Lys-Lys-Phe-Leu-Leu-Lys-Gln)	3.1-6.2	6.2-12.5	>100	15 ± 2.2
BPC108	c(Leu-Lys-Lys-Lys-Lys-Phe-Leu-Leu-Lys-Gln)	6.2-12.5	6.2-12.5	75-100	2 ± 0.2
BPC114	c(Lys-Leu-Lys-Lys-Leu-Phe-Lys-Leu-Lys-Gln)	6.2-12.5	6.2-12.5	75-100	24 ± 1.8
BPC116	c(Leu-Lys-Lys-Lys-Leu-Phe-Lys-Leu-Lys-Gln)	6.2-12.5	6.2-12.5	25-50	13 ± 0.9
BPC122	c(Lys-Leu-Leu-Lys-Lys-Phe-Lys-Leu-Lys-Gln)	6.2-12.5	6.2-12.5	75-100	4 ± 1.3
BPC132	c(Lys-Leu-Lys-Lys-Leu-Phe-Leu-Lys-Lys-Gln)	6.2-12.5	6.2-12.5	75-100	4 ± 0.9
BPC136	c(Lys-Lys-Leu-Leu-Lys-Phe-Leu-Lys-Lys-Gln)	6.2-12.5	6.2-12.5	75-100	41 ± 1.8
BPC140	c(Leu-Lys-Lys-Lei-Lys-Phe-Leu-Lys-Lys-Gln)	6.2-12.5	6.2-12.5	25-50	23 ± 1.1
BPC142	c(Lys-Leu-Leu-Lys-Lys-Phe-Leu-Lys-Lys-Gln)	6.2-12.5	6.2-12.5	25-50	14 ± 1.6
BPC144	c(Leu-Lys-Leu-Lys-Lys-Phe-Leu-Lys-Lys-Gln)	3.1-6.2	6.2-12.5	50-75	9 ± 1.9
BPC146	c(Leu-Leu-Lys-Lys-Lys-Phe-Leu-Lys-Lys-Gln)	6.2-12.5	6.2-12.5	75-100	3 ± 0.5
BPC154	c(Lys-Leu-Leu-Lys-Leu-Phe-Lys-Lys-Lys-Gln)	6.2-12.5	6.2-12.5	25-50	11 ± 1.7
BPC158	c(Leu-Leu-Lys-Lys-Leu-Phe-Lys-Lys-Lys-Gln)	6.2-12.5	6.2-12.5	50-75	9 ± 0.7

[a]Results excerpted from Table 1 in Monroc et al., 2006c
[b]Percent hemolysis at 375 μM
[c]Ps stands for *P. syringae*; [d]Xv stands for *X. vesicatoria*; [e]Ea stands for *E. amylovora*

In order to elucidate if there is a common substructure at the most active peptides of library I against each bacterium, a frequency analysis was carried. The selected cyclic peptides had to have a minimal inhibitory concentration against each pathogen below MIC of parent peptide BPC10L namely MICs below 12.5 μM against *P. syringae*, 6.2 μM against *X. vesicatoria* and 100 μM against *E. amylovora* (Table 15). It was then determined the most occurring residues at each position for each pathogen. First, all Leu and Lys residues of each selected decapeptide were codified as 1 and 2, respectively. Gln and Phe were not considered in this study given their fixed positions in all the decapeptides. The most occurring residue at each position was then determined by calculating the Lys/Leu proportion for each position, and comparing the values obtained with the 5/3 theoretical proportion (all decapeptides contain five Lys and three Leu). The quotient of these two proportions indicates if the considered residue was significant for the antibacterial activity. The more this quotient was faraway from 1 the more significant the residue was. The frequency analysis against *P. syringae* gave c(X^1-X^2-X^3-Lys-Lys-Phe-X^6-Lys-X^8-Gln) as general structure for the best antibacterial candidates. The amino acids of the positions labelled X^1, X^3, X^4 and X^6 appeared to be much more difficult to identify as the proportion quotients were inferior to 1.4 (Table 16). Against the other two bacteria, the preferred amino acid sequences were c(X^1-X^2-X^3-X^4-Lys-Phe-Lys-Lys-Leu-Gln) for *X. vesicatoria* and c(X^1-X^2-X^3-Lys-X^5-Phe-X^6-Lys-X^8-Gln) for *E. amylovora* (Table 16). The predicted sequences against the three bacteria were very similar. Besides, peptides from library I with general structure c(X^1-X^2-X^3-X^4-Lys-Phe-Lys-Lys-Leu-Gln) (BPC088-BPC098) exhibited low MIC values against the three bacteria. Consequently, the general structure c(X^1-X^2-X^3-X^4-Lys-Phe-Lys-Lys-Leu-Gln) was selected for further study.

Table 16. Frequency analysis the most active peptides of library I against *X. vesicatoria*

Code	Peptide sequence	c(	X^1	-	X^2	-	X^3	-	X^4	-	X^5	-	**Phe**	-	X^6	-	X^7	-	X^8	-	**Gln**)
BPC088	c(Lys-Lys-Leu-Leu-Lys-**Phe**-Lys-Lys-Leu-**Gln**)		2[a]		2		1[b]		1		2				2		2		1		
BPC090	c(Lys-Leu-Lys-Leu-Lys-**Phe**-Lys-Lys-Leu-**Gln**)		2		1		2		1		2				2		2		1		
BPC092	c(Leu-Lys-Lys-Leu-Lys-**Phe**-Lys-Lys-Leu-**Gln**)		1		2		2		1		2				2		2		1		
BPC096	c(Leu-Lys-Leu-Lys-Lys-**Phe**-Lys-Lys-Leu-**Gln**)		1		2		1		2		2				2		2		1		
BPC098	c(Leu-Leu-Lys-Lys-Lys-**Phe**-Lys-Lys-Leu-**Gln**)		1		1		2		2		2				2		2		1		
Leu[c]			3		2		2		3		0				0		0		5		
Lys[d]			2		3		3		2		5				5		5		0		
ratio Lys/Leu			0.67		1.50		1.50		0.67		-				-		-		-		
dominant residue[e]			1[b]		1		1		1		2[a]				2		2		1		
factor[f]			0.40		0,90		0.90		0.40		-				-		-		-		
general structure[g]		c(	X^1	-	X^2	-	X^3	-	X^4	-	Lys	-	**Phe**	-	Lys	-	Lys	-	Leu	-	**Gln**)

[a]2 stands for lysine.
[b]1 stands for leucine.
[c]Total number of leucine at each position
[d]Total number of lysine at each position
[e]The dominant residue depends on the ratio Lys/Leu. If the ratio is less than 1.67, the dominant factor is leucine, otherwise the dominant factor is lysine
[f]The factor is the quotient of the ratio Lys/Leu by 1.67, the ratio Lys/Leu in BPC10L.
[g]The general structure depends on the factor. If the factor is more than 1.40, the residue is the dominant residue of the considered position, otherwise the residue is undetermined

Library II was designed to improve the antibacterial and hemolytic activities of the library I (Figure 39, Table 15). Considering the general selected structure c(X^1-X^2-X^3-X^4-Lys-Phe-Lys-Lys-Leu-Gln) for the most effective cyclic peptides against the three bacteria, the residues at positions 1-4 remained to determine. Therefore, a library of 16 cyclodecapeptides was designed incorporating the substructure Lys^5-Phe-Lys-Lys-Leu-Gln^{10} and all possible combinations of Leu and Lys at positions 1-4. Thus, library II included BPC088, BPC090, BPC092, BPC094, BPC096 and BPC098 from the library I and, to allow better comparison, these peptides were re-synthesized.

Peptides with optimised activity profiles were identified (Table 17). BPC194 and BPC198 displayed low MIC values against *P. syringae* (3.1-6.2 µM) and against *X. vesicatoria* (3.1-6.2 µM), and maintained a low level of hemolysis (around 15%). In addition, BPC194 exhibited improved activity against *E. amylovora* (MIC of 6.2-12.5 µM) compared to the most active peptides of library I.

All cyclodecapeptides of library II showed a disordered structure in phosphate buffer and became structured at 50% TFE with gramicidin S-like CD spectra (Figures 36 and 40).

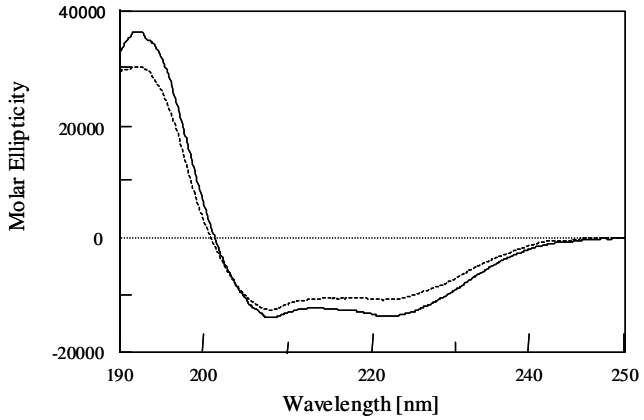


Figure 40. CD spectra of BPC194 (—) and BPC198 (– – –) in 50% TFE in 10 mM sodium phosphate buffer at pH 7.4

Table 17. Sequences, retention time, molar ellipticity and biological activities of the peptides of library II

Code	Peptide sequence	t_R[a] (min)	$[\theta]_{208}$[b]	MIC intervals (μM)			Hemolysis (%)[c]
				Ps[d]	Xv[e]	Ea[f]	
BPC088	c(Lys-Lys-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	4.11	–14 721	6.2-12.5	1.6-3.1	25-50	33 ± 3.3
BPC090	c(Lys-Leu-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	4.08	–6 663	6.2-12.5	3.1-6.2	25-50	30 ± 4.1
BPC092	c(Leu-Lys-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	3.97	–13 578	6.2-12.5	3.1-6.2	>100	7 ± 0.7
BPC094	c(Lys-Leu-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	4.53	–12 385	6.2-12.5	6.2-12.5	25-50	75 ± 1.6
BPC096	c(Leu-Lys-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	4.12	–13 650	6.2-12.5	3.1-6.2	12.5-25	24 ± 4.3
BPC098	c(Leu-Leu-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	4.13	–13 692	6.2-12.5	1.6-3.1	12.5-25	28 ± 2.4
BPC184	c(Lys-Leu-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	4.70	–13 876	12.5-25	3.1-6.2	50-75	89 ± 5.3
BPC186	c(Lys-Lys-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	3.68	–13 843	6.2-12.5	6.2-12.5	>100	0 ± 0.4
BPC188	c(Leu-Leu-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	4.73	–17 357	12.5-25	6.2-12.5	25-50	87 ± 6.5
BPC190	c(Leu-Lys-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	3.68	–13 821	6.2-12.5	6.2-12.5	>100	0
BPC192	c(Leu-Lys-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	4.34	–16 108	12.5-25	3.1-6.2	25-50	49 ± 7.7
BPC194	c(Lys-Lys-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	3.90	–13 874	3.1-6.2	3.1-6.2	6.2-12.5	17 ± 1.7
BPC196	c(Leu-Leu-Lys-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	4.36	–11 774	12.5-25	6.2-12.5	>100	47 ± 7.2
BPC198	c(Lys-Leu-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	3.90	–12 704	3.1-6.2	3.1-6.2	12.5-25	14 ± 1.4
BPC200	c(Leu-Leu-Leu-Leu-Lys-Phe-Lys-Lys-Leu-Gln)	3.80	–16 139	25-50	12.5-25	>100	71 ± 11.7
BPC202	c(Lys-Lys-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln)	3.41	–10 861	12.5-25	6.2-12.5	>100	2 ± 0.2

[a]HPLC retention time
[b]Determined in 50% TFE in 10 mM sodium phosphate buffer (pH = 7.4)
[c]Percent hemolysis at 375 μM plus confidence interval (α = 0.05)
[d]Ps stands for *P. syringae*
[e]Xv stands for *X. vesicatoria*
[f]Ea stands for *E. amylovora*

In order to identify the four leftmost residue influences from the general structure c(X^1-X^2-X^3-X^4-Lys-Phe-Lys-Lys-Leu-Gln) on both biological properties, an experimental design methodology was made from the experimental library II. The leucine and lysine residues could be present at every position of the four leftmost positions labelled X^1, X^2, X^3 and X^4. Consequently, DOE calculations constituted a full two-level factorial design, 2^4 possible substitution variations. Among the antibacterial activities, five series of experiments were selected to be statistically analysed by DOE. These series were the percent of bacterial growth inhibition at peptide concentrations of 6.2 µM for *P. syringae*, 3.1 and 6.2 µM for *X. vesicatoria*, and 25 and 50 µM for *E. amylovora*. For hemolysis, three series of experiments, corresponding to peptide concentrations of 125, 250 and 375 µM, showed a significant variation and were statistically investigated. All these experiments were performed in triplicate to take into account the standard errors.

The DOE study started by first arbitrarily assigning numerical levels to each residue. The MINITAB program [MINITAB version 14 for Windows. Minitab Inc., State College, PA, 2004] was then used to perform statistical functions. It allowed establishing which factors and which individual effect of each factor as well as the two-, three- and four-factor interactions, had a significant influence on the selected series for antibacterial and hemolytic activities. From the experimental library II data of each series, the software has plotted graphical data representations in a normal probability paper. Therefore, four graphical data representations in a normal probability paper showed the magnitude of the combination (single, double, triple and quadruple interactions) effect according to the corresponding point position respective the straight line of the random Gaussian distribution (Figures 41, 42, 43 and 44). As points move far away from this line, more statistically significant is the effect for the considered activity. Consequently, the main rule corresponded to the farest point of the random Gaussian distribution line and the second farest points corresponded to secondary rules with the single effects and double interactions taking precedence over the triple and quadruple interactions

The graphical data representations showed at any concentration that while the two-factor interaction between residues at the positions X^2 and X^3 was the most important to a higher antibacterial activity towards the three bacteria, and two relevant single

effects corresponding to residues at the positions X^2 and X^3 gave a lower hemolytic activity (Figures 41, 42, 43 and 44).

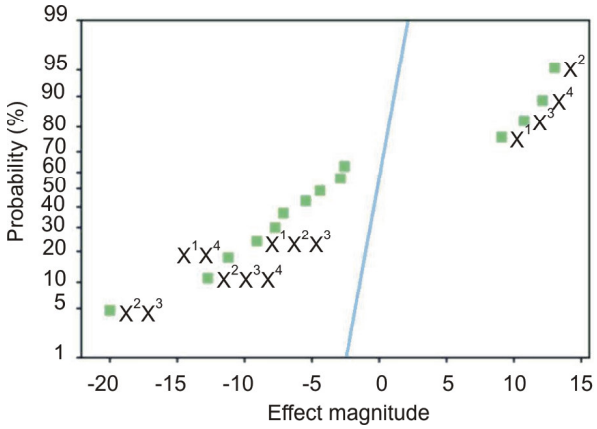


Figure 41. Effects plot for *P. syringae* at 6.2 µM (■ ■)

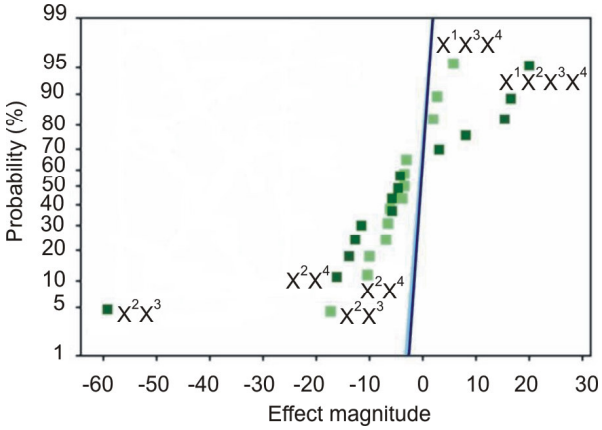


Figure 42. Effects plot for *X. vesicatoria* at 3.1 µM (■ ■) and 6.2 µM (■ ■)

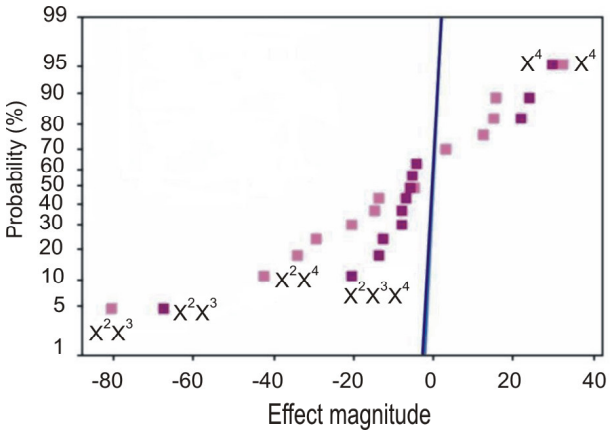


Figure 43. Effects plot for *E. amylovora* at 25 µM (■ ■) and 50 µM (■ ■)

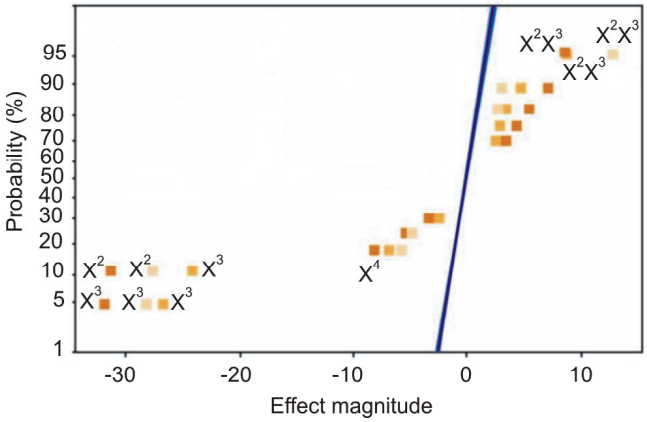


Figure 44. Effects plot for hemolysis at 375 µM (■ ■), 250 µM (■ ■) and 125 µM (■ ■)

In the next step, MINITAB program evaluated the interaction plots and the single effects plots for both biological activities, and generated graphs to represent them. In the interaction plot graphs such as in figure 45, the substitution of one of the two considered positions, namely the two factors, was identified in the abscissas axis. The considered effect percentage was represented in Y-axis. Therefore, an ascending line for a given amino acid residue (Leu or Lys) of a given position meant the considered effect increase when leucine is substituted for lysine at the other position. A descending line, for a given amino acid residue (Leu or Lys) of a given position meant the considered effect decrease when leucine is substituted for lysine at the other position. In the one-factor effects plots, also called main effects plots or single effects plots, the substitution of the considered position was identified in the abscissas axis and the considered effect percentage was represented in Y-axis such as in figure 48. Therefore, an ascending line for of a given position meant the considered effect increase when leucine is substituted for lysine at this position while a descending line meant the effect decrease.

The interaction plots where the X^3 substitution was identified in the abscissas axis indicated that the residues at the positions X^2 and X^3 had to be different for all the bacteria (Figures 45, 46 and 47). With the main rule, different residues at the positions X^2 and X^3, other secondary rules characteristic for each bacterium were also determined (Figures 41, 42 and 43). For *P. syringae*, the better antibacterial activity would be obtained when the residues at the positions X^2 and X^4, as single effects, are lysine (Figure 48). For *X. vesicatoria* at both concentrations and *E. amylovora* at 50 µM, leucine as residue at the position X^2 and lysine as residue at the position X^4 in the double interaction X^2X^4 should be involved a better antimicrobial activity (Figures 49 and 50).

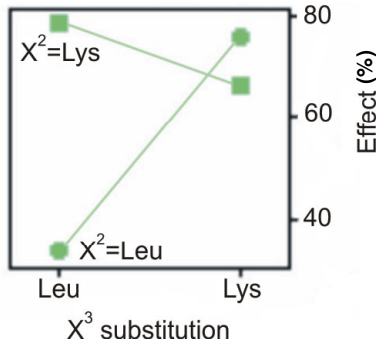


Figure 45. Interaction plot for *P. syringae* at 6.2 µM (■ ■)

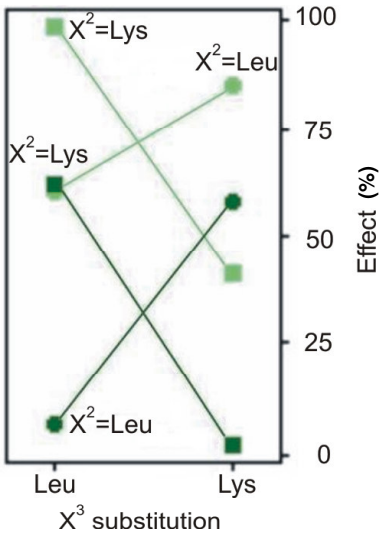


Figure 46. Interaction plots for *X. vesicatoria* at 3.1 µM (■ ■) and 6.2 µM (■ ■)

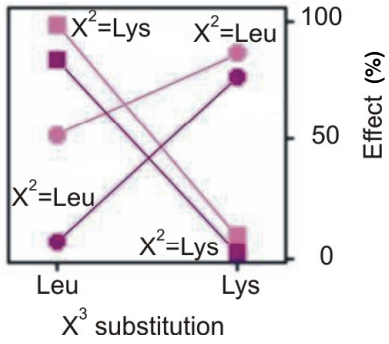


Figure 47. Interaction plots for *E. amylovora* at 25 µM (■ ■) and 50 µM (■ ■)

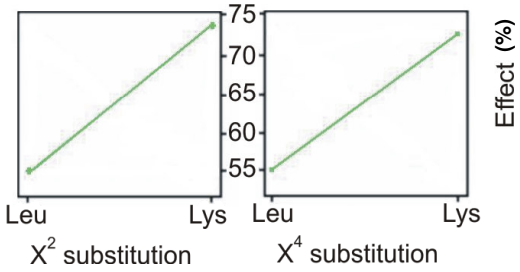


Figure 48. Single effect plot for *P. syringae* at 6.2 µM

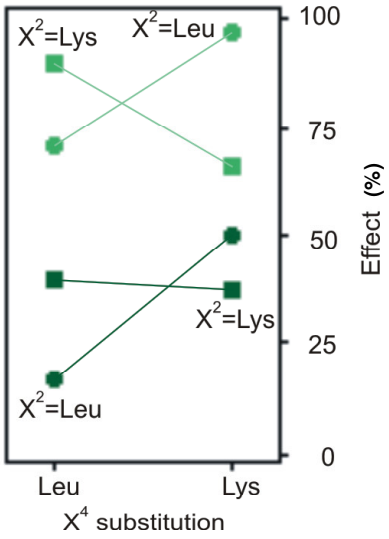


Figure 49. Interaction plots for *X. vesicatoria* at 3.1 µM (■) and 6.2 µM (■)

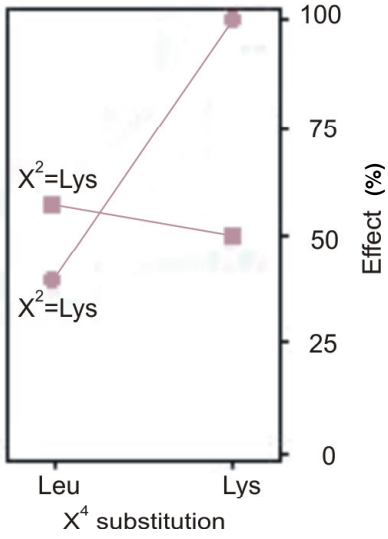


Figure 50**.** Interaction plot for *E. amylovora* at 50 µM

As the main effects plots, the lowest hemolysis would be obtained when residues at the positions X^2 and X^3 are lysine (Figures 44 and 51). A secondary rule, considering the double interaction X^2X^3, determined that low hemolysis would be obtained when residues at the positions X^2 and X^3 are lysine, specially the subset with lysine residue at the position X^4 (Figures 44, 52 and 53). However, if the residues at the positions X^2 and X^3 considering the double interaction X^2X^3 are different, specially the subset with lysine residue at the position X^4, the hemolysis was expected to be higher but reasonable (Figures 44, 52 and 53).

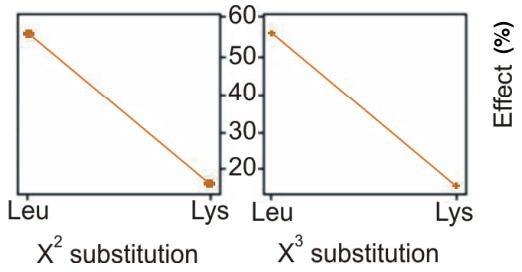


Figure 51. Single effect plots for hemolysis at 375 µM

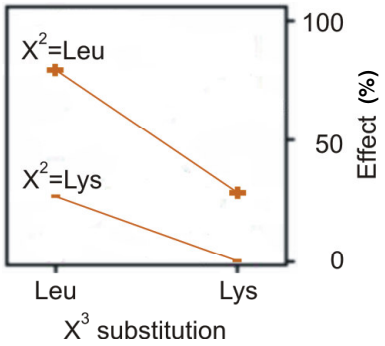


Figure 52. Interaction plots for hemolysis at 375 µM

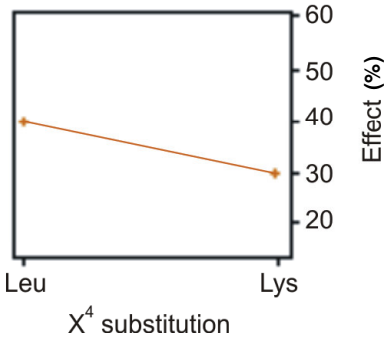


Figure 53. Single effect plot for hemolysis at 375 µM

Patent

Péptidos cíclicos antimicrobianos

P2005-03137

The present PhD thesis results have been protected in a request patent at the "Oficina Española de Patentes y Marcas". Here is included the first page of the document.

SOLICITANTE : Universitat de Girona

INVENTORES : Eduard Bardají Rodríguez, Emili Montesinos Seguí, Marta Planas Grabuleda, Esther Badosa Romañó, Lidia Feliu Soley, Sylvie Martine Colette Monroc

NUMERO DE SOLICITUD : P200503137

TITULO : Péptidos cíclicos antimicrobianos

RESUMEN : La presente invención se refiere a nuevos péptidos cíclicos que tienen propiedades antimicrobianas. Dichos péptidos tienen un número par de aminoácidos comprendido entre 4 y 10, y un contenido molar de lisina comprendido entre el 40% y el 60%. La invención describe la síntesis y la utilización de los mencionados péptidos como agentes antimicrobianos frente a bacterias y hongos patógenos para plantas. También se refiere a composiciones que comprenden dichos péptidos y un agente auxiliar, y a un método para prevenir y tratar infecciones y enfermedades de las plantas provocadas por bacterias y hongos patógenos.

General discussion

The head-to-tail cyclic cationic antimicrobial peptides from 4 to 10 residues presented in this work were designed as synthetic peptides with different Leu/Lys amounts and proportion. The aim was to improve their specificity for the microorganisms over human erythrocytes taking into account several facts that regulate biological activities of cationic peptides. Essentially, a good balance between the ability to adopt secondary structures, either preformed or inducible in lipid-mimicking environments, the amphipathicity and the overall hydrophobicity are required for the most desirable biological properties i.e. high antimicrobial activity coupled with low hemolytic activity (Kondejewski et al., 2002; Lee et al., 2004, Yount et al., 2006). While leucine was selected for its hydrophobic nature, lysine was selected for its cationic nature and for its easy and efficient handling in peptide synthesis compared to arginine. Furthermore, few reports showed the interest of Leu- and Lys-rich peptides as antimicrobial peptides (Blondelle and Houghten, 1992; Oren et al., 1997; Papo et al., 2002; Epand et al., 2003; Papo and Shai, 2005). For all molecules initially designed, a phenylalanine residue was included to facilitate HPLC analysis. Its bulky nonpolar aromatic side chain and consequently its lipophylic anchor character should make the Phe incorporation as positive element for antimicrobial activity . Finally, glutamic acid residue was also added to facilitate the solid-phase cyclization step. This amino acid residue would become a glutamine residue, an hydrophilic neutral amino acid, in the final molecules.

Irrespective of the procedure used, cyclization depended on the ring size and the phenylalanine configuration. Higher difficulties arose for the cyclization of linear L-Phe peptides of 4 and 6 residues than for the linear precursors of 8 and 10 residues. These could be explained by strains in the small peptides, while the ring closure of the octa- and decapeptides should be more flexible and should be less entropically disfavoured. According to the studies of Ehrlich and co-workers, the set reversing by L-Phe substitution for its enantiomer could be due to the incorporation of the D-amino acid residue, a turn-inducing element (Ehrlich et al., 1996). This D-amino acid incorporation would stabilize a turn structure promoting the cyclization of the open-

chain precursor of 4 and 6 residues while it would be much more troublesome for the longer peptide sequences removing the terminal amino group from the cyclization site.

The purities obtained depending of the solid support used and the need to increase the reagent excess with growing stacked SynPhase Lantern disks could be attributed to problems of SynPhase Lantern diffusion.

According to the Schwyzer hypothesis and the gramicidin S studies, the cyclic peptide CD results, in particular the octapeptide BPC8L CD spectrum, suggested the formation of β-sheet structure without β-hairpin turn (Gibbs et al., 1998) Unlike gramicidin S, they should not then form cyclic β-hairpins.

The improved antibacterial activity of cyclic peptides compared to their linear counterparts was associated to an increase of the hydrophobicity, represented as RP-HPLC retention time (t_R), and secondary structure content which are related to an enhanced amphipathicity. A decrease of antibacterial and hemolytic activities was observed when a D-Phe or a L-Leu was introduced into the cyclic sequences, which was attributed to their low amphipathicity as shown by their low secondary structure content and the low D-Phe cyclopeptide t_R. Interestingly, the efficacy of BP10L against the fungi *Verticillium dahliae* and *Fusarium oxysporum* did not depend to this structural exigency. Similar results was published with linear antibacterial androctonin variant (Bulet et al., 2004). This antifungal unstructured BP10L activity could indicate that there is a receptor-type interaction with a specific target. It is worth noting that its structured cyclic counterpart BPC10D had a similar antifungal activity against both fungi.

The cyclodecapeptide BPC10L was chosen to be the framework to improve the antibacterial activity and specificity. Although BPC10L exhibited high activity against bacteria and eukaryotic cells compared with the cyclooctapeptide BPC8L, its on-SynPhase Lantern cyclization step did not need microwave use. This element was made easier by the combinatorial library designs. The sequence changes were not expected to lead to significant structural changes due to the conformational constraint of these head-to-tail cyclic peptides. Indeed, the CD spectrum results of the

cyclodecapeptides tested showed β-sheet structures. The systematic modifications of the BPC10L amphipathicity as well as of the overall BPC10L hydrophobicity, indicated by the retention behaviour in RP-HPLC, did not correlate with antibacterial activity. In contrast, an increase of the amphipathicity and of the overall hydrophobicity were, in general, associated with an enhanced hemolytic activity.

The peptide susceptibility difference to *Xanthomonas vesicatoria*, *Pseudomonas syringae* and *Erwinia amylovora* explained by the different charge and lipid composition of the bacterial cell membranes which would influence the rates of peptide binding. It could also result from different mechanisms of action of the cyclic peptides depending on the bacteria. On the other hand, it is worth noting that the slow time kinetics of *P. syringae* and *X. vesicatoria* were similar to those obtained by antimicrobially active dermcidin peptides. Targets allowing receptor-type interaction could explain these slow bactericidal effects of cyclic peptide.

The rules determined by DOE accounted for compounds exhibiting the highest antibacterial activity against the three bacteria, in particular, peptides BPC088, BPC098, BPC194 and BPC198. The only exceptions were compounds BPC192 and BPC196 that did not display high antibacterial activity despite obeying the constraint which determined that the residues at the positions X^2 and X^3 had to be different. Furthermore, the cyclic decapeptides BPC092, BPC186, BPC190 and BPC202 with the lowest hemolytic activity obeyed to the main rules determined by DOE (lysine as residue at the positions X^2 and X^3). In addition, peptides BPC088, BPC090, BPC096, BPC098, BPC192, BPC194, BPC196 and BPC198 which fulfilled the secondary rule (different residues at the positions X^2 and X^3) showed really a low hemolytic level, specially the subset with lysine as residue at the position X^4 (BPC096, BPC098, BPC194 and BPC198). The use of the MINITAB software then allows quick generation of DOE and an easy interpretation of results. This design of experiments permits the simultaneous study of both effects of each residue and residue interactions on the biological properties that can not be observed by the methods commonly used in the design of peptide libraries.

De novo cyclic peptides significantly active against *Erwinia amylovora*, *Pseudomonas syringae* and *Xanthomonas vesicatoria* displaying a low eukaryotic

cytotoxicity, and *de novo* linear peptide against *Verticillium dahliae* and *Fusarium oxysporum* have been identified. Two peptides, BPC194 and BPC198, are especially, effective against the three devastating bacteria with MIC values ranging from 3 to 25 µM and show a low eukaryotic cytotoxicity at concentration 30-120 times higher than the MIC values. The *in vivo* efficacy and *in vitro* activity determination of these peptides compared to antibiotics used in agriculture for bacterial disease control such as streptomycin have not been yet performed. However, taking in to consideration the *in vitro* streptomycin activity of 2 to 9 µM for bacterial disease control, it seems possible to consider BPC194 and BPC198 as suitable candidates for the development of antibacterial agents for use in plant protection. And, though the in vivo efficacy is an important aspect to consider, several other aspects have to be considered before the development of AMPs as antimicrobial agents: their toxicity; microbial targets; stability; the acquisition of resistance of the microorganisms to such peptides; the modes of application; the social acceptance and the cost of manufacturing.

The peptides as amino acid sequences are degraded and, therefore, they are not expected to be any ecological risk. This constitutes an obvious advantage on commercialised pesticides. Furthermore, with their low eukaryotic cytotoxicity at concentration 375 µM (17% and 14%, respectively), BPC194 and BPC198 are not expected to present toxic effects at operational streptomycin doses for field treatment, namely around 100 µM. This is an important advantage compared with available pesticides.

An important disadvantage of natural peptides is their potential instability to proteases. Indeed, proteases from epiphytic microorganisms or intrinsic to the plant in internal tissues may degrade the peptides. The augmentation in peptide stability by cyclization largely allows the resolution of this problem and, consequently to increase the half-life of these peptides in the plant environment.

According to reported microbial resistance to antimicrobial peptides, the microbial resistance to the peptides presented in this work could occur. But if this unlikely event did occur it would be sometime in the distant future.

The sequence of BPC194, BPC198 and BP10L have a good solubility in water without the need to add extra organic solvent. Therefore, the solution could be

applied via the conventional techniques of spray, immersion or irrigation. The requisite peptide concentrations could be evaluated using microbiologic techniques and could depend on the affected plant, the pathogen, the disease spread, etc.

Another way to control some plant diseases could be transgenic plants capable of a self generating BP10L. This method had showed good results. However, recent surveys prove that a significant consumer percentage, mainly in Europe, are still not ready to accept transgenic food. It is for this reason that research and development on different management tools such as transgenic plants and effective antimicrobial peptides had to continue concurrently.

An important problem of antimicrobial peptide synthesis is the cost of manufacturing which is often expensive. However, BPC194, BPC198 and BP10L are small 10-amino acid peptides with simple structure of natural amino acids and their manufacture is simple with the usual protecting groups and reagents. These items contribute to the cost effectiveness of the manufacturing process.

The development of the cationic peptides presented in this work as commercial antimicrobial agents therefore appears to be, nowadays, a good option against phytopathogens of plant diseases of difficult control.

Conclusions

1. The solid-phase synthesis of head-to-tail cyclic peptides of 4 to 10 residues bearing cationic (Lys) and hydrophobic (Leu and Phe) amino acids has been made using resin and SynPhase Lanterns as solid support. A general protocol for the allyl group removal on SynPhase Lanterns has been established. An efficient and rapid head-to-tail cyclization of linear peptides of 6, 8 and 10 residues on SynPhase Lanterns under microwave irradiation has been set up. The cyclization protocol presented in this work is the first example of peptide cyclization on SynPhase^{TM} Lanterns under microwave irradiation.

2. The set of cyclic peptides of 4 to 10 residues were evaluated against their antimicrobial activity against *Erwinia amylovora*, *Pseudomonas syringae*, *Xanthomonas vesicatoria*, *Clavibacter michiganensis*, *Verticillium dahliae*, *Fusarium oxysporum*, *Penicillium expansum* and *Aspergillus niger*, and the hemolytic activity was determined. It has been shown that for these peptides:
    i. Conformational constraints imposed by cyclization are a requirement for bacterial activity and also increase peptide stability.
    ii. Higher activity has been observed for cyclic peptides containing an even number of residues.
    iii. Introduction of a D-amino acid has resulted in lower antibacterial and hemolytic activities, and higher stability to protease degradation.
    iv. Degree of structuring influences amphipathicity implying differences in activity between diastereoisomers.
    v. Modification of the hydrophobicity affects the balance between the antibacterial potency and cytotoxicity.

3. While the peptide BP10L exhibited antifungal activity against *V. dahliae* and *F. oxysporum* with MIC values ranging from 1.0-6.2 μM, its cyclic analogue BPC10L was the most active and displayed antibacterial activity at 12.5 μM and 6.25 μM against *P. syringae* and *X. vesicatoria*, respectively, as well as

an antifungal activity against *V. dahliae* and *F. oxysporum* with MIC values under 6.2 μM.

4. The improvement of the peptide BPC10L has been performed using a combinatorial chemistry approach, and cyclodecapeptides with better biological properties than BPC10L have been identified.

    i. The sequence pattern for high antibacterial activity and low hemolysis has been determined by DOE and is c(X^1-X^2-X^3-X^4-Lys-Phe-Lys-Lys-Leu-Gln) with different residues at the position X^2 and X^3 (main rule) and lysine as residue at the position X^4 (secondary rule).

    ii. BPC194 and BPC198 are the peptides with the better activity profile. They are active against *E. amylovora*, *X. vesicatoria* and *P. syringae*, with MIC values ranging from 3 to 25 μM, and display a low hemolytic activity.

    iii. The small size, simple structure, microcidal effect, and stability to protease degradation of the best peptides make them potential candidates for the development of antimicrobial agents for use in plant protection. In this aim, a patent was registered (P2005-03137).

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