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Structure-Activity Relationship of CbnB2 and LeuA, and the Search for the Putative Receptor of LeuA

by

Liang Zeng Yan 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

DEPARTMENT OF CHEMISTRY

Edmonton, Alberta

Spring 2000

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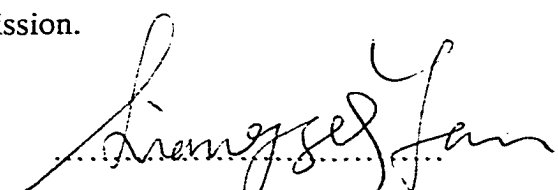

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Structure-Activity Relationship of CbnB2 and LeuA, and the Search for the Putative Receptor of LeuA** by **Liang Zeng Yan** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy.**

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To my parents, my wife Qingwen and my daughter Zixin

ABSTRACT

The redox state of carnobacteriocin B2 (CbnB2) was investigated and found to readily form a disulfide bond which had full antimicrobial activity. These results facilitated the construction of a solution structure of CbnB2 determined by NMR techniques.

Besides isolation from its natural producer *Carnobacterium piscicola* LV17B, CbnB2 was also prepared by overexpression as a fusion with a maltose-binding protein. Similar methodology permitted production of its precursor (CbnB2P), six mutants: CbnF3 (Y3F), CbnS33 (F33S), CbnI34 (V34I), CbnI37 (V37I), CbnG46 (R46G), Cbn28 (CbnB2(1-28)+ELTHL) and uniformly labelled ^{15}N-CbnB2. CbnB2, CbnI34, CbnI37, CbnG46 and ^{15}N-CbnB2 were fully active; CbnB2P, CbnF3, CbnS33, Cbn28 and the fusion proteins had greatly reduced or no activity. Induction experiments indicated that all were able to induce bacteriocin production except Cbn28. These results demonstrated that the induction function is more tolerant of structural modification. That growth of Bac$^-$ cultures was inhibited by its own bacteriocin, suggesting that production of bacteriocin and the immunity protein were concomitant.

Photoaffinity labelling of leucocin A (LeuA, from *Leuconostoc gelidum*) sensitive bacteria with biotinylated Bpa-LeuA (synthetic replacement of Phe22 of LeuA with *p*-benzoylphenylalanine) revealed two strongly labelled bands (*ca.* 14 and 20 kDa) for all tested bacteria. Bpa-LeuA could be connected to dodecylphosphocholine by UV irradiation. Photoaffinity labelling experiments using LeuA derivatives bearing a benzophenone photophore at various positions indicated no improvement in the labelling specificity.

Studies of the activity of those LeuA derivatives demonstrated that, while all the charged residues on the peptide chain were important for activity, some of them have stronger impact than others. These results indicated not only electrostatic interactions, but also specific molecule-molecule recognition might be involved in the antimicrobial function.

D-LeuA was manually synthesized using D-amino acids. D- and L-LeuA formed opposite helical structrures in TFE as indicated by CD spectra. D-LeuA had no antimicrobial activity. Agonistic or antagonistic effects between different bacteriocins and peptide fragments were also investigated.

Subtilosin A from *Bacillus subtilis* JH642 was found to be closely resembled a bacteriocin produced by *Bacillus subtilis* 168. An efficient method for production and isolation of subtilosin was established. Analyses of the two active components isolated from the supernatant of *Carnobacterium piscicola* UAL26 indicated that the two small peptides might share the same sequence with different oxidation states at the Met residue.

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LIST OF ABBREVIATIONS

[α]	specific rotation
Å	angstrom
A or Ala	alanine
ABC	ATP-binding cassette
ABq	AB coupling pattern (NMR)
Ac	acetyl
Anal.	analysis
APT	attached proton test
Ar	aryl
ATP	adenosine triphosphate
Boc	*tert*-butoxycarbonyl
Bpa	benzoylphenylalanine
br	broad
tBu	*tert*-butyl
c, or conc.	concentration
C or Cys	cysteine
calcd	calculated
CbnB2	carnobacteriocin B2
CD	circular dichroism
CI	chemical ionization
COSY	correlated spectroscopy
δ	chemical shift in parts per million downfield from TMS
d	doublet
D or Asp	aspartic acid

Da	dalton
DCC	1,3-dicyclohexylcarbodiimide
DMAP	4-*N,N*-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DPC	dodecylphosphocholine
DSS	2,2-dimethyl-2-silapentane-5-sulfonic acid
DTT	dithiothreitol
E or Glu	glutamic acid
EDTA	ethylenediaminetetraacetic acid disodium salt
EI	electron impact ionization
ES-MS	electrospray mass spectrometry
F or Phe	phenylalanine
FAB	fast atom bombardment
Fmoc	9-fluorenylmethyloxycarbonyl
G or Gly	glycine
H or His	histidine
HATU	2-(1*H*-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HPLC	high-performance liquid chromatography
I or Ile	isoleucine
IR	infrared
J	coupling constant
K or Lys	lysine
L or Leu	leucine
LAB	lactic acid bacteria

LeuA	leucocin A
lit.	literature reference
m	multiplet (for NMR) or medium (for IR)
m	meta
M or Met	methionine
MBP	maltose-binding protein
MeCN	acetonitrile
MHz	megahertz
MIC	minimal inhibition concentration
mp	melting point
MS	mass spectrometry
MW	molecular weight
m/z	mass to charge ratio
N or Asn	asparagine
NHS-biotin	biotinamidocaproate *N*-hydroxysuccinimide ester
NHS-SS-biotin	3-sulfosuccinimidyl 2-biotinamidoethyl-1,3-dithiopropionate
NMM	*N*-methylmorpholine
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
p	pentet
p	para
P or Pro	proline
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Ph	phenyl
Pmc	2,2,5,7,8-pentamethylchroman-6-sulphonyl
ppm	parts per million

q	quartet
Q or Gln	glutamine
quant.	quantitative yield
R or Arg	arginine
R_f	retardation factor
RMS	root mean square
RP	reverse phase
RT	retention time
s	singlet (for NMR) or strong (for IR)
S or Ser	serine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
t	triplet
t or *tert*	tertiary
T or Thr	threonine
TBS	Tris buffered saline
TFA	trifluoroacetic acid
TFE	trifluoroethanol
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
Tris	tris-(hydroxymethyl)aminomethane
Trt	trityl
UV	ultraviolet
V or Val	valine
W or Trp	tryptophan
Y or Tyr	tyrosine

Chapter I Carnobacteriocin B2 (CbnB2) and its Structural Variants

INTRODUCTION

1.1 Antimicrobial peptides in nature

Lipophilic antimicrobial peptides and proteins occur widely in virtually all living systems,[1-5] being produced by mammals, birds, amphibians, insects, plants, and micro-organisms. Although they form a diverse group of peptides as judged by their primary structures, they are often cationic and amphipathic. In mammals, antimicrobial peptides are expressed in phagocytes and mucosal epithelial cells.[6] In insects, bacterial infection induces the release of antibacterial peptides into the haemolymph.[2] In frogs, the skin contains glands which produce antimicrobial peptides enabling frogs with wounds to thrive in water dense with bacteria.[7] Antimicrobial peptides in animals are, therefore, thought to be key effector molecules in the innate immunity that is particularly important in early defense against invading microorganisms. As pointed out by Boman,[8] antimicrobial peptides are an ideal first line of defense because small peptides are made much faster than large proteins assuming a constant rate of peptide bond formation. Moreover, small peptides diffuse more rapidly in tissues and blood than large proteins and immune cells. The production of antimicrobial peptides by bacteria may also be thought of as a type of defense because the peptides kill invading bacteria that compete with the producer for nutrients.[8]

Most ribosomally-synthesized, antimicrobial peptides presently known have been identified during the past 20 years. The insect cecropins and the defensins isolated in the early 1980s being the first animal antimicrobial peptides thoroughly characterized.[9,10] Since then there has been an increasing interest in these antimicrobial peptides. This is in part due to the importance of developing new types of antimicrobial agents because of the increase in antibiotic-resistant bacterial strains resulting from the extensive use of antibiotics.[11] Interest in antimicrobial peptides is also due to their general biological and biochemical importance. By studying these peptides, insight has been gained into host defense systems, membrane-protein interactions, and protein modification and secretion. Some antimicrobial

peptides contain D-amino acids and can be used as a model system for studying how D-amino acids may be formed in ribosomally-synthesized polypeptides.[12]

Antimicrobial peptides of eukaryotes and prokaryotes are produced and function in entirely different settings. However, apparently diverse biological systems often have many elements in common at a molecular level. The prokaryote has been an especially useful model system from which much of our general and fundamental knowledge in biochemistry and molecular biology has been obtained.[12] Bacterial ribosomally-synthesized antimicrobial polypeptides are generally referred to as bacteriocins. Bacteriocins are produced by both Gram-positive and Gram-negative bacteria.[13]

1.2 Antimicrobial peptides from lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are Gram-positive, non-spore-forming cocci, coccobacilli, or rods with a DNA base composition of less than 50 mol% guanosine + cytidine. They need a fermentable carbohydrate for growth; glucose is converted mainly to lactic acid (homofermentatives) or to lactic acid, carbon dioxide, and ethanol or acetic acid or both (heterofermentatives). LAB are widely distributed in a variety of habitats. The ecological niches of LAB include not only foods such as dairy products, sourdough bread, fermented vegetables and meats, and beverages, but also they can be found in silage, sewage, on plants, and in the genital, intestinal and respiratory tracts of humans and animals.[14]

In recent years LAB have become the focus of an increasing amount of fundamental and applied research. This trend is mainly due to the importance of many of these organisms in food and feed technology.[15] These organisms also show promise towards human and animal health applications such as probiotics.[16] LAB are utilized for the manufacture of a wide variety of fermented food and beverage products. LAB contribute to the organoleptic properties of the fermented products and they are mainly associated with the release into the environment of a variety of metabolic products generated at the expense

of food components. They also contribute to the improvement of shelf-life and safety of the product by inhibiting the growth of food-spoiling bacteria through the production of growth inhibiting substances (bacteriocins) and other substances.[17]

The early definition of bacteriocins was based on, and referred to, proteins like colicins which are produced by several Gram-negative microorganisms.[18] The identification of antimicrobial proteins and peptides among Gram-positive bacteria which shared characteristics with colicins, but showed clear differences from them, called for a re-definition of bacteriocins. Tagg *et al.*[19] proposed six criteria to define an antimicrobial compound as a bacteriocin:

a. a narrow spectrum of inhibitory activity centered about the homologous species;

b. the presence of an essential, biologically-active protein moiety;

c. a bactericidal mode of action;

d. attachment to a specific cell receptor;

e. plasmid-borne genetic determinants of bacteriocin and of host cell bacteriocin immunity;

f. production by lethal biosynthesis (*i.e.* commitment of the bacterium to bacteriocin production will ultimately lead to cell death).

Understanding the diversity already existing among these antimicrobial compounds, Tagg suggested that an antimicrobial compound should meet at least criteria (b) and (c) to be recognized as a bacteriocin. Later, Klaenhammer[20] defined bacteriocins as "proteins or protein complexes with bactericidal activity against species which are usually closely related to the producer bacterium".

Bacteriocins from LAB are currently classified into three main groups: class I consisting of modified bacteriocins (the lantibiotics); class II consisting of unmodified, small, heat-stable peptides; and class III consisting of large, heat-labile proteins.[21] Based on common features, some of the class II bacteriocins can be further divided into separated sub-groups:

IIa -- pediocin-like bacteriocins with strong anti-*Listerial* activity;

IIb -- two-peptide bacteriocins;

IIc -- *sec*-dependent secreted bacteriocins.

These antimicrobial compounds show promise towards human and animal health applications, such as new approaches for dealing with antibiotic-resistant bacteria.[12,16] Bacteriocins could play a role in the microbial ecology of food by adversely affecting sensitive microorganisms and conferring a competitive advantage to the producer strains.[20] The antimicrobial activity of bacteriocins against foodborne pathogens and organisms with high spoilage capability makes these compounds candidates for use as biopreservatives in conjunction with or in place of, traditional chemical preservatives. The best example of the application of a bacteriocin as a biopreservative is the use of the class I lantibiotic nisin A, the most characterized bacteriocin produced by LAB. Nisin A is produced by strains of *Lactococcus lactis* subsp. *lactis*, and it is active against a wide range of Gram-positive bacteria.[22] The use of nisin A as a food preservative is approved in over 40 countries and applications have been developed for processed cheese, dairy desserts, milk, fish, bacon, frankfurters, fermented beverages and several canned foods.[17]

1.3 Biosynthesis of class II bacteriocins from LAB

With the exception of the very few bacteriocins containing a *sec*-dependent signal sequence,[23] class II bacteriocins are synthesized in a pre-form containing an N-terminal double-glycine leader. The double-glycine leader-containing bacteriocins are processed concomitantly with externalization by a dedicated ABC-transporter which has been shown to possess an N-terminal proteolytic domain (see Figure 1).[21,24] The synthesis of LAB bacteriocins rests upon a general genetic structure encompassing four different genes which encode the basic functions required for production of the extracellular antimicrobial activity. These four genes are:

a. the structural gene for encoding the pre-bacteriocin;

b. a dedicated immunity gene which is always located next to the bacteriocin gene and on the same transcription unit;

c. a gene encoding for a dedicated ABC-transporter which externalizes the bacteriocin concomitant with processing of the leader;

d. a gene encoding for an accessory protein essential for the externalization of the bacteriocin, the specific role of which is yet to be elucidated.

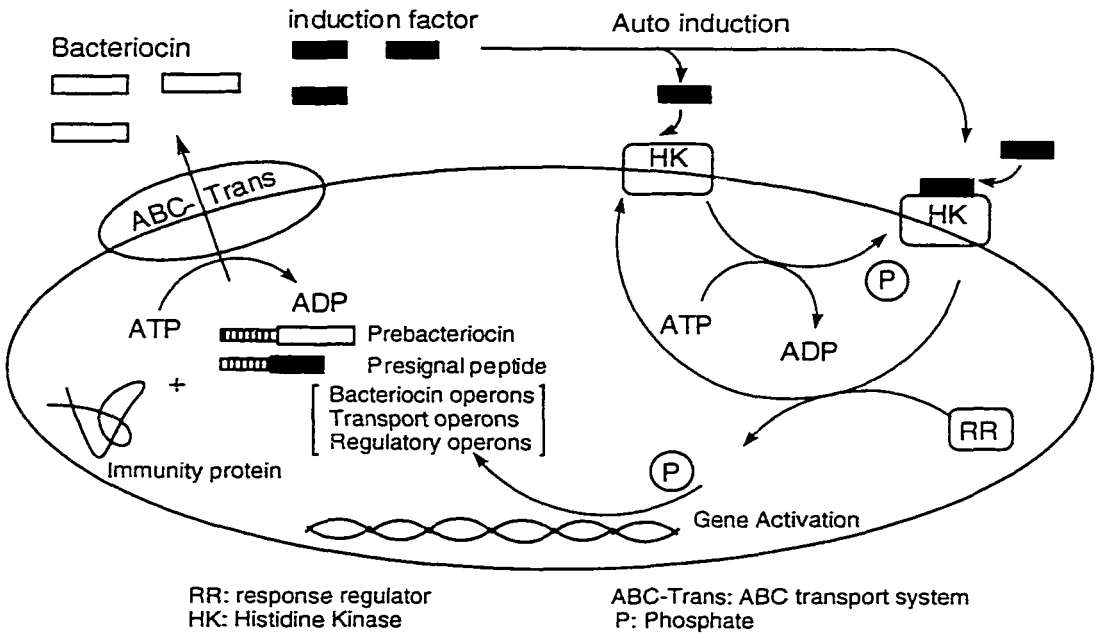


Figure 1. Regulation and production of class II bacteriocins in the cell[21, 24]

The structural gene encodes the pre-form of the bacteriocin containing an N-terminal leader sequence whose function seems to prevent the bacteriocin from being biologically active while detained inside the producer and to provide the recognition signal for the transporter system. It has been found that pre-bacteriocins show much less potency than the active bacteriocin, or even no activity.[25,26] However, recently it was reported that the pediocin AcH precursor is biologically active.[27] Stiles and co-workers[28] fused the leader peptides of leucocin A (LeuA), lactococcin A or colicin V, respectively, to divergicin A, a

bacteriocin from *Carnobacterium divergens* which is secreted *via* the cell's general secretion pathway. The different leader peptides directed the production of divergicin in the homologous hosts. In some cases production of divergicin was also observed when the leader peptides were used in heterologous hosts. These observations demonstrated that leader peptides are involved in bacteriocin production and translocation as well as other biological functionalities.

Bacteriocin producers have developed a protection system against their own bacteriocin. Each bacteriocin has its own dedicated protein conferring immunity, which is expressed concomitantly with the bacteriocin. In all bacteriocin operons studied so far, potential immunity genes have been identified next to and downstream of the bacteriocin structural genes. Whereas synthesis of extracellular bacteriocin requires a dedicated secretion/processing system, the immunity protein is functionally expressed in the absence of transport and processing.[29] The immunity proteins are fairly small, ranging from 50 to approximately 150 amino acids. The homology between various immunity proteins is surprisingly low considering that class IIa bacteriocins show 38-55% identity. For example, LeuA and mesentericin Y105 (MesY105) differ only at two residues (Phe22-Ala and Val26-Ile), but their immunity proteins only share 74% homology.[30,31] This lack of similarity is particularly strong between the immunity proteins of the two identical bacteriocins, sakacin A and curvacin A,[32,33] where the putative immunity proteins are 90 and 51 amino acids, respectively. This observation may suggest that no direct interaction occurs between bacteriocins and their immunity proteins.[34] It has also been proposed that the immunity protein of lactococcin A is closely associated with a postulated bacteriocin receptor.[35]

As mentioned above, most bacteriocins are synthesized in a preform containing an N-terminal extension. The conservation of the cleavage site of the leaders strongly suggests a common processing mechanism. It is well established that secretion of the double-glycine leader-containing bacteriocins is mediated by a dedicated trans-membrane translocator

belonging to the ATP-binding cassette (ABC) transporter superfamily.[36] The ABC-transporter gene is usually part of the bacteriocin operon or found on a separate operon in the vicinity of the bacteriocin-containing operon. It has been generally recognized[21] that all ABC-transporters contains three domains: an N-terminal proteolytic domain (~150 amino acids), a membrane spanning domain (~300 amino acids) and a C-terminal ATP-binding domain (~250 amino acids). It has been demonstrated that the ABC-transporter is a cysteine protease.[36] The two processes of cleavage and translocation are integrated and the leader peptide serves as a recognition signal for the transmembrane transport process of the bacteriocin. Further studies have shown that the ABC-transporter proteins from different LAB share high sequence homology, including the conserved cysteine residues at their N-terminal proteolytic domain.[30,36-38]

The production of some class II bacteriocins has been shown to be transcriptionally regulated through a signal transduction system which consists of three components: an induction factor (IF), histidine protein kinase (HK), and a response regulator (RR). This is an extension of the more common two-component signal transduction system found in prokaryotic organisms.[21] It has been demonstrated that some bacteriocin producers also are self-regulated by their own bacteriocin, like nisin,[39] sakacin P,[40] and carnobacteriocin B2 (CbnB2).[25,41] This suggests that some bacteriocins have a dual function.

1.4 Mode of action and structure-activity relationship

It is still not very clear how bacteriocins from Gram-positive bacteria exert their effect. It has been proposed that some bacteriocins kill target bacteria by forming pores in the cytoplasmic membrane. The pores would allow the passage of hydrophilic solutes with molecular masses of up to 0.5 kDa,[42] but little is known of the actual mechanism of pore formation. Although class IIa bacteriocins share high sequence homology, especially at their N-termini, their antimicrobial spectra are quite different. Some experiments demonstrated that specific membrane receptor proteins might be involved in bacteriocin

function.[35,43] The antimicrobial activity of pediocin PA-1 was specifically inhibited by a 15-mer fragment derived from its own sequence.[44] This kind of antagonistic effect was also found between nisin and a nisin(1-12) fragment.[45] These results suggest that this area of the peptide chain is involved in receptor binding. However, the presence of such a receptor is still speculative and the molecular entity has yet to be identified.

Structure-function correlations of some antimicrobial peptides from eukaryotes have been extensively studied.[46] Some of these peptides could be truncated to a certain length without compromising their antimicrobial activity, but charged residues were found to be critically important for their biological function. Little work has been done on structure-activity relationship of class II bacteriocins. A recent report on synthetic versions of MesY105, a 37 amino acid YGNGV bacteriocin, demonstrated that this peptide cannot be truncated at either the C- or N-terminus without loss of activity.[47] It was also shown that replacement of cysteine residues at position 9 and 14 with serine drastically compromises its activity. The dithiothreitol-reduced pediocin PA-1, in which the cysteine residues were present in the reduced form, has been shown to be inactive, suggesting that intact disulfide linkages are essential for the activity of this bacteriocin.[48] The two cysteine residues are one of the most conserved sequences in this class of bacteriocin. However, It may be that the disulfide bridge of LeuA is not critically important for activity.[49,50]

It is interesting to note that different bacteria may produce the same bacteriocin. As shown in Figure 2, pediocin PA-1[51] produced by *Pediococcus acidilactici* PAC 1.0 is the same as pediocin AcH[52] produced by *Pediococcus acidilactici* H. The same is observed for piscicocin V1a[53] produced by *Carnobacterium piscicola* V1 isolated from fish *vs.* piscicolin 126[54] produced by *Carnobacterium piscicola* JG126 isolated from spoiled ham; piscicocin V1b[53] produced by *Carnobacterium piscicola* V1 *vs.* CbnBM1[37] produced by *Carnobacterium piscicola* LV17B, a *Lactobacillus* type organism isolated from chill-stored meats; sakacin P[55] produced by *Lactobacillus sake* LTH673 *vs.* sakacin 674[56] produced by

Lactobacillus sake Lb674; sakacin A[55] produced by *Lactobacillus sake* Lb706 *vs.* curvacin A[33] produced by *Lactobacillus curvatus* LTH1174.

	1	11	21	31	41
LeuA[49,50]	KYYGN GVHCT	KSGCS	VNWGE	AFSAG VHRLA	NGGNG FW
MesY105[31]	KYYGN GVHCT	KSGCS	VNWGE	AASAG IHRLA	NGGNG FW
Pediocin PA-1[51,52]	KYYGN GVTCG	KHSCS	VDWGK	ATTCI INNGA	MAWAT GGHQG NHKC
Piscicocin V1a[53,54]	KYYGN GVSCN	KNGCT	VDWSK	AIGII GNNAA	ANLTT GGAAG WNKG
Sakacin P[55,56]	KYYGN GVHCG	KHSCT	VDWGT	AIGNI GNNAA	ANWAT GGNAG WNK
Mundticin[57]	KYYGN GVSCN	KKGCS	VDWGK	AIGII GNNSA	ANLAT GGAAG WSK
Bifidocin B[58]	KYYGN GVTCG	LHDCR	VDRGK	ATCGI INNGG	MWGDI G
Sakacin A[55,59]	ARSYGN GVYCN	NKKCW	VNRGE	ATQSI IGGMI	SGWAS GLAGM
CbnBM1[37]	AISYGN GVYCN	KEKCW	VNKAE	NKQAI TGIVI	GGWAS SLAGM GH
CbnB2[37]	VNYGN GVSCS	KTKCS	VNWGQ	AFQER YTAGI	NSFVS GVASG AGSIG RRP

Figure 2. Sequence homology of selected class IIa bacteriocins (conserved sequences are underlined)

The YGNGVXC motif of type IIa bacteriocins usually has the cysteine as part of a disulfide bridge.[12] These bacteriocins may require an unidentified membrane-bound protein receptor for activity, and both the N-terminal motif as well as the C-terminal portion have been suggested as recognition sequences for the putative receptor protein.[47] Thus far the only type II bacteriocin whose three-dimensional structure has been solved is LeuA,[60] a 37 residue peptide isolated from *Leuconostoc gelidum* UAL187.[49,50] NMR studies show that LeuA adopts a well-defined tertiary structure which is composed of a three-stranded antiparallel β-sheet (residues 2 to 16) followed by an amphiphilic α-helix (residues 17 to

31) in both TFE and dodecylphosphocholine (DPC) micelles. Interestingly, the same peptide is essentially unstructured in water or aqueous DMSO.[60]

1.5 Preparation of antimicrobial peptides

Access to a large quantity of purified bacteriocins is important both for practical applications of these antimicrobial peptides and for studies of their structures. The isolation of bacteriocins from their natural producers often proves to be time-consuming and frequently gives a low yield. Solid-phase peptide synthesis is an alternative means to prepare small to medium sized peptides and proteins.[47,61] In recent years there has also been considerable progress in solution phase protein synthesis.[62,63] Modern molecular biology is also a good alternative for the production of large amounts of protein. This involves the overexpression of the target protein in a foreign host; for simplicity of handling this is usually *Escherichia coli*. Many methods have been developed to accelerate purification of the recombinant protein from the foreign host. For example, the introduction of a histidine tail to the recombinant protein permits a one-step purification by metal affinity chromatography.[64] Another successful example is the maltose-binding protein (MBP) fusion expression system.[65-67] A plasmid vector is constructed to direct the synthesis of high levels of fusions between a target protein and MBP in *E. coli*. The MBP domain is used to purify the fusion protein in a one step procedure by affinity chromatography to crosslinked amylose resin (starch).[66] The fusion protein contains the recognition sequence (Ile-Glu-Gly-Arg) for blood coagulation factor Xa protease between the two domains.[67] Cleavage by factor Xa separates the two domains and the target protein domain can then be purified by repeating the affinity chromatography step or by other chromatographic techniques, such as RP-HPLC.[25,35]

1.6 Carnobacteriocin B2 (CbnB2)

CbnB2 is a well-characterized, heat-stable, cationic bacteriocin which is present in the culture supernatant of *Carnobacterium piscicola* LV17B, a *Lactobacillus*-type organism isolated from chill-stored meats.[37] CbnB2 targets the cytoplasmic membrane of sensitive cells and causes dissipation of the proton motive force with leakage of intracellular components, possibly through formation of pores. Its spectrum of antimicrobial activity includes many LAB, as well as strains of potentially pathogenic *Enterococcus* and *Listeria* species.[37] A second and unexpected biological role of CbnB2 is to function as an inducer peptide. Addition of the bacteriocin to cultures of *C. piscicola* LV17B which had lost the ability to produce bacteriocin upon dilution below 1 x 10^4 cells per milliliter induced the rescue of the Bac^+ phenotype.[41] CbnB2 is ribosomally synthesized as pre-carnobacteriocin B2 (CbnB2P), which contains 66 amino acids.[37] This undergoes post-translational cleavage at a Gly^{-2}-Gly^{-1} site to remove an 18 amino acid leader sequence from the N-terminus to yield the mature bacteriocin of 48 amino acids which is found in the culture supernatant. The genetic determinant of CbnB2 (*cbnB2*) is located in a gene cluster present on a 61 kb plasmid in *C. piscicola* LV17B.[37] This cluster has four other genes which are required for the wild type Bac^+Imm^+ phenotype which, based on sequence homology, function as: (i) an ATP-binding cassette type transporter; (ii) an "accessory protein" of the bacterial *sec*-independent secretion system; (iii) a histidine protein kinase; (iv) a response regulator of the bacterial two-component signal transduction system.[68] A fifth gene in the cluster, *cbiB2*, encodes for an immunity protein (111 amino acids) that protects the producer organism from the antimicrobial effect of CbnB2.[34]

Although CbnB2 shows high sequence similarity to other type IIa bacteriocins near the N-terminus (Figure 2, page 10), uniquely, CbnB2 was obtained without a disulfide bond between the two conserved cysteines, Cys9 and Cys14.[37] This disulfide was deemed to be critical for antimicrobial activity of MesY105,[47] a peptide which differs from LeuA only at positions 22 and 26. Further work is needed to rationalize the variation.

One goal of this thesis research was to ascertain the structural consequences and importance of the disulfide functionality in CbnB2 and to assess whether there is a common "receptor-binding" structural motif.[47] To achieve this, the three-dimensional solution structure of CbnB2 is determined using NMR spectroscopy in a collaborative effort. The redox chemistry of the two conserved cysteines in CbnB2 and model peptides is examined using mass spectrometry and chemical modification experiments. Comparison of the structures of LeuA and CbnB2 shows that high sequence conservation of the N-terminal region does not generate identical structural motifs, whereas the more variable sequences of the central region result in similar amphiphilic α-helices that may be involved in receptor recognition.

RESULTS AND DISCUSSION

1.7 Investigations into the redox state of CbnB2

Mass spectrometric analysis of CbnB2 after its initial isolation[37] indicated that it was apparently unique among type IIa bacteriocins in that it did not possess a disulfide bond between the conserved cysteines. Preliminary NMR investigation showed that the chemical shift values for residues in the N-terminal region were quite different from those of LeuA, whose sequence is similar but does possess a disulfide linkage. Because such a linkage was reported to be essential for the biological activity of MesY105,[47] and because LeuA could not be easily kept in a reduced (dithiol) state under aerobic conditions, the oxidation state of the two cysteine residues in antimicrobially active CbnB2 was re-examined in detail as a prelude to the determination of its three dimensional structure. The approaches employed a synthetic truncated peptide fragment CbnB2(1-22) in conjunction with mass spectrometry and thiol modification experiments.

Following the literature procedure,[37] CbnB2 was successfully isolated and purified. The identity of the newly purified CbnB2 from its natural producer *C. piscicola* LV17B was confirmed by mass spectrometry and co-injection on HPLC with the previously-isolated sample of CbnB2[37] (hereafter referred to as an authentic sample), as well as by biological assays. The molecular weight was determined to be 4968.00 +/- 0.49 (cal. 4969.51 for reduced form). It was noted, however, that this value varied up to one unit from run to run due to instrumental error. Because the molecular weights of the oxidized form and reduced form of CbnB2 are different only in two units, mass spectral data alone could not confirm the oxidation state of the cysteine residues. Co-injection of the newly prepared CbnB2 and the authentic sample on HPLC gave a single peak with the same retention time as that of the authentic sample. The specific activity of the purified CbnB2 was determined to be 50 AU/μg (1 AU is the amount of bacteriocin required to produce

visible clearing on a lawn of the indicator strain) towards *C. divergens* LV13 using spot-on-lawn technique,[69, 70] which was also the same as that of the authentic sample.[37]

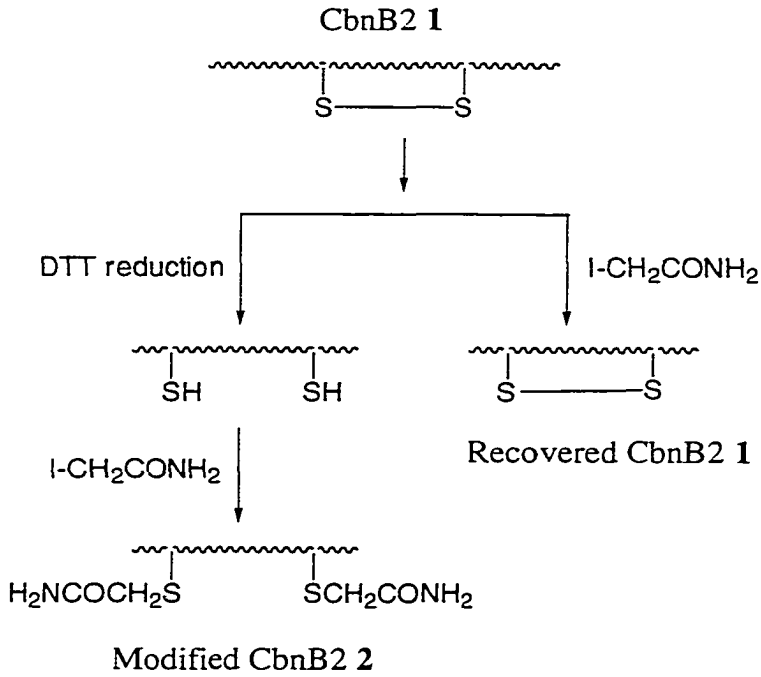
Under the assumption that CbnB2 exists in a reduced form.[37] we investigated the oxidation of CbnB2, *i.e.* the formation of a disulfide bridge between Cys9 and Cys14 at its N-terminus. Attempts at oxidizing CbnB2 in 0.1 M ammonium bicarbonate buffer, pH 8.0 did not produce any conclusive information regarding the redox state of CbnB2. The molecular weights of the isolated products after oxidization of different batches of protein were not consistent. Although the variation was only one to two units, as mentioned above, such an error is too large to provide conclusive information about the existence or absence of a disulfide bridge. Prolonged exposure of CbnB2 to oxygen in the basic buffer resulted in degradation of the peptide and no CbnB2 could be recovered. It is known that LeuA, a similar class IIa bacteriocin, is not stable in basic environments.[49]

Ellman's reagent has been widely used for the quantification of thiols in peptides and proteins.[71-73] It reacts selectively with free thiols to form a mixed disulfide. Unfortunately, attempts at directly derivatizing CbnB2 with Ellman's reagent were unsuccessful.

These results forced a re-evaluation of the assumption that CbnB2 exists in a reduced form. Thus, if the two cysteine residues exist in an oxidized form, prior reduction would be required in order to derivatize the thiols.

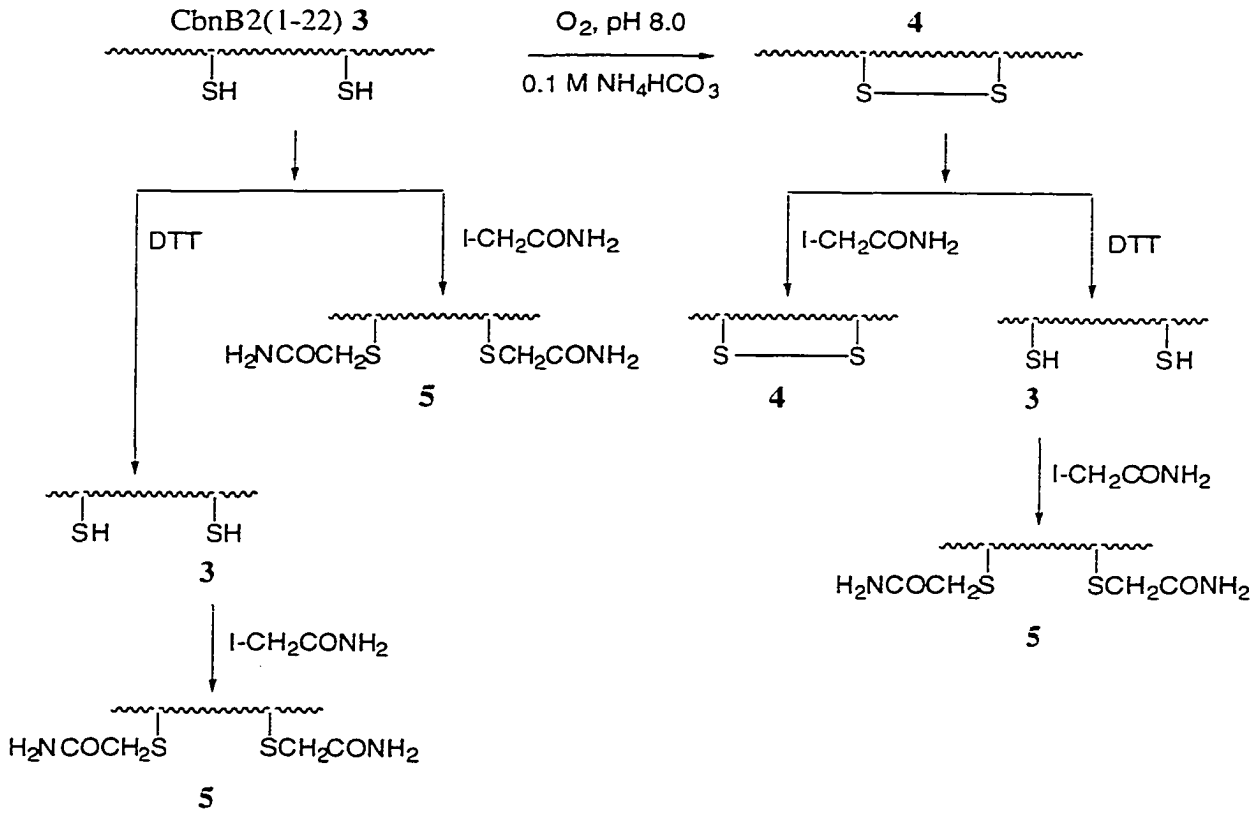
The redox status of CbnB2 was further investigated by attempted carboamidomethylation of the CbnB2 cysteines (Scheme 1). The molecular weights of the CbnB2 products clearly indicated that CbnB2 could be carboamidomethylated only if the sample was first reduced with DTT. This demonstrated that a disulfide bond existed between the two cysteines of CbnB2 (Cys9 and Cys14) prior to reduction for samples isolated by our current protocol. Furthermore, activity studies showed that the recovered unmodified CbnB2 maintained its antimicrobial activity, whereas there was no detectable activity of the modified product, *S,S'*-bis(carboamidomethyl) CbnB2 **2**. Disruption of the

disulfide bridge and modification of the thiol groups resulted in the loss of its antimicrobial activity.



Scheme 1

In order to verify the above results, an N-terminal peptide fragment (VNYGN GVSCS KTKCS VNWGQ AF) of CbnB2 was synthesized. The synthetic 22-mer peptide fragment CbnB2(1-22) **3** gave the expected molecular weight and it was shown to be homogeneous by RP-HPLC. After the peptide was cleaved from the resin, it was always kept in acidic environments, and therefore the cysteine residues could stay in the reduced form. After being oxidized in ammonium bicarbonate buffer overnight, this peptide fragment gave two main fractions on RP-HPLC. The retention times of these two fractions differed by about one minute and the two components were easily separated. One of the fractions was the starting material **3** (same retention time and MS), and the other fraction gave a molecular weight which was about two units less than that of the starting synthetic material. The second fraction was, therefore, assumed to be the oxidized form **4** of the 22-mer peptide fragment.



Scheme 2

Both the oxidized form **4** and the reduced form **3** of the 22-mer peptide were subjected to carboamidomethylation in the same way as that described for CbnB2 (Scheme 2). The two samples obviously showed different modification patterns. For the oxidized synthetic 22-mer **4**, the same modification patterns as those seen for CbnB2 were observed. Specifically, the two cysteine thiol groups were carboamidomethylated only if the sample was first reduced with DTT. For the reduced form of the 22-mer **3**, the two cysteines could be carboamidomethylated regardless of whether or not DTT was used. These carboamidomethylation patterns further confirmed that CbnB2 was isolated in an oxidized form, and that **4** was the oxidized form of **3**.

1.8 Three-dimensional solution structure of CbnB2 and comparison with that of LeuA

1.8.1 Chemical shift assignment, secondary and tertiary structures of CbnB2 (done by Drs. Y. Wang, S. Chai and N. F. Gallagher)

An understanding of the three dimensional structure of bacteriocins is a critical part of the study of structure-activity relationships. Hence, a description of the determination of the structure of CbnB2 by NMR experiments is provided here even though a major portion of the work was done by other researchers in our laboratory and that of Professor David Wishart (Faculty of Pharmacy). A substantial portion of the following discussion is from a manuscript that has been accepted by *Biochemistry* for publication.

The ^{1}H NMR chemical shift assignments, including the $^3J_{HNH\alpha}$ coupling constants and amide proton temperature coefficients for CbnB2, are attached in the appendix. The observed medium-range (i, i+2; i, i+3) NOEs, strong amide proton (i, i+1) NOEs and characteristically small $^3J_{HNH\alpha}$ coupling constants from Trp18 to Ser39 indicate that CbnB2 adopts a helical configuration in this region.

It has been shown that ^{1}H NMR chemical shifts are strongly dependent on the character and nature of protein secondary structure.[74] Specifically, it has been observed that α-H chemical shifts experience an upfield shift (with respect to the random coil value) when in a helical configuration and a comparable downfield shift when in a β-strand or extended configuration.[74-76] The almost continuous set of upfield α-proton chemical shifts from Trp18 to Ser39 clearly indicates the existence of an α helix in this region. Small amide temperature coefficients (<3 ppb/°C), also found over the same region, confirm the presence of extensive hydrogen bonding and are further suggestive of a well-defined α-helical structure.

Some weak NOEs between the Val7 γ CH_3 protons and the Tyr3 ring protons were observed, but no other significant medium or long-range NOEs could be found at the N-terminus. These data, together with the random coil-like $^3J_{HNH\alpha}$ coupling constants (~ 7 Hz) measured throughout this region, demonstrate that the N-terminus of CbnB2 is essentially unstructured. It is also worth noting that no NOEs (at any temperature) could be detected between Cys 9 and Cys 14 or any other residues involved in the N-terminal disulfide bond. The lack of NOE data in this region made it impossible to determine by NMR whether CbnB2 was, in fact, reduced or oxidized. Confirmation of the oxidation state of the NMR sample by subsequent mass spectrometry and chemical modification experiments eventually allowed a disulfide constraint to be built into the final model. Evidently the rapid motions experienced by the N-terminus lead to a time-averaged dissipation of the expected NOEs.[77] As for the C-terminus of CbnB2 (residues 41-48), the absence of any medium or long range NOEs, the "random-coil" $^3J_{HNH\alpha}$ coupling constants (~ 7 Hz) and featureless net structural α-proton chemical shifts suggest that this region is largely unstructured.

MOLMOL[78] was used to visualize and superimpose structures, and also to calculate average root mean square (RMS) deviation values. Twenty of the initial 30 structures were taken into the final ensemble of CbnB2 solution structures. Closer examination of the helical region reveals that the side chains of several hydrophobic residues Trp18, Phe22, Tyr26, Ile30, Phe33, Val34 and Val37 form a long, well-defined hydrophobic surface on one side of the helix (Figure 3). In contrast, the side-chains of several hydrophilic residues Asn17, Gln20, Glu24, Arg25, Asn31, Ser35, and Ser39 comprise a well-defined hydrophilic surface on the opposite side. The solution structure of CbnB2 together with the refined structure of LeuA are shown in Figure 4.

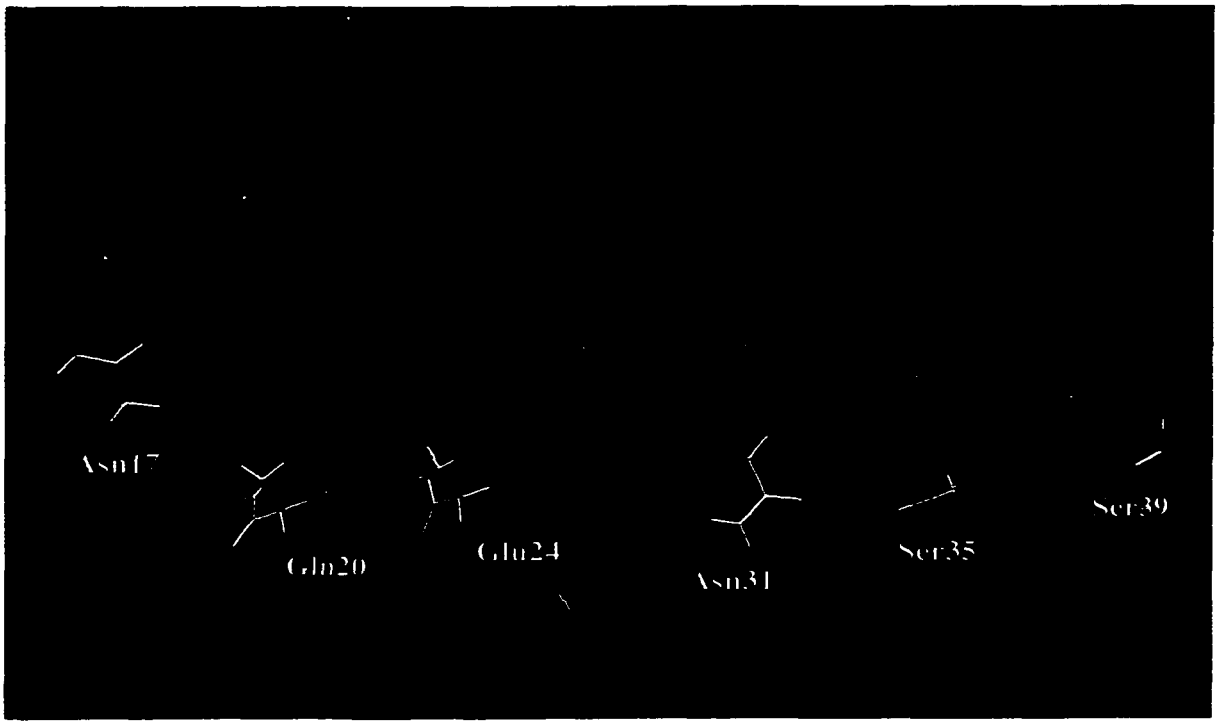


Figure 3. Ribbon diagram of amphipathic helical section of CbnB2 (by Dr. Y. Wang)

Figure 4. Superimposition of ribbon diagrams of CbnB2 and LeuA based on the alignment of backbone atoms from Trp18 to Phe22 (by Dr. Y. Wang)

1.8.2 Comparison of solution structures of LeuA and CbnB2

Like other antimicrobial peptides, LAB bacteriocins are believed to disrupt membranes of target bacteria and lead to leakage of cellular contents.[12-44] Some, such as the lantibiotic nisin A, do not require a receptor protein in the target,[79] but others may recognize a receptor, as yet unidentified, in target organisms. Several lines of evidence support the existence of such a putative receptor protein for type IIa bacteriocins. There is a high level of specificity for particular target organisms among groups that are closely-related. Interchange of large domains of different type IIa bacteriocins, such as pediocin, sakacin P and curvacin A (sakacin A) (Figure 2, page 10), gives chimeric antimicrobial compounds whose specificity corresponds to the C-terminal portion.[30] Recent work shows that a peptide fragment (residues 20-34) of pediocin PA-1 specifically inhibits pediocin PA-1 activity, though it is not antimicrobially active and does not effectively inhibit closely related bacteriocins such as LeuA, sakacin P and curvacin A.[44] Although the latter studies indicate that the more sequence-variable C-terminal portion is involved in receptor recognition, other workers suggest recognition of the highly-conserved N-terminal portion.[47] It is clear that the entire peptide is essential for overall activity. For example, large fragments of these bacteriocins are inactive and deletion of a single amino acid from the C-terminus of MesY105 leads to a drop in activity by 10^5 fold.[47] Detailed knowledge of the three dimensional structure of these bacteriocins is an essential step in understanding their interactions with a protein receptor.

Since the YGNGVXC sequence had been suggested to be a recognition element for a membrane-bound protein receptor,[47] it was of interest to compare the three-dimensional structure of this region in CbnB2 and LeuA. It is clear that the CbnB2 N-terminus is highly disordered, whereas the corresponding region in LeuA forms a well defined antiparallel β-sheet (Figure 4). For CbnB2, the NOEs observed between the Val7 γ CH_3 protons and the Tyr3 ring protons indicate the existence of a weak, or poorly defined reverse turn in this region. The proximity of these side chains provides a small hydrophobic surface that may

be important for biological activity. In this regard, it has been shown that the substitution of Tyr3 with Phe can substantially reduce the antimicrobial activity of CbnB2.[25] Interestingly, both peptides share a well-defined amphipathic α-helix located near their *C*-termini (residues 19-39 for CbnB2; residues 17-31 for LeuA) despite much greater variability in sequence (Figure 4). This result is in accord with the proposal that this region of the bacteriocin determines target specificity through binding of the putative receptor protein.[44,80]

In structural biology it is generally believed that shared sequence homology implies shared structural homology.[81] The actual level and extent of sequence homology is critical in determining the level and extent of observed structural homology.[82,83] Normally, short regions of sequence homology require higher levels of sequence identity to confer structural conservation.[83] There are many instances where identical penta- and hexapeptides have been found to have completely different tertiary structures in large proteins.[82,84] There are also several reports that single amino acid substitutions over longer stretches of amino acids have led to significantly different structures. For example, bovine and porcine pancreatic phospholipase share a segment of 13 amino acids that differs by just one residue (LDSCKV(F)LVDNPYT) but X-ray analysis of these two closely related enzymes reveals that this stretch of amino acids assumes two quite different conformations.[82,85] However, empirical studies by Sander and Schneider[86] demonstrate that for an alignment spanning 24 residues, a level of sequence identity of 48% should be enough to give structural homology. Hence, the 66% sequence identity between CbnB2 and LeuA over a similar segment length at the N-terminus would be expected to give close structural homology (see Figure 5).

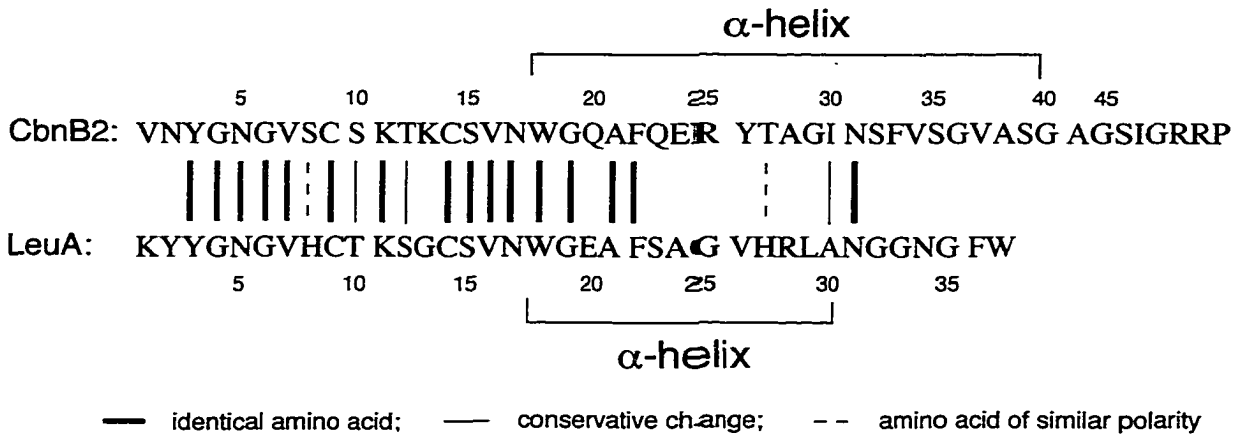


Figure 5. Primary sequence homology of CbnB2 and LeuA

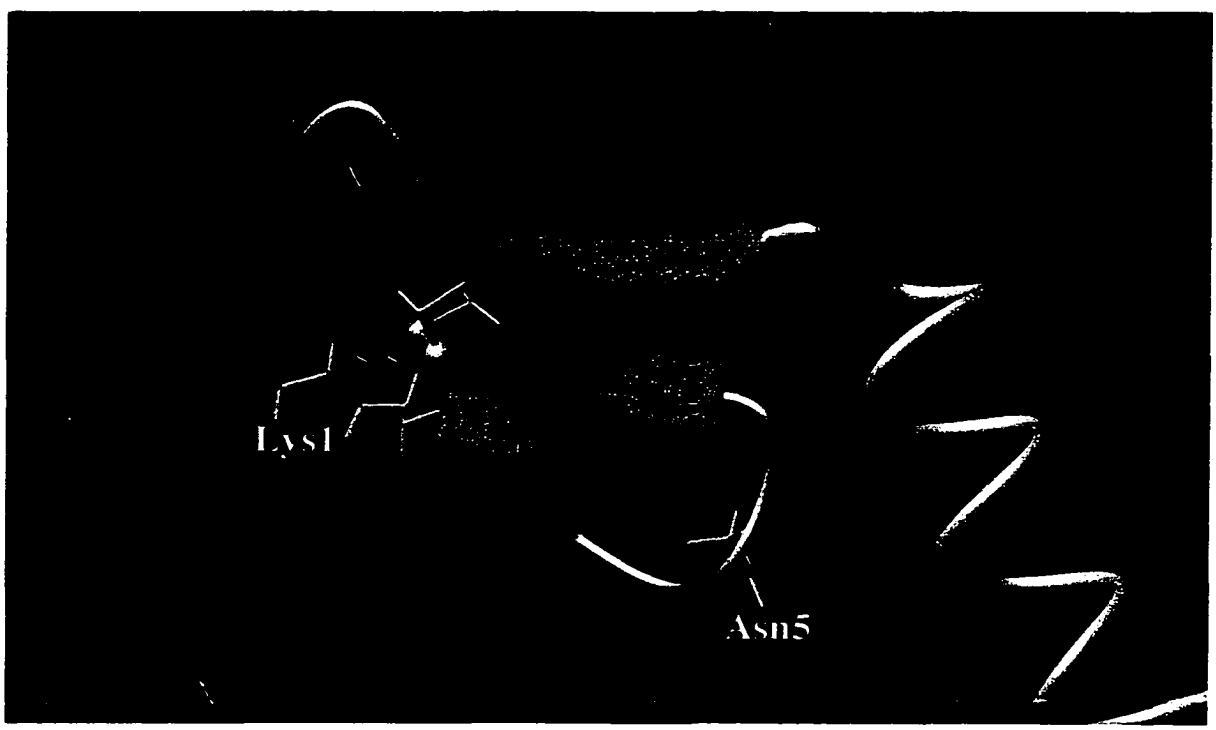


Figure 6. Amphipathic β-sheet at the N-terminus of LeuA (by Dr. Y. Wang)

To rationalize these structural differences in the N-termini of CbnB2 and LeuA, it is important to rule out any sources of experimental error which may have led to the generation of incorrect or unreasonable structures. Three possibilities have been investigated: (1) differential thermal stability between LeuA and CbnB2; (2) errors in the assignments or structure of LeuA; and (3) errors in the assignments or structure of CbnB2. Differential thermal stability was examined by checking the NMR data collected for CbnB2 at two lower temperatures (10 °C and 15 °C) and by searching for evidence of additional or previously undetected structure. No significant differences in chemical shifts, coupling constants or NOE patterns were found, which suggests that the disorder seen in the N-terminus of CbnB2 is not a consequence of thermal denaturation or low thermal stability. Similarly, the temperature sensitivity (i.e. thermal instability) of the 3-stranded β-sheet in LeuA was also studied. Chemical shift and NOE measurements at 25 °C and 35 °C indicated that this structure in LeuA is quite stable. These results indicate that differences in thermal stability are not the principal reason for the observed structural differences in LeuA and CbnB2.

Because the structural differences could not be attributed to differential thermal stability, the published assignments and structure of LeuA[60] were re-examined. Although the structural refinement of LeuA could be improved, it closely resembles the previously published version - including the well-defined N-terminal three-stranded β-sheet. In light of this result, we decided to examine the last possible source of error: incomplete structural sampling during the generation of the CbnB2 structures.

When creating the solution structure of a relatively flexible region of a peptide, it is always feasible that the structure generation protocol does not completely sample all parts of conformation space. Therefore, it may be possible that a structure in the N-terminus of CbnB2, identical to the one in LeuA, is completely compatible with the observed NMR data. To study this possibility we decided to "thread"[87] the CbnB2 sequence into the LeuA structure and to compare the calculated NMR data for this LeuA-like structure with the

observed NMR data. Hence, model structures of the first 37 residues of CbnB2 were built (*via* WHATIF)[88] using our previously refined LeuA structure as a template. The reliability of this "re-modelled" CbnB2 was then evaluated by comparison of its predicted ^{1}H chemical shifts with the observed values using the program TOTAL.[89] The recently developed methods (such as TOTAL) for chemical shift calculation have made it possible to calculate ^{1}H chemical shifts for proteins having well resolved structures with an accuracy of ±0.25 ppm.[89,90] The calculated α-proton chemical shifts, together with the observed values for these "re-modelled" CbnB2 structures clearly indicates that unrealistically large α-proton chemical shift differences (greater than two standard deviations) are predicted for residues Val7, Ser8, Cys9, Cys14, and Val16 of the "re-modelled" CbnB2 structure. Similarly, significant $^3J_{HNH\alpha}$ coupling constant differences are also predicted for residues Asn5 and Lys13 in the same re-modelled structure. These results demonstrate that a three-stranded β-sheet (such as the one found in LeuA) is structurally incompatible with the observed NMR data for CbnB2. Therefore, the possible cause of incomplete conformational sampling can be ruled out for the structural differences observed between LeuA and CbnB2.

What contributes to the structural differences at the N-termini of LeuA and CbnB2? Although there are reports that single amino acid substitutions in peptides and proteins have led to profound changes in function,[25,91-96] these substitutions usually lead to complete denaturation or loss of activity. What is particularly interesting about the case with LeuA and CbnB2 is that the observed structural differences do not seem to affect their strong antimicrobial activity. Since probable sources of experimental error have been excluded, we believe that these structural differences must lie in the intrinsic nature of the two peptides and/or their surrounding solvent (TFE).

Concerning the intrinsic nature of these peptides, analysis of the secondary structural propensity of the N-terminus of LeuA and CbnB2 results in three things. Firstly, the average β-sheet propensity[97] from residues 2 to 16 is slightly higher for LeuA than for

CbnB2 (1.05 vs. 1.00). Secondly, standard secondary structure prediction methods[98-102] obtained from PepTool[75] and the secondary structure server[103,104] predict that LeuA is more likely to form the central β-strand (7-10) than CbnB2. Finally, it has been observed that β-turn propensity can have a strong effect on the stability of both β-hairpins and β-sheets.[76,105,106] Regarding this point, the differing amino acids at positions 12 and 13 (Ser12-Gly13 for LeuA; Thr12-Lys13 for CbnB2), corresponding to the i+2 and i+3 positions of the LeuA β-turn may play an important role. More specifically, the β-turn probability[99] for this particular turn is calculated to be 1.88×10^{-4} in LeuA while it is only 0.85×10^{-4} for CbnB2.

Although linear sequence analysis can give some interesting differences, a much clearer picture can be gained by analyzing the tertiary structure. A close look at the β-sheet formed at the N-terminus of Leu A reveals that it is quite amphipathic. Specifically, four residues: Tyr2, His8, Thr10 and Ala24 form a hydrophobic cluster on one side of the β-sheet, while four residues: Lys1, Asn5, Cys9 and Cys14 form a weakly hydrophilic cluster on the opposite side (see Figure 6, page 23). However, such an amphipathic β-sheet structure can not form in CbnB2 if it adopts the same fold as LeuA because all four hydrophobic residues (Tyr2, His8, Thr10 and Ala24) in LeuA are replaced by hydrophilic or neutral residues (Asn2, Ser8, Ser10 and Glu24) in CbnB2. The obvious hydrophobic/hydrophilic differences arising from these four key residue substitutions could account, at least in part, for the observed structural difference between LeuA and CbnB2.

The amphipathic nature of the LeuA β-sheet may also be important for TFE to maintain or to induce the confined tertiary structure of this peptide. Solvent effects are known to have significant effects in determining the secondary structure of peptides and proteins. Indeed, solvent can often prevail over the intrinsic secondary structural propensity for many given sequences.[106-108] It has been suggested that the structure stabilizing properties of TFE result from two important physical properties, namely the hydrophobicity of the trifluoromethyl group and the hydrophilicity (or hydrogen bonding

character) of the hydroxyl group.[109] TFE offers a more hydrophobic and less basic environment than pure water. The compromised capacity of TFE to accept hydrogen bonds is thought to weaken the peptide-solvent interactions, resulting with stronger intramolecular interactions and increased formation of secondary structure.[110-112] Because of its intrinsic amphipathic or bipolar character, TFE is particularly effective at inducing or stabilizing amphipathic structures, such as α-helices, β-sheets and β-hairpins.[60,76,105,113-119]

If a protein sequence has very little potential to form an amphipathic structure or has very little intrinsic secondary structure propensity, TFE will not be able to induce a well-defined structure.[109,115,120-123] These results indicate that the effects of TFE on β-sheet stability is both sequence and structure dependent, with the structure dependence being based on a sequence's ability to form an amphipathic surface. In respect of this point, the amphipathic structure formed at the N-terminus of LeuA could be a major reason why the observed β-sheet is stabilized in TFE. This also suggests that the LeuA β-sheet, which is only seen in TFE, may be an artifact of the solution conditions. This further implies that the N-terminal structure of type IIa bacteriocins may not be central to their function. Detailed structural studies of other type IIa bacteriocins will likely be needed to resolve this issue.

1.9 Production of CbnB2, its precursor and six structural variants

1.9.1 Introduction

CbnB2 has considerable sequence similarity to other bacteriocins produced by LAB, especially near the N-terminus, where a characteristic motif of "Tyr-Gly-Asn-Gly-Val" (YGNGV) is highly conserved. Because many of these bacteriocins have quite different spectra of antimicrobial activity, the specificity for particular target organisms may in part be determined by the much more variable C-terminal region. Access to substantial quantities of specifically mutated LAB bacteriocins of this type would allow detailed examination of structure-activity relationships. The insights gained into the structural requirements governing specificity of action, potency, and role as an inducer of bacteriocin production, could serve as a guide to functional requirements for a host of closely-related bacteriocins.

The CbnB2 structural gene or that of its precursor, CbnB2P, was specifically inserted into a pMAL-c2 vector downstream from the *malE* gene, which encodes maltose-binding protein (MBP). This resulted in the cytoplasmic expression of an MBP-fusion protein.[66,67] The fusion protein was then purified by one-step affinity chromatography using cross-linked amylose resin. Since the vector also included a sequence coding for the recognition site of the protease factor Xa (which cleaves after the amino acids Ile-Glu-Gly-Arg) between the *malE* and the target protein (CbnB2 or CbnB2P) structural gene, the purified fusion protein was digested with factor Xa and the protein of interest was cleaved from MBP without adding any vector-derived residues to the protein. In this study, we describe the successful overexpression, production, and purification from *Escherichia coli* of: CbnB2; its precursor, CbnB2P; and six CbnB2 variants. The results show that certain peptides with reduced or no antimicrobial effect can still act as inducers of bacteriocin production, although one example lacks both antimicrobial and bacteriocin inducing

activity. Our study also demonstrates that single amino acid substitution or C-terminal truncation can have a profound effect on the biological activity of the purified peptides.

1.9.2 Construction of recombinant proteins and identification of CbnB2 mutants (done by Dr. L. Quadri)

In order to provide appropriate perspective on subsequent isolation and characterization of CbnB2 and its variants, molecular biological experiments done by Dr. Luis Quadri are briefly described here.

The PCR-amplified DNA fragments encoding the precursor CbnB2P **6** or the mature bacteriocin CbnB2 **1** were ligated to the maltose-binding protein gene (*malE*) to create the translational fusions using the established method.[124] To confirm the inducibility of the expression system, several clones which contained inserts of the expected size were screened for the production of the recombinant proteins. Analysis of the overexpressed proteins containing CbnB2P by SDS-PAGE indicated that all of the clones produced a protein of the expected size. One of these clones, referred to hereafter as pLQP, was sequenced to verify that there were no errors in the nucleotide sequence. This was then used for the large scale production of CbnB2P. In the case of the clones expressing the fusion protein containing CbnB2, smaller products were observed in a few cases. The presence of smaller recombinant proteins could indicate an unexpectedly high frequency of mistakes during the PCR amplification step, therefore several clones were sequenced. Analysis indicated that the majority of the clones contained the correct nucleotide sequence. One of these, pLQM, was used for the large scale production of CbnB2. Analysis of the DNA sequence allowed the identification of six clones containing mutations in the coding region for CbnB2. Five of these mutants had only one amino acid substitution: pLQF3 (Y3F), pLQS33 (F33S), pLQI34 (V34I), pLQI37 (V37I) and pLQG46 (R46G); and another one, pLQ28, had a frame shift mutation that replaced the last 20 amino acid

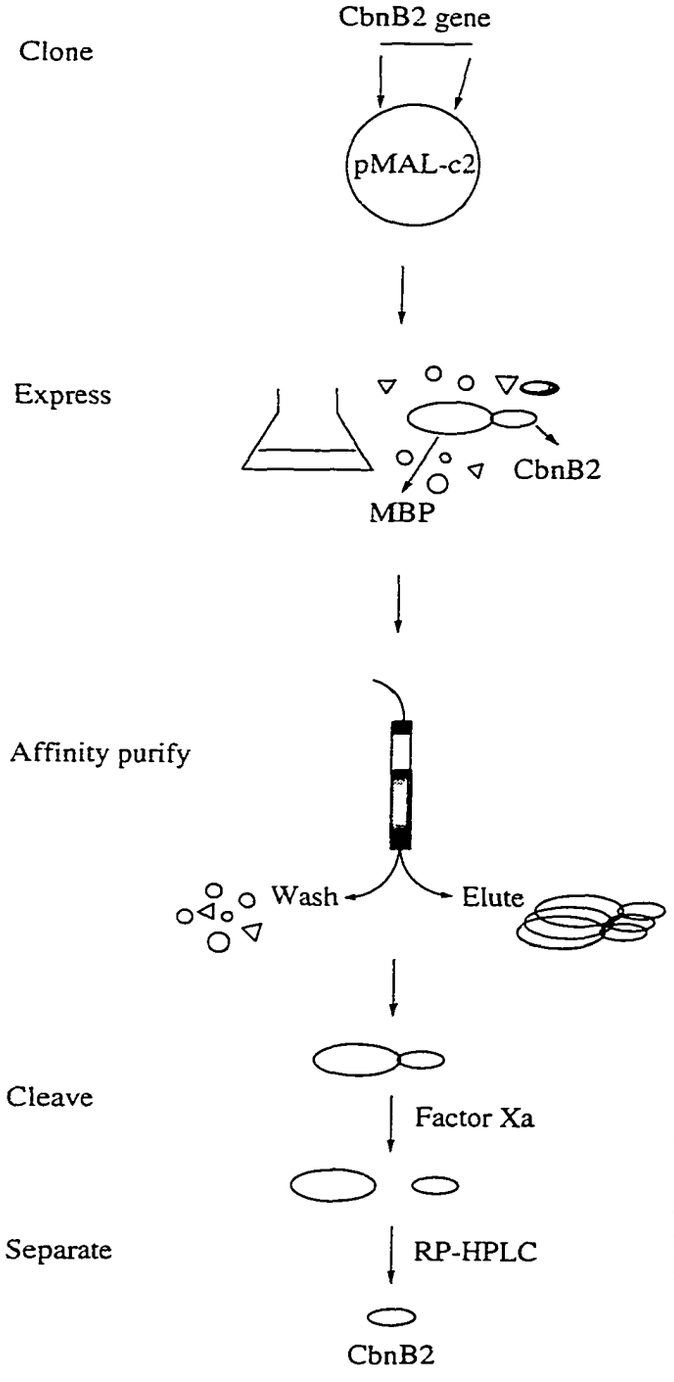
residues of CbnB2 with five other residues (ELTHL) from a different coding frame. These clones were used for the production of the CbnB2 variants.

1.9.3 Preparation and characterization of CbnB2P, CbnB2 and its variants

CbnB2 **1**, CbnB2P **6** and the six mutants: CbnF3 **7**, CbnS33 **8**, CbnI34 **9**, CbnI37 **10**, CbnG46 **11** and Cbn28 **12** were expressed in *E. coli* as recombinant proteins fused to the maltose-binding protein encoded on pMAL-c. The whole process is shown in Scheme 3. After purification on an amylose column, between 32 and 86 mg of the fusion proteins containing CbnB2P, CbnB2, CbnF3, CbnI34, CbnI37, CbnG46 or Cbn28, were recovered per liter of fermentation culture. The purified recombinant proteins represent approximately 10 to 25% of the total cellular protein. In the case of CbnS33, only 3.3 mg of the corresponding fusion protein could be purified from one liter of culture, representing approximately 1.6% of the total cellular proteins.

The presence of a recognition sequence for Factor Xa allowed the cleavage of the recombinant proteins to release the carnobacteriocin-related peptides without any extra amino acids at the N-terminus. During this reaction only 12 to 37% of the substrate was cleaved as indicated by the intensity of the bands visualized in 16.5% Tris-tricine SDS-PAGE[125,126] (Figure 7) and by the peak areas in HPLC separation. More prolonged exposure to Factor Xa was deleterious and led to side products, presumably because of non-specific fission of other peptide bonds.

After the cleavage reaction, the carnobacteriocin-related peptides were separated and purified by RP-HPLC to homogeneity. The retention times of the maltose-binding protein and the undigested fusion proteins were 28 and 27 min, respectively. Under identical conditions, the retention times of CbnB2, CbnB2P, CbnF3, CbnS33, CbnI34, CbnI37, CbnG46 and Cbn28 were 19.5, 19.1, 19.1, 13.9, 19.8, 19.5, 18.4 and 16.9 min, respectively. The approximate amounts of bacteriocins isolated in milligrams per liter of culture are: CbnB2, 1.40; CbnB2P, 0.24; CbnF3, 1.40; CbnS33, 0.04; CbnI34, 0.56; CbnI37, 1.40; CbnG46, 0.73; and Cbn28, 0.74.



The gene of CbnB2 or CbnB2P is cloned into the pMAL vector, creating a gene fusion with the MBP-encoding *malE* gene.

Transformed *E. coli* is grown and the culture is induced to produce MBP fusion protein.

The crude cell extract is loaded onto the amylose resin column. The fusion protein is purified by binding to the column, while other proteins flow through. The fusion protein is then eluted in purified form with maltose.

The purified fusion protein is cleaved with the specific protease factor Xa at the C-side of Ile-Glu-Gly-Arg and then the target protein (CbnB2P, CbnB2 or its variants) is purified by RP-HPLC.

Scheme 3

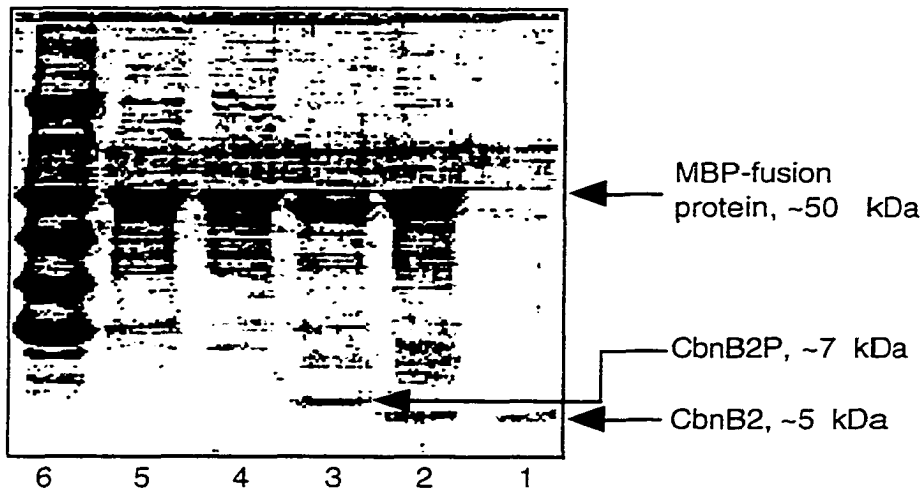


Figure 7. Factor Xa digestion of MBP fusion proteins of CbnB2 and CbnB2P

Lane 1: CbnB2 sample purified from *C. piscicola* LV17B;

Lane 2: MBP fusion of CbnB2 digested with Factor Xa;

Lane 3: MBP fusion of CbnB2P digested with Factor Xa;

Lane 4: MBP fusion of CbnB2P;

Lane 5: MBP fusion of CbnB2;

Lane 6: Molecular weight standards.

For each of the purified peptides, the first five amino acids were determined by Edman degradation. The amino acid sequences were the same as those predicted from the DNA sequences, thereby confirming that the correct cleavage had occurred with Factor Xa at the N-terminus of the carnobacteriocin-related peptides. To ascertain whether the peptides were fully translated and to determine whether they were post-translationally modified in *E. coli*, the molecular weight of each was determined by electrospray mass spectrometry. The calculated and the observed molecular weights of all peptides are in agreement (Table 1). Mass spectrometry data also indicated that the peptides were obtained with the cysteine residues in an oxidized state. For CbnI37 and CbnI34, it was not possible to determined the redox state of the cysteine residues due to the error limits in the molecular mass determination. The oxidation state of the cysteines of these CbnB2 variants was not determined by further experiments.

The antimicrobial activities of the purified carnobacteriocin-related peptides and of the fusion proteins were determined against the sensitive indicator strain, *C. divergens* LV13. The specific activities of CbnB2 (parent bacteriocin), CbnI34, CbnI37 and CbnG46 were 50 AU/μg for each peptide. The pre-bacteriocin, CbnB2P, and CbnF3 had specific activities of 0.4 and 7 AU/μg, respectively. No activity was detected for Cbn28 or CbnS33, or for any of the fusion proteins under the conditions of the assay, which has a limit of detection of 0.05 AU/μg (Figure 8). *C. divergens* LV13 transformed with pLQ400i is immune to the bacteriocin CbnB2 produced from *E. coli*, as previously reported for the corresponding compound isolated from *C. piscicola* LV17B.[34] The peptides CbnI34, CbnI37, CbnG46 and CbnF3 were not active against this strain of *C. divergens* LV13 expressing CbnB2 immunity protein. This indicates that the immunity protein protects the strain from the antimicrobial activity of these CbnB2 variants.

Table 1. Characterization of CbnB2, CbnB2P, and the six variants

	Yield of fusion protein (mg/L)	HPLC retention time[a] (min)	Factor Xa cleavage efficience (%)[b]	Calcd MW (Da)[c] (oxidized form)	MW (Da) (ES-MS)	Specific activity (AU/μg)[d]	The first 5 cycles of N-terminal sequencing
CbnB2 **1**	86.3	19.5	28.1	4969.51 (4967.51)	4967.15 +/- 0.46	50	VNYGN
CbnB2P **6**	32.3	19.1	12.3	6993.93 (6991.93)	6991.11 +/- 0.63	0.4	MNSVK
CbnF3 **7**	81.8	19.1	28.1	4953.53 (4951.53)	4951.25 +/- 0.79	7	VNFGN
CbnS33 **8**	3.3	13.9	14.7	4909.43 (4907.43)	4907.38 +/- 0.91	< 0.05	VNYGN
CbnI34 **9**	34.6	19.8	28.3	4983.55 (4981.55)	4981.74 +/- 2.06	50	VNYGN
CbnI37 **10**	59.1	19.5	35.7	4983.55 (4981.55)	4982.30 +/- 3.27	50	VNYGN
CbnG46 **11**	43.6	18.4	27.2	4870.39 (4868.39)	4867.73 +/- 0.75	50	VNYGN
Cbn28 **12**	30.3	16.9	37.3	3692.12 (3690.12)	3689.48 +/- 0.34	0	VNYGN

Notes:

a. Under identical chromatographic conditions.

b. Calculated from HPLC peak integration.

c. Calculated using Sherpa 3.1.1 (U. of Washington, Seattle, WA).

d. Specific activity was measured by spot-on-lawn test.[69,70]

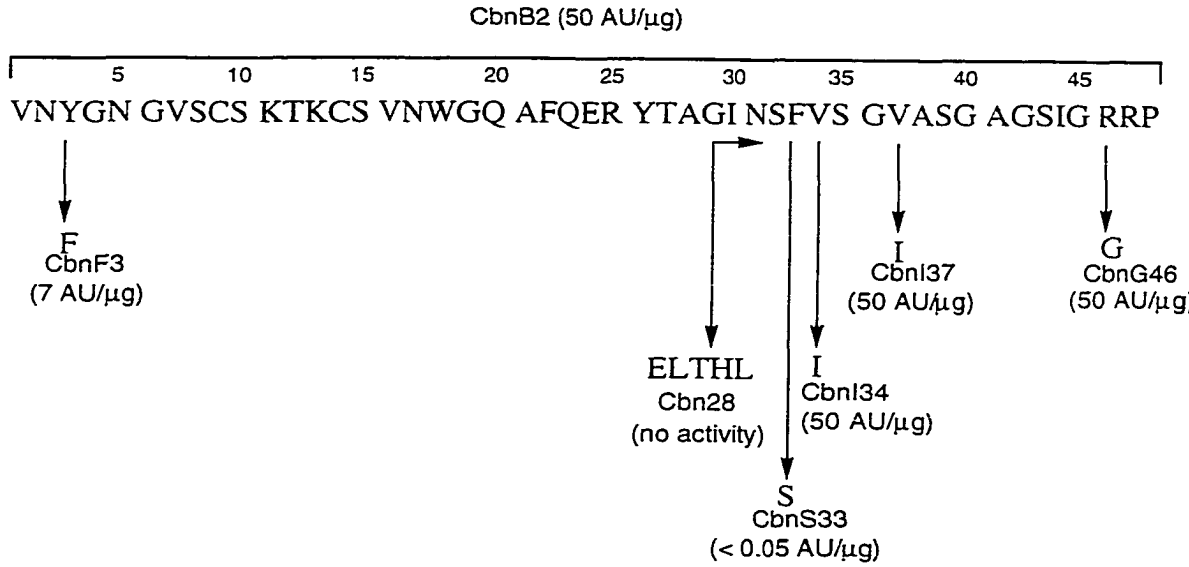


Figure 8. Amino acid sequences and specific activities of CbnB2 and its variants (AU: arbitrary unit of antimicrobial activity)

1.9.4 Induction of bacteriocin production of *C. piscicola* LV17B with CbnB2 and its structural variants

The ability of the purified peptides to function as inducers of bacteriocin production was also determined according to the literature procedure.[41] Induction was marginal (50 AU/mL of culture supernatant) when CbnB2, CbnI34, CbnI37 and CbnG46 were added at a final concentration of approximately 0.1 μg/mL (5 AU/mL) or CbnF3 was added to a final concentration of 0.7 μg/mL (5 AU/mL). However, bacteriocin production was induced to the level of the control (Bac^+ culture or Bac^- culture induced with 2% supernatant of Bac^+ culture) when the peptides were added to a final concentration of 0.3 μg/mL (15 AU/mL), or 2.2 μg/mL (15 AU/mL) for CbnF3. The bacteriocin precursor, CbnB2P, and CbnS33 induced bacteriocin production at a final concentration of 2.5 and 7.5 μg/mL. However, Cbn28, the C-terminal truncated peptide, did not induce bacteriocin

production at a final concentration of up to 30 μg/mL. There were no differences for the inducing function of CbnB2 from different sources (*e.g.* from its natural producer or from *E. coli*).

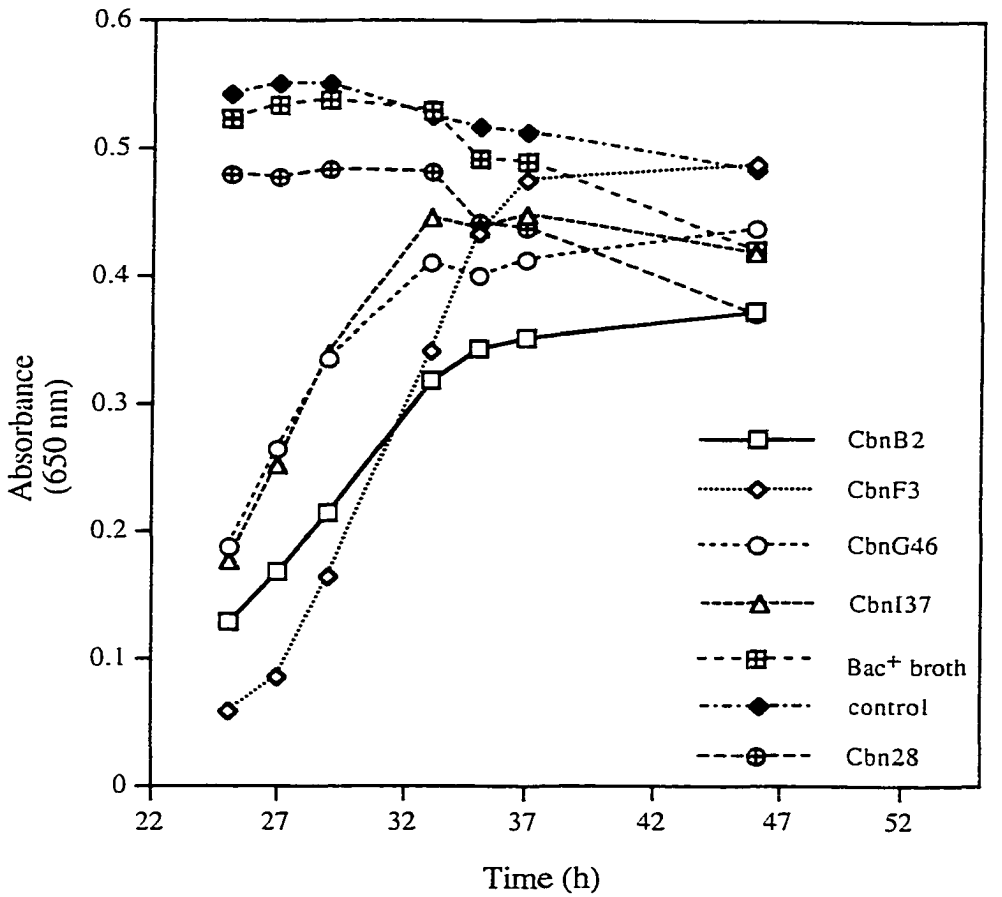


Figure 9. Induction of bacteriocin production by CbnB2 and its variants

During the induction studies, it was observed that the growth of *C. piscicola* LV17B (Bac$^-$) was inhibited by the peptides which were active towards *C. divergens* LV13 (CbnB2, CbnI34, CbnI37, CbnG46 and CbnF3) (Figure 9). The cells needed 10 to 15 h more to reach a stationary phase of growth compared with the control or the culture growing in the medium with inactive peptides. It is interesting to note that the growth of *C.*

piscicola LV17B (Bac^-) was even inhibited by its own bacteriocin CbnB2, which indicated the absense of an immunity protein. Hence, it is speculated that the lag-time in reaching a stationary phase growth is the induction period or the transformation process from Bac^- to Bac^+. This observation also demonstrated that the immunity protein was produced concomitantly with bacteriocin production.

1.9.5 Discussion and conclusions

We have over-expressed the mature CbnB2 **1**, six engineered variants **7-12** and the bacteriocin precursor, CbnB2P **6**, as fusions with maltose-binding protein in *E. coli*, and have specifically cleaved these to release the parent peptides. Because some of the purified peptides have little or no antimicrobial activity, direct detection of these target compounds in a complex fermentation mixture would be difficult and would have made their purification particularly troublesome. Their expression as maltose-binding protein fusions greatly facilitated their purification through affinity chromatography on amylose resin and afforded substantial quantities of the proteins. The cleavage reaction with Factor Xa also proceeded reasonably to generate useful quantities of the parent peptides. Typically, the yield of HPLC-purified peptide is about 1.4 to 0.6 mg per liter, except for CbnB2P **6** and CbnS33 **8**. The wild type mature bacteriocin, CbnB2 **1**, produced in this manner possessed characteristics (HPLC retention time, mass spectra, N-terminal amino acid sequence) identical to the compound purified from the natural host, *Carnobacterium piscicola*,[37] and also displayed the expected specific antimicrobial activity and induction of bacteriocin production.

The fusion proteins had no detectable antimicrobial activity, a result which was not surprising since the maltose-binding protein component imposes a huge size (42,700 Daltons) and polarity difference relative to the attached hydrophobic bacteriocin. It has the potential to alter both the preferred three dimensional conformation and the accessible surface of the parent peptide. Recently, it was reported that both pediocin AcH and pre-

pediocin AcH maltose-binding protein fusions showed antimicrobial activity. This may have been due to the slight variation in the expression system[27,127] and/or the intrinsic properties of the bacteriocin itself. A 14 amino acid peptide linker was inserted between the mature bacteriocin (pediocin AcH) or pre-bacteriocin (pre-pediocin AcH) and the maltose-binding protein. Pediocin AcH (also called pediocin PA-1, see Figure 2, page 10) differs from other class IIa bacteriocins in several aspects: it has two disulfide bonds instead of the one which is common for other class IIa bacteriocins; its pre-bacteriocin, pre-pediocin, possesses a comparable activity to its parent bacteriocin, which is contrary to the function of a leader peptide suggested by Håvarstein.[36]

Examination of the sequences of known LAB bacteriocins having the "YGNGV" motif[37] and consideration of the specificities (antimicrobial spectra) of closely related members of this group suggest that occurrence of at least one key molecular recognition event is essential for bacteriocin antimicrobial activity.[31,128] This probably involves binding to an unidentified protein receptor in the bacterial membrane, since it is difficult to rationalize the observed specificity of antimicrobial action by simple self-association and pore formation. Occurrence of such a receptor has been suggested in studies on the mode of action of pediocin PA-1, a structurally-related bacteriocin of this class.[48] The immunity protein which, when expressed within the cell, protects sensitive organisms from CbnB2 is probably also involved in a molecular recognition event (perhaps with the same receptor), although it does not show significant, direct binding to this bacteriocin and affords no protection if co-administered with CbnB2 from outside the sensitive cell.[34] The variants of CbnB2 produced in the present study offer interesting insights into structure-activity relationships. The presence of the 18 amino acid leader peptide in the purified precursor, CbnB2P, reduces the antimicrobial activity by a factor of 125. This may be due to alteration of the preferred three dimensional conformation and/or exposed surfaces available for binding to cellular constituents. The residual activity of this intracellular bacteriocin precursor, though low, may necessitate the observed co-expression of immunity protein in

wild type *C. piscicola* which is encoded on the same operon.[68] Reduction of antimicrobial properties for precursors of other types of bacteriocins has previously been suggested.[36]

The other purified bacteriocin variants with reduced or no activity are: CbnF3 (Tyr residue at position 3 replaced by Phe); CbnS33 (Phe residue at position 33 replaced by Ser); and Cbn28 which has a frame shift mutation which replaces the last 20 amino acid residues of CbnB2 by five other residues from a different coding frame. The lack of activity of Cbn28 is not surprising because it represents a major structural change from the parent bacteriocin. However, it is interesting that although the only difference between the wild type CbnB2 and CbnF3 is the replacement of a hydroxyl group by hydrogen, the antimicrobial specific activity is reduced seven fold. This small change in a peptide of 48 amino acids with a molecular weight of 4967 demonstrates that the tyrosine residue in the 'YGNGV' conserved motif is important for full biological activity. The effect of this amino acid substitution is particularly relevant because it could potentially be extended to natural bacteriocins which contain this sequence motif (see Figure 2, page 10).

Recent studies on synthetic analogs of MesY105 proposed that the highly conserved residues 1-14 of this 37 amino acid bacteriocin form part of a recognition sequence for a membrane-bound receptor. They also demonstrated that the entire structure, including the N- and C-termini, played a critical role in antimicrobial activity.[47] Removal of Trp37 or of the N-terminal residues 1-3 from MesY105 reduced the inhibitory activity of MesY105 by a factor of 10^4 or more. In the case of CbnB2, the Phe residue at position 33 is also critical for the antimicrobial activity. Its replacement by the more polar amino acid, Ser, in CbnS33 results in complete loss of activity. This amino acid substitution also decreases the HPLC retention time (13.9 min vs 19.5 min for CbnB2) on a reversed-phase C_8 column, indicating that the change significantly reduces the overall hydrophobicity of this variant. As mentioned above, the Phe residue is located in the amphipathic structure of CbnB2 forming part of the hydrophobic face of the helix (see Figure 3, page 20). Replacement of Phe33 with Ser would destroy the integrity of the hydrophobic face and

amphipathic structure, which might be detrimental to its biological function. These structural changes may account for the dramatic variation in the chromatographic properties.

In contrast, three other variants with single amino acid substitutions are fully active: CbnI34 (Val residue at position 34 replaced by Ile); CbnI37 (Val residue at position 37 replaced by Ile); CbnG46 (Arg residue at position 46 replaced by Gly). Although Val37 is also part of the hydrophobic surface of the helix (Figure 3, page 20), its replacement with Ile did not bring any change regarding the peptide's specific activity and chromatographic behavior. Such a conservative change is likely not to be structurally significant. Because Val and Ile are very similar in hydrophobicity and the variation is small (i.e. the addition of a methylene group), it is not surprising that the CbnI34 and CbnI37 bacteriocin mutants are fully active. The differences between the "YGNGV" bacteriocins, LeuA[49] and MesY105,[31] namely replacement of Phe22 with Ala and substitution of Val26 with Ile in a 37 amino acid peptide, result in only slight variation in levels of activity against identical organisms.[47] The much more drastic alteration of Arg46 in CbnB2 to Gly in CbnG46 did not affect the antimicrobial activity of the peptide, and this indicates that the side chain of this residue is not critical. As shown in Figure 4 on page 20, the C-terminus of CbnB2 from residue 41 onwards is largely unstructured.

In order to examine influences of structural variation in the bacteriocin on the efficacy of the immunity protein which protects against the action of the parent CbnB2, the activity of the peptides CbnI34, CbnI37, CbnG46 and CbnF3 was tested against a previously sensitive indicator strain (*C. divergens* LV13) which had been modified to express the immunity protein. The resulting strain was immune to these peptides, as well as the naturally-occurring CbnB2, thereby indicating that the changes introduced in their amino acid sequences did not compromise the mechanism of immunity. This result is in accord with the previous observation that the isolated immunity protein does not bind significantly to the parent bacteriocin CbnB2 *in vitro*.[34]

CbnB2 not only acts as an antimicrobial peptide, but it also induces bacteriocin production,[41] probably by a signal transduction mechanism. In order to examine the influence of structural variation in CbnB2 on bacteriocin induction, we investigated the ability of CbnB2, CbnB2P, CbnF3, CbnS33, CbnI34, CbnI37, CbnG46, and Cbn28 to induce bacteriocin production. All peptides, except Cbn28, induced Bac^- cultures to re-establish their Bac^+ phenotype. Because CbnS33 was not active but still functioned as an inducer, it is possible to conclude that the antimicrobial activity of the bacteriocin is not required for its function as inducer, and that the antimicrobial activity and the induction function are exerted through different biological pathways. Furthermore, the fact that Cbn28 failed to induce bacteriocin production, despite having the 28 N-terminal amino acids present in CbnB2, indicates that the C-terminal portion of the peptide is essential for this function. The ability of the precursor, CbnB2P, which has an 18 amino acid extension at the N-terminus of CbnB2, to induce bacteriocin production suggests that a free N-terminus in the bacteriocin is not required. The inhibition of the growth of *C. piscicola* LV17B (Bac^-) by its own bacteriocin CbnB2 indicated that when bacteriocin production was turned off, the production of the immunity protein was also stopped. Hence, the immunity protein is produced concomitantly with its bacteriocin.

The effective production of bacteriocins and their variants by the current strategy has allowed facile isotopic labelling of CbnB2 with ^{15}N in *E. coli* using [^{15}N]-ammonium chloride (^{15}N 98+%, Cambridge Isotope Laboratories). This has proved valuable for three-dimensional structure elucidation in lipophilic environments by NMR methods. The molecular weight of the purified ^{15}N-CbnB2 **13** (5030.67 +/- 1.29) matched the calculated one (calcd mass for $C_{213}H_{332}N_{66}O_{68}S_2$, 5033.54). There were no differences between the labelled or unlabelled samples in the specific antimicrobial activity, bacteriocin induction ability and the chromatographic properties. Although such labelling of parent bacteriocins can be done with wild-type LAB organisms using a multi-stage procedure involving preparation of labelled cyanobacterial peptone,[129] the present method for generation of

bacteriocins and their precursors affords much easier access to substantial quantities of ^{15}N labelled peptides. Furthermore, using the same methodology, uniformly ^{13}C labelled protein could in principle be prepared with [U-^{13}C] glucose as a starting material in the fermentation.

The expression of bacteriocin variants as maltose-binding protein fusions will also allow production of peptides which cannot be directly engineered into a natural host. For example, variants with amino acid substitutions which prevent their secretion or which make them toxic to the producer could be potentially expressed and purified as fusions in *E. coli.* In addition to providing probes for the biological machinery involved in bacteriocin production and mode of action, the present methodology could assist in the design of new antimicrobial agents with an improved spectrum of activity and with properties suitable for commercial food applications. CbnB2 precursor peptide is the native substrate of the ABC transporter protein. The availability of the genetic information for the ABC transporter protein of *C. piscicola* LV17B[37,68] and current access to CbnB2P make it feasible to further study the biosynthesis of this bacteriocin. For example, these tools would allow examination of the proteolytic processing of CbnB2P and CbnB2 translocation.

Chapter II Structure-activity Relationship of LeuA and the Search for its Putative Receptor

INTRODUCTION

2.1 Photoaffinity labelling and the benzophenone photophore

Investigation of receptor-ligand interaction remains a formidable challenge for chemists and biologists. Structural exploration of biological receptors is the starting point for a better understanding of how they function. As discussed in the previous chapter, it is generally believed that non-lantibiotic bacteriocins form pore complexes in target cell cytoplasmic membranes and induce the leakage of various small intracelluar substances from sensitive cells.[42] The bacteriocin permeates target cell membranes *via* a multi-step process, which may include binding, insertion, pore-formation, etc. Specific protein-protein recognition might be involved in one of these steps, which confers specificity on the antimicrobial peptide.[60,130] The mechanism of pore complex formation and whether there is a receptor protein involved in this process are the major topics of ongoing research. We employed the widely used photoaffinity labelling methodology to investigate the mode of LeuA function and to search for its putative receptor.

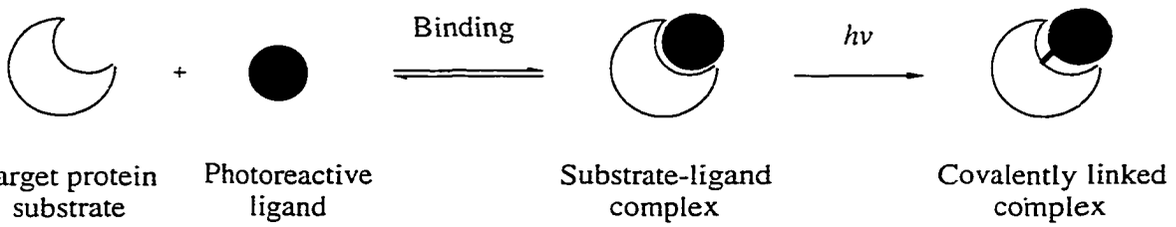


Figure 10. Photoaffinity labelling

Photoaffinity labelling is a technique used primarily to gain information about the location of the binding site of a reversibly bound enzyme-substrate complex. In this technique, a covalent bond between the substrate and the enzyme is formed by activating a photoreactive group on the substrate or substrate analogue (Figure 10).[131] In this context,

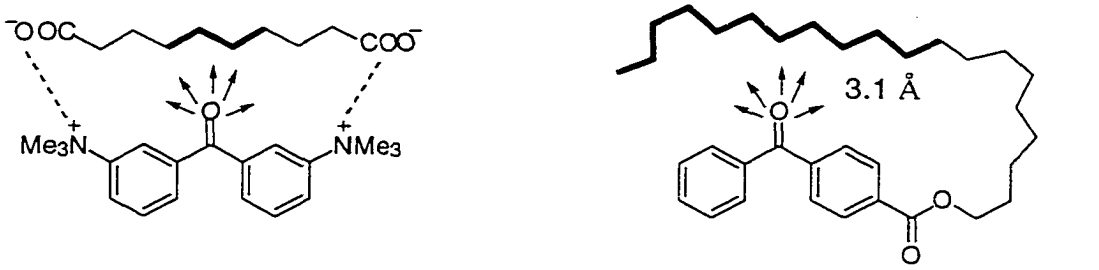
we are studying the interaction between a bacteriocin and its receptor protein. With a photoactivatable bacteriocin, if the bacteriocin and its receptor protein could form a stable bacteriocin-receptor complex, irradiation might create a covalent linkage between the bacteriocin and its receptor.

This technique requires chemical modification of the ligand (bacteriocin) so that irradiation of any resultant complex will produce a reactive intermediate that will bond covalently to its receptor protein. Many photoreactive functional groups have been exploited for photoaffinity labelling, such as azides, diazirines, diazo compounds, benzophenone derivatives, enones and diazonium salts.[131-133]

The benzophenone (BP) moiety was chosen as a photophore for this study because it has several distinct chemical and biochemical advantages as a probe to study peptide-protein interactions.[133] Firstly, among the most commonly used photophores, benzophenones are chemically more stable than diazo compounds, aryl azides, and diazirines. Secondly, benzophenones can be manipulated in ambient light and can be activated at 330-360 nm, avoiding wavelengths that may damage proteins. Thirdly, benzophenones react preferentially with unreactive C-H bonds in the target protein, even in the presence of solvent water and bulk nucleophiles and the resultant covalent adducts are stable to chemical and enzymatic polypeptide cleavage. The activation is not photodissociative and the labelling occurs preferentially at hydrophobic binding regions.

For benzophenone photophores, absorption of a photon at ~350 nm results in the formation of diradicals in triplet state.[134] In the absence of an abstractable hydrogen in the diradical's immediate vicinity, the triplet excited state reverts to the ground state and regenerates the photophore. The lifetime of the excited state may last 80-120 μs in the absence of an abstractable proton, but it may be 100 times shorter in the presence of a suitably oriented C-H bond. The electron-deficient oxygen is electrophilic and abstracts a hydrogen atom from an adjacent alkyl group. The resulting ketyl and alkyl radicals readily recombine to generate a new C-C bond, producing benzpinacol-type compounds. This

suggested mechanism is based on the observation that the diradical species can only attack geometrically accessible C-H bonds.[135,136] On the basis of modelling and experimental data, the reactive volume of the BP moiety was approximated as a sphere with a radius of 3.1 Å centered on the ketone oxygen (Figure 11). For example, when symmetrical 3,3'-bistrimethyl ammonium BP was photolyzed in the presence of a long-chain dicarboxylic acid, the H-abstraction occurred from the internal methylenes of the diacid substrate (Figure 11A).[135] Intramolecular BP photochemistry with long flexible chains provides multiple sites for attack (Figure 11B).[136] The reactivity order for H abstraction by the benzophenone triplet diradical is: $NCH_x > SCH_x > CH > C{=}CCH_2 > CH_2 > CH_3$.[134,137]



A. Sites of H-abstraction in organized media

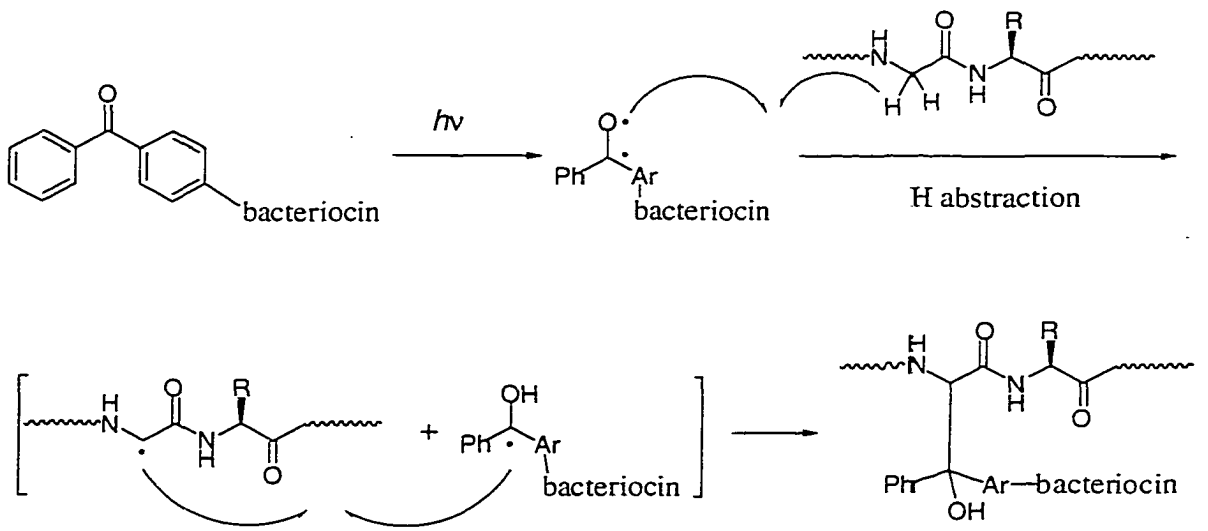
B. Intramolecular photochemical cyclization of benzophenone derivative

— Possible sites of H-abstraction

Figure 11. Possible H-abstraction sites of the benzophenone photophore

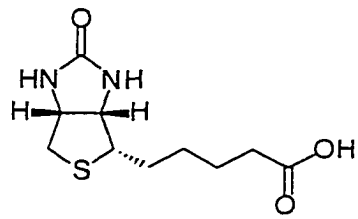
Since photoaffinity labelling implies the formation of a covalent bond between a labelled peptide ligand analogue and a receptor binding site (Scheme 4), it is theoretically possible to isolate and sequence the labelled peptides and then synthesize the corresponding oligonucleotide probes based on this sequence. The primary structure of a receptor protein could be obtained by reverse genetics using this synthetic oligonucleotide probe. Photoaffinity labelling might avoid the critical solubilization and purification steps of the classical approach. However, no such examples of primary structure determination based on photoaffinity labelling experiments have been reported, mainly due to the fact that non-

specific labelling complicates this technique. Nevertheless, successful applications of the benzophenone photophore in photoaffinity labelling have been reported with purified proteins.[138,139]



Scheme 4

Photoaffinity labelling methodology usually requires the incorporation of a tracking group, such as a radioactive atom like ^{125}I, ^{3}H or ^{14}C. Radioactive moieties can be incorporated into peptide or protein by solid-phase peptide synthesis using the appropriate labelled amino acids.[133,134] Alternatively, radioactive iodine (^{125}I) can be introduced to the protein or peptide chain using the method developed by Bolton and Hunter,[140] in which a radioactive tyrosine derivative is attached to the primary amino group of the target protein. An example of a non-radioactive tracking group is biotin, which is based on the well developed biotin-avidin chemistry.[141]



Biotin

Biotin is a growth factor present in every living cell. Avidin is a basic glycoprotein, usually composed of four identical subunits. The strong interaction between avidin and biotin was recognized as early as 1941;[141] the dissociation constant of the avidin-biotin complex (K_d) is 1.3 x 10^{-15} M at pH 5.0. The high affinity is due to the extensive hydrogen-bonding network, as well as van der Waals interactions.[142,143] This strong interaction has been exploited in many useful biochemical tools.[141] The major drawback of the widely used avidin-biotin affinity chromatography is the harsh conditions necessary to dissociate the avidin-biotin complex. Typically, dissociation of the complex requires 8 M guanidine at pH 1.5 or 120 °C for 15 min. Ligands with relatively weak interactions have been developed, such as streptavidin and monomeric avidin. The availability of various enzyme conjugated avidins or biotins makes it possible to utilize the strong avidin-biotin interaction for affinity blotting.[141, 144]

2.2 Leucocin A (LeuA)

Leucocin A (LeuA) is a 37-amino acid antimicrobial peptide isolated from the fermentation supernatant of the lactic acid bacterium (LAB) *Leuconostoc gelidum* UAL187.[49, 50] It belongs to the expanding group of hydrophobic bacteriocins produced by lactic acid bacteria with a conserved YGNGV motif at its N-terminal end.[145] The recently published three dimensional structure of L-LeuA in lipophilic environments shows that its N-terminus assumes a three-strand antiparallel β-sheet (residues 2-16) anchored by the

disulfide bridge between Cys9 and Cys14, and its C-terminal region (encompassing residues 17-31) exists as an essentially amphipathic α-helix conformation (see Figure 4, page 20).[60] It is interesting to note that CbnB2, another YGNGV class IIa bacteriocin, exhibits different structural features at its N-terminus, but very similar structural features at the C-terminus even though they share more than 60% sequence homology for the first 24 resides, but a varied C-terminal sequence (see Chapter I). Hybrid bacteriocins containing N- and C-terminal regions from different YGNGV bacteriocins have shown that the C-terminal half of these bacteriocins is an important determinant of the target cell specificity.[30]

Optical antipodes have been used to study whether ligand-receptor binding involves chiral interaction.[146,147] If nonchiral interactions are sufficient for function, the two enantiomers should be equally active. Hauge and colleagues reported the synthesis of truncated all-D plantaricin A, an induction factor originally produced by *Lactobacillus plantarum* C11.[147] It was found that the synthetic peptide composed of all D-amino acids is equally as active as the natural antimicrobial peptide (L-enantiomer); however, this synthetic D-isomer lost its biological function as a pheromone. These results demonstrate that the antimicrobial activity of plantaricin A does not require chiral interactions and is mediated through the formation of a strongly amphiphilic α-helical structure. In contrast, the pheromone activity is dependent on a chiral interaction between the peptide and a receptor protein. Hence, the activity of the D-isomer of LeuA will provide much information regarding the way it exerts its antimicrobial activity.

In order to identify the putative receptor protein and study the antimicrobial activity of L-LeuA, a practical assay is of critical importance. Since the bacteriocin-receptor interaction is specific, a possible starting point would be to look for an agonist or antagonist of the bacteriocin. It was reported that the bactericidal activity of pediocin PA-1 could be specifically inhibited by a 15-mer fragment that encompasses the bacteriocin from the center toward the C-terminus. The presence of the fragment in the medium increased the

minimum inhibition concentration (MIC) of pediocin PA-1 by more than 20 fold. However, this 15-mer fragment shows no or low inhibition activity towards other class IIa bacteriocins, such as LeuA and sakacin A.

Like all other class II bacteriocins, LeuA is produced as a precursor which has an N-terminal leader peptide. Most of the leader peptides of class II bacteriocins share similarities in amino acid sequence and contain a conserved processing site of two glycine residues in positions -1 and -2. A dedicated ATP-binding cassette (ABC) transporter is responsible for the proteolytic cleavage of the leader peptides and translocation of the bacteriocins across the cytoplasmic membrane.[30] Recently it has been demonstrated that the leader peptides function as a recognition sequence for the dedicated ABC transporter. Hybrid pre-bacteriocins (a leader peptide of one bacteriocin fused to a different mature bacteriocin) could be used for bacteriocin production in homologous hosts. In some cases bacterocin productions were observed in heterologous hosts.[28] In order to study the dual functions of the dedicated ABC transporter, several LeuA leader peptide analogues bearing a chromophore were synthesized as possible substrates for the proteolytic transporter protein.

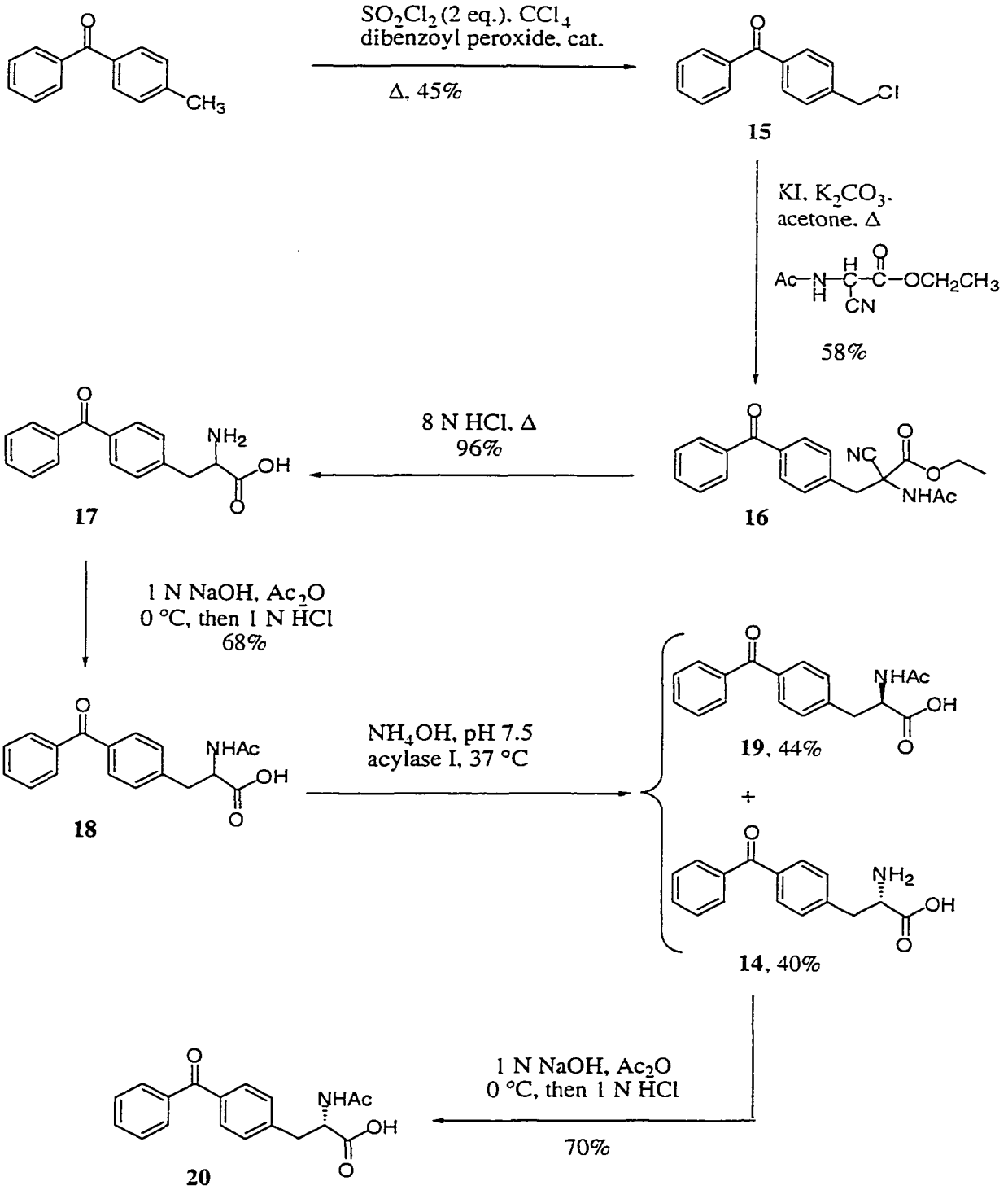
We have attempted to confirm the idea that the C-terminal end of the peptide is involved in the specific receptor binding through different approaches. We sucessfully incorporated the benzophenone photophore onto different sites of the LeuA peptide chain without abolishing its antimicrobial activity. The biotin moiety was attached to the photoactive LeuA as a tracking group, which made it possible to develop an assay to track the modified LeuA in biological systems. We used this bifunctionally labelled LeuA to study the interaction of LeuA with its sensitive bacteria, in an attempt to search for its putative receptor protein. With LeuA bearing a benzophenone photophore, we also studied its interaction with lipids. The effect of structural modifications on the antimicrobial activity, together with the role of charged residues in the peptide's antimicrobial functions, were also investigated.

RESULTS AND DISCUSSION

2.3 Syntheses of phenylalanine derivatives

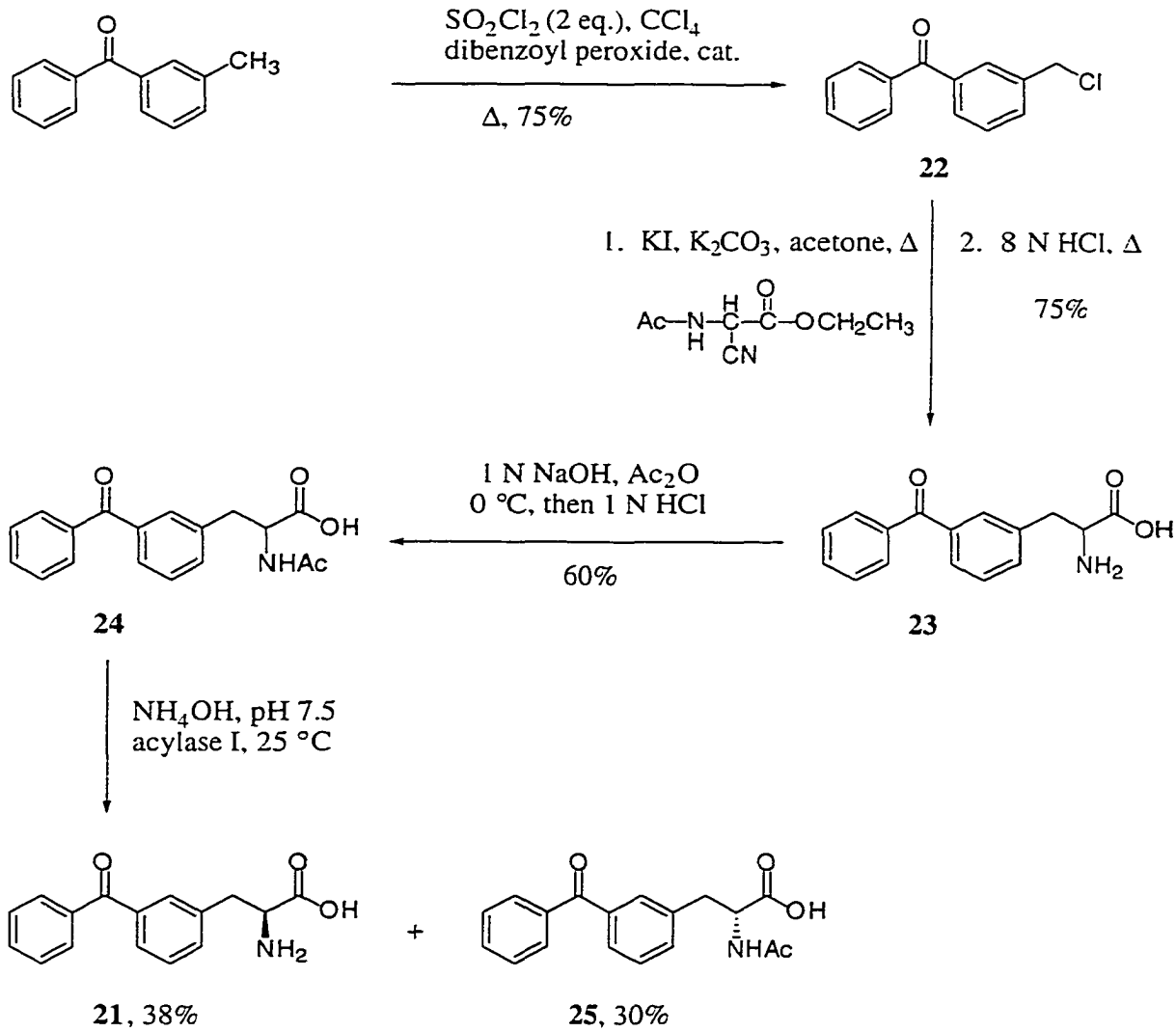
As discussed in the introduction, benzophenone is a widely used photophore for the study of interactions between proteins and peptides. The investigation of the interactions between LeuA and its target bacteria started with the syntheses of amino acid derivatives bearing the benzophenone photophore, such as benzoylphenylalanine (Bpa). The photoactive amino acid was then incorporated into LeuA by solid-phase peptide synthesis.

The method of synthesis of *p*-benzoyl-L-phenylalanine (*p*-Bpa) **14** (Scheme 5) was established by Kauer *et al.*[148] Commercially available *p*-methylbenzophenone was converted to *p*-chloromethylbenzophenone **15** through a radical process using dibenzoyl peroxide as an initiator. Nucleophilic attack on **15** by ethyl acetamidocyanoacetate in dry acetone gave **16**, which was hydrolyzed and simultaneously decarboxylated in hydrochloric acid to give *p*-benzoyl-D,L-phenylalanine **17** in good yield. In order to resolve the racemic mixture, **17** was acylated using acetic anhydride to give **18**. Selective hydrolysis of the L-isomer amide bond using acylase I at pH 7.5 afforded *p*-benzoyl-L-phenylalanine **14**, which was precipitated from the reaction mixture and was easily separated with good purity. Acidification of the aqueous filtrate from the acylase hydrolysis to pH 2.4 precipitated unreacted *N*-acetyl-*p*-benzoyl-D-phenylalanine **19** which was recovered by filtration. In order to confirm the optical purity of *p*-benzoyl-L-phenylalanine **14**, it was further derivatized with acetic anhydride to give *N*-acetyl-*p*-benzoyl-L-phenylalanine **20** to compare directly with **19**. The optical rotations of **14**, **19**, and **20** were the same as or higher than those in the literature.[148]



Scheme 5

The patented method of Kauer[148,149] was used to synthesize *m*-benzoyl-L-phenylalanine **21** (Scheme 6). The synthetic strategy is similar to the synthesis of *p*-benzoyl-L-phenylalanine **14**.



Scheme 6

Commercially available *m*-methylbenzophenone was chlorinated using sulfuryl chloride in the presence of a catalytic amount of dibenzoyl peroxide to give *m*-chloromethylbenzophenone **22**. Nucleophilic attack on **22** by ethyl acetamidocyanoacetate

in the presence of KI and K_2CO_3 in dry acetone gave a viscous yellow oil which was hydrolyzed and decarboxylated in 8.0 N hydrochloric acid without isolation to afford *m*-benzoyl-D,L-phenylalanine **23** in 75% yield. Resolution of the acylated racemic mixture of **24** with acylase I at pH 7.5 afforded *m*-benzoyl-L-phenylalanine **21** and unreacted *N*-acetyl-*m*-benzoyl-D-phenylalanine **25**. The reaction was a little slower compared with that of *N*-acetyl-*p*-benzoyl-D,L-phenylalanine **18**. The optical rotations of **21** and **25** were the same as or higher than those reported in the literature.[148,149]

Optically pure compounds **14** and **21** can be used in solid phase peptide synthesis after being protected with Boc or Fmoc.[148,149] During the course of this work, protected *p*-benzoyl-L-phenylalanine **14** became commercially available. Therefore this work was not continued.

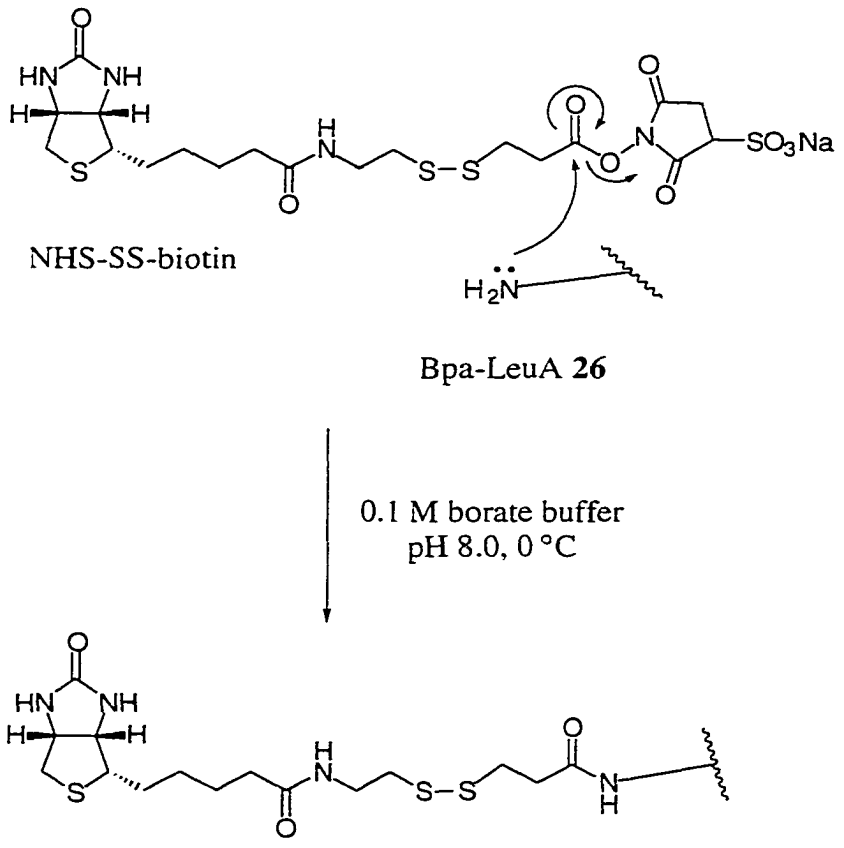
2.4 Synthesis and biotinylation of Bpa-LeuA

With the protected *p*-benzoyl-L-phenylalanine (Bpa) in hand, the next step was to incorporate this photoactive amino acid into the peptide chain. The goal was to replace Phe22 of LeuA with *p*-benzoyl-L-phenylalanine (see Figure 2, page 10 for sequence). Residue Phe22 was chosen since the resulting three dimentional structural change is likely to be minimal. In addition, it has been shown that Phe22 is part of the hydrophobic surface of LeuA's amphipathic α-helical structure in membrane-mimicking environments, which may determine antimicrobial specificity.[60] Another closely related class IIa bacteriocin, CbnB2, has the same residue at the same position. There is also conservative structural homology to the hydrophobic surface of the amphiphilic α-helical structure of CbnB2 which encompassed residues from Trp18 to Ser39 (see Figure 3, page 20). Because the C-terminal ends of class IIa bacteriocins are claimed to be responsible for the specificity of the antimicrobial peptides,[44,80] they are more likely to be involved in the specific receptor binding.[60] Nevertheless, this critical residue could tolerate some conservative structural

changes. Replacement at Phe22 and Val26 of LeuA with Ala and Ile, respectively, generates another antimicrobial peptide, MesY105.[31] There is little difference in activity between these two peptides.[47] Therefore, we believed that the replacement of Phe22 of LeuA with Bpa would be tolerable with repect to the changes in its structure and activity.

Bpa-LeuA **26** was synthesized at Harvard Medical School using Fmoc protected amino acids, with Fmoc-*p*-benzoylphenylalanine used at position 22 instead of Fmoc-Phe. The synthetic peptide was purified to homogeneity by RP-HPLC and the identity of the peptide was confirmed by electrospray mass spectrometry. The molecular weight was determined to be 4034.17 +/- 2.51. which is in agreement with the calculated mass of 4034.44 (Sherpa 3.1.1, University of Washington, Seattle, WA). As expected, this peptide has a comparable specific activity to the natural peptide, LeuA.

The synthetic peptide Bpa-LeuA **26** was successfully derivatized with 3-sulfosuccinimidyl 2-biotinamido ethyl-1,3-dithiopropionate (NHS-SS-Biotin) at 0 °C in sodium borate buffer, pH 8.0 (Scheme 7). In addition to recovered starting material, two major modified products were found, which corresponded to a singly biotinylated substance **27** and a doubly biotinylated substance **28** as determined by ES-MS (Table 2).



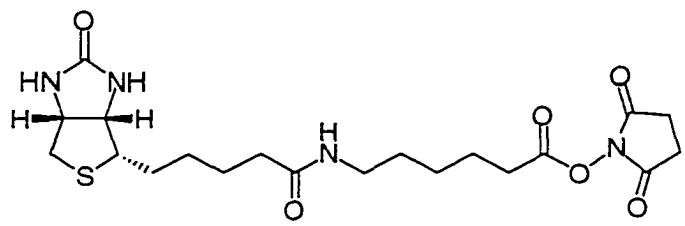
Scheme 7

Table 2. Biotinylated products of Bpa-LeuA **26** with NHS-SS-biotin

	26	**27**	**28**
RT (min)	26.2	27.3	28.6
MW (Da)	4032.50 +/- 2.18	4425.25 +/- 0.95	4814.08 +/- 1.97
calcd MW (Da)	4034.44	4426.00	4817.56
Activity (AU/μg)	205	21.3	4.0

LeuA bears several primary amino groups which can possibly be modified: the α-amino group, the ε-amino group of Lys1, the ε-amino group of Lys11 and the guanidinium primary amino group of Arg28. It is likely that the monosubstituted fraction **27** and the disubstituted fraction **28** are a mixture of different products. Although **27** and **28** could be separated from each other, the mixture of mono- or di-substituted products was practically inseparable on RP-HPLC. The mixture of mono-substituted products **27** was therefore used for photoaffinity labelling in the next step. Both **27** and **28** are active toward *C. divergens* LV13. However, the modification reduced the specific antimicrobial activity by almost 10 fold for the monosubstituted products and 50 fold for the disubstituted products as shown in Table 2.

In the above modification, the linkage between biotin and Bpa-LeuA has a reducible disulfide bond. Linkage without this kind of disulfide bond was also prepared. Commercially available biotinamidocaproate *N*-hydroxysuccinimide ester (NHS-biotin) was used to modify Bpa-LeuA in the same way as that described for NHS-SS-biotin in 0.1 M sodium borate buffer at pH 8.0. Modified products were separated from the starting material by RP-HPLC (Table 3). By comparison with the calculated molecular weights, it was found that **29** was a monosubstituted product and **30** was a disubstituted product. Both **29** and **30** are active towards the indicator *C. divergens* LV13. A 1:4 ratio of **29** and **30** as determined by HPLC peak integrations was used for the photoaffinity labelling in the next step.



Biotinamidocaproate *N*-hydroxysuccinimide ester (NHS-biotin)

Table 3. Biotinylated products of Bpa-LeuA **26** with NHS-biotin

	26	**29**	**30**
RT (min)	24.6	25.5	26.6
MW (Da)	4034.17 +/- 2.51	4373.40 +/- 1.01	4712.50 +/- 0.73
calcd MW (Da)	4034.44	4374.90	4715.36
Activity	+	+	+

2.5 Photoaffinity labelling of LeuA sensitive bacteria

We employed a biotin label as a way to track the bacteriocin peptide (Bpa-LeuA). UV irradiation was done following the method of Leeman and co-workers.[150] The process to visualize the labelled protein bands is shown in Figure 12.[141]

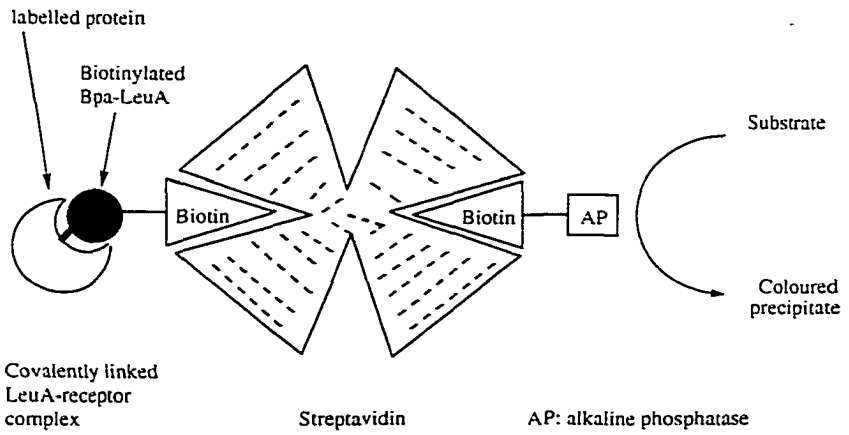
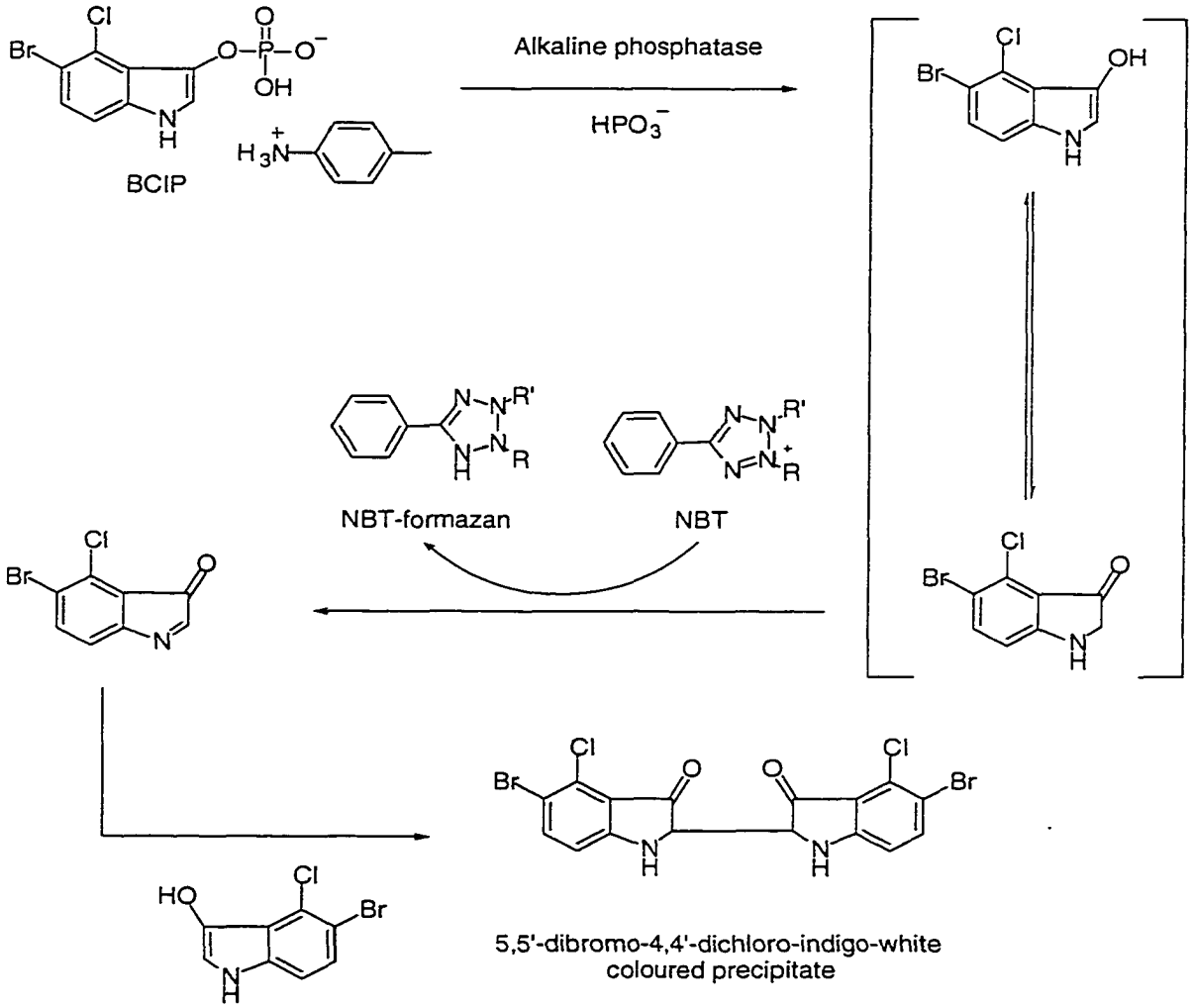


Figure 12. Assay of biotinylated proteins

We anticipated that the biotinylated Bpa-LeuA **27** or **30** would bind to its receptor protein and the photoreactive benzophenone group at positon 22 would likely form a covalent linkage between Bpa-LeuA and the receptor protein by UV irradiation. The biotin

moiety made it possible to locate the covalently linked receptor-bacteriocin complex through an alkaline phosphatase assay. As shown in Scheme 8, hydrolysis of substrate NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt) by alkaline phosphatase gives an unstable intermediate, a hydroxy indole derivative which dimerizes to give an insoluble black-purple precipitate of indigo-white.[141, 144]



Scheme 8

Since UAL9 (*C. divergens* LV13) is the most commonly used indicator strain to detect the activity of LeuA, it was initially chosen for the photoaffinity labelling. Cells from an overnight culture of UAL9 were collected by centrifugation and twice washed with 0.1 M PBS, pH 7.0. Varying amounts of biotinylated peptide were mixed with the washed culture and incubated at 4 °C for 1 h with gentle shaking. To determine whether excess peptide in the binding medium interferes with the labelling, additional reactions were performed as follows: one was irradiated directly in the binding medium; the duplicate was centrifuged and the cells were washed once with 0.1 M PBS, then the cell pellet was resuspended in the same volume of buffer and irradiated. The irradiation was performed with a UV lamp (100 W, maximum irradiation at 360 nm) at a distance of 6 cm for 15 min at 4 °C. After irradiation, the cells were pelleted by centrifugation. The cell pellet was then mixed with gel electrophoresis sample buffer and vortexed vigorously to lyse the cells. The samples were left at room temperature for 1 to 2 h with occasional vortexing.

The above samples were denatured in a boiling water bath for 5 min before loading onto a 12% polyacrylamide gel. The mini-slab gels (12%, with 4% stacking) were cast 2 h before use. The gels were loaded with 35 μL of above prepared samples and developed at 150 volts for 60 min at room temperature. The resolved proteins from the SDS-PAGE were transferred to a Nylon membrane using the Mini-Trans-Blot electrophoretic transfer cell. The membranes with the blotted proteins were then soaked in a blocking buffer containing 3% BSA for 1 h to block the non-specific binding sites. The well washed membranes from the blocking buffer were incubated in a buffer containing 2 μg/mL streptavidin for 1 h. Biotinylated alkaline phosphatase (0.1%) and a commercially available substrate (Pierce) were used to visualize the proteins containing a biotin moiety.

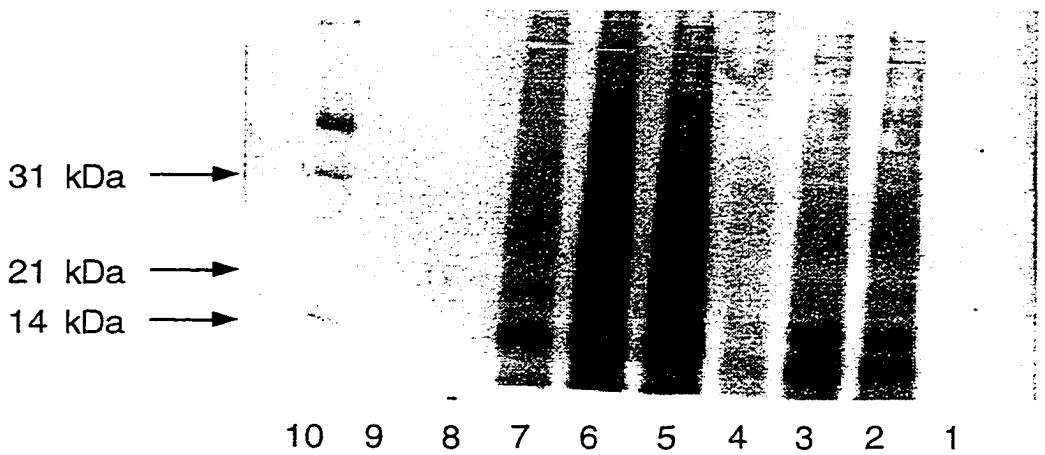


Figure 13. Photoaffinity labelling of UAL9 using biotinylated Bpa-LeuA

Lane 1: Blank, no sample loaded;

Lane 2: Labelled UAL9 with **27**. Cells were washed before irradiation;

Lane 3: Same as lane 2, cells were not washed;

Lane 4: Same as lane 2, the peptide added was one tenth;

Lane 5: Labelled UAL9 with **29+30**, cells were washed before irradiation;

Lane 6: Same as lane 5, cells were not washed before irradiation;

Lane 7: Same as lane 6, the peptide added was one tenth.

Lane 8: Control, UAL9 cells only, no peptide added, UV irradiated.

Lane 9: Control, same as lane 8, no UV irradiation.

Lane 10: Biotinylated molecular weight standards.

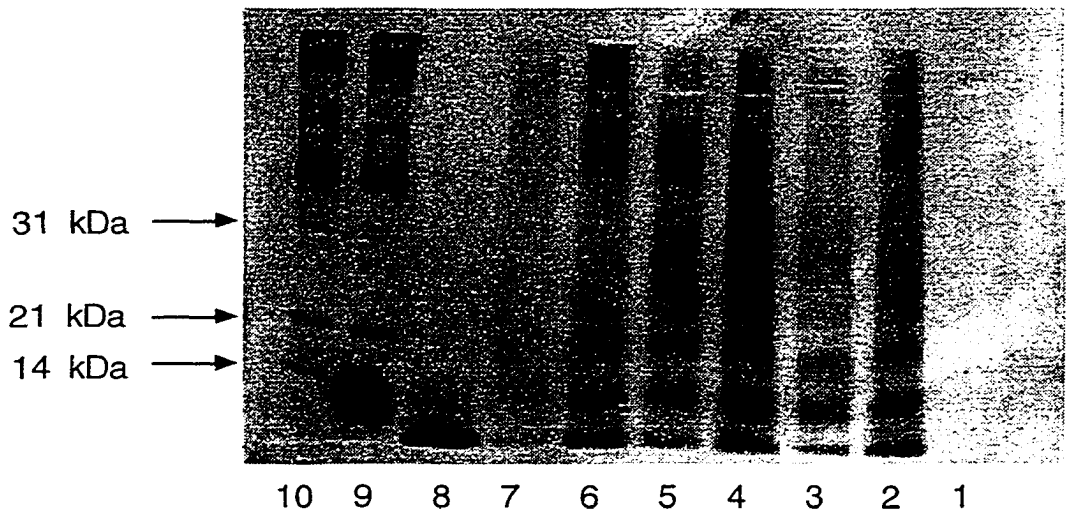


Figure 14. Photoaffinity labelling of UAL9 using biotinylated Bpa-LeuA

Lane 1: Control, UAL9 cells;

Lane 2: Labelled UAL9, 40 AU of **29** + **30**;

Lane 3: Same as lane 2, sample buffer contained 2 % of mercaptoethanol;

Lane 4: Same as lane 2, the peptide added was half;

Lane 5: Same as lane 2, the peptide added was one fourth;

Lane 6: Same as lane 2, no UV irradiation was performed;

Lane 7: LeuA (2 μg);

Lane 8: **29** + **30** (2 μg), no cells;

Lanes 9/10: Biotinylated protein standards.

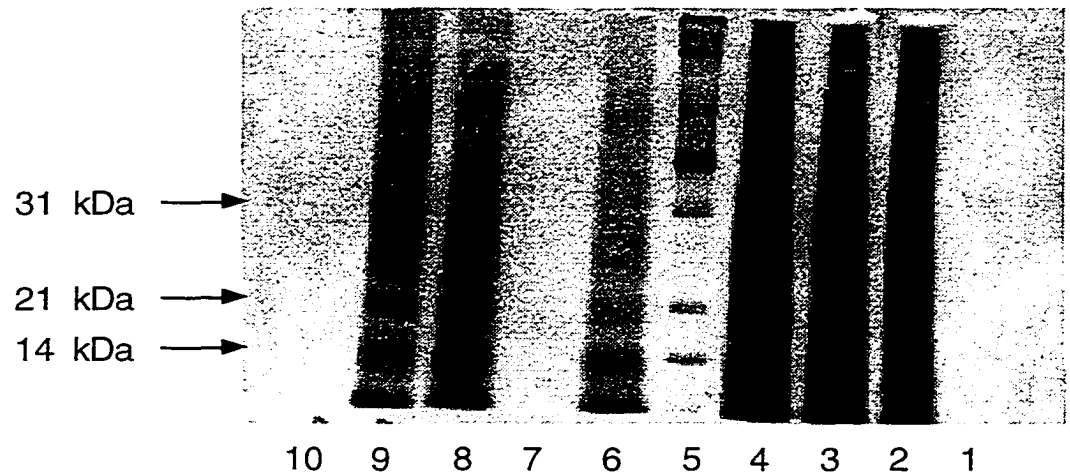


Figure 15. Photoaffinity labelling of UAL26 and 278 using biotinylated Bpa-LeuA

Lane 1: Control of UAL278-R (a LeuA resistant strain);

Lane 2/3/4: Labelled UAL9 with **29** + **30** (old samples);

Lane 5: Biotinylated protein standards;

Lane 6: Labelled UAL278-R with **29** + **30**;

Lane 7: Control of UAL278 (a LeuA sensitive strain);

Lane 8: Labelled UAL278 with **29** + **30**;

Lane 9: Labelled UAL26 with **29** + **30**;

Lane 10: Control of UAL26.

From the blotted membranes (Figures 13 and 14), it can be easily seen that two major labelled bands, one at *ca.* 14 kDa and one at *ca.* 20 kDa, are shown for all of the labelled samples, with the band at *ca.* 14 kDa being more intense than the one at *ca.* 20 kDa. Although the specificity was moderate, the two bands were clearly readable. By comparison of lane 2 and lane 4 in Figure 13, it was found that reducing the amount of ligand (biotinylated Bpa-LeuA) did not improve the specificity. The amount of photoactive peptide used for lane 4 was ten times less than that for lane 2. This trend was also observed in lanes 6 and 7 in Figure 13. The peptide used for lane 7 was ten times less than that for lane 6. The intensity of all labelled bands was proportional to the amount of peptide added. There were no apparent differences between lanes 2 and 3, or between lanes 5 and 6. This indicates that removing the cells from the binding medium and washing did not affect the labelling process and the labelling pattern as shown by the blotted membranes. The excess photoactive peptides in the binding media did not interfere with the labelling.

On Figure 13, biotinylated Bpa-LeuA used for lanes 2, 3 and 4 was **27** and for lanes 5, 6 and 7 was a mixture of **29** and **30** (1 : 4). The two major labelled bands of lanes 2 and 3 are lower in position and hence have a molecular weight smaller than those of lanes 5, 6 and 7. This is consistent with the fact that the molecular weight of **30** (main component) is *ca.* 300 Da larger than that of **27**, or might possibly be due to the disulfide linkage on **27**, which could be reduced or derivatized during cellular photoaffinity labelling process. Lanes 8 and 9 of pure cell proteins without any labelled peptides added were completely blank, which demonstrated that only proteins containing the biotin moiety could be developed.

For all of the samples containing biotinylated Bpa-LeuA, there was an obvious band at the bottom of each lane. This band is likely to be unreacted isolated biotinylated Bpa-LeuA, as supported by the results of lane 8 in Figure 14 where only biotinylated Bpa-LeuA was loaded. On the same figure, lane 7 was the normal LeuA and it appeared the same as the blank control. Under reducing conditions (2% of mercaptoethanol), the two

major labelled bands were lower (lane 3 of Figure 14) compared with nonreducing conditions (lane 2), which indicated that the labelled proteins might be reduced by mercaptoethanol. It is unclear why the two major bands at *ca.* 14 kDa and *ca.* 20 kDa (lane 6) were still visible even though photoirradiation was not performed. However, the intensity of the bands was weaker. Samples on lane 4 and lane 6 were from similar preparations, but the cells of lane 6 were not UV irradiated.

Under identical conditions, photoaffinity labelling of UAL278 (*C. divergens*), UAL26 (*C. piscicola*) and UAL278-R (*C. divergens*) was also performed. Both UAL278 and UAL26 are sensitive to LeuA. UAL278-R is a LeuA resistant isolate. The results of photoaffinity labelling of these strains are shown in Figure 15. The labelled samples of UAL9 were included for comparison (lanes 2, 3 and 4). As previously observed, the lanes of all of the controls were blank, *i.e.* lane 1 for UAL278-R; lane 7 for UAL278; and lane 10 for UAL26. For the photoaffinity labelled samples, it is interesting to note that all three strains showed similar labelling patterns as UAL9, which gave two major labelled bands at *ca.* 14 kDa and *ca.* 20 kDa.

Besides the functional domain(s), normal membrane-associated receptor proteins usually have several membrane-spanning segments. Hence, they are relatively large in size.[151] One of the two major labelled bands at *ca.* 14 kDa and *ca.* 20 kDa from the above experiments might be the putative receptor protein of LeuA, even though they are smaller in size than what is typical for a receptor protein. However, the same or similar labelling pattern for the several different bacterial strains indicated that either the receptor protein is common for all those tested strains or the above two bands are not the receptor protein. Alternatively, the two bands might be the common functional forms which biotinylated Bpa-LeuA assumes while it functions as an antimicrobial peptide in these bacteria. From this perspective, the two bands would possibly be the different aggregation states of Bpa-LeuA, such as a trimer (12 kDa) and a pentamer (20 kDa), respectively, which are covalently linked following photo irradiation. A pore formation model which functions

through bacteriocin molecule aggregation on the target cell membrane has been suggested in the literature (Figure 16).[42]

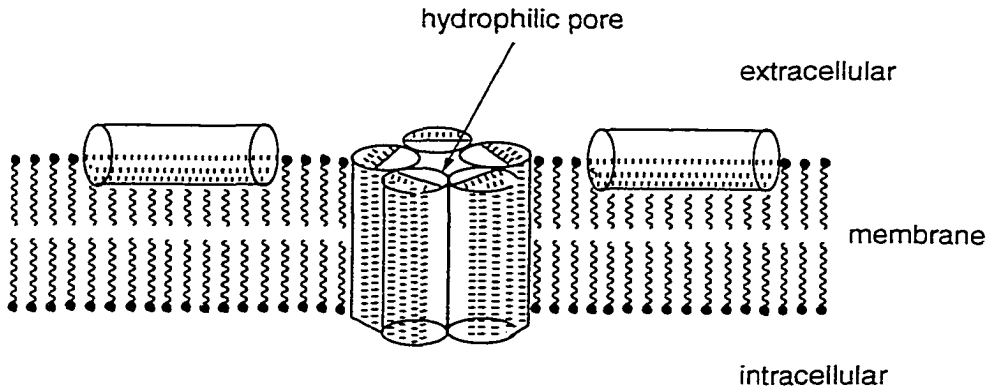


Figure 16. A pore-formation model for antimicrobial function of bacteriocins

The mode of antimicrobial action is a very complex process and there are still many unanswered questions regarding the details. The first question is whether there is a receptor protein involved in this process. There are conflicting reports regarding this putative receptor protein.[43,152] Even for the same bacteriocin, such as pediocin PA-1, different results were obtained from different studies.[48,153] If a putative receptor does exist, how bacteriocin binds to the receptor protein leading to cell death and the nature of the interaction between receptor protein and bacteriocin are presently unknown. A pore or channel forming process has been suggested as a common mechanism for many cationic antimicrobial peptides to kill target bacteria.[11,42] For class IIa bacteriocins, alternatively, both receptor binding and pore formation might be involved in the process of their antimicrobial function. The interaction between bacteriocin and its receptor or, the binding of bacteriocin to its receptor might be only the starting point of antimicrobial action. The binding itself may not be able to directly lead to cell death. A possible cascade of this process might be the aggregation of monomers at the surface of the target cell membrane to form water-filled pores, which may also be a critical part of the integrated process of

antimicrobial action. The water-filled pores are detrimental to the cell function and integrity by providing an outlet for the cell contents. On the other hand, if the binding of bacteriocin to its receptor protein is transient, there will be no stable bacteriocin-receptor complex formed during this process. Therefore, it would be very difficult to observe when bacteriocin and receptor closely associate with each other. What the receptor protein does in this process might be only to assist the binding and/or aggregation of bacteriocin molecules on the surface of target bacteria membranes. The receptor protein might play a role in translocation of bacteriocin molecules into target cells or onto the target cell membrane, which is similar to the function of the ABC transporter protein involved in bacteriocin production which functions in the reverse direction. The binding of bacteriocin to its putative receptor itself would not directly lead to cell death. Hence, if LeuA molecules aggregate and form pores on the cell membrane, they might covalently connect to each other instead of to its receptor protein when they are UV irradiated. The bands at *ca.* 14 kDa and *ca.* 20 kDa found in the above experiments might be different forms of this kind of aggregate. Nevertheless, the results do not exclude the possible existence of the receptor protein even though the aggregates could be isolated.

2.6 Preparative electrophoresis

Attempts to isolate the 14 kDa band were made using a preparative electrophoresis column gel. The sample preparation procedure was simply a scale up of the analytical process. After photoirradiation, the cell lysate was separated on a 15% polyacrylamide column gel using a Bio-Rad Mini Prep Cell. The 15% polyacrylamide concentration was chosen based on the optimization curve provided by the instrument supplier, this was expected to give a better resolution for small proteins with a molecular weight around 14 kDa. Elution was done with a flow rate of 0.08 mL/min. Fractions were collected and aliquots were taken from every five fractions to analyze its components as before. The results are shown in Figures 17 and 18.

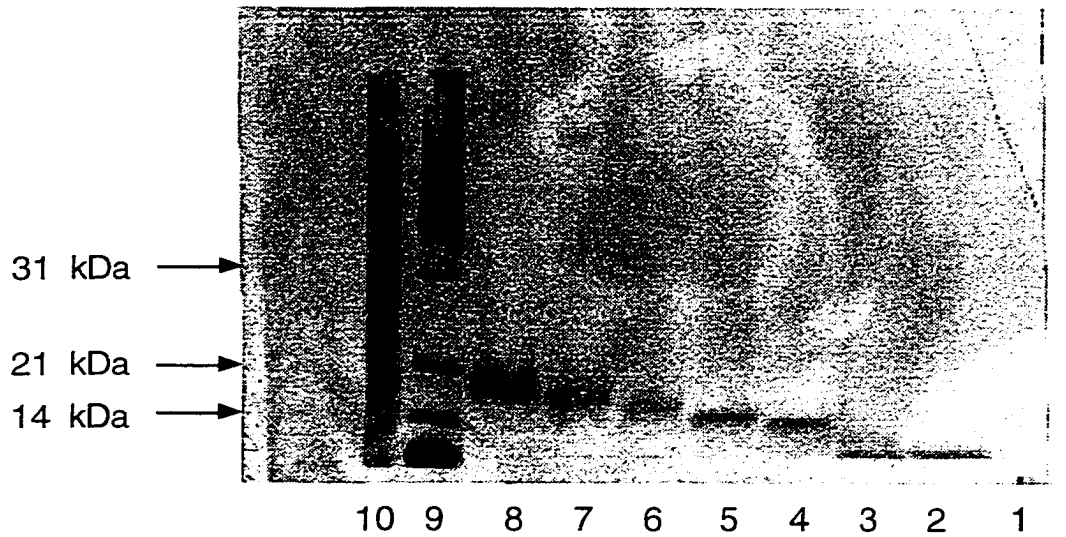


Figure 17. Blotting results of fractions from the preparative electrophoresis

Lane 1: Fraction #15;

Lane 2: Fraction #20;

Lane 3: Fraction #25;

Lane 4: Fraction #30;

Lane 5: Fraction #35;

Lane 6: Fraction #40;

Lane 7: Fraction #45;

Lane 8: Fraction #50;

Lane 9: Biotinylated protein standard (low range);

Lane 10: The mixture before the resolution.

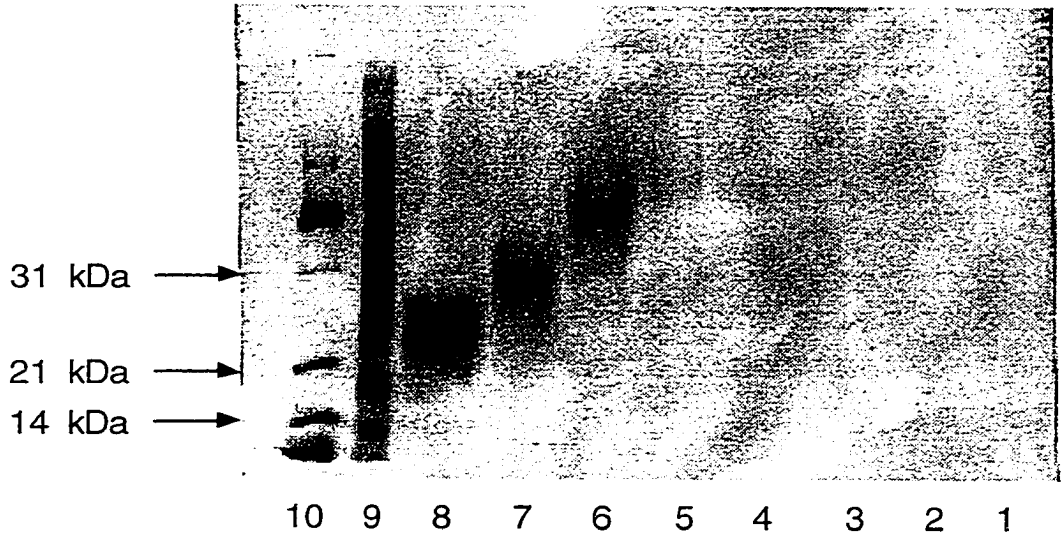


Figure 18. Blotting results of fractions from the preparative electrophoresis

Lane 1: Fraction #90;

Lane 2: Fraction #85;

Lane 3: Fraction #80;

Lane 4: Fraction #75;

Lane 5: Fraction #70;

Lane 6: Fraction #65;

Lane 7: Fraction #60;

Lane 8: Fraction #55;

Lane 9: The mixture before the resolution;

Lane 10: Biotinylated protein standard (low range).

As shown on Figure 17, the first major band at *ca.* 14 kDa was eluted in fractions 30 to 35 (lanes 4 and 5), and this was followed by the second band with a molecular weight of 20 kDa in fractions 45 to 50 (lanes 7 and 8). The rest of the material was in fractions 55 to 65 (lanes 6, 7 and 8 of Figure 18).

Fractions with a molecular weight *ca.* 14 kDa were combined and concentrated using a Centricon membrane. The concentrated sample was then further purified on RP-HPLC. A pure sample was obtained and the molecular weight was determined to be 7209.73, much smaller than the expected 14 kDa. N-Terminal amino acid sequencing of this sample **31** revealed that the first 11 amino acids were Met-Glu-Gln-Gly-Thr-Val-Lys-Trp-Phe-Asn-Ala.

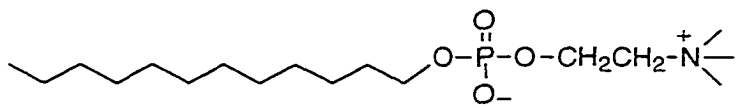
It was expected that the sequence of LeuA or a mixed sequence which included part of the sequence of LeuA could be elucidated from the purified protein sample. If the isolated product was the aggregate of Bpa-LeuA molecules, it would have still given the LeuA sequence. It is possible that the aggregate dissociated during the manipulation if they were not covalently linked, although, if this protein was labelled with biotinylated Bpa-LeuA, this covalently linked receptor-bacteriocin complex should have at least two N-termini, therefore it should give at least two sequences, a new sequence plus the sequence of LeuA. It is possible that the labelled protein may have been lost during the purification process due to the small quantity involved since the assay was very sensitive (1 to 10 pg range).[144] However, another possibility is that only a very small portion of this protein was labelled by Bpa-LeuA, and the majority is unlabelled. If this was the case, the sequencing would only have given the sequence of the unlabelled protein and the signals of the labelled product would be obscured by that of the unlabelled one.

A sequence homology search using PepTools™ 1.0 (BioTools, Inc., Edmonton, Alberta) found that the isolated protein had a high sequence homology with cold shock protein from *Bacillus subtilis*[154,155] or cold shock-like protein from *E. coli*.[156] These proteins are small in size (~7 kDa) and are produced by bacteria in response to the cold

shock from their environments. The second common property of these proteins is that they all have more acidic amino acids than basic amino acids and their pI values are lower than 6. Because LeuA, like other antimicrobial peptides, is a cationic peptide with a pI of 9.5, the isolated protein might be bound to LeuA in some way through an electrostatic interaction.

2.7 Labelling of lipids with Bpa-LeuA

It has been demonstrated that LeuA forms a defined amphipathic structure at its N-terminus in lipophilic environments or in membrane-mimicking environments, such as TFE or dodecylphosphocholine (DPC), while it is mainly unstructured in water or DMSO.[60] This indicates that the environments provided by the lipids are important for the stability of helical structure. Therefore the peptide molecules might interact with the lipid molecules in a specific way to form defined secondary and tertiary structure.



dodecylphosphocholine (DPC)

With the photoactive peptide Bpa-LeuA, we studied the interaction between the peptide and lipids. Irradiation of a solution of Bpa-LeuA in 180 mM DPC gave a new fraction on RP-HPLC, in addition to recovered starting material. The new substance **32** had a longer retention time on RP-HPLC. Under identical described chromatographic conditions, the retention time of this new fraction was 25.5 min, over four minutes longer than that of the starting material which eluted at 21.1 min. The molecular weight was determined to be 4385.18, which was in agreement with the calculated molecular weight of Bpa-LeuA plus DPC (4385.90). Attempts to further characterize this new molecule and to

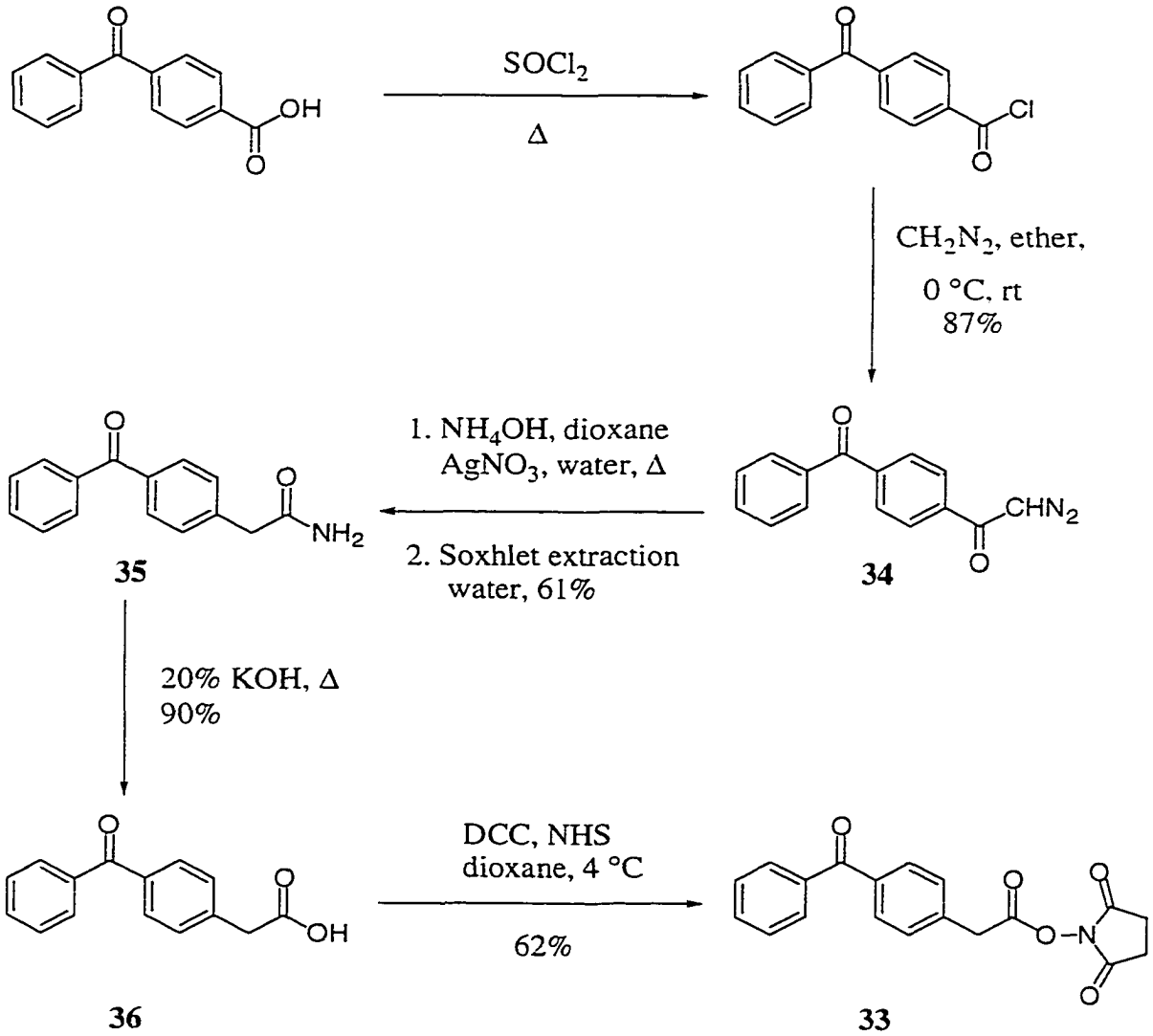
locate the exact connecting site between the photophore of the peptide and the lipid hydrocarbon chain were unsuccessful due to lack of material.

It is interesting to note that Bpa-LeuA did not attach to TFE or to itself under the same experimental conditions as that of the DPC experiment, even though LeuA assumes a well defined solution structure in 90% aqueous TFE. This observation indicates that, although both DPC and TFE can induce LeuA to form a defined structure in solution, LeuA molecules do not associate with each other in such a way as to form photo-linked polymers. Of course, the benzophenone diradical species is unable to abstract a hydrogen from the TFE molecule, and the result is most easily observed in this solvent.

2.8 Syntheses of *p*-benzoylphenylacetic acid *N*-hydroxysuccinimidyl ester 33 and the corresponding acylated LeuA derivatives

In order to introduce a benzophenone photophore at different positions of LeuA, *p*-benzoylphenylacetic acid *N*-hydroxysuccinimidyl ester **33** was prepared in five steps as outlined in Scheme 9.[157,158] The acyl chloride prepared from *p*-benzoylbenzoic acid was converted to the corresponding acyl chloride *in situ* using thionyl chloride. After the excess thionyl chloride was evaporated, the acyl chloride was directly reacted with freshly prepared diazomethane. Flash chromatography of the reaction mixture afforded **34** in good yield. Wolff rearrangement of **34** in the presence of silver salt and ammonia gave the corresponding amide **35**, which was hydrolyzed to the acid **36** in good yield. DCC coupling of *N*-hydroxysuccinimide to **36** in 1,4-dioxane gave the target compound **33**.[158]

LeuA **37** was synthesized by American Peptide Company, Inc. The identity of the synthetic peptide was confirmed by mass spectrometry, specific antimicrobial activity test, and co-injection on RP-HPLC with the peptide sample purified from its natural producer *L. gelidum* UAL187.[49]



Scheme 9

Modification of free amino groups of LeuA **37** with **33** was done in a mixture of water/DMF (1 : 1) with triethylamine as the base. The resulting products were purified by RP-HPLC. In addition to the recovery of starting material, four new fractions (**38**, **39**, **40**, **41**) were isolated and further purified to homogeneity by analytical RP-HPLC (Table 4). The ratio of the four new fractions was roughly 2 : 2 : 2 : 1 (**38** : **39** : **40** : **41**) according to integration of HPLC peaks. Mass spectrometry on these fractions revealed that the first three fractions have the same molecular weight, which matched the calculated

molecular weight corresponding to the monosubstituted LeuA. As mentioned earlier, there are two ε-amino groups from Lys1 and Lys11, one α-amino group at the N-terminus and one primary amino group from Arg28 present on LeuA which could possibly be modified. Each of the first three isolated fractions (**38**, **39**, **40**) might correspond to monosubstitution product of different sites. The observed molecular weight of the fourth fraction (**41**) was in agreement with a calculated molecular weight of a disubstituted product.

Table 4. Products of LeuA **37** modification with **33**

	37	**38**	**39**	**40**	**41**
RT (min)	26.7	29.4	29.8	30.3	32.8
MW (Da)	3930.07	4151.70	4151.60	4152.20	4373.50
	+/- 0.81	+/- 0.60	+/- 0.10	+/- 0.20	+/- 0.10
calcd MW (Da)	3932.33	4152.58	4152.58	4152.58	4374.83
Yield (%)	36	17	18	20	8
Activity (AU/μg)	512	42.7	18.7	5.3	1.2

N-Terminal amino acid sequencing on these modified products revealed the exact position of the substitute on the peptide chain. Edman degradation used for sequencing cleaves amino acid residues one by one from the peptide N-terminus using phenylisothiocyanate in conjunction with acidic hydrolysis.[159] This forms phenylthiocarbamyl amino acids, which are then separated by RP-HPLC and quantified from their UV absorbance. Each amino acid phenylisothiocyanate derivative has a characteristic retention time on RP-HPLC which is determined by its intrinsic structural features. By comparing the retention time of each cycle eluant with that of standard amino

acid derivatives, the peptide sequence is thus obtained. However, if the *N*-terminal α-amino group is derivatized, the degradation will not proceed and the peptide will not be sequenced by this method.

For **38**, Edman degradation revealed a sequence of XYYGNGVHCTKS, where X is an unknown residue. This indicated that the modification site was the ε-amino group of Lys1. This could be determined because that the first cycle of sequencing was "blank" (X) instead of Lys (K) being found as expected from the sequence of LeuA, while all of the following cycles of sequencing afforded the normal sequence of LeuA. For **39**, Edman degradation revealed a sequence of KYYGNGVHCTXS, which suggested that the ε-amino group of Lys11 was modified because the eleventh sequencing cycle was blank (X) and all of the degradation cycles before this revealed the normal sequence of LeuA. The third new fraction (**40**) could not be sequenced and therefore the modification site was likely to be the α-amino group based on the mechanism of Edman degradation.[159] The ratio of these products as mentioned above was 2 : 2 : 2 (**38** : **39** : **40**), which indicated that there was no selectivity among the several primary amino groups under these reaction conditions. The structures of **38**, **39** and **40** are shown in Figure 19.

The fourth new fraction (**41**), which is a disubstituted derivative of LeuA as indicated by its molecular weight, is likely to be a mixture of disubstituted products from a combination of varied modification sites since the selectivity among these primary amino groups is negligible. This fraction could be partially sequenced, which indicated that not all of the α-amino groups were modified since the blocked α-amino groups would make sequencing impossible. However, there was no Lys signal in the first cycle, but the second and the third cycle gave the normal Tyr signal, which indicated that the ε-amino group of Lys1 was modified in some cases. These fractions were all antimicrobially active even though the potencies varied, which will be discussed below.

The above monosubstituted products (**38**, **39**, **40**) were biotinylated in a mechanism similar to the one shown in Scheme 7 (page 56). The modification was performed in 0.1 M borate buffer, pH 8.6. The modified products were purified by RP-HPLC. The biotinylated products of **38**, **39** and **40** were **42**, **43** and **44**, respectively, and were confirmed by mass spectrometry. During the modification of **39**, a minor fraction of dibiotinylated product **45** was also isolated as indicated by electrospray MS.

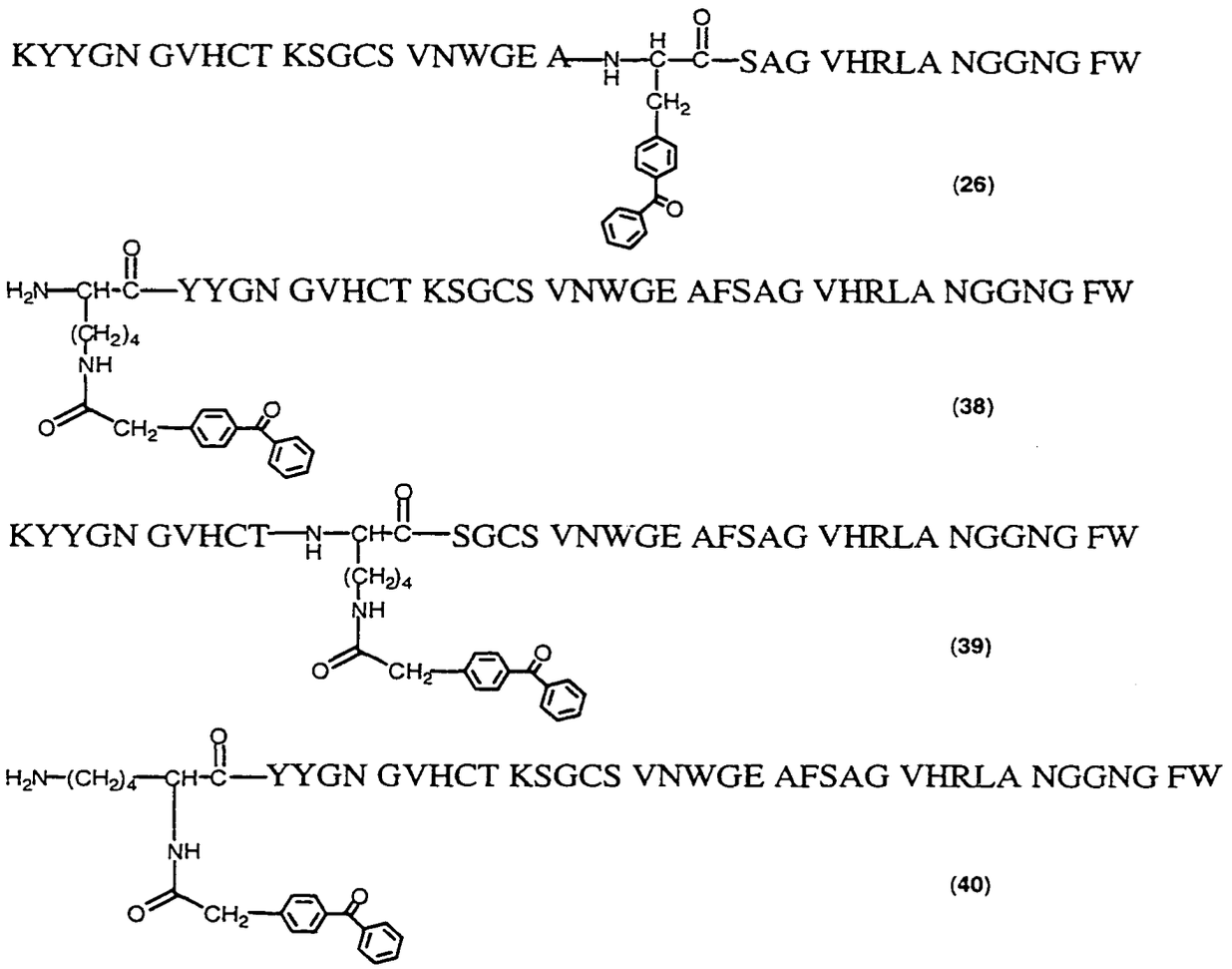


Figure 19. Structures of LeuA derivatives

2.9 Photoaffinity labelling of UAL9 and DPC with *N*-(*p*-benzoylphenyl-acetyl)-LeuA

Biotinylated compounds **42**, **43**, **44** and **45** were added to LeuA sensitive cells (UAL9) and irradiated under the same conditions to those described earlier for **27** and **29**. The results are shown in Figure 20. Compound **43** (lane 5), which bears the benzophenone photophore on the ε-amino group of Lys11, showed a similar labelling pattern as **27** and **29**. However, labelling bands in lanes 4 and 6 were of much weaker intensity. The efficiency of labelling was not as good as previously found in the case of biotinylated Bpa-LeuA. One possible reason for the observation is that the introduction of *p*-benzoylphenylacetyl group caused too much disturbance to the overall secondary structure and hydrophobicity of the peptide, as indicated by changes in its specific antimicrobial activity and the retention time on RP-HPLC.

Labelling of DPC with **38** was done in the same way as that described for **26** on page 71. The products were first desalted on an Econo-Pac 10 DG pre-packed column and further purified by RP-HPLC after UV irradiation. A new fraction **46** with a retention time of 34.7 min was isolated in addition to the recovery of starting material which was eluted at 30.7 min. The molecular weight of **46** was determined to be 4501.3 +/- 0.3, which corresponds to the adduct of **38** and DPC (calculated molecular weight 4503.5).

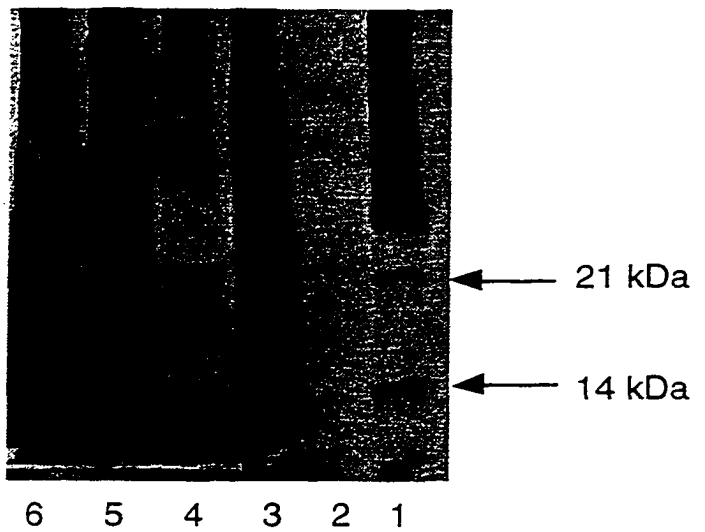


Figure 20. Photoaffinity labelling of UAL9 using biotinylated *N*-(*p*-benzoylphenylacetyl)-LeuA

Lane 1: Biotinylated molecular weight standards;

Lane 2: Control, cell proteins of UAL9;

Lane 3: Labelled UAL9 with biotinylated LeuA **45**;

Lane 4: Labelled UAL9 with biotinylated LeuA **44**;

Lane 5: Labelled UAL9 with biotinylated LeuA **43**;

Lane 6: Labelled UAL9 with biotinylated LeuA **42**;

2.10 Biotinylation of LeuA and avidin-biotin affinity chromatography

As an alternative to purification and identification of the putative LeuA receptor protein, the possibility of employing an avidin-biotin affinity chromatography was also explored. Monomeric avidin-agarose resin is commercially available and biotinylated LeuA was prepared in a similar way to the preparation of **27** shown in Scheme 7 (page 56).

The modification of the free amino groups of LeuA was done in 0.1 M borate buffer, pH 8.0 at 0 °C for 1 to 2 h. The reaction products were then purified by RP-HPLC. The activity assay was performed using *C. divergens* LV13 as an indicator. Biotinylation of LeuA with NHS-SS-biotin afforded **47**, **48** and **49** (Table 5). By comparison of the calculated molecular weights with the observed ones, it was found that **47** was a monosubstituted product; **48** and **49** were disubstituted products. Since it was previously found that there was no selectivity during the modification of the primary groups on the LeuA chain, **47** is likely to be a mixture of the three possible monoacylated products and **48** is likely to be a mixture of the disubstituted products. It is unclear why **49** showed such a large error between the calculated mass and the observed one.

Table 5. Biotinylated products of LeuA **37** with NHS-SS-biotin

	37	**47**	**48**	**49**
RT (min)	20.9	21.9	22.9	23.2
MW (Da)	3933.34	4325.00	4716.09	4708.80
	+/- 1.41	+/- 0.79	+/- 0.21	+/- 0.00
calcd MW (Da)	3932.33	4323.89	4715.45	4715.45
Activity	+	+	+	+

Similarly, biotinylation of LeuA using NHS-biotin afforded **50** and **51** (Table 6). Their molecular weights were in agreement with calculated ones. For the same reason as

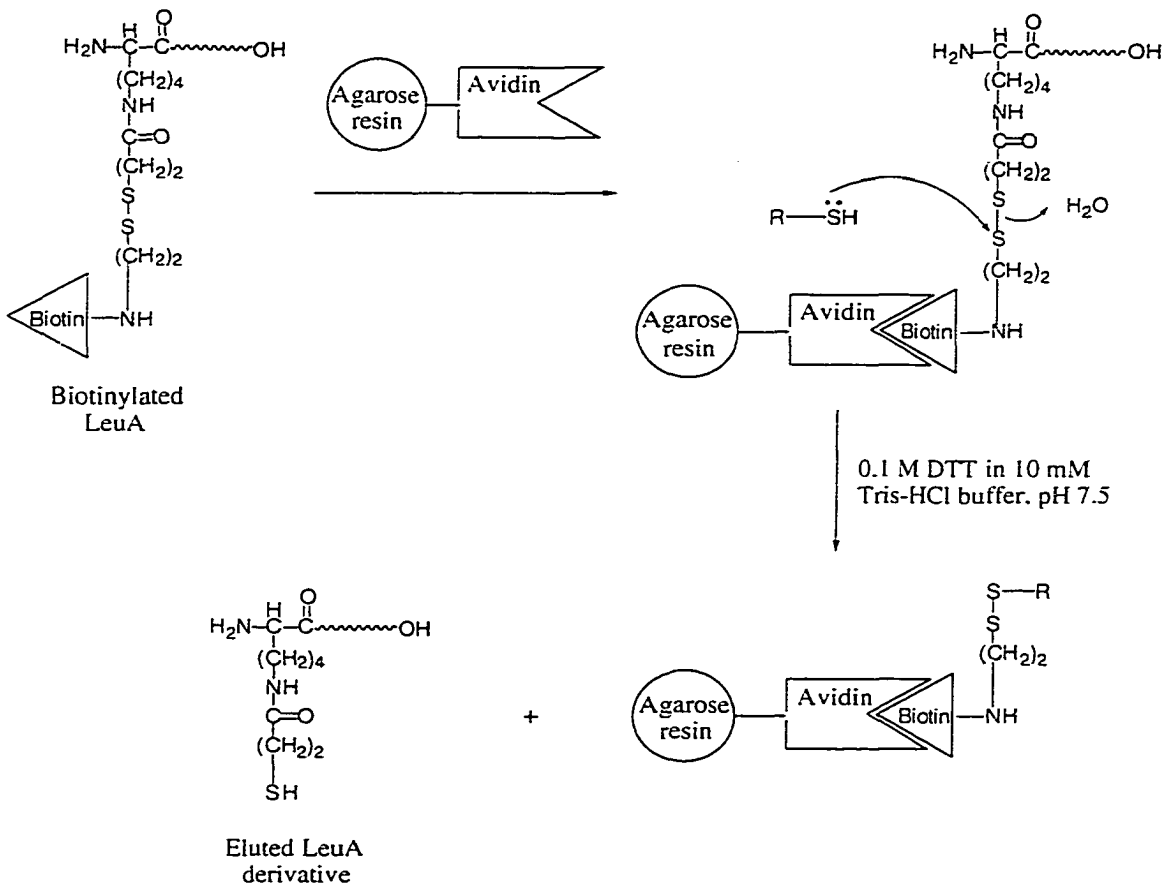
mentioned above, **50** is likely a mixture of monosubstituted products and **51** is a mixture of disubstituted products.

Table 6. Biotinylated products of LeuA **37** with NHS-biotin

	37	**50**	**51**
RT (min)	19.5	20.6	22.1
MW (Da)	3932.75 +/- 2.91	4271.52 +/- 1.12	4611.46 +/- 1.60
calcd MW (Da)	3932.33	4272.79	4613.25
Activity	+	+	+

A mixture of **47**, **48** and **49** in 0.1 M PBS, pH 7.0 was loaded onto the newly regenerated monomeric avidin-agarose resin. Attempts to elute the biotinylated peptides with biotin (1 mg/mL) in 0.1 M PBS, pH 7.0 or in 0.1 M glycine-HCl buffer, pH 2.0 were unsuccessful, possibly because binding between avidin and biotin was too tight to be dissociated under those conditions. However, the peptides could be eluted with 0.1 M DTT in 10 mM Tris-HCl buffer, pH 7.5 as shown in Scheme 10. The addition of DTT disrupted the disulfide linkage between biotin and LeuA, causing the release of LeuA from the agarose resin. The eluted peptides were desalted and purified by RP-HPLC.

Three fractions (**52**, **53** and **54**) (Table 7) were isolated. Their molecular weights were in agreement with the calculated ones, which was 4020.33 for a monosubstituted product (**52** and **53**) and 4108.33 for a disubstituted product **54**. It is interesting to note that the original biotinylated monosubstituted products could not be separated, but when the biotin moiety was cleaved through disruption of the disulfide linkage, the resulting monosubstituted products could be resolved (**52** and **53**). All three fractions were active towards *C. divergens* LV13 as detected by spot-on-lawn test.



Scheme 10

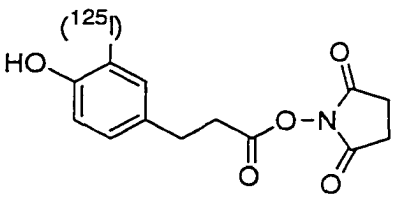
Table 7. Recovered LeuA derivatives from the avidin-agarose resin column

	52	**53**	**54**
RT (min)	21.6	22.2	22.8
MW (Da)	4019.99 +/- 0.68	4020.25 +/- 0.13	4108.63 +/- 0.51
calcd MW (Da)	4020.33	4020.33	4108.33
Activity	+	+	+

The experiments described above demonstrate that biotinylated LeuA can strongly bind to the avidin-agarose resin. Although the bound peptide could not be eluted through a commonly used process (a biotin solution or 0.1 M glycine-HCl, pH 2.0), it could be recovered by disrupting the disulfide linkage between biotin and LeuA with DTT. This technique could potentially be used for the isolation of the putative LeuA receptor.

2.11 Modification of LeuA with Bolton-Hunter Reagent

The possibility of incorporating a radioactive iodine moiety into LeuA as a tracking group was investigated by a model reaction. There are two ways to introduce ^{125}I into proteins. If the protein contains a Tyr residue, ^{125}I can be introduced directly by oxidative iodination of the Tyr residue with Chloramine-T.[160] Because Chloramine-T is considered a strong oxidizing agent, the application of this method is limited. Another method for iodine incorporation is using Bolton-Hunter reagent which has been developed for incorporating radioactive ^{125}I into peptides and proteins[140] that contain Lys residues. This method avoids the strong oxidation-reduction reaction conditions associated with the Chloramine-T procedure.



Bolton-Hunter Reagent

Commercially available Bolton-Hunter reagent (non-radioactive) was reacted with LeuA. The modified products (**55**, **56**, **57** and **58**) (Table 8) were purified by RP-HPLC. As indicated by the observed molecular weights, **55** was a monosubstituted product; **56** and **57** were disubstituted products; and **58** was a trisubstituted product.

N-Terminal amino acid sequencing revealed that **55** was a mixture of monosubstituted products, with roughly half of the sample acylated at the ε-amino group of Lys1, the other half was acylated at ε-amino group of Lys11. For compound **56**, both ε-amino groups of Lys1 and the ε-amino group of Lys11 were modified since it was found that the cycles of Lys1 and Lys11 were deleted on sequencing. Compound **57** could not be sequenced which indicates the α-amino group was derivatized.

Table 8. Modified products of LeuA **37** using Bolton-Hunter reagent

	37	**55**	**56**	**57**	**58**
RT (min)	21.5	22.5	23.5	23.8	24.9
MW (Da)	3930.97	4078.84	4228.20	4226.90	4378.59
	+/- 0.34	+/- 0.43	+/- 0.79	+/- 0.21	+/- 0.01
calcd MW (Da)	3932.33	4080.49	4228.65	4228.65	4376.81

Compounds **55**, **56** and **57** were active towards *C. divergens* LV13 and marginal activity was detected for **58**. The overall specific activity of a mixture of these modified peptides was approximately half of the value of their original peptide, LeuA.

2.12 Stucture-activity relationship of LeuA

Specific antimicrobial activity of bacteriocins is governed by many structural aspects and varies from one bacterial strain to another. Each bacteriocin has its own antimicrobial spectrum and does not possess the same specific activity for all of its sensitive bacteria.[13,37] As discussed in Chapter I, whereas a single amino acid substitution of CbnB2 (F33S, CbnS33) abolished its antimicrobial activity, conservative replacements of Val34 and Val37 with Ile did not bring about any significant changes.

PCR random mutagenesis of DNA encoding pediocin AcH (also named pediocin PA-1) produced a collection of pediocin AcH (see Figure 2, page 10 for sequence) structural variants.[161] Seven mutants (N5K, C9R, C14S, C14Y, G37E, G37R and C44W) were completely inactive against pediocin AcH-sensitive strains. Nine other mutants (K1N, W18R, I26T, M31T, A34D, N41K, H42L, K43N, and K43E) retained between 1% and 60% of the wild-type activity. One mutant, K11E, displayed 2.8 fold-higher activity. These results indicate that the residues of Cys9, Cys14 and Cys44 of pediocin AcH were among those required for activity since substitution of these residues led to a loss of activity. The basic amino acids as well as nonpolar amino acids located within the hydrophobic C-terminal region were also critical for full activity.

Synthetic studies of MesY105[47] revealed that an integral peptide chain is mandatory for high activity. Truncation of the last Trp residue (see Figure 2, page 10 for sequence) of MesY105 produced a dramatic 10,000-fold decrease in the anti-*Listerial* activity of the peptide. The replacement of Cys9 and Cys14 with Ser produced an analogue with a specific activity reduced by a factor of 20,000 relative to its parent compound. It has also been reported that the inhibitory potency and antimicrobial spectra of synthetic LeuA and MesY105 were roughly the same.[47] Through chemical modifications, we studied the correlation beween the charged residues and specific activity of LeuA as well as the importance of the disulfide bridge, since similar modification of MesY105 resulted in a dramatic change in activity.

2.12.1 Reduction and modification of the disulfide bridge of LeuA

In order to study the importance of the disulfide bridge between Cys9 and Cys14 on antimicrobial activity, the disulfide bond was first reduced with DTT in Tris-HCl buffer, pH 8.6 followed by *S*-carboamidomethylation with iodoacetamide. The resulting product **59** was purified by RP-HPLC and its identity was confirmed by mass spectrometry. The specific antimicrobial activity of the modified product, *S,S'*-dicarboamidomethyl-LeuA **59** was determined to be 5.3 AU/μg, about one hundred times less than the original peptide, LeuA. The indicator strain was *C. divergens* LV13. In contrast, as described in the previous chapter, *S,S'*-dicarboamidomethyl-CbnB2 **2** showed no detectable activity, which indicated that the disulfide bridge is essential for the activity of CbnB2.

2.12.2 Importance of charged residues in LeuA

Many derivatives of LeuA have been prepared by chemical modifications. These compounds are antimicrobially active but with varied potencies. As previously described, modification of LeuA with *p*-benzoylphenylacetic acid *N*-hydroxysuccinimidyl ester (**33**) gave three isomers (**38**, **39**, **40**), which carried the hydrophobic *p*-benzoyl phenylacetyl group at different positions (Figure 19, page 76). The specific activity of **26** is 205 AU/μg (Table 2, page 56) and that of **38**, **39** and **40** are 42.7, 18.7, 5.7 AU/μg, respectively (Table 4, page 74). The specific activity of the parent peptide (LeuA) is 512 AU/μg. The modification of the ε-amino group of Lys1 produced the least change in LeuA activity, suggesting that this residue plays a less crucial role in activity of the peptide. Derivatization of the α-amino group afforded a significant loss in activity suggesting that it was of great importance. Acylation of the α-amino group decreases its activity by 100-fold, which is of an equivalent magnitude to that observed for the disruption and modification of the

disulfide bridge (see above section 2.12.1). The modification of the ε-amino group of Lys11 created an effect somewhere in magnitude between the modifications of the α-amino group and the ε-amino group of Lys1. These antimicrobial assays demonstrated that although this modification of LeuA resulted in three compounds in which a different amino group had been acylated in each, the effects on the antimicrobial activity were different. The charges on the peptide chain are therefore not equally important for the antimicrobial function of the parent peptide. In other words, antimicrobial activity is not exerted *via* purely electrical charge interactions, otherwise all of the charges on the peptide chain would have had the same effect on the peptide's activity. The specific location of the charged residues also plays a role in the overall peptide activity.

These modifications also produced different effects on the overall hydrophobicity of the peptide as seen from their chromatographic properties. All three isomers (**38**, **39** and **40**) have the same net charges, but they were eluted at different times by RP-HPLC. For instance, the modification of the α-amino group increased the retention time by 4 min (**40**). Hydrophobic residues at the C-terminal end of class IIa bacteriocins are of critical important for their antimicrobial activity.[25,161] However, the above experiments demonstrated that introduction of hydrophobicity into the N-terminal end drastically compromised antimicrobial activity. A free N-terminal α-amino group is essential for the normal activity of LeuA.

Bpa-LeuA **26** has a similar specific antimicrobial activity to wild type LeuA and a similar retention time on reverse phase column chromatography. It has been shown that Phe22 of LeuA is located on the hydrophobic surface of the amphipathic α-helix at the C-terminal region.[60] These facts suggest that Bpa-LeuA might be a good mimic of LeuA.

Compared with the *p*-benzoyl phenylacetyl group, the biotin moiety is less hydrophobic. However, the bulk of the biotin moiety likely alters the secondary structure

of LeuA and biotinylation neutralizes the charged residues. Modification of Bpa-LeuA with NHS-SS-biotin reduced the specific antimicrobial activity of Bpa-LeuA by 10 fold for the monobiotinylated products and 50 fold for the disubstituted products (Table 2, page 56). These results further confirmed that the charged residues are important for the peptide activity.

2.13 Attempts to search for an agonist or an antagonist

In searching for the putative protein receptor of LeuA, an assay to identify the receptor is of critical importance. Since the bacteriocin-receptor interaction or the binding of bacteriocin to its receptor is specific, it should be possible to find an agonist or antagonist of this bacteriocin.

It was reported that the bactericidal activity of pediocin PA-1 could be specifically inhibited by a 15-mer fragment that encompasses the sequence of this bacteriocin from the center toward the C-terminus.[44] The presence of this fragment in the medium increased the MIC of pediocin PA-1 by more than 20 fold. However, this fragment shows little or no inhibitory activity towards other class IIa bacteriocins, such as LeuA and sakacin A. This observation may or may not be general for all class IIa bacteriocins, since pediocin PA-1 is different by having a second disulfide bridge at its C-terminal region. Since class IIa bacteriocins share a very similar N-terminal sequence, they might be agonistic or antagonistic towards each other.

As discussed in the previous chapter, class IIa bacteriocins are first biosynthesized as pre-bacteriocins which carry an N-terminal extension called a leader peptide. Although the exact biological role of this leader is not known, it seems that it prevents the bacteriocin from being biologically active while still inside the producer organism.[25,26] The mechanism by which its works is presently unknown. We investigated whether the leader peptide interferes with the activity of the mature bacteriocin *in vitro*.

2.13.1 Syntheses of LeuA fragments and inhibition studies of these peptides towards their parent bacteriocin LeuA

Three peptide fragments derived from the sequence of LeuA were synthesized: LeuA(18-32) **60**, LeuA(18-37) **61** and *N*-acetyl-LeuA(18-37) **62**. Inhibition studies of LeuA activity by the above fragments are shown in Figures 21, 22 and 23. When an equal amount of the peptide fragment was mixed with the parent peptide, weak inhibition of LeuA activity was detected for all three fragments (Figures 21, 22 and 23). The MIC (minimum inhibition concentration) of LeuA was doubled in the presence of an equal amount of LeuA(18-37) **61** in comparison with the control (Figure 22) which indicates the presence of **61** inhibits the activity of LeuA. However, this effect is much lower than that of the pediocin PA-1 fragment, which inhibited its parent peptide activity by 20 fold.[44] Increasing the amounts of the peptide fragments did not increase the inhibition towards LeuA for all the three synthetic peptides. On the contrary, the MIC of LeuA was slightly reduced, which was presumably due to the residual activity of the peptide fragments themselves. There was no obvious effect towards the activity of LeuA (Figure 23) in the presence of the *N*-acetylated fragment **62**.

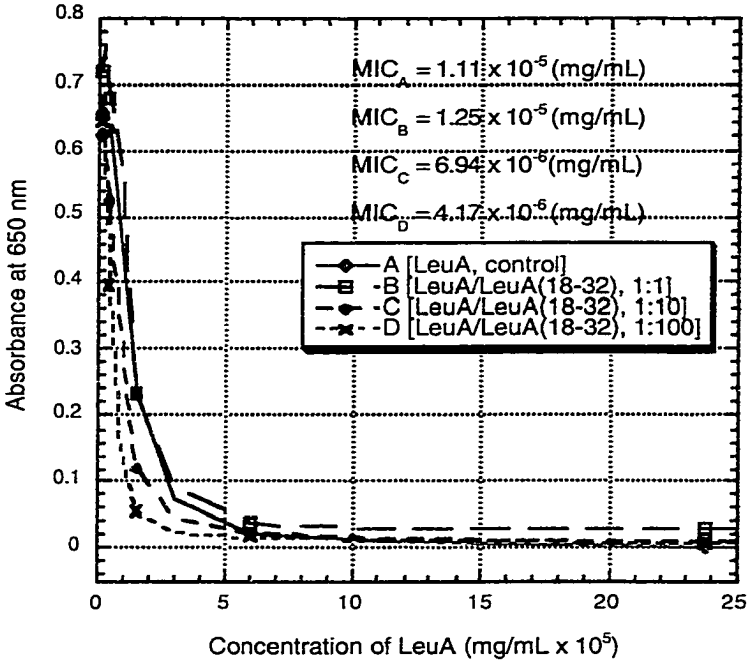


Figure 21. Inhibition studies of LeuA(18-32) **60** towards LeuA **37**

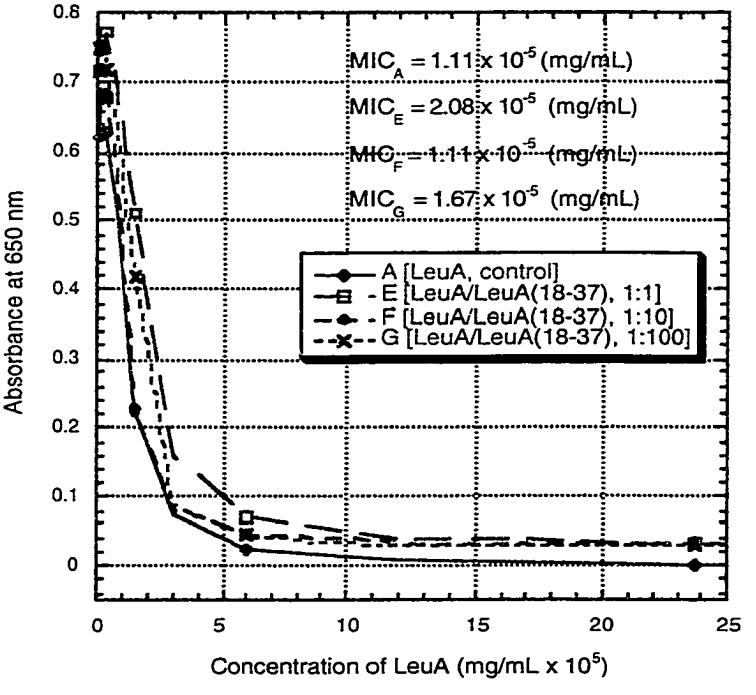


Figure 22. Inhibition studies of LeuA(18-37) **61** towards LeuA **37**

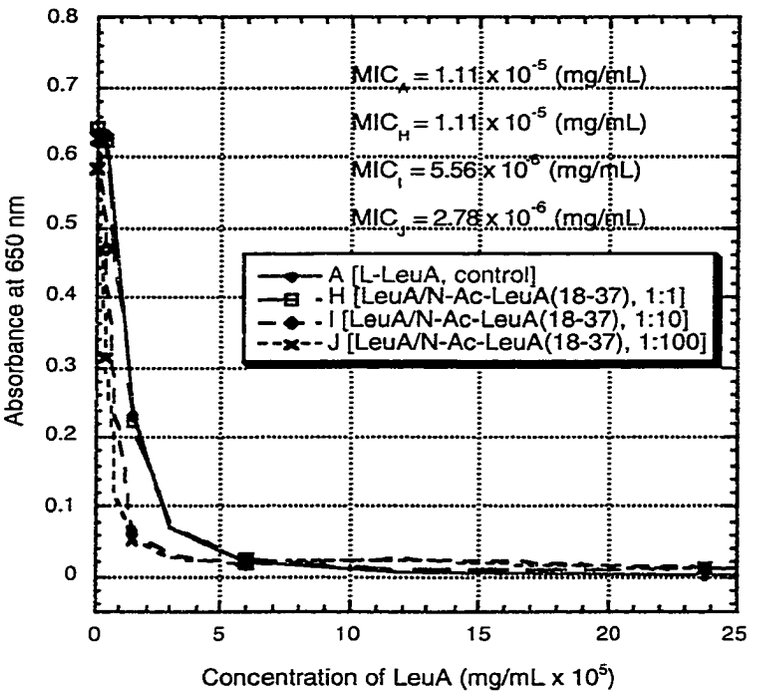


Figure 23. Inhibition Studies of *N*-Ac-LeuA(18-37) **62** towards LeuA **37**

2.13.2 Competitive studies of CbnB2 towards LeuA

CbnB2 and LeuA share a high sequence homology at their N-termini (Figure 5, page 23) and very similar C-terminal stuctures (Figure 4, page 20). Although they have different antimicrobial spectra (Table 9), they might affect each other's activity if they were present together. In order to study the possible agonistic/antagonistic effects between CbnB2 and LeuA, several bacterial strains which are sensitive to LeuA, but insensitive to CbnB2, were screened with LeuA solutions supplemented with different amounts of CbnB2. The antimicrobial activity of each combination was measured with the spot-on-lawn technique. The plates were incubated at 25 °C overnight before the inhibition zone was measured. A solution of LeuA at the same concentration without CbnB2 was used as a control. The change of the specific activity of LeuA reflects the influence of CbnB2. The results of these assays are listed in Tables 10 to 16.

Table 9. Selected antimicrobial spectra of LeuA and CbnB2

	LeuA	CbnB2
Enterococcus ATCC 19433	+	+
Leuconostoc UAL 187.13	+	+
Leuconostoc UAL187	-	-
Lactococcus ATCC 43921	-	-
Carnobacterium UAL26/8B	+	-
Enterococcus ATCC 11576	+	-
Enterococcus BEF900	+	-
Carnobacterium N5	+	-

The presence of up to ten times the concentration of CbnB2 in the solution of LeuA did not inhibit the antimicrobial activity of LeuA towards all of the tested strains. However, for *C. piscicola* UAL26/8B, when CbnB2 was present in 1000 times the concentration of LeuA, the specific activity of LeuA was reduced by 16 fold (Table 14). Due to the availability of material, only one strain was tested. Weak agonistic effects were found for the stains of *Lactococcus mesenteroides* 23386 and *Lactobacillus sake* 20017. The presence of ten times the amount of CbnB2 increased the specific activity of LeuA by 2 to 4 fold. CbnB2 did not interfere with the activity of LeuA for all of the tested *Enterococcus* strains and *C. piscicola* N5.

Table 10. Competitive inhibition studies of CbnB2 and LeuA towards *Lactococcus mesenteroides* 23386

Peptide conc. (mg/mL)	Inhibition zone (mm) at different dilutions						
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}
LeuA/CbnB2 0.1 : 0.1	17	16	14	11	10	-	-
LeuA/CbnB2 0.1 : 1.0	17	16	14	12	11	10	-
LeuA/CbnB2 0.1 : 0.01	17	15	13	12	10	10	9
LeuA 0.1 (no CbnB2)	16	15	13	11	10	-	-

Table 11. Competitive inhibition studies of CbnB2 and LeuA towards *Lactobacillus sake* 20017

Peptide conc. (mg/mL)	Inhibition zone (mm) at different dilutions						
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}
LeuA/CbnB2 0.1 : 0.1	18	17	15	12	10	-	-
LeuA/CbnB2 0.1 : 1.0	19	17	15	15	13	10	8
LeuA/CbnB2 0.1 : 0.01	17	16	15	13	11	11	10
LeuA 0.1 (no CbnB2)	18	16	14	11	10	-	-

Table 12. Competitive inhibition studies of CbnB2 and LeuA towards *Carnobacterium piscicola* N5

Peptide conc. (mg/mL)	Inhibition zone (mm) at different dilutions						
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}
LeuA/CbnB2 0.1 : 1.0	15	14	13	12	12	11	-
LeuA/CbnB2 0.1 : 0.1	14	14	13	12	10	-	-
LeuA/CbnB2 0.1 : 0.01	13	13	12	11	11	11	10
LeuA 0.1 (no CbnB2)	14	13	11	11	10	-	-

Table 13. Competitive inhibition studies of CbnB2 and LeuA towards *Enterococcus faecium* BFE900

Peptide conc. (mg/mL)	Inhibition zone (mm) at different dilutions						
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}
LeuA/CbnB2 0.1 : 0.1	14	13	11	-	-	-	-
LeuA/CbnB2 0.1 : 1.0	15	12	11	-	-	-	-
LeuA/CbnB2 0.1 : 0.01	13	12	10	-	-	-	-
LeuA 0.1 (no CbnB2)	13	11	10	-	-	-	-

Table 14. Competitive inhibition studies of CbnB2 and LeuA towards *Carnobacterium piscicola* UAL26/8B

Peptide conc. (mg/mL)	Inhibition zone (mm) at different dilutions						
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}
LeuA/CbnB2 0.1 : 10^2	n/d	-	-	-	-	-	-
LeuA/CbnB2 0.1 : 10	n/d	13	10	-	-	-	-
LeuA/CbnB2 0.1 : 1.0	17	17	14	12	11	-	-
LeuA/CbnB2 0.1 : 0.1	17	15	14	12	-	-	-
LeuA/CbnB2 0.1 : 0.01	17	15	14	11	9	-	-
LeuA 0.1 (no CbnB2)	16	15	12	9	-	-	-

Table 15. Competitive inhibition studies of CbnB2 and LeuA towards *Enterococcus* ATCC 11576

Peptide conc. (mg/mL)	Inhibition zone (mm) at different dilutions						
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}
LeuA/CbnB2 0.1 : 0.1	15	14	13	12	-	-	-
LeuA/CbnB2 0.1 : 1.0	16	14	13	12	-	-	-
LeuA/CbnB2 0.1 : 0.01	15	13	13	11	-	-	-
LeuA 0.1 (no CbnB2)	15	14	12	10	-	-	-

Table 16. Competitive inhibition studies of CbnB2 and LeuA towards *Enterococcus* ATCC 19432

Peptide conc. (mg/mL)	Inhibition zone (mm) at different dilutions						
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}
LeuA/CbnB2 0.1 : 0.1	14	14	11	-	-	-	-
LeuA/CbnB2 0.1 : 1.0	15	14	11	-	-	-	-
LeuA/CbnB2 0.1 : 0.01	15	13	11	-	-	-	-
LeuA 0.1 (no CbnB2)	15	12	11	-	-	-	-

2.13.3 Effects of CbnB2(1-22) 3 and CbnB2 leader peptide 63 on the antimicrobial activity of CbnB2 1

The leader peptide of CbnB2 (MNSVK ELNVK EMKQL HGG) **63** was synthesized at the Alberta Peptide Institute (Edmonton, Alberta). The identity of this peptide was confirmed by mass spectrometry. The effects of the 22-mer peptide fragment and the CbnB2 leader peptide towards its parent bacteriocin CbnB2 were analyzed in the same way as that outlined above for the fragments of LeuA. The culture medium was supplemented with different amounts of CbnB2 or a mixed sample of CbnB2 and the leader peptide **63** or CbnB2 and the 22-mer fragment CbnB2(1-22) **3**. *C. divergens* LV13 was used as the indicator strain. The growth of the culture was monitored spectrophotometrically at 650 nm using a microplate reader. The culture absorbance was then plotted against CbnB2 concentration and the MIC was read from the graph (Figures 24 and 25).

The leader peptide of CbnB2 **63** showed a very minor antagonistic effect towards CbnB2 when it was present at a high concentration (Figure 24). There was no detectable effect when its concentration was equal to or ten times that of the parent peptide. When the leader peptide is covalently attached to its parent bacteriocin, it can drastically affect the bacteriocin's activity (see Chapter I). However, the leader peptide could not effectively reduce the antimicrobial activity of its parent bacteriocin when it was free from the N-terminus of its bacteriocin as demonstrated by the above experiments.

CbnB2(1-22) **3** did not inhibit the activity of CbnB2 at all of the tested concentrations. When it was present at higher concentrations, it slightly reduced the MIC of CbnB2, which was possibly due to the residual activity of the peptide fragment. These results indicate that the whole peptide chain, or at least not only the N-terminus, is involved in bacteriocin action and that the N-terminal region is not solely responsible for receptor binding which confers the specificity of class IIa bacteriocins.

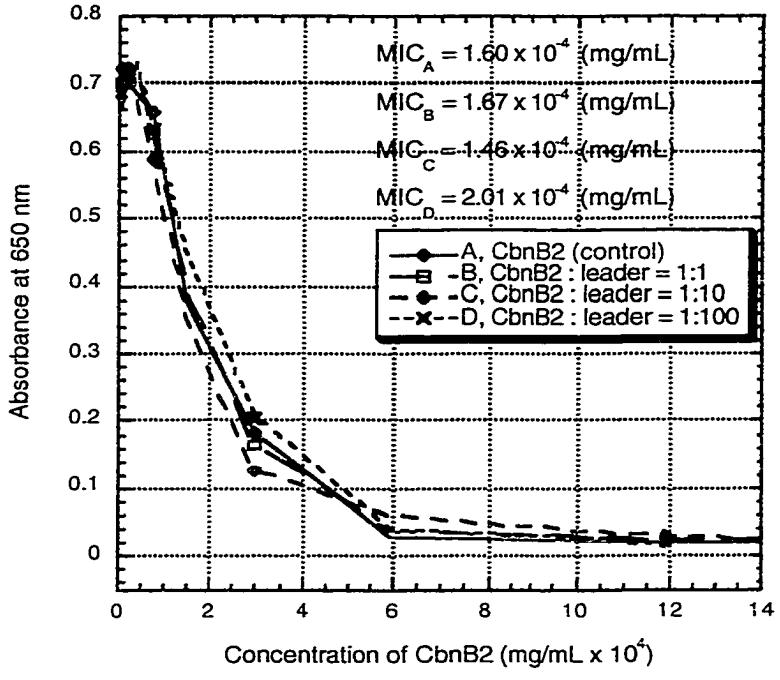


Figure 24. Effect of CbnB2 leader peptide **63** on the activity of CbnB2 **1**

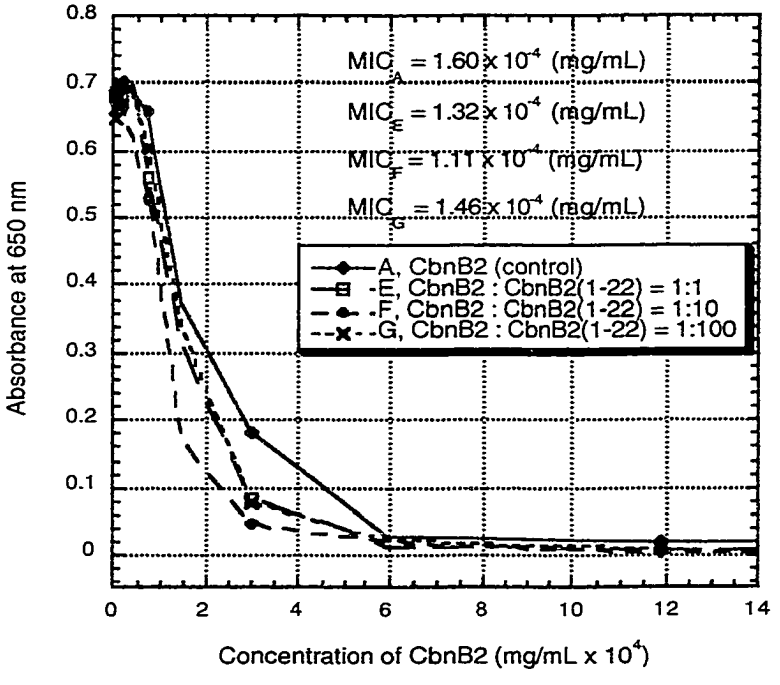


Figure 25. Effect of CbnB2(1-22) **3** fragment on the activity of CbnB2 **1**

2.14 D-LeuA synthesis and characterization

2.14.1 Synthesis and structural characterizations of D-LeuA

D-LeuA was successfully synthesized by the solid phase method using Fmoc protected D-amino acids. This work was done with assistance of Mr. Al Gibbs (Faculty of Pharmacy). The side chains of sensitive Fmoc-D-amino acids were protected as follows: Arg (Pmc); Asn (Trt); Cys (Trt); Glu (OtBu); His (Trt); Lys (Boc); Ser (tBu); Thr (tBu); Trp (Boc); Tyr (tBu). This is the first total synthesis of the enantiomer of a naturally occurring class IIa bacteriocin. Throughout the synthesis, test cleavages were performed and intermediates were characterized by mass spectrometric analysis; the desired molecular weights were obtained in each case. When the chain assembly was complete, the crude peptide was cleaved from the resin and simultaneously deprotected, and then purified to homogeneity by RP-HPLC. The molecular weight of the purified peptide was determined to be 3932.11 ± 0.35 (Figure 26. calcd mass 3932.33) by ES-MS.

After the cleavage and during the purification, the peptide was mainly present in the reduced thiol form as shown by a carboxyamidomethylation test using iodoacetamide. Following oxidation with oxygen in ammonium bicarbonate buffer, the molecular weight was determined to be 3929.95 ± 0.59. There was no obvious difference in retention time on reverse phase HPLC for the oxidized and reduced species. Oxidization was complete in 16 h at room temperature and was confirmed by iodoacetamide treatment. For the oxidized sample, the two cysteine residues could be carboxyamidomethylated only if it was previously treated with DTT. The yield of D-LeuA was *ca.* 6% overall with more than 95% purity (Figure 27). On reverse phase HPLC, the synthetic D-LeuA was identical to its L-isomer (L-LeuA) and a 1 : 1 mixture of the two enantiomers gave a single peak (Figure 28). The N-terminal amino acid sequencing on the purified peptide gave the expected sequence KYYGN for the first five amino acids.

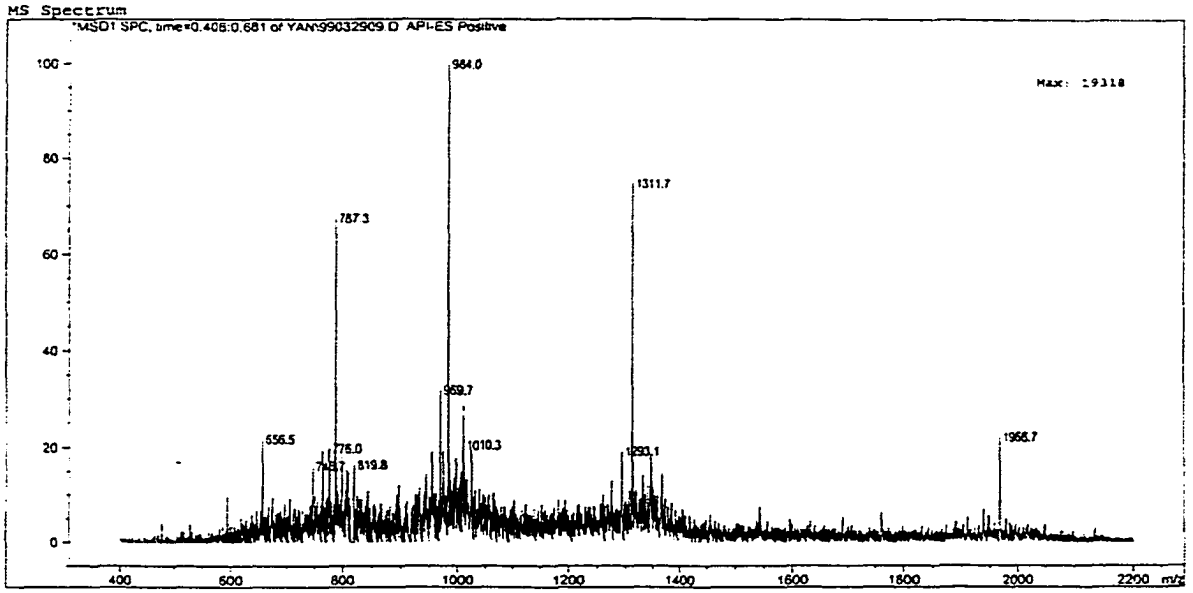


Figure 26. Mass spectrum of synthetic D-LeuA

(Multiple ions $[M + nH]^{n+}$ at *m/z* values of 1966.7, 1311.7, 984.0, 787.3, 656.5 are corresponding, respectively, to n = 2, 3, 4, 5, 6 protonated species. Determined molecular weight after deconvolution 3932.11 +/- 0.35)

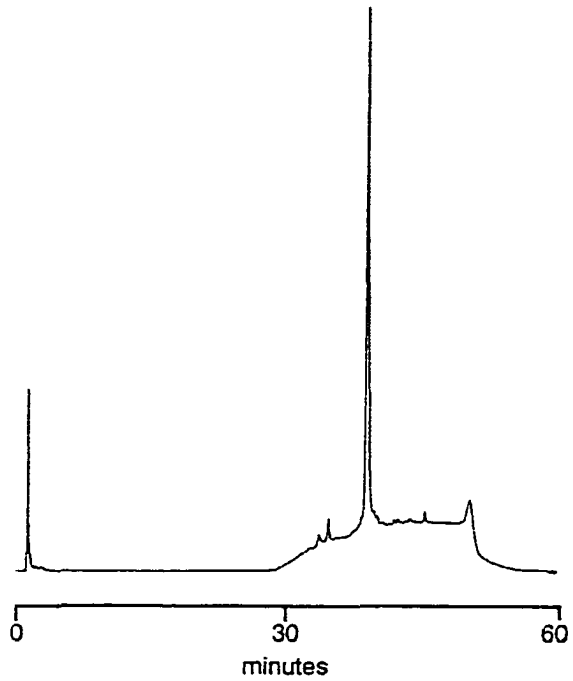


Figure 27. HPLC trace of the purified D-LeuA

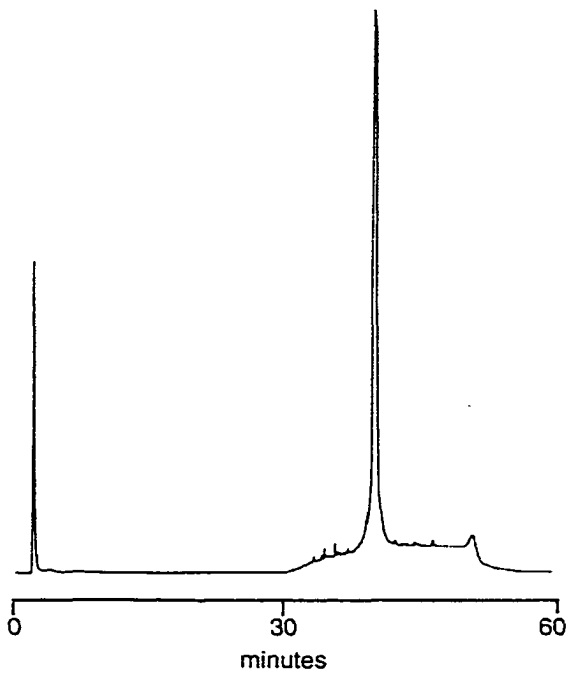


Figure 28. HPLC trace of a 1:1 mixture of L-LeuA and D-leuA

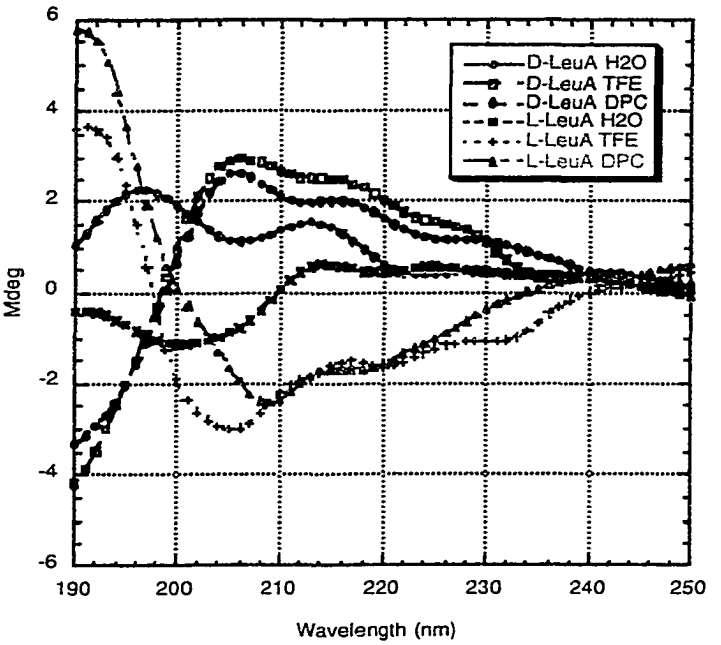


Figure 29. CD Spectra of D- and L-LeuA (0.1 mM) in various solvents

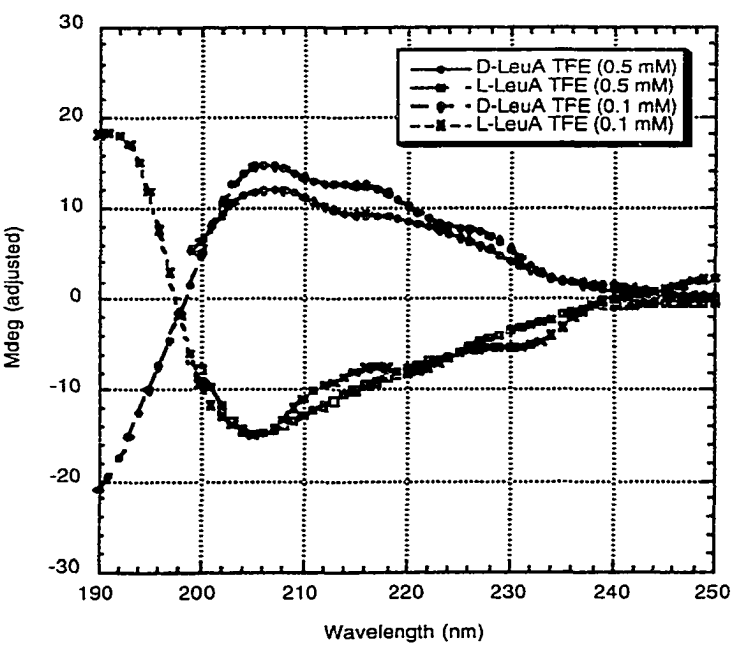
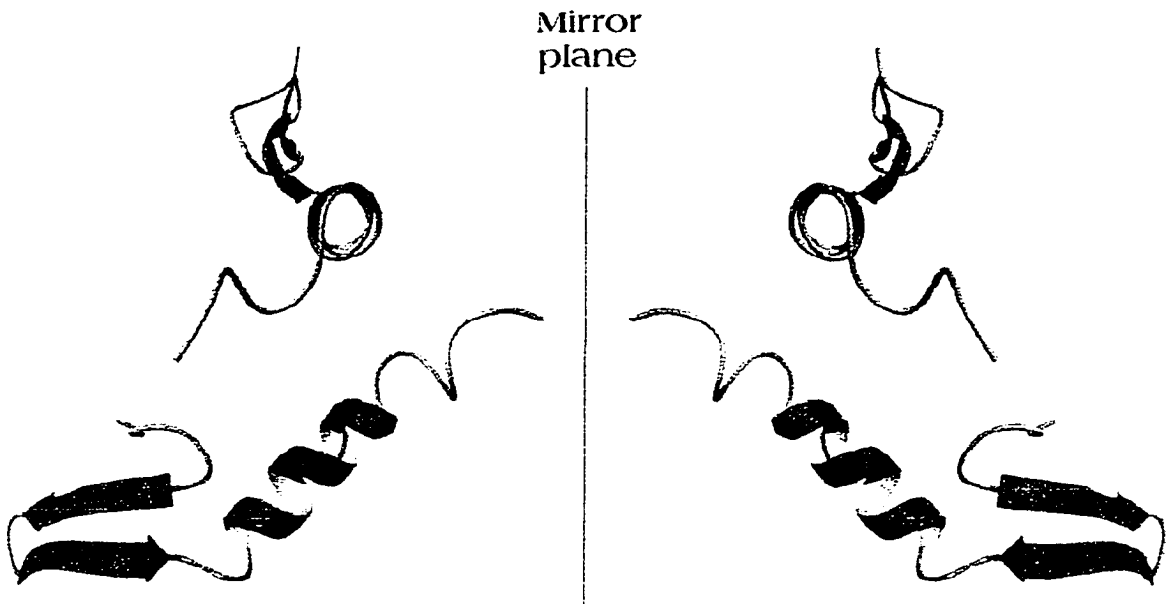


Figure 30. CD Spectra of D- and L-LeuA in TFE



L-LeuA[60] **D-LeuA (a model)**

Figure 31. The model structure of D-LeuA compared with the solution structure of L-LeuA[60]

(Top parts of the figure shows a view along the helices. Lower parts of the figure are the side view of the helices and the β-sheets. MolMol was used for the ribbon rendering.)

Circular dichroism spectra of the synthetic D-LeuA and L-LeuA in various solvents are shown in Figure 29. Both peptides are mostly unstructured in aqueous 0.1% TFA. In 90% TFE or in 4 mM DPC, they assume an α-helical secondary structure as indicated by the high optical rotation at 206 nm. However, the helices are of opposite handedness. D-LeuA forms a left-handed helix and L-LeuA forms a right-handed one. Both peptides give a better helical structure in 90% TFE than in 4 mM DPC. At higher peptide concentration (0.5 mM) in 90% TFE, CD spectra of the two enantiomers are a perfect mirror image of each other as shown in Figure 30. According to the CD spectra and preliminary NMR studies, a model structure of D-LeuA was constructed by our collaborator Mr. A. Gibbs at Faculty of Pharmacy. The three dimensional solution structure of L-LeuA reported by our colleagues[60] is shown in Figure 31 along with the model structure of D-LeuA.

2.14.2 Tests for antimicrobial activity of D-LeuA and competitive studies of L-LeuA and D-LeuA

While L-LeuA was active towards all of the screened strains (except its producer strain *L.* UAL187), D-LeuA had no detectable activity (< 0.25 AU/μg) for the following tested strains: *C. divergens* LV13, *C. piscicola* N5, *C. piscicola* LV17A, *C. piscicola* LV17B (Bac+ and Bac-), *Enterococcus faecium* BFE 900, *L. gelidum* UAL187, *L. gelidum* UAL187.13, *Lactococcus messenteroides* 23386, *Lactobacillus sake* 20017, and *Listeria monocytogenes* LI0502. Results of these assays are listed in Tables 17 to 25. At high concentration, D-LeuA had a residual activity of *ca.* 0.05 AU/μg towards *C. divergens* LV13. Under identical conditions, the specific activity of L-LeuA was higher by a factor of 10^5. For D-LeuA, before and after the formation of the disulfide bridge between Cys9 and Cys14, there was no obvious changes in its residual activity towards *C. divergens* LV13.

In order to test possible agonistic/antagonistic effects of these two enantiomers, differing amounts of L-LeuA and D-LeuA were mixed together in various ratios and tested

for changes in the specific activity of L-LeuA. The results varied from strain to strain and are given in Tables 17 to 25. Compared with the controls, the presence of up to one hundred times of D-LeuA showed no effect on the specific activity of L-LeuA towards the strains of *C. divergens* LV13, *C. piscicola* N5, *C. piscicola* LV17A, *C. piscicola* LV17B (Bac+), *L.* UAL187 and *Lactococcus messenteroides* 23386. However, weak agonistic effects were found in the other tested strains: *L.* UAL187.13, *C. piscicola* LV17B (Bac-), *Enterococcus faecium* BFE 900, *Lactobacillus sake* 20017. For *Lactobacillus sake* 20017, the specific activity of L-LeuA was increased by two dilutions (Table 18). In no case did D-LeuA inhibit the activity of its L-isomer.

Table 17. Competitive inhibition studies of D- and L-LeuA towards *C. divergens* LV13

Peptide conc.	Activity at different dilutions									
(mg/mL)	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.004 (D) : 0.04 (L)	+	+	+	+	+	+	+	+	+/-	-
0.04 (D) : 0.04 (L)	+	+	+	+	+	+	+	+	+/-	-
0.4 (D) : 0.04 (L)	+	+	+	+	+	+	+	+	+/-	-
0.4 (D) : 0.004 (L)	+	+	+	+	+/-	-	-	-	-	-
0.04 (L)	+	+	+	+	+	+	+	+	+/-	-
0.004 (L)	+	+	+	+	+/-	-	-	-	-	-
0.4 (D)	-	-	-	-	-	-	-	-	-	-
0.04 (D)	-	-	-	-	-	-	-	-	-	-
0.004 (D)	-	-	-	-	-	-	-	-	-	-

+, +/- and - refer to positive, borderline and negative activity, respectively.

Table 18. Competitive inhibition studies of D- and L-LeuA towards *Lactobacillus sake* 20017

Peptide conc.	Activity at different dilutions									
(mg/mL)	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.004 (D) : 0.04 (L)	+	+	+	+	+	+	+	+	+	+*
0.04 (D) : 0.04 (L)	+	+	+	+	+	+	+	+	+	+*
0.4 (D) : 0.04 (L)	+	+	+	+	+	+	+	+	+	-
0.4 (D) : 0.004 (L)	+	+	+	+	-	-	-	-	-	-
0.04 (L)	+	+	+	+	+	+	+	+	-	-
0.004 (L)	+	+	+	-	-	-	-	-	-	-
0.4 (D)	-	-	-	-	-	-	-	-	-	-

* Further dilutions all showed negative activity.

Table 19. Competitive inhibition studies of D- and L-LeuA towards *C. piscicola* LV17A

Peptide conc.	Activity at different dilutions									
(mg/mL)	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.004 (D) : 0.04 (L)	+	+	+	+	+	+/-	-	-	-	-
0.04 (D) : 0.04 (L)	+	+	+	+	+	+	-	-	-	-
0.4 (D) : 0.04 (L)	+	+	+	+	+	+	+/-	-	-	-
0.4 (D) : 0.004 (L)	+	+	+	-	-	-	-	-	-	-
0.04 (L)	+	+	+	+	+	+/-	-	-	-	-
0.004 (L)	+	+	+/-	-	-	-	-	-	-	-
0.4 (D)	-	-	-	-	-	-	-	-	-	-

Table 20. Competitive inhibition studies of D- and L-LeuA towards *C. piscicola* LV17B

Peptide conc.	Activity at different dilutions									
(mg/mL)	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.004 (D) : 0.04 (L)	+	+	+	+	-	-	-	-	-	-
0.04 (D) : 0.04 (L)	+	+	+	+	+	-	-	-	-	-
0.4 (D) : 0.04 (L)	+	+	+	+	-	-	-	-	-	-
0.4 (D) : 0.004 (L)	+	-	-	-	-	-	-	-	-	-
0.04 (L)	+	+	+	+	+	-	-	-	-	-
0.004 (L)	+	-	-	-	-	-	-	-	-	-
0.4 (D)	-	-	-	-	-	-	-	-	-	-

Table 21. Competitive inhibition studies of D- and L-LeuA towards *C. piscicola* LV17B (Bac$^-$ strain)

Peptide conc.	Activity at different dilutions									
(mg/mL)	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.004 (D) : 0.04 (L)	+	+	+	+	+	+	-	-	-	-
0.04 (D) : 0.04 (L)	+	+	+	+	+	+	+	-	-	-
0.4 (D) : 0.04 (L)	+	+	+	+	+	+	-	-	-	-
0.4 (D) : 0.004 (L)	+	+	+	-	-	-	-	-	-	-
0.04 (L)	+	+	+	+	+	-	-	-	-	-
0.004 (L)	+	+	-	-	-	-	-	-	-	-
0.4 (D)	-	-	-	-	-	-	-	-	-	-

Table 22. Competitive inhibition studies of D- and L-LeuA towards *C. piscicola* N5

Peptide conc.	Activity at different dilutions									
(mg/mL)	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.004 (D) : 0.04 (L)	+	+	+	+	+	+	+	-	-	-
0.04 (D) : 0.04 (L)	+	+	+	+	+	+	+	-	-	-
0.4 (D) : 0.04 (L)	+	+	+	+	+	+	+	-	-	-
0.4 (D) : 0.004 (L)	+	+	+	-	-	-	-	-	-	-
0.04 (L)	+	+	+	+	+	+	+	-	-	-
0.004 (L)	+	+	+	-	-	-	-	-	-	-
0.4 (D)	-	-	-	-	-	-	-	-	-	-

Table 23. Competitive inhibition studies of D- and L-LeuA towards *Enterococcus faecium* BFE 900

Peptide conc.	Activity at different dilutions									
(mg/mL)	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.004 (D) : 0.04 (L)	+	+	+	+	+	+	+	-	-	-
0.04 (D) : 0.04 (L)	+	+	+	+	+	+	+	-	-	-
0.4 (D) : 0.04 (L)	+	+	+	+	+	+	-	-	-	-
0.4 (D) : 0.004 (L)	+	+	+	+	-	-	-	-	-	-
0.04 (L)	+	+	+	+	+	+	-	-	-	-
0.004 (L)	+	+	+	-	-	-	-	-	-	-
0.4 (D)	-	-	-	-	-	-	-	-	-	-
0.04 (D)	-	-	-	-	-	-	-	-	-	-

Table 24. Competitive inhibition studies of D- and L-LeuA towards *Lactococcus mesenteroides* 23386

Peptide conc.	Activity at different dilutions									
(mg/mL)	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.004 (D) : 0.04 (L)	+	+	+	+	+	+	+	+	-	-
0.04 (D) : 0.04 (L)	+	+	+	+	+	+	+	+	+	-
0.4 (D) : 0.04 (L)	+	+	+	+	+	+	+	+	-	-
0.4 (D) : 0.004 (L)	+	+	+	+	-	-	-	-	-	-
0.04 (L)	+	+	+	+	+	+	+	+	-	-
0.004 (L)	+	+	+	+	-	-	-	-	-	-
0.4 (D)	-	-	-	-	-	-	-	-	-	-

Table 25. Competitive inhibition studies of D- and L-LeuA towards *L. gelidum* UAL187.13 (plasmidless L-LeuA producer strain)

Peptide conc.	Activity at different dilutions									
(mg/mL)	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
0.004 (D) : 0.04 (L)	+	+	+	-	-	-	-	-	-	-
0.04 (D) : 0.04 (L)	+	+	+	-	-	-	-	-	-	-
0.4 (D) : 0.04 (L)	+	+	+	-	-	-	-	-	-	-
0.4 (D) : 0.004 (L)	-	-	-	-	-	-	-	-	-	-
0.04 (L)	+	+/-	-	-	-	-	-	-	-	-
0.004 (L)	-	-	-	-	-	-	-	-	-	-
0.4 (D)	-	-	-	-	-	-	-	-	-	-

2.14.3 Tests for bacteriocin induction towards *C. piscicola* LV17B (Bac$^-$) with L-LeuA and D-LeuA

Bacteriocin production of *C. piscicola* LV17B is regulated by its own bacteriocin.[41] It has been shown that the structural variants of CbnB2 could also re-establish this biological process (see section 1.9.4, page 35). Since LeuA and CbnB2 have a high sequence homology, LeuA might share this function as is the case with CbnB2 variants.

Table 26. Induction of bacteriocin production of *C. piscicola* LV17B with L-LeuA[a]

Peptide conc.	Activity of supernatant at different dilutions									
(mg/mL x 10^3)	$1/2^1$	$1/2^2$	$1/2^3$	$1/2^4$	$1/2^5$	$1/2^6$	$1/2^7$	$1/2^8$	$1/2^9$	$1/2^{10}$
10.0[b]	+	+	+	+	+	-	-	-	-	-
5.00	+	+	+	+	+/-	-	-	-	-	-
2.50	+	+	+	+/-	-	-	-	-	-	-
1.25	+	+	+/-	-	-	-	-	-	-	-
0.62	+	+/-	-	-	-	-	-	-	-	-
0.31	+	-	-	-	-	-	-	-	-	-
0.16	-	-	-	-	-	-	-	-	-	-
10.0 (control)[c]	+	+	+	+	+	+	+/-	-	-	-
Control (+)[d]	+	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Control (-)[d]	-	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d

a. *C. Divergens* LV13 was the indicator. The plates were read after incubation at 25 °C for 16 h.

b. The concentration of L-LeuA in the culture medium.

c. Test of background activity due to the added L-LeuA in the medium.

d. Positive control: 1% supernatant of Bac$^+$ culture, which induces bacteriocin production. Negative control was the direct subculture of Bac$^-$ culture.
n/d: not determined; +/- : borderline activity

As shown in Table 26, L-LeuA was unable to re-establish the bacteriocin production of *C. piscicola* LV17B (Bac$^-$). Since the activity of the culture supernatant with inducing peptide (L-LeuA) was lower than the corresponding control, the activity of added peptide (L-LeuA) was lost during the induction, which might be due to the binding of the peptide to the cell surface or degradation of the peptide itself in the culture medium. The same experiments were carried out with D-LeuA and similar results were obtained (data not shown).

2.14.4 Discussion and conclusions

It has been demonstrated that bacteriocin producing bacteria protect themselves by co-transcription of an immunity protein.[13] Immunity proteins associated with two-peptide bacteriocins consist of 110 to 154 amino acids, containing several (usually four) potential transmembrane helices, while those of one-peptide bacteriocins are generally smaller in size (51 to 113 amino acids) and contain few or no potential transmembrane helices.[162] Immunity proteins with no potential transmembrane helices are still thought to be associated with the membrane.[162] It is understandable that bacteria produce an immunity protein to protect themselves, however, the existence of this kind of receptor protein would be very inefficient on an evolutionary basis. Specific receptor-ligand interactions are essential in efficient signal transduction in biological systems and these intricate systems evolve over long time periods for the benefit of living creatures themselves.[151] To evolve a bacteriocin receptor protein would be unfavourable for its host and have no driving force in nature. However, it is likely that receptor protein on the target cell membrane may have been developed for other biological purposes. Without the involvement of a specific bacteriocin-receptor interaction, it is difficult to rationalize the high level of specificity for certain bacteriocins towards closely-related target organisms. Furthermore, each bacteriocin has a specific corresponding immunity protein, which implies that protein-protein interaction is

also involved in the killing process since this process can be counteracted by another protein-protein interaction.

Bacteriocins are most active towards closely related species.[20] Hence, the so-called receptor protein or close analog may also be present on the bacteriocin producing organism. The bacteriocin does not kill the producer because it also has an unique immunity protein, which is absent in sensitive cells. The immunity protein may be associated with the receptor protein. It is known that all of the immunity proteins discovered so far share very low homology.[23,34,37,38,152,163] The putative receptor protein might play an enhancing role in modulating bacteriocin action as suggested by Montville and Chen.[164] If, for instance, the ABC transporter protein was the receptor, the interaction of an immunity protein with the transporter protein could make the translocation of the bacteriocin molecule from the outside to the inside impossible, thereby protecting the producer. However, target organisms that have the ABC transporter protein but not the immunity protein may translocate the bacteriocin through the membrane into the cell.

Receptor-ligand interactions usually involve chiral molecular recognition. The peptide chains of naturally occurring bacteriocins are generally composed only of L-amino acids. Many of these bacteriocins are likely to form a right-handed α-helical structure in lipophilic solvents or in membrane mimicking environments.[47,60,165] If a receptor protein is involved in bacteriocin function, and the interaction is chiral, its enantiomer, the all-D amino acid peptide would likely be inactive. We chose a well characterized and typical class II bacteriocin, leucocin A (L-LeuA) as a model to investigate this hypothesis.

Since L-LeuA forms a right-handed amphipathic α-helix,[60] D-LeuA assumes a left-handed one, as confirmed by CD spectra. The negligible antimicrobial activity of D-LeuA demonstrates that the backbone conformation is critical to its biological function. The backbone and its side chains of L-LeuA are probably involved in the interaction with a receptor protein on the target cell. This confirms our previous suggestion that the α-helical

part towards the peptide C-terminus determines target specificity (*i.e.* its antimicrobial spectrum) through receptor binding.[60]

Our observations contrast the recent report by Hauge *et al.*[147] that both plantaricin A-22L (synthetic peptide with L-amino acids) and plantaricin A-22D (synthetic peptide with D-amino acids) have equal biological activity, even though the first four N-terminal residues of the parent plantaricin A (26-mer) were truncated. However, plantaricin A is different from class IIa bacteriocins in both the primary structure and the relative potency.[166] Further studies found that plantaricin A is an induction factor (pheromone) for the production of two two-component bacteriocins. No genes necessary for its processing, maturation, translocation, and/or immunity have been found, which is not the case for L-LeuA or other class IIa bacteriocins. The gene encoding plantaricin A is co-transcribed with genes encoding a histidine kinase and two response regulators, which together make up a complete two-component regulatory system.[167] It is likely that, plantaricin A acts through a different antimicrobial mechanism than LeuA or other class IIa bacteriocins. The integral peptide chain is critical for the full activity of class IIa bacteriocins. An analogue of L-LeuA, MesY105 which differs only at residues 22 and 26, has similar potency against identical organisms. However, deletion of a single amino acid from the C-terminus of MesY105 drastically reduces its activity,[47] whereas plantaricin A could be truncated without compromising its antimicrobial activity. Antimicrobial peptides from different sources, such as cecropin A, magainin 2 and melittin, can partially be truncated without losing their potency.[168] Interestingly, the D-enantiomers of these peptides are equally active, indicating that their function does not depend on chiral interactions.[169] In contrast, peptide hormones, such as angiotensin, oxytocin and bradykinin function through interacting with chiral receptors, and their D-enantiomers are biologically inactive.[146,170] Furthermore, D-LeuA displays no antagonistic or agonistic effects on the enantiomer, a natural bacteriocin, L-LeuA.

There are conflicting results regarding whether pediocin PA-1, another YGNGV class IIa bacteriocin, needs a receptor for its function. Nes *et al.*[48] suggested that a protein receptor mediates pediocin PA-1 pore formation in pediococcal cells. However, other studies claim that a protein receptor is not essential for the action of pediocin PA-1 in other organisms,[153] suggesting that a bacteriocin may act through different mechanisms.

A 15-mer peptide fragment corresponding to residues 20 to 34 of pediocin PA-1 efficiently inhibited its parent bacteriocin, but all the other fragments from its N-terminus or C-terminus had little or no effect.[44] Thus, the specificity-determining region is localized within the C-terminal half of pediocin PA-1. In similar experiments, corresponding fragments derived from L-LeuA were unable to inhibit its activity. Although LeuA and pediocin PA-1 have very high sequence homology at their N-terminus, the latter has two essential disulfide bridges instead of one.[48] The second disulfide bridge of pediocin PA-1 forms a 20-amino acid ring at its C-terminus and restricts available conformations. In contrast, corresponding fragments from L-LeuA may show increased conformational moblility and random coil structure. In similar studies, the *N*-terminal peptide fragment of CbnB2(1-22) **3** was not able to inhibit the antimicrobial activity of CbnB2, even when present in a one thousand fold excess.

Bacteriocin production is not always consistent in bacteria such as *Lactobacillus plantarum* C11, *Lactobacillus sake*, *C. piscicola* LV17.[41,171,172] It can be inducible by a dedicated regulator (inducer factor) or self-regulated by its own bacteriocin.[41] In the previous chapter it was reported that CbnB2 could re-establish the Bac^+ phenotype of *C. piscicola* LV17B, and that several of its specific mutants could also induce bacteriocin production. This suggests that inducing activity can tolerate more structural variations.[25] However, neither L-LeuA nor D-LeuA could induce the Bac^- cultures of *C. piscicola* LV17B to re-establish the bacteriocin production. Although L-LeuA and CbnB2 have very similar sequences at their N-termini, the C-termini vary considerably, suggesting that the latter portion of the peptide is essential for this function. This is consistent with our

previous findings that the C-terminal truncated peptide Cbn28 does not possess this function.

To summarize, we synthesized D-LeuA, the enantiomer of the natural L-LeuA. The lack of activity of this D-peptide demonstrates that the antimicrobial function depends on chiral interaction, thereby suggesting the existence of a putative receptor protein. There is also no agonistic/antagonistic effect between L-LeuA and D-LeuA, and neither is able to effect the "self-induction" that CbnB2 displays in *C. piscicola* LV17B.

2.15 Synthesis of substrate analogues for the ABC transporter

Like all other class II bacteriocins, LeuA is initially synthesized as a prebacteriocin, pre-LeuA (Figure 32).[49] The prebacteriocin is then proteolytically processed and translocated by a dedicated ABC transporter.[21,30] It has been demonstrated that the ABC transporter proteins are conservative for class II bacteriocin producers and are likely also to be cysteine proteases.[36] The leader peptide is the recognition sequence for the ABC transporter protease.[28] Since most cysteine proteases that cleave large proteins recognize a short sequence (4-10 amino acids) on either side of the cleavage site,[173] we synthesized short pentapeptide fragments representing the C-terminus of the pre-LeuA leader bearing variable length linkers with nitrophenyl chromophores as potential substrates for the ABC transporter of *L. gelidum* UAL187.

Using Fmoc protected amino acids, the *N*-acetylated pentapeptide **65** was synthesized. As outlined in Scheme 11, reaction of 2,4-dinitrofluorobenzene with Boc protected diamines gave the corresponding 2,4-dinitrophenyl derivatives **66**, **67** and **68**, which were deprotected in 40% TFA to afford quantitatively **69**, **70** and **71**, respectively. Coupling of **69**, **70** or **71** to pentapeptide **65** in the presence of HBTU and Et_3N in DMF yielded the potential substrates **72**, **73**, **74**, respectively. These compounds should be valuable tools for assay, isolation and substrate specificity studies for the ABC transporter

of the LeuA operon. Studies to clone and express bacteriocin ABC transporters and portions thereof (cysteine protease sections) are currently in progress.

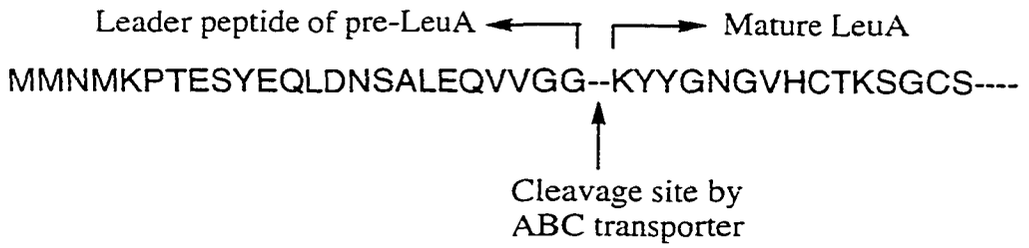
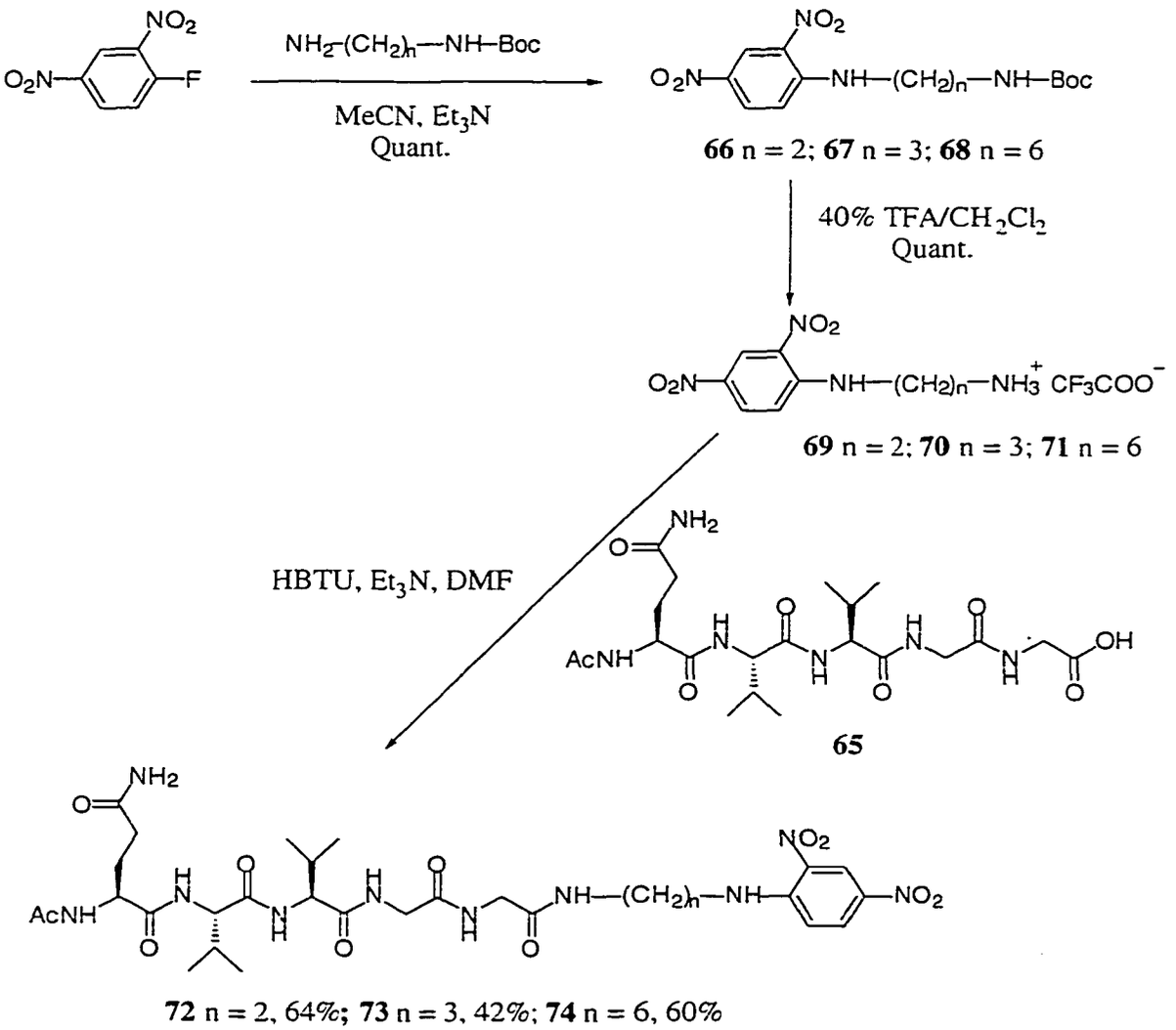


Figure 32. Pre-LeuA processing by ABC transporter



Scheme 11

Chapter III Production and Preliminary Structural Analysis of Subtilosin A and UAL26

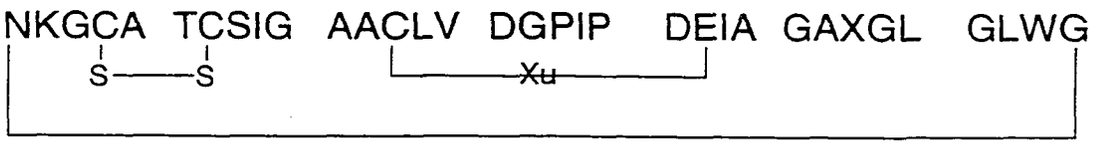
INTRODUCTION

3.1 Subtilosin A from *Bacillus subtilis* JH642

Polypeptide antibiotics are frequently found as metabolites of Gram-positive bacteria such as *Bacillus* species.[13,20,174,175] In *Bacillus subtilis*, bacteriocins are synthesized ribosomally, while others are produced non-ribosomally by large peptide synthases.[175] As demonstrated in studies of subtilin (*Bacillus subtilis*), nisin (*Lactococcus lactis*), pediocin (*Pediococcus acidilactici*) and other bacteriocins, in Gram-positive bacteria ribosomal peptides are typically synthesized as extended precursors having reduced biological activity.[21,25,177,178] The *N*-terminal leader sequences are then cleaved to yield the mature, active bacteriocins. In some cases the polypeptide may undergo extensive post-translational modifications.[179,180] Transport to the external environment is accomplished by ATP-dependent efflux protein complexes which are membrane-associated.[36] Genes involved in the biosynthesis of bacteriocins are typically organized into operons,[178,181] which include the structural gene and genes whose products function in maturation, export, immunity, and, in some cases, the regulation of expression. The operons for lantibiotic biosynthesis also contain genes encoding signal transduction systems composed of two-component regulatory proteins.[182,183]

Antimicrobial substances produced by a wild-type of *B. subtilis* isolated from a Chinese fermented food[184,185] are currently under investigation in our laboratory. One of these was initially identified as a bacteriocin, termed subtilosin A, endowed with activity against *Listeria monocytogenes* and *Bacillus cereus*.[185] An operon required for the observed activity was identified by insertion mutagenesis.[186] The operon (*alb*, for anti-Listerial bacteriocin) consists of seven genes and is preceded by the gene *sbo* which encodes for subtilosin A, a modified antimicrobial peptide. This structural gene encodes for a mature peptide composed of 35 amino acids, which closely resembles subtilosin-168 produced by *Bacillus subtilis* 168.[187] A partial structure for subtilosin-168 has been

reported (Figure 33).[187] The *sbo* gene resides in the vicinity of *fnr* and *argS* (arginyl-tRNA synthetase).[188] Further information on purified subtilosin A is needed to elucidate the structure and post-translational modifications of this antimicrobial peptide.



X and Xu are unknown residues

Figure 33. The partial structure of subtilosin-168 reported by Babasaki *et al.*[187]

3.2 UAL26 from *C. piscicola* UAL26

Carnobacterium piscicola UAL26 was discovered to inhibit the growth of a wide range of organisms, during screening of lactic acid bacteria isolated from vacuum-packaged beef for a potential biological preserving agent.[189] Inhibitory activity was attributed to the production of a proteinaceous substance, termed bacteriocin UAL26, which is released to the culture medium during fermentation. UAL26 is active against species of *Pediococcus, Lactobacillus, Streptococcus, Leuconostoc, Bacillus, Clostridium* and *Listeria*, but not against Gram-negative organisms tested. On APT agar, UAL26 is produced at temperatures as low as 1 °C. Extensive effort has been expended to determine the molecular identity of this bacteriocin, but the structure has yet to be elucidated.

RESULTS AND DISCUSSION

3.3 Structural studies on subtilosin A

3.3.1 Mass spectrometry, amino acid sequencing and analysis

A sample of subtilosin A **75** provided by our collaborator, Professor Peter Zuber (Oregon Graduate Institute of Science and Technology, Portland, OR) was purified to homogeneity on RP-HPLC. The molecular weight of the purified peptide was determined by electrospray mass spectrometry to be 3401.30 +/- 0.30, which differs by approximately 24-25 units from the calculated molecular weight (3425.96, average mass) based on the genetic sequence (Figure 34). The molecular weight is also different from that reported by Babasaki and co-workers (3398.9 Da, FAB).[187]

NKGCA TCSIG AACLV DGPIP DFEIA GATGL FGLWG

Figure 34. Genetic sequence of subtilosin A (by Dr. Peter Zuber)

N-Terminal amino acid sequencing was unsuccessful, which indicates that the *N*-terminal end of this peptide is blocked.[159] Complete hydrolysis, derivatization with phenylisothiocyanate for analysis of amino acid composition by RP-HPLC gave the results in Table 27. In general, the peptide sample was hydrolyzed using 6 N HCl with 0.1% phenol at 160 °C for one hour. Cysteine residues were measured as cysteic acid by hydrolysis in the presence of 5% DMSO. To measure the tryptophan residues, the sample was hydrolyzed using 4 M methanesulfonic acid in the presence of 0.2% tryptamine. The amino acid composition of subtilosin A matches the genetic sequence for the Ser, Glx, Pro, Gly, Ala, Cys, Val, Ile, Leu and Lys residues. The extra tryptophan content may be the result of post-translational modification of the Phe residues. Two Phe residues are

predicted from genetic sequencing, but no such residues were found in the amino acid analysis. Similarly, genetic sequencing predicted a second threonine (Thr) residue, which was also not detected.

Table 27. Amino acid analyses of subtilosin A, predicted composition from the genetic sequencing, and results reported by Babasaki.[187]

Amino Acid	Residues in subtilosin from our preparation	Residues from the genetic sequence	Results reported by Babasaki and co-worker[187]
Asx*	3	3	3.0
Thr	1	2	0.7
Ser	1	1	0.8
Glx*	1	1	1.0
Pro	2	2	1.9
Gly	7	7	7.1
Ala	5	5	5.0
Cys	3	3	2.5
Val	1	1	1.0
Ile	3	3	3.0
Leu	3	3	3.0
Lys	1	1	1.0
Trp	3 ~ 5	1	0.5
Phe	0	2	0

* Asx refers to Asn or Asp; Glx refers to Gln or Glu.

3.3.2 Trypsin digestion

It was found that the sample was not stable under the slightly basic conditions (pH 8.0) required for trypsin digestion. Thus, in the digestion buffer (2 M urea, 0.1 M NH_4HCO_3, pH 8.0), in addition to the recovery of starting material, five major fractions were seen on RP-HPLC. Interestingly, all five fractions gave the same mass spectra, even though they had quite different retention times (25.01, 25.93, 26.95, 27.87, 29.08 min). The molecular weight of the decomposed products was 3357.5, which corresponds to approximately 44 units less than the original peptide (MW 3401.4). Attempts to sequence the new fractions were unsuccessful, which suggests that the *N*-terminal ends of these peptides are still blocked and that the structural changes occur on the side-chains. Amino acid analysis was performed on one (29.08 min) of these fractions. The amino acid composition, including Cys residues, was very similar to that of the original subtilosin A sample, except that no tryptophan residues could be detected. This suggests that Trp and the modified Phe residues undergo further transformation in pH 8 buffer.

It is not clear why trypsin failed to digest subtilosin A, because there is a Lys residue in this peptide, as confirmed by both amino acid analysis and genetic sequencing (Table 27). Typically trypsin should cleave the peptide at the C-side of a Lys residue. It is possible that subtilosin A exists in a rigid conformation which is resistant to such digestion. It has been suggested by Babsaki *et al.*[187] that subtilosin-168 exists in a cyclic form with its *N*- and *C*-termini connected.

3.3.3 Partial hydrolysis with hydrochloric acid

Subtilosin A was partially hydrolyzed using concentrated hydrochloric acid and the hydrolyzed products were isolated by RP-HPLC. Since this peptide is post-translationally modified, it is difficult to match the fragments with the parent peptide sequence based on molecular weights determined by mass spectrometry.

N-Terminal amino acid sequencing of some of the fragments revealed that the partial amino acid sequence of the fragment eluting at 31.88 min (MW 820.00) is AAXLVDGP (calcd mass 744.86). There was no obvious signal in the third sequencing cycle, suggesting that X is a cysteine residue in accord with the genetic sequence. The fraction which eluted at 32.54 min (MW 1510.70) gave a sequence of AXLVDGPI (calcd mass 786.94). Attempted sequencing of several other components was unsuccessful, probably due to lack of material. Nevertheless, if larger amounts of material could be isolated, this should allow additional amino acid sequencing.

3.3.4 Partial hydrolysis with thermolysin

A sample of subtilosin A was also digested with thermolysin. Only one major fraction was observed after 24 h digestion at 60 °C in a weakly acidic buffer. Its molecular weight was determined to be 3419.27 Da (mass of original sample was 3401.40 Da). *N*-Terminal amino acid sequencing revealed that the first five residues are GPIPD. The sequencing signal disappeared after the fifth residue for unknown reasons.

3.3.5 Attempts at reduction and derivatization of the cysteine residues

The thiol moieties of the cysteine residues could not be modified with iodoacetamide in Tris-buffered 6.0 M guanidium hydrochloride solution with or without prior treatment with an excess amount of DTT. In both cases, starting material was recovered based on HPLC and mass spectrometry. The lack of modification of the thiol groups indicated that they are covalently modified, and if a disulfide was present, it was inaccessible to DTT.

3.3.6 Production and purification of subtilosin A

Initial attempts at structural elucidation of subtilosin A using 800 μg of the sample provided by Professor Zuber were problematic due to the limited amount of material. Hence, we decided to isolate more material for further studies. Subtilosin A was isolated and purified to homogeneity from the fermentation supernatant of *B. subtilis* JH642 based on modification of the method of Zuber and co-workers.[186] Analysis of the purified sample by HPLC gave a single peak with a retention time identical to that of the subtilosin A sample provided by Professor Zuber. Ten milligrams of pure subtilosin A can be isolated per liter of culture using the improved method. The main differences in the improved procedure are:

a. Short purification process without ammonium sulfate precipitation and Sephadex LH-20 chromatography.[187]
b. Nutrient sporulation medium (NSM)[190] replaced yeast extract-glucose medium and no glucose was added to NSM.
c. Butanol was added directly to the culture without separation of the cells which extracted bacteriocin molecules associated with the cells.[191]

3.3.7 Conclusions and future work on subtilosin A

Genetic studies on *B. subtilis* JH642 done at Professor Zuber's laboratory reveal that the *sbo* gene encodes for subtilosin A and the other genes of the *alb* operon function in the production of subtilosin A. The *alb* operon (*albABCDEFG*) encodes proteins which may function in the processing and export of peptides, such as an ABC transporter complex (*albC*) and two putative processing peptidases, AlbE and AlbF.[188] The product of *albE*, AlbE, shows significant sequence similarity to PqqF, a protein required for the synthesis of the co-factor pyrroloquinoline quinone or PQQ.[192,193]

Immediately upstream of the *alb* operon is the *sbo* gene encoding the 43 amino acid precursor of the bacteriocin, pre-subtilosin: MKKAVIVE NKGCA TCSIG AACLV

DGPIP DFEIA GATGL FGLWG. The underlined sequence is not found in the mature bacteriocin and is presumably the leader peptide. The sequence contains no obvious recognition motif normally required for peptide export. Nor are there any of the typical motifs associated with processing of pre-lantibiotics. The results indicate that subtilosin A and subtilosin-168 are probably the same compound, and that the structure proposed by Babasaki *et al.* is incorrect (Figure 33).[187]

The peptide appears to undergo some unique modifications during maturation which includes proteolytic cleavage at the Asn9 residue, cyclization of *C*- and *N*-termini and modifications of residues of Cys12, Cys15, Cys21, Phe30, Phe39, Thr36, and possibly Glu31.

The calculated molecular weight of subtilosin A based on the genetic sequence (Figure 34) is 3425.96, which is 24 ~ 25 units more than the determined value of 3401.30 +/- 0.30. Removing 18 units (H_2O) for the cyclic structure, there is still a six unit difference. This could be due to a disulfide bond between Cys4 and Cys7 suggested by Babasaki et al.[187] and two other intramolecular oxidative connections as shown in Figure 35.

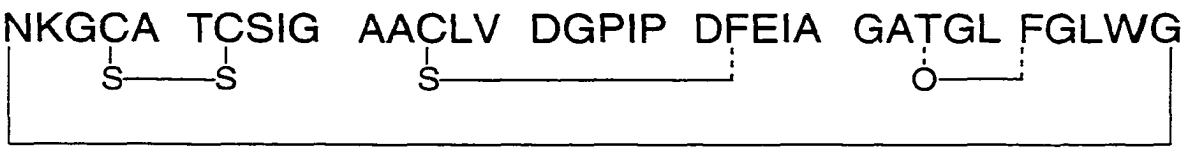


Figure 35. Proposed structure for subtilosin A **75**

As large quantities of subtilosin A are now available, further structure elucidation should be possible by partial hydrolysis or selective enzymatic digestion. The presence of two Phe residues or their post-translationally modified products could be determined by feeding experiments with isotopically labelled Phe. With experience in solution structures

of LeuA and CbnB2,[60,165] it should also be possible to elucidate the solution structure of subtilosin A by NMR techniques.

3.4 Isolation, purification and partial characterization of UAL26 76

The purification of UAL26 was accomplished *via* a series of chromatographic methods. UAL9 (*C. divergens* LV13) was used as an indicator to track the activity of UAL26. The hydrophobic components in the supernatant were first bound to Amberlite XAD-8 resin and were eluted with increasing concentrations of ethanol. Highest activity was detected in the fraction eluted with 60% ethanol. This was concentrated and loaded onto a Sephadex G-50 column. The active fractions from the G-50 column were pooled, concentrated *in vacuo* and further purified on Sepharose SP fastflow cation exchange column at pH 5.0. The protein component was eluted with increasing concentrations of sodium chloride solution in 20 mM sodium acetate buffer. The most active fraction (500 mM sodium chloride) was desalted and concentrated by a C_{18} Sep-PAK. The active components were eluted by 60% of aqueous MeCN from the Sep-PAK cartridge and was finally purified to homogeneity by RP-HPLC.

The first active fraction (UAL26A, **76A**) was eluted at 35 min, and gave a molecular weight of 1844.66 +/- 0.31; the second active fraction (UAL26B, **76B**) was eluted at 38 min and its molecular weight was determined to be 1828.92 +/- 0.34. *N*-Terminal amino acid sequencing revealed that UAL26A had an *N*-terminal sequence of FARLMA. The signal dropped off significantly after cycle 6. UAL26B has a *N*-terminal sequence of FARLM ACIGL VK. It is interesting to note that the two peptides gave the same *N*-terminal sequences. Since the difference of the molecular weights of these two peptides is 16 and there is a Met residue at the 5th position, we speculate that the two peptides were the same but with a different oxidation state of the Met residue. Oxidization of the sulfur in Met to sulfoxide would increase the molecular weight by 16 units and enhance the molecule's hydrophilicity. The oxidized peptide is eluted several minutes

earlier than the un-oxidized form. Bacteriocins with Met residue(s) existing as both forms have been previously reported for carnobacteriocin B1 and BM1.[37]

Although we managed to isolate enough material to perform preliminary studies (activity assay, mass spectrometry and amino acid sequencing) on UAL26, not enough material has been prepared for detailed structural studies. The low production of UAL26 is still a problem yet to be solved. While the production and purification strategy demands further optimization, genetic work using a gene disruption plasmid library[185] might be an alternative to solve this puzzle and reveal the molecular identity of UAL26.

Experimental Procedures

General procedures

All reactions requiring anhydrous conditions were performed under an atmosphere of dry argon using oven-dried glassware. All solvents for anhydrous reactions were dried according to Perrin *et al.*.[194] THF, dioxane and diethyl ether were distilled over sodium and benzophenone under an argon atmosphere. CCl_4 was distilled over CaH_2. Water was obtained from a Milli-Q reagent water system (Millipore Corp., Milford, MA). Solvent evaporation was performed under reduced pressure, below 40 °C, using a Büchi rotary evaporator attached to a Welch dry vacuum system, followed by evacuation to constant sample weight. Unless otherwise specified, solutions of NH_4Cl, $NaHCO_3$, KOH and NaOH refer to aqueous solutions.

All reagents employed were of American Chemical Society grade or finer, and were used without further purification unless otherwise mentioned. All commercially available labelled compounds were purchased from Cambridge Isotope Laboratories (Woburn, MA). Unless otherwise specified, all peptides and proteins were synthesized from commercially available protected L-amino acids or purified from natural source. In order to differentiate LeuA from its enantiomer D-LeuA, which was prepared from all D-amino acids, LeuA is occassionally referred to as L-LeuA.

Where possible all reactions were monitored by thin layer chromatography on Merck 60 F-254 silica plates and were visualized using UV fluorescence or iodine staining. In solid phase reactions, the primary amino group was detected using a solution composed of 75 μL of each of the following three pre-prepared solutions: 5.0 g ninhydrin in 100 mL of 95% ethanol; 20.0 g phenol in 20 mL of ethanol; 2.0 mL of 0.66 mg/mL aqueous KCN was mixed with 98 mL of pyridine. A small amount of resin was mixed with the above solution and heated at 105 °C for 5 min. A brown or light yellow colour indicated no free amino groups. Flash chromatography was performed according to Still *et al.*[195] using

Silicycle silica gel (Quebec City, PQ). High performance liquid chromatography (HPLC) was performed on a Beckman System Gold instrument equipped with a model 166 variable wavelength UV detector or Bio-Rad Value Chrom chromatography system equipped with a Bio-Rad Model 1305A UV monitor, with monitoring at 218 nm unless otherwise indicated. HPLC grade MeCN (190 nm cutoff) and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). All HPLC solvents were prepared fresh daily and filtered with a Millipore filtration system under vacuum before use.

Melting points were determined on a Thomas-Hoover apparatus using open capillary tubes and are uncorrected. Optical rotations were measured on a Perkin Elmer 241 polarimeter (10.0 cm path length, 0.9 mL) at ambient temperature. All specific rotations reported were measured at the sodium D line and were referenced against air. Infrared spectra (IR) were recorded on a Nicolet 7199 or 20SX FT-IR spectrometer. Cast refers to the evaporation of a solution on a NaCl plate. The intensity of IR signals are reported as s (strong) or m (medium). Mass spectra (MS) were recorded on Kratos AEI MS-50 for high resolution, electron impact ionization (EI), or MS-9 for fast atom bombardment (FAB) instruments, or HP1100 LC-MSD for electrospray mass spectra. Cleland matrix used in FAB refers to a 5:1 mixture of dithiothreitol and dithioerythritol. Microanalyses were done on Perkin Elmer 240 or Carlo Erba 1180 elemental analyzers.

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AM-300, Bruker WM-360, Bruker WH-400, Varian INOVA600 or Varian UNITY500 instruments. ^{1}H NMR chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS) using the residual proton resonance of solvents as the reference: $CDCl_3$ δ 7.25, CD_2Cl_2 δ 5.32, D_2O δ 4.72, CD_3OD δ 3.30, $(CD_3)_2SO$ δ 2.49, $(CD_3)_2CO$ δ 2.04. ^{13}C NMR shifts are reported relative to $CDCl_3$ δ 77.0, CD_2Cl_2 δ 53.8, CD_3OD δ 49.0, $(CD_3)_2SO$ δ 39.5, $(CD_3)_2CO$ δ 29.8, CD_3CN δ 117.7 (CN). Selective homonuclear decoupling attached proton test (APT) and ^{1}H-^{1}H correlation spectroscopy (COSY) were

occasionally used for signal assignments. ^{1}H NMR data are tabulated in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; p, pentet, and m, multiplet), number of protons, coupling constant(s) in Hertz (Hz), and assignment.

All cultures used were obtained from Professor M. Stiles laboratory culture stock, except *Bacillus Subtilis* JH642 which was provided by Professor P. Zuber at Oregon Graduate Institute of Science and Technology, Portland, OR.

NMR assignment and 3-dimensional structure modeling of CbnB2 were done by Drs Y. Wang, S. Chai and N. F. Gallagher. The expression system for bacteriocin fusions with maltose-binding protein were constructed by Dr. L. Quadri.

VNYGN GVSCS KTKCS VNWGQ AFQER YTAGI NSFVS GVASG AGSIG RRP

Production and isolation of CbnB2 1. Production and isolation of CbnB2 was done as previously described.[37] Thus, an 18 h culture of *Carnobacterium piscicola* LV17B was inoculated (1%) into 5 liters of CAA medium maintained at pH 6.2 by addition of 1 N NaOH (controlled by Chem-Cadet®, Cole-Parmer, Chicago, IL), and gently stirred under a N_2 atmosphere at 25 °C for 26 h. The semidefined CAA medium used for the production includes the following (per liter of solution): casamino acids, 15.0 g; yeast extract, 5.0 g; D-glucose, 20.0 g; dipotassium phosphate, 2.0 g; diammonium citrate, 2.0 g; magnesium sulfate, 0.10 g; manganous sulfate, 0.05 g; Tween 80, 0.10 mL. The supernatant was loaded directly onto a Superlite DAX-8 (Sigma-Aldrich Co.) column (4.5 x 50 cm) pre-equilibrated with 0.1% TFA in water, and sequentially eluted with increasing concentrations of ethanol (2.0 liters each of 0, 20, and 35%; 1.5 liters of 50%; and 1.0 liter of 80%). The most active fraction (50% ethanol) was concentrated *in vacuo* at 30 °C to about 20 mL, diluted with an equivalent volume of MeCN, and applied onto a Sephadex LH-60 (Sigma) column (5 x 25 cm) pre-equilibrated with 0.1% TFA in 1 : 1 MeCN : water. After elution, the active fractions were pooled and concentrated to *ca.* 10 mL. The separation of active compounds was accomplished by RP-HPLC with a VYDAC C_8 column (10 x 250 mm, 10-μm particle size, 300-Å pore size; Scientific Products and Equipment (Concord, ON); flow rate 2.5 mL/min) using a gradient from 20 to 60% MeCN in 0.1% aqueous TFA over a period of 30 min. Active fractions were pooled and purified to homogeneity on the same column. The antimicrobial activity of the peptides was determined by spot-on-lawn test.[69,70] The purified peptide was concentrated *in vacuo,* lyophilized and stored at -20 °C. The identity of purified CbnB2 was confirmed by an activity test, mass spectrometry and co-injection on RP-HPLC with an authentic CbnB2 sample.[37] The molecular weight was 4968.00 +/- 0.49 (calcd 4969.51, reduced form).

Attempted oxidization of the two cysteine residues of CbnB2. Typically CbnB2 (0.1 mg) was dissolved in 1 mL of 0.1 M ammonium bicarbonate buffer, pH 8.0. The solution was then stirred vigorously under an oxygen atmosphere at room temperature for 16 h. The peptide product was desalted and purified by RP-HPLC using an aqueous MeCN solvent system as described for CbnB2 purification. Each time an activity test and mass spectrometry indicated the recovery of starting material. The molecular weight was determined to be 4969.10 +/- 1.03. Prolonged exposure of the basic solution to oxygen decomposed the peptide.

Attempted derivatization of CbnB2 with Ellman's reagent (DTNB). CbnB2 (0.1 mg) was dissolved in 1 mL of 0.1 M Tris-HCl, pH 8.0. To this was added 0.1 mL of a freshly prepared 5.5 mM solution of DTNB (21 mg DTNB dissolved in 10 mL of 0.1 M Tris-HCl, pH 8.0).[71] The mixture was gently shaken at room temperature for 1 h, after which time the mixture was purified by RP-HPLC under the same conditions as described above for CbnB2 purification. CbnB2 was recovered every time without any modification; MS (electrospray): 4967.21 +/- 0.52.

Reduction and carboamidomethylation of CbnB2.[71] Two identical samples, each containing 50 μg of CbnB2, were dissolved in 0.5 mL of buffer (6 M guanidinium hydrochloride in 0.6 M Tris-HCl, pH 8.6). To one of the samples was added 20 μL of freshly prepared 280 mM DTT. The sample was vortexed briefly, and incubated at 37 °C for 30 min. Freshly prepared 580 mM iodoacetamide (150 μL) was then added dropwise over a period of 2 min, with stirring under argon, and the sample was kept at 37 °C in the absence of light for 1 h. The second sample was treated with iodoacetamide under exactly the same conditions, but without DTT reduction. The products of each reaction were isolated by RP-HPLC with a VYDAC C_8 column (4.6 x 250 mm, 5-μm particle size, 300-Å pore size; Scientific Products and Equipment (Concord, ON); flow rate 1 mL/min) using

a gradient from 20 to 60% MeCN in 0.1% aqueous TFA over a period of 30 min. The molecular weights of modified product and the starting material are shown in Table 28. The results demonstrated that if CbnB2 was not previously reduced with DTT, the thiols could not be modified. There was no detectable activity for the carboamidomethylated CbnB2 (**2**).

Table 28. Molecular weights (Da) of products from iodoacetamide reaction.

	Starting material	with DTT reduction found (calcd)	without DTT reduction found (calcd)
CbnB2 **1**	4967.09	5083.46 (5083.21)	4969.14 (4967.51)
22-mer **3**	2348.93	2464.12 (2463.75)	2463.81 (2463.75)
22-mer **4**	2346.73	2464.34 (2463.75)	2348.22 (2349.63)

VNYGN GVSCS KTKCS VNWGQ AF

Synthesis of CbnB2(1-22) 3 and its derivatization. In order to confirm the above results, a model peptide with the *N*-terminal sequence of CbnB2(1-22) **3** was synthesized using standard Fmoc chemistry (BioTools, Inc., Edmonton, AB). The molecular weight of the peptide was determined by electrospray mass spectrometry (Fisons VG Trio 2000 ESMS) to be 2346.21 +/- 2.63 (calcd mass 2349.6).

The synthetic peptide **3**, containing free cysteine thiols, was dissolved in 0.1 M ammonium bicarbonate buffer, pH 8.0 and stirred under an atmosphere of oxygen overnight. The oxidized product **4** was easily separated from the starting material by RP-HPLC with a C_8 VYDAC column (4.6 x 250 mm, 5-μm particle size, 300-Å pore size; Scientific Products and Equipment (Concord, ON); flow rate 1 mL/min) using a gradient from 12 to 44% MeCN in 0.1% aqueous TFA over a period of 30 min. The retention time

of the reduced form **3** was 15.2 min and that of oxidized form was 16.8 min. About one third of starting material was oxidized according the peak integration on HPLC.

Two identical samples of both oxidized and reduced forms of the 22-mer peptide were reacted with iodoacetamide under the same conditions as those described for CbnB2 modification. Molecular weights of the reaction products are shown in Table 28. The modification patterns of this model peptide confirmed that the two cysteine residues of CbnB2 exist as oxidized form, since attempts at carboamidomethylation were unsuccessful without prior treatment with DTT.

Production of CbnB2, its variants and precursor protein using MBP fusion

1. Bacterial strains, culture conditions and determination of antimicrobial activity. *Carnobacterium* and *Escherichia coli* cultures were grown in APT and Luria-Bertani (LB) medium, respectively, as previously described.[69,124] Ampicillin (100 μg/mL) was added to LB media for growth and selection of *E. coli* transformants. The antimicrobial activity of the peptides was determined by spot-on-lawn test.[69] Thus, plates containing approximately 20 mL of APT agar were overlaid with 10 mL of soft APT agar (0.75% agar) inoculated with a 24 h culture of the sensitive indicator strain, *Carnobacterium divergens* LV13 (1% inoculum). Serial two-fold dilutions of the stock solutions of purified peptides (1 mg/mL, see below) were prepared in 0.1% aqueous TFA, and up to 20 μL of each stock and its dilutions were spotted (20 μL) onto the bacterial lawn. The plates were kept at 24 °C for approximately 16 h before the presence of inhibition zones was recorded. The results were expressed in arbitrary units (AU) of bacteriocin - one AU is the minimal amount of peptide required to produce a visible clearing on the lawn of the indicator strain. Inhibition was recorded as a positive if a distinct clearing was observed. The limit of detection was 0.05 AU/μg at the tested concentration. *C. divergens* LV13 transformed with pLQ400i, a plasmid expressing CbnB2 immunity

protein, which protects the strain against CbnB2,[34] was also challenged with the purified peptides in the same way as *C. divergens* LV13. No inhibition of the indicator strain was detected when 0.1% aqueous TFA alone was used in the assay.

2. Construction of recombinant protein genes. Analysis of the DNA sequence allowed the identification of six clones containing mutations in the coding region for CbnB2. Five of these mutants had only one amino acid substitution: pLQF3 (Y3F), pLQS33 (F33S), pLQI34 (V34I), pLQI37 (V34I) and pLQG46 (R46G) and another one had a frame shift mutation that replaced the last 20 amino acid residues of CbnB2 with five other residues (ELTHL) from a different coding frame (pLQ28). These clones were used for the purification of the CbnB2 variants, and thereafter these structural variants of CbnB2 are referred to as CbnF3 **7**, CbnS33 **8**, CbnI34 **9**, CbnI37 **10**, CbnG46 **11**, Cbn28 **12**, respectively.

3. Screening for overproduction and purification of fusion proteins. For screening and large scale production of fusion proteins, *E. coli* strains were grown in rich broth containing: tryptone, 10.0 g; yeast extract, 5.0 g; NaCl, 5.0 g; glucose, 2.0 g; and 2.0 mL of ampicillin solution (50 mg/mL) per liter, or in a complete minimal medium containing: Na_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; NaCl, 0.5 g; NH_4Cl, 1.0 g; 2.0 mL of 1.0 M $MgSO_4$, 20.0 mL of 20% D-glucose, 1.0 mL of 1.0 M thiamine, 2.0 mL of ampicillin solution (50 mg/mL) per liter. Production of recombinant proteins was induced with IPTG (0.3 mM) when the cultures reached an optical density of 0.5 at 600 nm. After induction, the cultures were incubated for 3 h before the cells were harvested by centrifugation. The presence of overexpressed recombinant proteins in *E. coli* was screened by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE). Total cell proteins from 100 μL of an IPTG-induced culture were loaded on SDS-PAGE. The strong band around 50 kDa indicated the efficient production of the fusion protein. In clear contrast, this band was

relatively much weaker in intensity for the total cell proteins of the culture before IPTG induction. In the large scale purification the induced cells (1.0 liter culture) were harvested and resuspended in column buffer (100 mL, containing: 20.0 mM Tris-Cl, 200 mM NaCl, 1.0 mM EDTA, 10 mM β-mercaptoethanol, 1.0 mM sodium azide, pH 7.4). After this step, all manipulations were done at 4 °C. Cells were lysed in a French pressure cell at 1300 p.s.i. and sonicated three times for 2 min. The lysates were centrifuged at 7,000 rpm for 20 min, and the resulting supernatant was diluted to 700 mL with column buffer. The lysates were loaded onto a 25 mL amylose resin column (flow rate 0.5 mL/min). After washing the column with 12 to 15 volumes of column buffer, the recombinant proteins were eluted with the same buffer containing 10.0 mM maltose. Fractions containing the recombinant proteins were dialyzed against Milli-Q water, lyophilized and stored at -20 °C. Protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories) using BSA as a standard.

4. Cleavage of fusion proteins and purification of bacteriocins. The cleavage of the fusion proteins with Factor Xa was done as recommended by the supplier (New England Biolabs) except as noted. The initial protein concentration was from 1 to 2 mg/mL. The cleavage time was from 7 to 9 h, except for the fusion proteins bearing CbnS33 and CbnB2P, which were cut for 10 and 12h, respectively. Selected digested protein mixtures were first analyzed by 16.5% Tris-tricine SDS-PAGE.[125,126] The mature CbnB2, the precursor peptide (CbnB2P), and the carnobacteriocin variants were purified by RP-HPLC with a VYDAC C_8 column (10 x 250 mm, 10-μm particle size, 300-Å pore size; flow rate 2.5 mL/min) using a gradient from 21 to 49% MeCN in 0.1% aqueous TFA over a period of 25 min, which was followed by 49 to 63% MeCN in 0.1% aqueous TFA over a period of 5 min. Purified peptides were lyophilized, resuspended in 0.1% TFA to a concentration of 1 mg/mL and stored at -20 °C. Protein concentration was determined using

Bio-Rad Protein Assay (Bio-Rad Laboratories) or by HPLC (monitoring at 218 nm) using 1 mg/mL of CbnB2 solution as a standard.

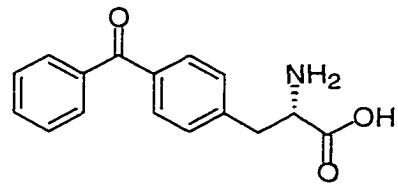
5. *N*-Terminal sequence analyses and mass spectrometry. Purified peptides were analyzed by the Alberta Peptide Institute (University of Alberta). The *N*-terminal amino acid sequences were obtained using Edman degradation on an automated gas phase sequencer (Applied Biosystems model 470A) with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems model 120A). The mass spectra of the purified peptides were done by direct injection of their solutions (50% aqueous MeCN, 0.1% TFA) using a VG Quattro triple quadrupole instrument with an electrospray ionization source (Fisons, Manchester, England).

6. Uniform ^{15}N-labelling of CbnB2. Uniformly ^{15}N labelled CbnB2 was prepared using the maltose-binding-protein fusion strategy oulined above. An *E. coli* strain which transcribes MBP-CbnB2 fusion was grown in a complete minimal medium containing: Na_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; NaCl, 0.5 g; $^{15}NH_4Cl$, 1.0 g; 2.0 mL of 1.0 M $MgSO_4$; 20.0 mL of 20% D-glucose; 1.0 mL of 1.0 M thiamine and 2.0 mL of ampicillin solution (50 mg/mL) per liter. All of the manipulations were same as those for the production of CbnB2 variants. The uniformly ^{15}N-labelled peptides were cleaved from their MBP fusion proteins under the same conditions used for preparing unlabelled peptides. Final products were characterized using mass spectrometry, automatic amino acid sequencing and activity test. A large amount of uniformly ^{15}N-labelled CbnB2 was prepared with this method to facilitate its NMR and biological studies. This methodology can be easily adapted for production of uniformly ^{13}C labelled protein. The chemical, biological (specific activity towards *C. divergens* LV13) and chromatographic properties of uniformly ^{15}N-labelled CbnB2 were identical to the corresponding unlabelled peptide. The molecular weight of ^{15}N-CbnB2 was 5030.67 +/- 1.29 **13** (calcd mass for

$C_{213}H_{332}N_{66}O_{68}S_2$, 5033.54, oxidized form). The error might be due to the incorporation of minor amounts of ^{14}N material from the preculture.

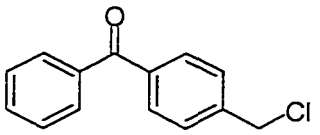
7. Induction of bacteriocin production of *C. piscicola* LV17B with CbnB2 and its variants. Induction experiments were done as previously described with the following modifications.[41] *C. piscicola* LV17B cultures with suppressed bacteriocin production (Bac^- cultures) were obtained by sub-culturing bacteriocin producing cultures (Bac^+ cultures) using an inoculum below 1 x 10^4 cells per milliliter of broth. Before sub-culture of the Bac^- cell, the medium was supplemented with CbnB2 or its antimicrobially active variants (CbnI34, CbnI37, CbnG46, CbnF3) to give final concentrations of 5 AU/mL, 15 AU/mL, and 30 AU/mL, respectively. There were three CbnB2 samples used in the induction experiment: CbnB2 **1** from its natural producer, *C. piscicola* LV17B; CbnB2 **1** from *E. coli* produced through MBP fusion; uniformally ^{15}N-labelled CbnB2 (**13**) from *E. coli* produced through MBP fusion. For the inactive peptides (Cbn28, CbnS33, and CbnB2P) induction experiments employed concentrations of 2.5 μg/mL and 7.5 μg/mL of medium. Cbn28 was also tested at 30 μg/mL. Bacteriocin activity present in the culture supernatant was evaluated when the cultures reached stationary phase of growth. The inhibitory activity of the heat-treated supernatant (65 °C for 30 min) was assayed by the spot-on-lawn technique using a bacteria lawn of *C. divergens* LV13. Background activity from the bacteriocins added to induce production was taken into account in determining induction at high concentration. Bac^+ supernatant was included as a positive control and the direct subculture of Bac^- cells was used as a negative control. The assay was done in a 96-well microtest tissue culture plate and the cell growth was monitored with a SOFTmax (version 2.32) microplate reader at 650 nm. CbnB2 purified from its natural producer *C. piscicola* LV17B was also included to study the possible function variation due to different sources. Apart from the *C*-terminal truncated peptide

Cbn28, all of the structural variants, CbnB2P, and CbnB2 could re-establish the bacteriocin production of Bac$^-$ culture. The cells in the medium containing active peptides (CbnB2, CbnI34, CbnI37, CbnG46, CbnF3) needed 10 to 15 h more to reach a stationary phase of growth compared with the control or the culture growing in the medium with inactive peptides. There were no differences for the inducing function of CbnB2 from different sources (*e.g.* from its natural producer or from *E. coli*) or the uniformly ^{15}N-labelled peptide.



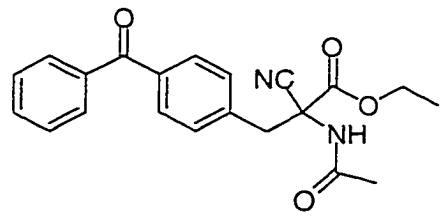
***p*-Benzoyl-L-phenylalanine 14.** Compounds **14** to **20** were prepared using the method of Kauer *et al.*[148] To a stirred suspension of **18** (1.0 g, 3.2 mmol) in water (170 mL) was added 4.7 N ammonium hydroxide (*ca.* 0.6 mL). The solution was filtered and acylase I (35 mg, Sigma) and toluene (0.08 mL) were added. The solution was stirred at 37 °C for 18 h and was cooled to room temperature and filtered (see below for filtrate processing to afford **19**). The solid product was dissolved in 0.5 N hydrochloric acid (13 mL) at 70 °C. Celite® was added and the solid material was removed by filtration. The solid was washed with hot water (13 mL). The clear filtrate was brought to pH 7.0 by the addition of 1 N sodium hydroxide solution (*ca.* 2 mL) at which point precipitation of the desired product occurred. The solid product was isolated by filtration, washed with water and a small amount of ethanol and air dried to give **14** sesquihydrate (0.4 g, 80%); mp 175-178 °C (lit.[148] mp 178-179 °C); $[\alpha]_D^{25}$ +3.2° (*c* 1.0, 1 N HCl) (lit.[148] $[\alpha]_D^{25}$ +3.0° (*c* 1.0, 1 N HCl)); IR (microscope) 3404 (br s), 1643 (s), 1612 (s), 1313 (s), 701 (s) cm^{-1};

^{1}H NMR (300 MHz, D_2O) δ 7.45-7.35 (m, 4 H, ArH), 7.27-7.10 (m, 5 H, ArH), 4.15 (dd, 1 H, J = 7.3, 6.1 Hz, CH), 3.42-3.23 (dABq, 2 H, J = 14.5, 7.3, 6.1 Hz, $ArCH_2CH$); ^{13}C NMR (75 MHz, $(CD_3)_2SO$), δ 196.19 (ArCOAr'), 170.19 (COOH), 140.55, 137.42, 136.40, 133.32, 130.33, 130.25, 130.04, 129.14 ($C_6H_5COC_6H_4$), 53.18 (CH), 35.79 ($ArCH_2$); FAB MS *m/z* (Cleland) 270.0 (MH^+, 100).

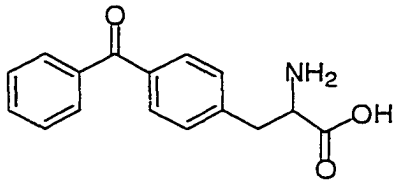


***p*-Chloromethylbenzophenone 15.** A solution of *p*-methylbenzophenone (20.0 g, 102 mmol), sulfuryl chloride (16.5 mL, 28.3 g, 204 mmol), carbon tetrachloride (26 mL), and dibenzoyl peroxide (12 mg, 0.05 mmol) was heated under reflux under an argon atmosphere. At 2, 4, 6 h, additional portions of dibenzoyl peroxide (12 mg, 0.05 mmol) were added. After 24 h, a final portion of dibenzoyl peroxide (12 mg, 0.05 mmol) was added and the heating was continued for a further 1 h. Unreacted sulfuryl chloride was removed by co-distillation with carbon tetrachloride. To the residue containing carbon tetrachloride, was added potassium carbonate (1.0 g) and basic alumina (1.0 g). The mixture was heated at reflux for 20 min with stirring, and solid material was removed by filtration. On cooling the filtrate, the desired product was obtained as a crystalline material. Recrystallization from ethanol yielded a colourless crystalline solid **15** (10.6 g, 45%); mp 90-92 °C (lit.[148] mp 95-96 °C); IR ($CHCl_3$ cast) 3059 (m), 1658 (s), 1277 (s), 701 (s) cm^{-1}; ^{1}H NMR (300 MHz, CD_2Cl_2) δ 7.81-7.77 (m, 4 H, ArH), 7.65-7.59 (m, 1 H, ArH), 7.55-7.47 (m, 4 H, ArH), 4.69 (s, 2 H, $ArCH_2$); ^{13}C NMR (75 MHz, CD_2Cl_2) δ 196.04 (ArCOAr'), 142.20, 137.88, 137.81, 132.85, 130.71, 130.26, 128.79, 128.68 ($C_6H_5COC_6H_4$), 45.92 ($ArCH_2$); HRMS (EI) *m/z* (relative intensity) calcd for $C_{14}H_{11}{}^{35}ClO$

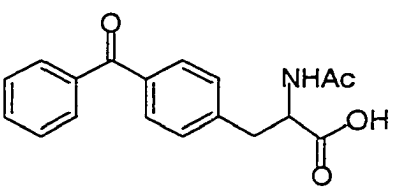
230.0498, found 230.0496; calcd for $C_{14}H_{11}{}^{37}ClO$ 232.0469, found 232.0471. Anal. calcd for $C_{14}H_{11}ClO$: C 72.92; H, 4.77; Cl, 15.39. Found: C, 72.96; H, 4.75; Cl, 15.24.



***N*-Acetyl-α-cyano-*p*-benzoyl-DL-phenylalanine ethyl ester 16.** A stirred mixture of *p*-chloromethylbenzophenone **15** (5.0 g, 22 mmol), ethyl acetamidocyanoacetate (3.7 g, 22 mmol), potassium iodide (380 mg, 2.3 mmol) and anhydrous potassium carbonate (2.1 g, 15 mmol) in dry acetone (35 mL) was heated under reflux overnight. The reaction mixture was cooled and the solid material was removed by filtration and washed with acetone. The combined filtrates were evaporated under reduced pressure. The residue was crystallized from ethanol (10 mL) by cooling to 5 °C and recrystallized from ethanol (100 mL) and hexane (300 mL) to afford solid **16** (4.6 g, 58%); mp 146-148 °C (lit.[148] mp 151-152 °C); IR ($CHCl_3$ cast) 3295 (m), 3027 (m), 2200 (m), 1658 (s), 1279 (s), 702 (s) cm^{-1}; ^1H NMR (300 MHz, CD_2Cl_2) δ 7.81-7.75 (m, 4 H, ArH), 7.65-7.59 (m, 1 H, ArH), 7.54-7.32 (m, 4 H, ArH), 6.44 (br s, 1 H, NH), 4.30 (q, 2 H, J = 7.1 Hz, OCH_2CH_3), 3.75-3.45 (dd, 2 H, J = 13.4, 13.3 Hz, $ArCH_2$), 2.05 (s, 3 H, $COCH_3$), 1.30 (t, 3 H, J = 7.1 Hz, OCH_2CH_3); ^{13}C NMR (75 MHz, CD_3COCD_3) δ 196.01 (ArCOAr'), 170.63, 166.59 ($NHCOCH_3$, $COOCH_2$), 138.30, 138.12, 137.95, 133.28, 131.28, 130.80, 130.58, 130.43, 129.23 ($C_6H_5COC_6H_4$), 117.47 (CN), 63.43 (CCN), 59.34 (OCH_2), 42.05 ($ArCH_2$), 22.06 ($COCH_3$), 14.06 (CH_2CH_3); HRMS (EI) *m/z* calcd for $C_{21}H_{20}N_2O_4$ 364.1423, found 364.1425. Anal. calcd for $C_{21}H_{20}N_2O_4$: C, 69.21; H, 5.53; N, 7.68. Found: C, 69.09; H, 5.54; N, 7.73.

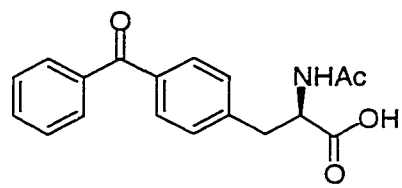


***p*-Benzoyl-DL-phenylalanine 17.** A suspension of **16** (4.0 g, 11 mmol) in 8.0 N hydrochloric acid (20 mL) was heated under argon at 100 °C for 20 h. The mixture was cooled, the solid was collected, washed with 8.0 N hydrochloric acid followed by ethanol, and dried to give the crude hydrochloric acid salt of **17**. The crude product was dissolved in boiling water (40 mL) and filtered hot. The hot filtrate was diluted with hot water (40 mL) and immediately neutralized to pH 7.0 with 1.0 N sodium hydroxide solution (*ca.* 10 mL). The mixture was cooled on ice and the resulting fine solid was collected by filtration, washed with water and vacuum-dried to afford **17** (2.9 g, 98%); mp 215 °C (dec.) (lit.[148] mp 217 °C (dec.)); IR (CH_2Cl_2/MeOH cast) 3472 (br s), 1645 (s), 1612 (s), 1314 (s), 701 (s) cm^{-1}; ^1H NMR (360 MHz, D_2O) δ 7.56-7.46 (m, 5 H, ArH), 7.33 (t, 2 H, J = 7.6 Hz, ArH), 7.25 (d, 2 H, J = 8.2 Hz, ArH), 4.38 (dd, 1 H, J = 7.5, 6.1 Hz, CH), 3.42-3.23 (dABq, 2 H, J = 14.5, 7.5, 6.1 Hz, $ArCH_2CH$); ^{13}C NMR (75 MHz, DMSO-D_6), δ 196.04 (ArCOAr'), 170.12 (COOH), 140.46, 137.32, 136.29, 133.18, 130.21, 130.14, 129.93, 129.01 ($C_6H_5COC_6H_4$), 53.07 (CH), 35.69 ($ArCH_2$); FAB MS *m/z* (Cleland) (relative intensity) 270.0 (MH^+, 100).



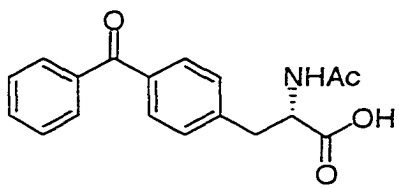
***N*-Acetyl-*p*-benzoyl-DL-phenylalanine 18.** To a stirred solution of **17** (1.5 g, 5.6 mmol) in 1 N sodium hydroxide (22 mL) was added ice (22 g) and acetic anhydride (2

mL). The mixture was stirred for 5 min and was then acidified to pH 3.0 by the slow addition of 1 N hydrochloric acid (*ca.* 18 mL). The fine crystalline product was filtered, washed with water and dried. Recrystallization from ethanol/hexane yielded **18** (1.2 g, 68%): mp 168-171 °C (lit.[148] mp 174-176.5 °C); IR (microscope) 3352 (m), 3338 (m), 1702 (s), 1646 (s), 1631 (s), 1607 (s), 1284 (s), 695 (s) cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 7.77-7.68 (m, 4 H, ArH), 7.65-7.59 (m, 1 H, ArH), 7.55-7.38 (m, 4 H, ArH), 4.65 (dd, 1 H, J = 9.1, 5.2 Hz, CH), 3.34-3.01 (dABq, 2 H, J = 13.9, 9.1, 5.2 Hz, $ArCH_2CH$), 1.85 (s, 3 H, $COCH_3$); ^{13}C NMR (75 MHz, CD_3OD), δ 198.31 (ArCOAr'), 174.35, 173.16 (COOH, $NHCOCH_3$), 144.10, 138.96, 137.30, 133.72, 131.22, 130.92, 130.43, 129.49 ($C_6H_5COC_6H_4$), 54.74 (CH), 38.44 ($ArCH_2$), 22.31 ($COCH_3$); HRMS (EI) *m/z* calcd for $C_{18}H_{17}NO_4$ 311.1157, found 311.1147. Anal. calcd for $C_{18}H_{17}NO_4$: C, 69.45; H, 5.46; N, 4.50. Found: C, 69.83; H, 5.71; N, 4.37.

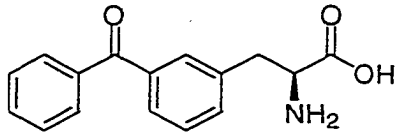


***N*-Acetyl-*p*-benzoyl-D-phenylalanine 19.** The aqueous filtrate from the acylase hydrolysis described in preparation of **14** was acidified to pH 2.4 with 1.0 N hydrochloric acid. The crystals were isolated and recrystallized from hot ethyl acetate (70 mL) to afford **19** (0.4 g, 88%); mp 187.5-188.5 °C (lit.[148] mp 186.5-187.0 °C); $[\alpha]_D^{25}$ -48.8° (*c* 1.1, 95% EtOH) (lit.[148] $[\alpha]_D^{25}$ -49.0° (*c* 1.0, EtOH)); IR (microscope) 3352 (m), 3335 (m), 1702 (s), 1646 (s), 1607 (s), 1285 (s), 696 (s) cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 7.77-7.68 (m, 4 H, ArH) 7.65-7.59 (m, 1 H, ArH), 7.55-7.37 (m, 4 H, ArH), 4.73 (dd, 1 H, J = 9.1, 5.1 Hz, CH), 3.34-3.01 (dABq, 2 H, J = 13.9, 9.1, 5.1 Hz, $ArCH_2CH$), 1.85 (s,

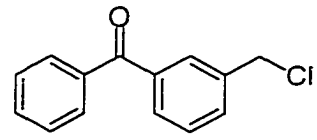
3 H, $COCH_3$); ^{13}C NMR (75 MHz, CD_3OD), δ 198.31 (Ar<u>C</u>OAr'), 174.34, 173.16 (<u>C</u>OOH, NH<u>C</u>OCH_3), 144.09, 138.95, 137.29, 133.72, 131.23, 130.92, 130.43, 129.49 ($\underline{C}_6H_5CO\underline{C}_6H_4$), 54.74 (<u>C</u>H), 38.44 (Ar<u>C</u>H_2), 22.31 (CO<u>C</u>H_3); FAB MS *m/z* (Cleland) (relative intensity) 311.9 (MH^+, 55). Anal. calcd for $C_{18}H_{17}NO_4$: C, 69.45: H, 5.46; N, 4.50. Found: C, 69.42; H, 5.54; N, 4.49.



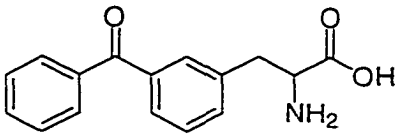
***N*-Acetyl-*p*-benzoyl-L-phenylalanine 20.** To a stirred solution of **14** (40 mg, 0.13 mmol) in 1.0 N sodium hydroxide (0.6 mL) was added ice (0.6 g) and acetic anhydride (0.05 mL). The mixture was stirred for 5 min and was then adjusted to pH 3.0 by the slow addition of 1.0 N hydrochloric acid (*ca.* 0.5 mL). The fine crystalline product was isolated by filtration, washed with water and dried to afford a crystalline solid **20** (29 mg, 70%); $[\alpha]_D^{25}$ +48.4° (*c* 1.0, 95% EtOH) (lit.[148] $[\alpha]_D^{25}$ +50.9° (*c* 1.0, EtOH)); mp 186-188 °C (lit.[148] mp 187-188 °C); IR (microscope) 3353 (m), 3337 (m), 1701 (s), 1645 (s), 1607 (s), 1284 (s), 695 (s) cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 7.70-7.60 (m, 4 H, Ar<u>H</u>), 7.60-7.55 (m, 1 H, Ar<u>H</u>), 7.50-7.28 (m, 4 H, Ar<u>H</u>), 4.65 (dd, 1 H, *J* = 8.9, 5.3 Hz, C<u>H</u>), 3.30-2.95 (dABq, 2 H, *J* = 13.8, 8.9, 5.3 Hz, ArC<u>H</u>$_2$CH), 1.85 (s, 3 H, CO<u>C</u>H_3); ^{13}C NMR (75 MHz, CD_3OD), δ 198.39 (Ar<u>C</u>OAr'), 174.34, 173.16 (<u>C</u>OOH, NH<u>C</u>OCH_3), 144.15, 138.95, 137.28, 133.74, 131.22, 130.92, 130.44, 129.49 ($\underline{C}_6H_5CO\underline{C}_6H_4$), 54.82 (<u>C</u>H), 38.47 (Ar<u>C</u>H_2), 22.32 (CO<u>C</u>H_3); FAB MS *m/z* (relative intensity) (Cleland) 311.9 (MH^+, 41). Anal. calcd for $C_{18}H_{17}NO_4$: C, 69.45; H, 5.46; N, 4.50. Found: C, 69.25; H, 5.47; N, 4.44.



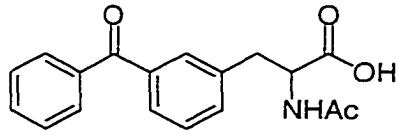
***m*-Benzoyl-L-phenylalanine 21.** Compounds **21** to **25** were prepared using the method of Kauer.[149] A suspension of **24** (1.0 g, 3.2 mmol) in water (170 mL) and concentrated ammonium hydroxide (0.3 mL) was adjusted to pH 7.5 by the dropwise addition of 0.5 N hydrochloric acid. Aspergillus acylase I (84 mg, Sigma) was added and the solution was stirred for four days at 25 °C. Filtration of the suspension (see below for recovery of **25** from the filtrate) afforded crude **21**. The crude product was dissolved in 0.5 N hydrochloric acid (7 mL) with heating. Celite® was added and the solid was removed by hot filtration. The filtrate was diluted by addition of hot water (12 mL), and 1.0 N sodium hydroxide (3.5 mL) was added. The solution was adjusted to pH 7.0 by addition of 0.1 N hydrochloric acid. The desired product was isolated by filtration to give a colourless solid **21** (0.38 g, 76%); $[\alpha]_D^{25}$ +2.7° (*c* 1.0, 1 N HCl) (lit.[149] $[\alpha]_D^{25}$ -2.9° +/- 0.8 (*c* 0.9, EtOH)); mp 228-230 °C (lit.[149] mp 230-232 °C); IR (MeOH/CH_2Cl_2 cast) 3416 (m), 1651 (s), 1628 (s), 1598 (s), 1321 (s), 726 (s), 703 (s) cm^{-1}; ^1H NMR (360 MHz, D_2O) δ 7.58-7.49 (m, 4 H, ArH), 7.48-7.33 (m, 5 H, ArH), 4.21 (dd, 1 H, *J* = 7.2, 6.2 Hz, CH), 3.25-3.07 (dABq, 2 H, *J* = 14.6, 7.2, 6.2 Hz, $ArCH_2CH$); ^{13}C NMR (75 MHz, DMSO-D_6) δ 196.69 (ArCOAr'), 170.39 (COOH), 137.99, 137.51, 136.04, 134.61, 133.67, 131.42, 130.44, 129.72, 129.44, 129.35 ($C_6H_5COC_6H_4$), 53.66 (CH), 35.92 ($ArCH_2$); FAB MS *m/z* (Cleland) 270.0 (MH^+, 8), 119. 0 (100).



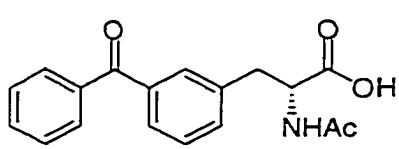
***m*-Chloromethylbenzophenone 22.** A solution of 3-methylbenzophenone (20.0 g, 102 mmol), sulfuryl chloride (16.5 mL, 28.3 g, 204 mmol), carbon tetrachloride (28 mL) and dibenzoyl peroxide (25 mg, 0.1 mmol) was heated under reflux under an argon atmosphere. Additional portions of dibenzoyl peroxide (25 mg, 0.1 mmol) were added at intervals of 0.5, 1, 3.5, and 17 h. Heating was continued for a futher 1 h and excess sulfuryl chloride was removed by co-distillation with several portions of carbon tetrachloride. The carbon tetrachloride solution obtained was treated with potassium carbonate (2.5 g) and basic alumina (2.5 g). The mixture was heated under reflux for 15 min and then the solid material was removed by filtration. The desired product was obtained by distillation of the filtrate under a reduced pressure (*ca.* 1 mm Hg) and the fraction boiling at 140-158 °C was collected. The distillate crystallized upon standing and the crystals obtained were recrystallized from hexane (65 mL) to give **22** (3.9 g, 17%); mp 45-47 °C (lit.[149] mp 45-46 °C); IR ($CHCl_3$ cast) 3060 (m), 1658 (s), 1263 (s), 705 (s) cm^{-1}; ^1H NMR (360 MHz, CD_2Cl_2) δ 7.83-7.70 (m, 5 H, ArH), 7.68-7.59 (m, 2 H, ArH), 7.55-7.48 (m, 2 H, ArH), 4.65 (s, 2 H, $ArCH_2$); ^{13}C NMR (75 MHz, CD_2Cl_2) δ 196.09 (ArCOAr'), 138.49, 138.37, 137.72, 132.89, 132.75, 130.26, 129.07, 128.71 ($C_6H_5COC_6H_4$), 46.07 ($ArCH_2$); HRMS (EI) *m/z* (relative intensity) calcd for $C_{14}H_{11}{}^{35}ClO$ 230.0498, found 230.0507; calcd for $C_{14}H_{11}{}^{37}ClO$ 232.0469, found 232.0477. Anal. calcd for $C_{14}H_{11}ClO$: C 72.92; H, 4.77; Cl, 15.39. Found: C, 72.88; H, 4.56; Cl, 15.17.



***m*-Benzoyl-DL-phenylalanine 23.** A mixture of **22** (3.5 g, 15 mmol), ethyl acetamidocyanoacetate (2.6 g, 15 mmol), potassium iodide (150 mg, 0.9 mmol), anhydrous potassium carbonate (1.4 g, 10 mmol) and dry acetone (28 mL) was heated under reflux overnight. The reaction mixture was allowed to cool, and solid material was removed by filtration through Celite®. The solids were washed with acetone, and the combined filtrates were concentrated *in vacuo*. The resulting viscous yellow oil was used in the next reaction without further purification. The residue was dissolved in 8.0 N hydrochloric acid (20 mL) and the mixture was heated under argon at 100 °C overnight. The solution was cooled and the solid material was removed by filtration. The filtrate was then neutralized with 1.0 N potassium hydroxide (*ca.* 200 mL) to pH 7.0. The resulting fine yellow crystals were collected by filtration, washed with water and dried under vacuum. The crude product was dissolved in a hot solution of 1.0 N hydrochloric acid (21 mL) and water (42 mL). The solution was filtered hot and 1.0 N sodium hydroxide solution (21 mL) was added to the filtrate to give a dark yellow crystalline solid **23** (3.0 g, 75%); mp 208-210 °C (lit.[149] mp 208-209 °C); IR (microscope) 3164 (s), 3049 (s), 1632 (s), 1599 (s), 1322 (s), 728 (s), 709 cm^{-1}; ^1H NMR (360 MHz, DMSO-D_6 + DCl) δ 7.67-7.56 (m, 3 H, ArH), 7.55-7.42 (m, 6 H, ArH), 4.09 (dd, 1 H, J = 6.6, 5.9 Hz, CH), 3.24-3.10 (dABq, 2 H, J = 14.2, 6.6, 5.9 Hz, $ArCH_2CH$); ^{13}C NMR (75 MHz, DMSO-D_6) δ 196.52 (ArCOAr'), 170.34 (COOH), 137.87, 137.41, 135.96, 134.50, 133.54, 131.34, 130.34, 129.57, 129.32 ($C_6H_5COC_6H_4$), 53.51 (CH), 30.00 ($ArCH_2$); FAB MS *m/z* (Cleland) (relative intensity) 270.0 (MH^+, 39). Anal. calcd for $C_{16}H_{15}NO_3$: C, 71.36; H, 5.61; N, 5.20. Found: C, 71.04; H, 5.67; N, 5.12.



***N*-Acetyl-*m*-benzoyl-DL-phenylalanine 24.** To a stirred solution of anhydrous **23** (2.5 g, 9.3 mmol) in 1.0 N sodium hydroxide (26 mL) was added ice (26 g) and acetic anhydride (2 mL). The mixture was shaken periodically for 5 min and was then acidified to pH 2.0 by the slow addition of 1.0 N hydrochloric acid (*ca.* 25 mL). The crystalline product was recovered by filtration, washed with water and dried. Recrystallization from ethyl acetate/hexane yielded a colourless or light yellow crystalline solid **24** (1.6 g, 60%); mp 148-151 °C (lit.[149] mp 152-153 °C); IR (microscope) 3308 (m), 3060 (m), 1710 (s), 1656 (s), 1560 (s), 1284 (s), 709 (s) cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 7.78-7.74 (m, 2 H, Ar<u>H</u>), 7.66-7.61 (m, 3 H, Ar<u>H</u>), 7.55-7.50 (m, 3 H, Ar<u>H</u>), 7.48-7.42 (m, 1 H, Ar<u>H</u>), 4.88 (br s, 1 H, N<u>H</u>), 4.69 (dd, 1 H, J = 8.9, 5.1 Hz, α-C<u>H</u>), 3.31-2.98 (dABq, 2 H, J = 13.9, 8.9, 5.1 Hz, Ar<u>CH</u>$_2$CH), 1.80 (s, 3 H, CO<u>CH</u>$_3$); ^{13}C NMR (75 MHz, CD_3OD), δ 198.51 (Ar<u>C</u>OAr'), 174.32, 173.10 (<u>C</u>OOH, NH<u>C</u>OCH$_3$), 139.09, 138.97, 138.80, 134.68, 133.82, 131.84, 131.04, 129.61, 129.54, 129.48 (<u>C</u>$_6$H$_5$CO<u>C</u>$_6$H$_4$), 54.91 (<u>C</u>H), 38.26 (Ar<u>C</u>H$_2$), 22.34 (CO<u>C</u>H$_3$); HRMS (EI) *m/z* calcd for $C_{18}H_{17}NO_4$ 311.1157, found 311.1154. Anal. calcd for $C_{18}H_{17}NO_4$: C, 69.45; H, 5.46; N, 4.50. Found: C, 69.17; H, 5.41; N, 4.48.



***N*-Acetyl-*m*-benzoyl-D-phenylalanine 25.** The aqueous filtrate from the acylase I hydrolysis described in preparation of **21** was acidified to pH 2.0 and extracted with ethyl

acetate. Solvent was removed *in vacuo* and the solid was recrystallized from ethyl acetate and hexane to afford a colourless solid **25** (0.3 g, 60%); $[\alpha]_D^{25}$ -49.7° (*c* 0.6, 95% EtOH) (lit.[149] $[\alpha]_D^{25}$ -46.8° (*c* 0.6, EtOH)); mp 145-147 °C (lit.[149] mp 145-147 °C); IR (CH_2Cl_2/MeOH cast) 3347 (s), 1728 (s), 1654 (s), 1546 (s), 1282 (s), 721 (s), 705 (s) cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 7.79-7.74 (m, 2 H, ArH), 7.67-7.61 (m, 3 H, ArH), 7.56-7.50 (m, 3 H, ArH), 7.49-7.44 (m, 1 H, ArH), 4.69 (dd, 1 H, *J* = 8.9, 5.1 Hz, α-CH), 3.34-2.98 (dABq, 2 H, *J* = 13.9, 8.9, 5.1 Hz, $ArCH_2CH$), 1.85 (s, 3 H, $COCH_3$); ^{13}C NMR (75 MHz, CD_3OD) δ 198.49 (ArCOAr'), 174.37, 173.08 (COOH, $NHCOCH_3$), 139.05, 138.89, 138.73, 134.68, 133.82, 131.82, 131.71, 131.03, 129.59, 129.52, 129.47, 127.98 ($C_6H_5COC_6H_4$), 54.92 (CH), 38.24 ($ArCH_2$), 22.37 ($COCH_3$); HRMS (EI) *m/z* calcd for $C_{18}H_{17}NO_4$ 311.1157, found 311.1161. Anal. calcd for $C_{18}H_{17}NO_4$: C, 69.45; H, 5.46; N, 4.50. Found: C, 69.28; H, 5.37; N, 4.38.

KYYGN GVHCT KSGCS VNWGE A(Bpa)SAG VHRLA NGGNG FW

Biotinylation of Bpa-LeuA 26. *p*-Benzoylphenylalanine22-LeuA (Bpa-LeuA) (**26**) was synthesized at Harvard Medical School. Electrospray mass spectrometry showed a major signal at 4034.17 +/- 2.51 (calcd mass 4034.44, oxidized form). This sample was further purified by RP-HPLC with a VYDAC C_{18} column (10 x 250 mm, 10-μm particle size, 300-Å pore size; Scientific Products and Equipment (Concord, ON); flow rate 2.5 mL/min) using a gradient from 25 to 50% MeCN in 0.1% aqueous TFA over a period of 25 min.

Biotinylation of Bpa-LeuA was done in 0.1 M sodium borate buffer, pH 8.0. Bpa-LeuA (1.0 mg, 0.2 μmol) was dissolved in 20% aqueous MeCN (0.5 mL), and sodium borate buffer (0.1 M, 4.5 mL) was then added. This solution was cooled on ice before

adding 3-sulfosuccinimidyl 2-biotinamido ethyl-1,3-dithiopropionate (NHS-SS-biotin) (0.45 mg, 0.7 μmol; Pierce). The mixture was left on ice for 1 to 2 h with occasional vortex mixing. The reaction products were separated by RP-HPLC under the same conditions as those used for Bpa-LeuA **26**. In addition to the recovery of starting material, two new compounds **27** and **28** were isolated. **26**: RT 26.2 min; ES-MS calcd 4034.44, found 4032.50 +/- 2.18. **27**: RT 27.3 min; ES-MS calcd 4426.00, found 4425.25 +/- 0.95. **28**: RT 28.6 min; ES-MS calcd 4817.56, found 4814.08 +/- 1.97. Specific activities determined by spot-on-lawn technique using *C. divergens* LV13 as an indicator were 205, 21.3, 4.0 AU/μg, respectively for **26**, **27** and **28**.

N-Biotinamidocaproate-Bpa-LeuA **29** and **30**, which differs in the degree of modification, were prepared in a similar way to **27** and **28** using biotinamidocaproate *N*-hydroxysuccinimide ester (NHS-biotin, Sigma) in 0.1 M sodium borate buffer, pH 8.0. Thus, Bpa-LeuA (0.3 mg, 0.06 μmol) was dissolved in 20% aqueous MeCN (0.3 mL), and sodium borate buffer (0.1 M, 0.7 mL) was added. The solution was cooled on ice before adding NHS-biotin (0.15 mg, 0.3 μmol). The mixture was left on ice for 2 h with occasional vortexing. The reaction products were purified by RP-HPLC on the same column as before using a gradient from 20 to 52% MeCN in 0.1% aqueous TFA over a period of 30 min to give **29**, **30**, plus recovered **26**. **26**: RT 24.6 min; ES-MS calcd 4034.44, found 4034.17 +/- 2.51. **29**: RT 24.6 min; ES-MS calcd 4374.90, found 4373.40 +/- 1.01. **30**: RT 26.6 min; ES-MS calcd 4715.36, found 4712.50 +/- 0.73. Spot-on-lawn test using *C. divergens* LV13 as an indicator revealed that both **29** and **30** were antimicrobially active.

Photoaffinity labelling. Two milliliters of 16 h culture of UAL9 (*C. divergens* LV13) were centrifuged at 14,000 rpm for 5 min. The cell pellet was washed twice with sterile

phosphate buffered saline (PBS, 0.1 M phosphate, 0.15 M NaCl, pH 7.0), and resuspended in 1 mL of the same buffer. Different amounts of biotinylated Bpa-LeuA (**27** or a mixture of **29** and **30**) were added to the above prepared culture. UV irradiation was done by following a literature method.[150] The sample was incubated at 4 °C with shaking for 1 h, then irradiated (Blak-Ray® long wave ultraviolet lamp, 100 W, Model B 100AP. Upland, CA. Maximum irradiation at 350-360 nm) at 4 °C for 15 min at a distance of 6 cm. After centrifugation at 14,000 rpm for 8 min, the pellet was washed once with PBS buffer, resuspended in 40 μL of gel electrophoresis sample buffer (made of 3.1 mL of 1.0 M Tris-HCl, pH 6.8; 0.5 mL of a 1.0% aqueous solution of bromophenol blue; 5.0 mL of glycerol; and water to make a 25.0 mL solution). The mixture was occasionally stirred by vortex mixing while it was left at room temperature for 1 to 2 h.

The samples were denatured in a boiling water bath for 5 min before loading onto a 12% polyacrylamide gel. The mini-slab gels (12% acrylamide) with 4% stacking were cast according to the instruction manual (Bio-Rad Laboratories, Richmond, CA) 2 h before use. For each of the above prepared samples, 35 μL was loaded onto the 10-well slab gel. The gels were developed at 150 volts for 50-60 min in Mini-Protean® II Dual Slab Cell (Bio-Rad) in a Tris-glycine buffer (10 times concentrated stock solution made of 30.25 g Tris, 144.0 g glycine, and water to make a 1-liter solution. SDS is added to a final concentration of 1% before use).

The gel slabs were soaked in transfer buffer (3.03 g Tris, 14.40 g glycine per liter, pH 8.3) for 30 min with gentle shaking. Transfer buffer contained 25 mM Tris, 192 mM glycine with a pH value in the range of 8.1 to 8.4, without adjustment by the addition of acid or base. The blotting membrane (Zeta-Probe® Nylon membrane, Bio-Rad), filter paper and the plastic fiber were all pre-soaked in transfer buffer for 30 min before use. Mini-Trans-Blot® electrophoretic transfer cell (Bio-Rad) was chosen for the blotting. The

apparatus and the slab gels were assembled according to the instruction manual of Bio-Rad mini blotting (Bio-Rad, cat. no. 170-3930). The ice tray used to cool the blotting system should be prepared one day before. The blotting was done at 100 volts for 30 min, then at 300 mA for 40 min in transfer buffer. Non-specific binding was blocked by soaking the membranes in blocking buffer for 1 h with mild shaking. Blocking buffer was TBS (Tris buffered saline) with 3% bovine serum albumin (BSA, Sigma). TBS contained 25 mM of Tris, 150 mM of sodium chloride at pH 8.0. The membranes were then washed 3 x 10 min with wash buffer (TBS with 0.3% BSA and 0.05% Tween 20). The washed membranes were incubated in 50 mL of wash buffer containing 2 μg/mL of streptavidin (Bio-Rad) for 1 h. After washing 3 x 10 min with wash buffer, the membranes were incubated in 50 mL of wash buffer containing 0.1% biotinylated alkaline phosphatase (Bio-Rad, cat. no. 170-6403) for 1 h, which was followed by a further three times washing as before. A ready-to-use substrate, 1-Step™ NBT/BCIP (Pierce, cat. no. 34042) was used to visualize the proteins labelled with biotin. The membranes were soaked in the substrate solution with swirling until enough colour had developed, usually 10 to 15 min. The membranes were then washed with a large amount of Milli-Q water and dried with filter paper.

Preparative electrophoresis for purification of the photoaffinity labelled proteins. Preparative electrophoresis was performed using a Bio-Rad Mini Prep Cell (Bio-Rad, cat. no. 170-2908). The concentration of acrylamide solution used to cast the column gel was 15% based on the optimization curve provided by the supplier. The length of the column gel was 8 to 8.5 cm with 4% stacking (0.5 cm). Normally it was cast overnight before use.

The sample preparation procedure was simply a scale up of the analytical electrophoresis. Fresh culture of UAL9 (60 mL) was washed twice with 0.1 M PBS, pH 7.0 then incubated in the same buffer (20 mL) with 900 AU biotinylated Bpa-LeuA (**27** or a mixture of **29** and **30**) for 1 h. After binding, the culture was irradiated as before under a

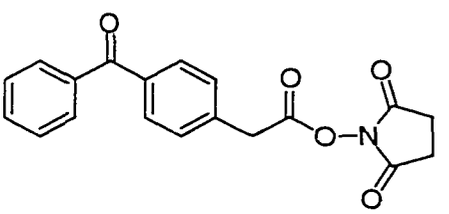
UV lamp (100 W, maximum irradiation at 350-360 nm) at a distance of 6-8 cm for 30 min at 4 °C. The cells were pelleted by centrifugation. The cell pellet was resuspended in SDS-reducing sample buffer (made of 11.2 mL water; 6.0 mL of 0.5 M Tris-HCl, pH 6.8; 8.0 mL of glycerol; 6.4 mL of 10% SDS; 0.8 mL of 0.5% aqueous bromophenol blue; and 5% β-mercaptoethanol). The column gel was developed at a constant voltage of 200 volts. Depending on the length and the concentration of the gel, the total running time ranged from 8 to 12 h. A flow rate of 0.08 mL/min (Econo Pump, Model EP-1, Bio-Rad) was used for the elution. After 3 h fractions were collected at 6 min intervals. An aliquot of 100 μL was taken from every five fractions. The samples were dried *in vacuo* (speedvac) and re-dissolved in 40 μL of gel sample buffer. These samples were run on analytical gel and blotted onto Nylon membrane as previously described to check the resolution of this process and to locate the fractions which contained the target protein(s).

The fractions with a molecular weight of approximately 14 kDa were pooled and a Centricon® membrane (3 kDa molecular weight cut-off) was used to clean detergent from the sample. Subsequently the sample was further purified by RP-HPLC with a VYDAC C_{18} column (4.6 x 250 mm, 5-μm particle size, 300-Å pore size; flow rate 1.0 mL/min) using a gradient from 16 to 80% MeCN in 0.1% aqueous TFA over a period of 80 min. Samples from several runs were combined in order to get enough material for characterization. A pure sample **31** was obtained with a retention time of 43 min and the molecular weight was determined to be 7209.73 +/- 0.74. *N*-Terminal automatic amino acid sequencing carried out at Alberta Peptide Institute (Edmonton, AB) indicated a sequence of Met-Glu-Gln-Gly-Thr-Val-Lys-Trp-Phe-Asn-Ala.

Labelling of lipid with Bpa-LeuA. Bpa-LeuA **26** (250 μg) was added to 0.5 mL of 180 mM dodecylphosphocholine (DPC) in 0.1% aqueous TFA and mixed thoroughly by

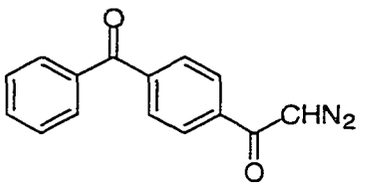
vortexing. The solution was deoxygenated by bubbling argon through it for 5 min, followed by sonication 3 x 30 sec. The mixture was irradiated as before under a UV lamp (100 W, maximum irradiation at 350-360 nm) at a distance of 6 cm for 15 min at 4 °C. The solution was diluted with a large amount of water containing 0.1% TFA and was concentrated by centrifugation using Centricon® membranes (3 kDa molecular weight cut-off). The concentrated sample was then purified by RP-HPLC with a VYDAC C_{18} column (4.6 x 250 mm, 5-μm particle size, 300-Å pore size; flow rate 1.0 mL/min) using a gradient from 24 to 48% MeCN in 0.1% aqueous TFA over a period of 30 min. Two major components were isolated: unreacted **26** (*m/z* 4037.74 +/- 0.27; retention time 21.1 min) and material **32** corresponding to **26** + DPC (*m/z* 4385.18 +/- 0.22, calcd mass 4385.90; retention time 25.5 min).

Similar labelling experiments were also done separately in 0.1 M PBS, pH 7.0; 90% TFE in 0.1% aqueous TFA; and 25 mM SDS in 0.1% aqueous TFA. Starting material **26** was recovered in each case. Bpa-LeuA **26** did not dimerize or polymerize and was not modified by these small molecules.



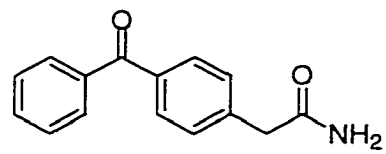
***p*-Benzoylphenylacetic acid *N*-hydroxysuccinimidyl ester 33.**[158,196] To a mixture of **36** (240 mg, 1 mmol) and *N*-hydroxysuccinimide (126 mg, 1.1 mmol) in 1,4-dioxane (5 mL) was added DCC (227 mg, 1.1 mmol) at 0 °C. After 1 h the reaction mixture was allowed to warm to room temperature and left to stir overnight. The mixture was then cooled on ice and the solid material was removed by filtration. The solvent of the above filtrate was removed *in vacuo* and the residue was purified by flash chromatography

(solvent: $CHCl_3$/ethyl acetate 2:1; $R_f = 0.5$). Recrystallization from CH_2Cl_2/petroleum ether to give a colourless crystalline solid **33** (264 mg, 78%); mp 103-105 °C; IR ($CHCl_3$ cast) 3328 (m), 1814 (s), 1783 (s), 1738 (s), 1657 (s), 1205 (s), 701 (m); ^1H NMR (360 MHz, $CDCl_3$) δ 7.80-7.75 (m, 4 H, ArH), 7.59-7.54 (m, 1 H, ArH), 7.48-7.43 (m, 4 H, ArH), 4.00 (s, 2 H, $ArCH_2$), 2.80 (s, 4 H, 2 CH_2); ^{13}C NMR (75 MHz, $CDCl_3$), δ 196.00 (ArCOAr'), 168.91, 166.18 ($ArCH_2COON$, 2 x CH_2CON), 137.35, 136.98, 135.92, 132.46, 130.48, 129.92, 129.43, 128.34 ($C_6H_5COC_6H_4$), 37.46 ($ArCH_2$), 25.53 ($COCH_2CH_2CO$); HRMS (EI) *m/z* calcd for $C_{19}H_{15}NO_5$ 337.0950, found 337.0947. Anal. calcd for $C_{19}H_{15}NO_5$: C, 67.65; H, 4.48; N, 4.15. Found: C, 67.31; H, 4.48; N, 4.34.



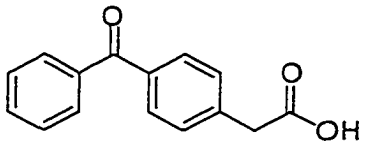
***p*-Benzoyl-diazoacetophenone 34.** The method of Zelinski *et al.*[157] was used to prepare **34**, **35** and **36**. A solution of *p*-benzoylbenzoic acid (3.6 g, 16 mmol) in thionyl chloride (50 mL) was heated under reflux for 1 h and the excess thionyl chloride was evaporated *in vacuo*. Traces of thionyl chloride were removed by azeotropic distillation with dry carbon tetrachloride. The resulting white solid was dissolved in dry THF (10 mL) and Et_2O (40 mL) to give a solution which was added dropwise to a freshly prepared solution of diazomethane over 20 min with magnetic stirring at 0 °C. Diazomethane was prepared by following a literature method[197] using a Diazomethane Generator (Aldrich). The solution was left at 0 °C for a further 3 h, warmed to room temperature and left to stand overnight. The solvent was removed *in vacuo* and the residue was purified by flash chromatography (solvent: ethyl acetate/hexane 1:3; R_f = 0.24) to afford a light yellow

crystalline solid **34** (3.5 g, 87%); mp 106-109 °C (lit.[157] mp 114-116 °C); IR ($CHCl_3$ cast) 3099 (m), 2106 (s), 1654 (s), 1596 (s), 1357 (s), 705 (s); ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.86 (s, 4 H, ArH), 7.81-7.78 (m, 2 H, ArH), 7.63-7.59 (m, 1 H, ArH), 7.50 (t, 2 H, J = 7.4 Hz, ArH), 5.95 (s, 0.8 H, CHN_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 195.86 (ArCOAr'), 185.38 ($ArCOCHN_2$), 141.05, 139.48, 136.98, 132.98, 130.18, 130.11, 128.51, 126.67 ($C_6H_5COC_6H_4$), 54.98 (CHN_2); HRMS (EI) *m/z* calcd for $C_{15}H_{10}N_2O_2$ 250.0742, found 250.0743. Anal. calcd for $C_{15}H_{10}N_2O_2$: C, 71.99; H, 4.02; N, 11.19. Found: C, 72.01; H, 3.95; N, 11.34.



***p*-Benzoylphenylacetamide 35.** To a solution of **34** (2.0 g, 8 mmol) in dioxane (15 mL) was added 28% ammonium hydroxide (10 mL) and 10% aqueous silver nitrate (5 mL). The mixture was heated under reflux for 6.5 h, then cooled to room temperature. The solid material was removed by filtration. The filtrate was diluted with water (150 mL) and left overnight at 4 °C. The pale yellow precipitate was collected by filtration. Extraction of the solid with a total of 200 mL of water for 16 h in a Soxhlet extractor, followed by cooling the aqueous solution, yielded crude **35**, which was recrystallized from water to give a shining snowflake-like crystalline solid (0.7 g, 39%); mp 131-132 °C (lit.[157] mp 136-137 °C); IR ($CHCl_3$ cast) 3364 (m), 3177 (m), 1656 (s), 695 (m); ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.81-7.78 (m, 4 H, ArH), 7.61-7.58 (m, 1 H, ArH), 7.51-7.48 (m, 2 H, ArH), 7.40 (d, 2 H, J = 8.2 Hz, ArH), 5.90 (br s, 1 H, one of NH_2), 5.58 (br s, 1 H, one of NH_2), 3.69 (s, 2 H, $ArCH_2$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 196.23 (ArCOAr'), 172.51

($\underline{C}ONH_2$), 139.46, 137.47, 136.72, 132.57, 130.73, 130.02, 129.36, 128.37 ($\underline{C}_6H_5CO\underline{C}_6H_4$), 43.15 ($\underline{C}H_2$); HRMS (EI) *m/z* calcd for $C_{15}H_{13}NO_2$ 239.0946, found 239.0943. Anal. calcd for $C_{15}H_{13}NO_2$: C, 75.32; H, 5.43; N, 5.84. Found: C, 75.34; H, 5.47; N, 5.92.



***p*-Benzoylphenylacetic acid 36.** A solution of **35** (2.0 g, 8 mmol) in 20% potassium hydroxide (50 mL) was heated under reflux for 2 h. After brief cooling, charcoal (1.0 g) was added and the reaction mixture was heated under reflux for 5 min. Charcoal was removed by hot filtration. The filtrate was cooled and acidified to pH 1.0 by slow addition of 6.0 N hydrochloric acid. The resulting precipitate was extracted with diethyl ether (3 x 50 mL). The ether extract was dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* and the residue was further purified by flash chromatography (solvent: $CHCl_3$: MeOH : AcOH 90 : 8 : 2; R_f = 0.23). Recrystallization from ethyl acetate/hexane a colourless crystalline solid **36** (1.6 g, 80%); mp 111-113 °C (lit.[157] mp 112-114 °C); IR ($CHCl_3$ cast) 3059 (m), 1734 (s), 1710 (s), 1657 (s), 699 (m); ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.81-7.78 (m, 4 H, Ar$\underline{H}$), 7.61-7.57 (m, 1 H, Ar$\underline{H}$), 7.51-7.46 (m, 2 H, Ar$\underline{H}$), 7.43-7.39 (d, 2 H, *J* = 8.3 Hz, Ar$\underline{H}$), 3.76 (s, 2 H, ArC$\underline{H}_2$); ^{13}C NMR (75 MHz, $CDCl_3$), δ 196.33 (Ar$\underline{C}$OAr'), 176.75 ($\underline{C}$OOH), 137.87, 137.54, 136.69, 132.51, 130.49, 130.05, 129.43, 128.34 ($\underline{C}_6H_5CO\underline{C}_6H_4$), 40.97 ($\underline{C}H_2$); HRMS (EI) *m/z* (relative intensity) calcd for $C_{15}H_{12}O_3$ 240.0786, found 240.0784. Anal. calcd for $C_{15}H_{12}O_3$: C, 74.99; H, 5.03. Found: C, 74.70; H, 5.35.

KYYGN GVHCT KSGCS VNWGE AFSAG VHRLA NGGNG FW

***N*-*p*-Benzoylphenylacetyl-LeuA (38, 39, 40, 41).** LeuA (**37**, above) was synthesized by American Peptide Company, Inc. (Sunnyvale, CA). The molecular weight was determined as 3930.54 (calcd mass: 3930.33, oxidized form) after oxidization of the two cysteine residues to form the disulfide bridge. The chromatographic behavior and specific antimicrobial activity of the synthetic LeuA were identical to the LeuA purified from the supernatant of its natural producer *L. gelidum* UAL187.[49]

LeuA **37** (1.1 mg, 0.28 μmol) was dissolved in DMF (1 mL). Water (1 mL) and triethylamine (50 μL of 1% aqueous solution) were added. The mixture was stirred at room temperature and **33** (80 μL of 2 mg/mL in DMF) was added dropwise over a few minutes. The solution was stirred for 3 h at room temperature and the reaction products were isolated by RP-HPLC with a VYDAC C_8 column (10 x 250 mm, 10-μm particle size, 300-Å pore size; flow rate 2.5 mL/min) using a gradient from 20 to 70% MeCN in 0.1% aqueous TFA over a period of 30 min to give **38**, **39**, **40** and **41**. These fractions were further purified on RP-HPLC to homogeneity using an analytical VYDAC C_8 column (4.6 x 250 mm, 5-μm particle size; flow rate 1 mL/min) under similar conditions. The identity of the products was determined by mass spectrometry. **37**: RT 26.7 min; ES-MS calcd 3930.33, found 3930.07 +/- 0.81. **38**: RT 29.4 min; ES-MS calcd 4152.58, found 4151.70 +/- 0.60. **39**: RT 29.8 min; ES-MS calcd 4152.58, found 4151.60 +/- 0.10. **40**: RT 30.3 min; ES-MS calcd 4152.58, found 4152.20 +/- 0.20. **41**: RT 32.8 min; ES-MS calcd 4374.83, found 4373.50 +/- 0.10.

N-Terminal amino acid sequencing of these modified peptides was performed to locate the site of *p*-benzoylphenylacetyl group attachment. Specific activities of these peptides were determined by spot-on-lawn technique using UAL9 as an indicator to be 512, 42.7, 18.7, 5.3, 1.2 AU/μg, respectively for **37**, **38**, **39**, **40**, **41**.

Biotinylations of 38, 39, 40. To a solution of modified LeuA (**38**, 50 μg) in DMF (50 μL) was added 500 μL of 0.1 M borate buffer, pH 8.6, followed by 80 μL of NHS-biotin (Sigma, 1 mg/mL in DMF) with stirring at room temperature. The mixture was stirred for 2 h before it was purified directly by RP-HPLC. The chromatographic conditions used were the same as those for the purification of **38**. The molecular weight of the main biotinylated product **42** was determined to be 4490.50 +/- 0.50 (calcd mass 4493.04) and it was biologically active towards *C. divergens* LV13. In addition to the recovery of some starting material, another minor fraction also was found with a molecular weight 4490.2 +/- 0.2, presumably due to modification at a different amino group.

Biotinylations of **39**, **40** were done in the same way to give **43** and **44.** The observed molecular weights (4490.4 +/- 0.4 for **43** and 4490.6 +/- 0.6 for **44**) were in agreement with the calculated value of 4493.04. During the modification of **39**, a minor fraction **45** was also isolated as a dibiotinylated product, as indicated by the observed molecular weight of 4830.76 +/- 0.6 (calcd mass 4833.50). Unreacted starting material was recovered each time.

Photoaffinity labelling with 42, 43, 44 and 45. Photoaffinity labellings of LeuA sensitive cells with **42**, **43**, **44** and **45** were done using similar conditions to those described for **27** and **29**. Thus, peptides (*ca.* 1 - 5 μg each) were added to 2 mL of washed culture *C. divergens* LV13 (UAL9). After 1 h incubation at room temperature, the culture was then irradiated under UV light. The cell lysate in gel electrophoresis sample buffer was loaded onto a 12% polyacrylamide ready-gel (Bio-Rad, cat. no. 161-1156). After the gel was resolved at 150 volts for 60 min, the proteins on the gel were then transferred to a Zeta-Probe® Nylon membrane and blotted as previously described.

Photolabelling of DPC with *N*-*p*-Benzoylphenylacetyl-LeuA. Peptide **38** (120 μg) was added to 0.5 mL of 180 mM dodecylphosphocholine (DPC) and thoroughly mixed by vortexing. The solution was deoxygenated by bubbling argon through it for 5 min, and was then sonicated 3 x 30 s. The mixture was irradiated under a UV lamp (100 W, maximum irradiation at 350-360 nm) at a distance of 6-8 cm for 60 min at 4 °C. The solution was desalted on a pre-packed column (Econo-Pac 10 DG. Bio-Rad, cat. no. 732-2010). The active fractions (indicator: *C. divergens*) were then purified by RP-HPLC with a VYDAC C_{18} column (4.6 x 250 mm, 5-μm particle size, 300-Å pore size; flow rate 1.0 mL/min) using a gradient from 20 to 50% MeCN in 0.1% aqueous TFA over a period of 30 min. Two major components were isolated: unreacted **38** (*m/z* 4151.90 +/- 1.70; retention time 30.7 min) and one component **46** corresponding to **38** + DPC (*m/z* 4501.30 +/- 0.30, calcd mass 4503.58; retention time 34.7 min).

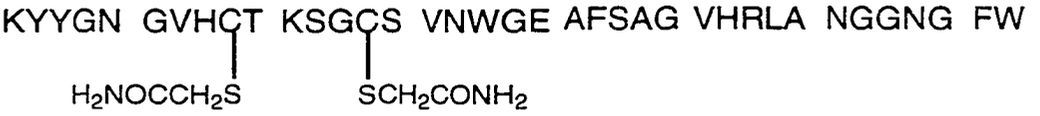
Biotinylation of LeuA. Biotinylated LeuA (**47, 48, 49**) with a disulfide linkage were prepared in a similar way to **27** and **28**. Thus, LeuA **37** (50 μg, 0.013 μmol) was dissolved in 100 μL of 0.1 M borate buffer, pH 7.5 at 0 °C, and NHS-SS-biotin (19 μg, 0.032 μmol; Pierce) was added. The mixture was left on ice for 1 h with occasional vortexing. The reaction products were separated by RP-HPLC under similar chromatographic conditions as described above for the purification of **27** and **28** to give **47**, **48** and, **49**, plus recovered **37**. **37**: RT 20.9 min; ES-MS calcd 3932.33, found 3933.34 +/- 1.41. **47**: RT 21.9 min; ES-MS calcd 4323.89, found 4325.00 +/- 0.79. **48**: RT 22.9 min; ES-MS calcd 4715.45, found 4716.09 +/- 0.21. **49**: RT 23.2 min; ES-MS calcd 4715.45, found 4708.80 +/- 0.00. The purified substances (**47**, **48** and, **49**) were antimicrobially active towards *C. divergens* LV13 as detected by spot-on-lawn test.

Biotinylated LeuA (**50**, **51**) without a disulfide linkage were also prepared in a similar way. Thus, LeuA (0.3 mg, 0.06 μmol) was dissolved in 20% aqueous MeCN (0.3 mL), sodium borate buffer (0.1 M, 0.7 mL, pH 8.0) was then added. The solution was cooled on ice before NHS-biotin (0.15 mg, 0.3 μmol; Sigma) was added. The mixture was left on ice for 2 h with occasionally vortexing. The reaction products were purified by RP-HPLC on the same column as before with a gradient of 20% to 60% MeCN in 30 min to give **50** and **51**, in addition to the recovery of **37**. **37**: RT 19.5 min; ES-MS calcd 3932.33, found 3932.75 +/- 2.91. **50**: RT 20.6 min; ES-MS calcd 4272.79, found 4271.52 +/- 1.12. **51**: RT 22.1 min; ES-MS calcd 4613.25, found 4611.46 +/- 1.60. Compounds **50** and **51** were antimicrobially active towards *C. divergens* LV13 as detected by spot-on-lawn test.

Binding to and elution of biotinylated LeuA from avidin-agarose column. Avidin (monomeric)-Agarose resin (Sigma) was regenerated before use as recommended by the supplier. Resin (1.5 mL, 5 x 20 mm) was washed with 6 mL of 0.1 M PBS, pH 7.0, containing 1.0 mg/mL of D-biotin, followed by 15 mL of 0.1 M glycine-HCl, pH 2.0. The resin was then equilibrated with PBS. About 200 μg of peptides (a mixture of **47**, **48**, **49**) in PBS were loaded onto the column followed by extensive washing with 18 mL of the same buffer. Peptides were eluted from the column with 0.1 M DTT in 10 mM Tris-HCl, pH 7.5. Eluted peptides (**52**, **53**, **54**) were further purified and desalted on RP-HPLC as previously described. **52**: RT 21.6 min; ES-MS calcd 4020.33, found 4019.99 +/- 0.68. **53**: RT 22.2 min; ES-MS calcd 4020.33, found 4020.25 +/- 0.13. **54**: RT 22.8 min; ES-MS calcd 4108.33, found 4108.63 +/- 0.51. Compounds **52**, **53** and **54** were antimicrobially active towards *C. divergens* LV13 as detected by spot-on-lawn test.

Modification of LeuA with Bolton-Hunter reagent. LeuA **37** (100 μg, 0.025 μmol) was dissolved in 100 μL of 0.1 M borate buffer, pH 7.5 at 0 °C. Bolton-Hunter reagent (3-(*p*-Hydroxyphenyl)propionic acid N-hydroxysuccinimide ester, Sigma) (16.5 μg, 0.0625 μmol in 3.3 μL of ethanol) was added. The mixture was left on ice for 45 min with occasional vortexing. The reaction was quenched by the addition of 27 μL of 1% TFA. Reaction products were separated by RP-HPLC under similar chromatographic conditions to those described for **52** to give **55**, **56**, **57**, **58**, plus recovered **37**. **37**: RT 21.5 min; ES-MS calcd 3932.33, found 3930.97 +/- 0.34. **55**: RT 22.5 min; ES-MS calcd 4080.49, found 4078.84 +/- 0.43. **56**: RT 23.5 min; ES-MS calcd 4228.65, found 4228.20 +/- 0.79. **57**: RT 23.8 min; ES-MS calcd 4228.65, found 4226.90 +/- 0.21. **58**: RT 24.9 min; ES-MS calcd 4376.81, found 4378.59 +/- 0.01. Compounds **55**, **56**, **57** and **58** were antimicrobially active towards *C. divergens* LV13 as detected by spot-on-lawn test.

N-Terminal amino acid sequencing was done by Alberta Peptide Institute (Edmonton, AB) on the modified peptides in order to locate the modification sites. The sequences were obtained using Edman degradation on an automated gas phase sequencer (Applied Biosystems model 470A) with on-line phenylthiohydantoin derivative identification by RP-HPLC (Applied Biosystems model 120A). The overall specific activity of the modified peptides was reduced to half (one dilution) amount of the original peptide.



Reduction and modification of LeuA 59. To a solution of LeuA (10 μg) in 0.4 mL of 6 M guanidinium Tris-HCl buffer (0.6 M, pH 8.6) was added 20 μL of freshly prepared

280 mM DTT. The mixture was incubated at 37 °C for 15 min and then cooled to room temperature. Freshly prepared aqueous iodoacetamide solution (200 μL, 584 mM) was added to the above solution under argon with stirring. The solution was incubated at 37 °C for 60 min and then the reaction products were purified by RP-HPLC using conditions described for the preparation of **38**. The isolated product **59** gave a molecular weight 4046.0 +/- 0.8 (calcd mass 4046.45). Its specific activity was determined to be 5.3 AU/μg using *C. divergens* LV13 as an indicator.

Syntheses of peptide fragments of LeuA and inhibition studies of these peptides towards its parent bacteriocin LeuA. Two peptide fragments of L-LeuA were synthesized at BioTools Inc. (Edmonton, AB) using standard Fmoc chemistry. The first fragment was a 15-mer peptide (WGEAF SAGVH RLANG) **60** spanning from residue 18 (Trp) to residue 32 (Gly) of L-LeuA with non-modified *N*- and *C*-termini. The second fragment was a 20-mer peptide (WGEAF SAGVH RLANG GNGFW) **61** having the *C*-terminal sequence of LeuA from residue 18 (Trp) to residue 37 (Trp). A sample of the 20-mer peptide was *N*-terminal acetylated (Ac-WGEAF SAGVH RLANG GNGFW) **62**. The peptides were purified to >95% homogeneity by RP-HPLC using an MeCN-H_2O gradient (0.05% TFA) using a Beckman System Gold Nouveau instrument. The molecular weights of the peptides were determined by electrospray mass spectrometry (Fisons VG Trio 2000 ESMS). The molecular weight of the 15-mer peptide **60** was 1571.78 +/- 0.37 (calculated mass 1571.76) and that of the 20-mer **61** was 2133.1 +/- 0.67 (calculated mass 2133.39). The molecular weight of the *N*-acetylated 20-mer **62** was 2174.69 +/-0.50 (calculated mass 2175.43).

Bacteriocin activity was measured with a 96-well microtiter plate assay system. LeuA solution at two-fold dilutions, various amounts of the 15-mer peptide fragment or the

20-mer peptide fragment, and 2 μL of 16 h culture (indicator strain, *C. divergens* LV13 or *Lactobacillus sake* 20017) were added to each microtiter plate well containing 200 μL of APT broth. The microtiter plate cultures were incubated for 16 h at 25 °C, after which time the growth of the indicator strain was measured spectrophotometrically at 650 nm with a microplate reader (Molecular Devices Inc., Softmax 2.3.2). The culture absorbance was then plotted against the peptide concentration and the minimal inhibition concentration (MIC) was read from the graph directly. The MIC was defined as the bacteriocin concentration that inhibited the growth of the indicator strain by 50% (50% of the absorbance of the control culture without bacteriocin at 650 nm).

Search for agonistic and antagonistic effects between CbnB2 and LeuA. In order to study the possible agonistic/antagonistic effects between CbnB2 and LeuA, the following strains, which are sensitive to LeuA but insensitive to CbnB2, were screened: *Lactococcus mesenteroides* 23386, *Lactobacillus sake* 20017, *Carnobacterium piscicola* N5, *Carnobacterium piscicola* UAL26/8B, *Enterococcus faecium* BFE900, *Enterococcus durans* ATCC 11576, *Enterococcus durans* ATCC 19432. Solutions were prepared with a standard concentration of LeuA and differing amounts of CbnB2. The antimicrobial activity of the mixture was measured with the spot-on-lawn technique. APT agar plates and APT culture medium were used throughout. The plates were incubated at 25 °C in an incubator overnight before the inhibition zone was measured. A solution of LeuA at the same concentration without CbnB2 was used as a control. The change of the specific activity of LeuA reflects the influence of CbnB2.

Effects of CbnB2(1-22) 3 and CbnB2 leader peptide 63 on the antimicrobial activity of CbnB2. The leader peptide of CbnB2 (MNSVK ELNVK EMKQL HGG) **63** was synthesized at Alberta Peptide Institute (Edmonton, AB). The molecular weight

was determined to be 2042.00 ± 0.15 (calculated mass: 2042.48) by electrospray mass spectrometry (HP1100, LC-MSD). The effects of the 22-mer peptide fragment and the CbnB2 leader peptide towards its parent bacteriocin CbnB2 were analyzed in the same way as that outlined above for the fragments of LeuA. *C. divergens LV13* was the indicator strain used for measuring the specific antimicrobial activity of CbnB2 or its mixture with its leader peptide or the 22-mer fragment.

KYYGN GVHCT KSGCS VNWGE AFSAG VHRLA NGGNG FW

Synthesis of D-LeuA 64. D-LeuA **64**, the enantiomer of a naturally occuring class IIa bacteriocin L-LeuA, was manually synthesized by stepwise solid phase synthesis[61] using D-amino acids. The following side chain protection was used: Arg (Pmc); Asn (Trt); Cys (Trt); Glu (OtBu); His (Trt); Lys (Boc); Ser (tBu); Thr (tBu); Trp (Boc); Tyr (tBu). The synthesis started with coupling of Fmoc-tryptophan (Boc) to 0.3 mmoles of Wang resin using 1,3-dicyclohexylcarbodiimide (DCC) as the activating agent in the presence of a catalytic amount of 4-*N,N*-dimethylaminopyridine (DMAP) in DMF. Following this, each amino acid was assembled in turn by cycles of deprotection, activation and coupling. Each step was followed by extensive washing sequentially with DMF, dichloromethane, isopropanol, dichloromethane and DMF. Freshly prepared 20% piperidine in DMF was used for the deprotection of Fmoc groups. Couplings were carried out in DMF by activating with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-methyl morpholine using a four-fold excess of the Fmoc protected amino acid between 1 to 2 h at room temperature. Coupling yields were quantitated by ninhydrin assay for residual free amine.[198] If the Kaiser test gave a positive result after double coupling, either more coupling under the same conditions or capping with acetic anhydride was performed. For difficult residues (Cys and most of the residues after position 20), elevated temperatures of up to 50 °C were used, and 2-(1*H*-azabenzotriazole-1-yl)-1,1,3,3-

tetramethyluronium hexafluorophosphate (HATU) was used as the coupling reagent instead of HBTU. A test cleavage was performed after each five residues were coupled and the desired product was given each time as was confirmed by electrospray mass spectrometry. Cleavage from the resin was done in a mixture of 90% TFA, 5% thioanisole, 3% DTT and 2% anisole for 2 h at room temperature with mechanical shaking. One third of the resin was removed after the first 20 amino acids had been attached.

After the chain assembly was complete, the crude peptide was cleaved from the resin and deprotected under the same conditions used for test cleavages. Following purification on a preparative RP-HPLC with a C_{18} column (21 x 250 mm, Zorbax 300 SB column; flow rate of 8 mL/min; monitored at 210 nm) using a gradient from 25 to 50% MeCN in 0.1% aqueous TFA over a period of 90 min. The fractions showing the desired mass (calcd mass 3932.33, reduced form) were pooled and lyophilized. The lyophilized peptide was further purified to homogeneity by RP-HPLC with a semi-preparative VYDAC C_8 column (10 x 250 mm, 10-μm particle size, 300-Å pore size; flow rate 2.5 mL/min) using a gradient from 20 to 40% MeCN in 0.1% aqueous TFA over a period of 50 min. The purified sample was then concentrated and lyophilized.

The lyophilized peptide was redissolved in 20 mM of ammonium bicarbonate solution (pH 8.1) with a peptide concentration of 0.5 mg/mL. The solution was stirred overnight under oxygen at room temperature. The oxidization state of the two cysteine residues was monitored by iodoacetamide reaction. After disulfide bond formation was complete, the solution was diluted with water and lyophilized. The oxidized pure peptide was stored at -20 °C or dissolved in water containing 0.1% TFA to make a 2 mg/mL solution for biological tests.

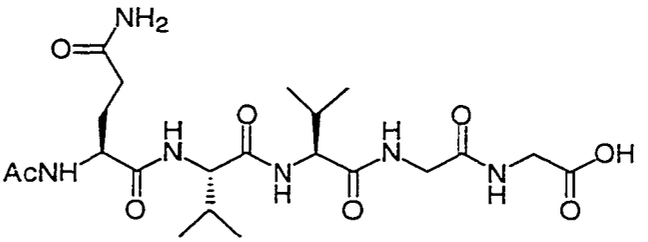
The structure of the purified D-LeuA was characterized by automatic amino acid sequencing, mass spectrometry, co-injection on analytical HPLC with an authentic L-LeuA sample and circular dichroism (CD) spectroscopy. The *N*-terminal amino acid sequences of the purified peptide were obtained using Edman degradation on an automated gas phase

sequencer (Applied Biosystems, model 470A) with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems, model 120A). The mass spectra of the purified peptides were obtained by direct injection of their solutions (50% aqueous MeCN, 0.1% TFA) using a HP1100 LC-MSD instrument. In order to confirm the stereochemistry of the peptide back bone and to obtain conformational information of D-LeuA, CD spectra were obtained on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) at 25 °C. The CD spectra were averages of four scans, collected at 0.1 nm intervals between 190 and 250 nm with a LeuA concentration of 0.1 mM. Spectra were recorded in: 90% trifluoroethanol (TFE) in 0.1% aqueous TFA; aqueous dodecylphosphocholine (DPC) with 0.1% TFA (1:40 ratio of LeuA:DPC); and 0.1% aqueous TFA. For L-LeuA, the CD spectra were collected under identical conditions as those of D-LeuA.

Tests for antimicrobial activity and the search for an agonistic/antagonistic effect between L-LeuA 37 and D-LeuA 64. The antimicrobial activity of the peptide or peptide mixtures was determined by spot-on-lawn test.[69] Thus, plates containing approximately 20 mL of APT agar were overlaid with 10 mL of soft APT agar (0.75% agar) inoculated with a 16 h culture of various sensitive indicator strains (1% inoculum). The following cultures were screened: *C. divergens* LV13, *C. piscicola* N5, *C. piscicola* LV17A, *C. piscicola* LV17B (Bac^+ and Bac^-), *Enterococcus faecium* BFE 900, *L. gelidum* UAL187, *L. gelidum* UAL187.13, *Lactococcus messenteroides* 23386, *Lactobacillus sake* 20017 and *Listeria monocytogenes* LI0502. The purified peptide was dissolved in water containing 0.1% TFA to give a concentration of 1.0 or 2.0 mg/mL. To test for an agonistic and/or antagonistic effect of L-LeuA and D-LeuA, solutions of the mixture of the two isomers were prepared with different molar ratios of D- to L-LeuA ranging from 0.1 to 100 (solution A, 0.004 : 0.04 μg/μL; solution B, 0.04 : 0.04 μg/μL; solution C, 0.4 : 0.04 μg/μL; solution D, 0.4 : 0.004 μg/μL). Pure peptides with the same concentration were used as controls and tested in the same way. Serial two-fold dilutions of these stock

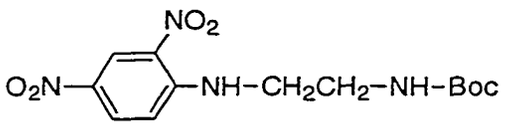
solutions were performed in water containing 0.1% TFA, and up to 10 μL of each concentrations were spotted onto the bacterial lawn. The plates were kept at 25 °C for approximately 16 h before the presence of inhibition zones was recorded. The results were expressed in arbitrary units (AU) of bacteriocin (one AU is the minimal amount of peptide required to produce a visible clearing on the lawn of the indicator strain). Inhibition was recorded as a positive if a distinct clearing was observed. No inhibition of the indicator strain was detected when 0.1% TFA alone was used in the assay.

Tests for bacteriocin induction towards *C. piscicola* LV17B (Bac⁻) with LeuA and D-LeuA. Bac⁻ cells of *C. piscicola* LV17B were prepared from Bac⁺ cells by serial one tenth dilutions.[41] The Bac⁻ cells were sub-cultured once before use and no bacteriocin production was found in the supernatant. Media were prepared containing differing amounts of L-LeuA or D-LeuA. The concentrations of the peptides in the culture media ranged from 10 μg/mL to 10 x 2^{-10} μg/mL obtained through series of dilutions. After the culture reached full growth, the inhibitory activity of the heat-treated supernatant (100 °C for 15 min) was assayed by the spot-on-lawn technique as mentioned above, on a bacteria lawn of *C. divergens* LV13. Background activity from the bacteriocins added to the culture medium was taken into account in determining induction at high concentration. The supernatant of Bac⁺ culture, which induces the production of bacteriocin(s), were included as a positive control. Negative control was the subculture of Bac⁻ cells.



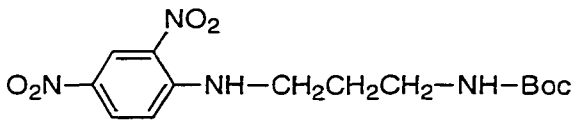
Ac-Gln-Val-Val-Gly-Gly-OH 65. Pentapeptide **65** was synthesized on a Rainin PS 3 peptide synthesizer using standard Fmoc solid phase peptide chemistry.[61] Specifically, Fmoc-glycine Wang resin (240 mg, 0.2 mmol; resin loading 0.83 mmol/g) was swollen in DMF, and thoroughly washed using DMF (5 x 10 mL). Each amino acid was assembled in turn by automated cycles of deprotection, activation and coupling. The amino acids were deprotected using 20% piperidine in DMF for 10 min followed by washing with DMF (5 x 10 mL). Each residue was activated by mixing a four molar excess of Fmoc protected amino acids (0.8 mmol in DMF) with HBTU (0.7 mmol in DMF) and a slight excess of neat *N*-methylmorpholine (NMM, 1.6 mmol) for 5 min. The activated amino acids were condensed to the peptide resin by agitation with N_2 bubbling for 60 min and then washed with DMF (5 x 10 mL). Double coupling was performed for the two Val residues. The *N*-terminal acetylation was accomplished by reacting with NMM (800 μL), acetic anhydride (500 μL) and DMF (15 μL) for 15 minutes. The peptide was cleaved with 10 mL of a cleavage cocktail (85% TFA : 7.5% H_2O : 7.5% anisole) for 1 h and precipitated with cold ether (30 mL). The crude peptides were purified by RP-HPLC with a μBondapak C_{18} column (25 x 100 mm, 10-μm particle size, 100-Å pore size; Waters; flow rate 15.0 mL/min; monitored at 220 nm) using a gradient from 20 to 60% MeCN in 0.1% aqueous TFA over a period of 24 min. The fractions were analyzed by mass spectrometry to confirm their identity. The fraction with the desired mass was concentrated and lyophilized to afford a fluffy solid **65** (66.7 mg, 67%); IR (microscope) 3280 (br s), 1733 (s), 1664 (s), 1226 cm^{-1}; ^1H NMR (600 MHz, DMSO-D_6) δ 8.25 (t, 1 H, J = 5.8 Hz, N<u>H</u>-Gly),

8.09 (t, 1 H, J = 5.8 Hz, NH-Gly), 8.03 (d, 1 H, J = 8.0 Hz, CH_3CONH), 7.80 (dd, 2 H, J = 8.5, 6.8 Hz, 2 NH-Val), 7.23 (s, 1 H, one of $CONH_2$), 6.72 (s, 1 H, one of $CONH_2$), 4.27-4.22 (m, 1 H, α-CH-Gln), 4.18 (dd, 1 H, J = 8.8, 6.6 Hz, α-CH-Val), 4.10 (dd, 1 H, J = 8.0, 7.1 Hz, α-CH-Val), 3.78-3.70 (m, 4 H, 2 CH_2-Gly), 2.02-2.12 (m, 2 H, γ-CH_2-Gln), 2.00-1.88 (m, 2 H, 2 β-CH-Val), 1.88-1.75 (m, 4 H, $COCH_3$ and one of β-CH_2-Gln), 1.72-1.60 (m, 1 H, one of β-CH_2-Gln), 0.87-0.77 (m, 12 H, 4 CH_3-Val) (600 MHz, ^{1}H-^{1}H COSY confirmed the assignment); ^{13}C NMR (100 MHz, APT, DMSO-D_6), δ 173.77, 171.50, 171.26, 171.10, 171.07, 169.30, 168.98, 58.11, 57.54, 52.26, 41.68, 40.57, 31.46, 30.41, 30.19, 27.75, 22.43, 19.15, 19.10, 18.27, 17.95; FAB MS *m/z* (Cleland) 501.0 (MH^+, 69), 522.8 (MNa^+, 15), 171.0 (100).

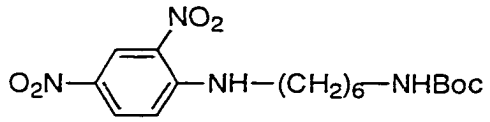


N^1-Boc-N^2-(2,4-Dinitrophenyl)-diaminoethane 66. A solution of *N*-Boc-ethylenediamine (0.46 g, 2.8 mmol), 2,4-dinitrofluorobenzene (0.53 g, 2.8 mmol) and triethylamine (0.3 g, 3 mmol) in MeCN (20 mL) was stirred at room temperature for 3 h. After the solvent was removed *in vacuo*, the residue was purified on silica gel (solvent: CH_2Cl_2/hexane 3 : 1 containing 1% Et_3N) to give a bright yellow solid **66** (0.94 g, quant.); mp 116-118 °C; IR ($CHCl_3$ cast) 3353 (s), 2976 (m), 1696 (s), 1619 (s), 1523 (s), 1336 (s), 744 (m) cm^{-1}; ^{1}H NMR (360 MHz, CD_2Cl_2) δ 9.08 (d, 1 H, J = 2.7 Hz, ArH), 8.73 (br s, 1 H, ArNH), 8.27 (dd, 1 H, J = 9.5, 2.7 Hz, ArH), 7.05 (d, 1 H, J = 9.5 Hz, ArH), 4.90 (br s, 1 H, NHBoc), 3.55 (dt, 2 H, J = 5.6, 5.4 Hz, $ArNHCH_2$), 3.47 (dt, 2 H, J = 5.9, 5.7 Hz, CH_2NHCO), 1.45 (s, 9 H, 3 CH_3); ^{13}C NMR (75 MHz, CD_2Cl_2) δ

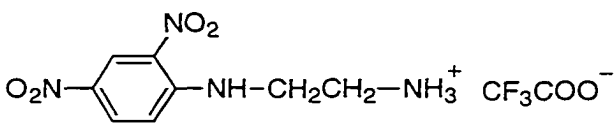
149.04 (CO), 130.53, 124.42, 114.53 (C_6H_3), 80.00 ($C(CH_3)_3$), 44.33 ($NHCH_2$), 39.67 ($NHCH_2$), 28.39 (3 CH_3); HRMS (EI) *m/z* calcd for $C_{13}H_{18}N_4O_6$ 326.1226, found 326.1221.



N^1-Boc-N^3-(2,4-Dinitrophenyl)-1,3-diaminopropane 67. Compound **67** was prepared in the same way as **66** using commercially available *N*-Boc-1,3-diaminopropane. Thus, a solution of *N*-Boc-1,3-diaminopropane (0.5 g, 2.8 mmol), 2,4-dinitrofluorobenzene (0.53 g, 2.8 mmol) and triethylamine (0.3 g, 3 mmol) in MeCN (20 mL) was stirred at room temperature for 3 h. After the solvent was removed *in vacuo*, the residue was purified on silica gel (solvent: CH_2Cl_2/hexane 3 : 1 containing 1% Et_3N) to give a yellow solid **67** (1.1 g, quant.); mp 96-98 °C; IR (microscope) 3326 (s), 2947 (m), 1671 (s), 1620 (s), 1586 (s), 1332 (s), 1316 (s), 819 (m), 744 (m) cm^{-1}; ^1H NMR (360 MHz, CD_2Cl_2) δ 9.09 (d, 1 H, J = 2.6 Hz, ArH), 8.71 (br s, 1 H, ArNH), 8.27 (dd, 1 H, J = 9.5, 2.6 Hz, ArH), 7.05 (d, 1 H, J = 9.5 Hz, ArH), 4.78 (br s, 1 H, NHCO), 3.49 (dt, 2 H, J = 6.8, 6.1 Hz, $ArNHCH_2$), 3.26 (dt, 2 H, J = 6.4, 6.4 Hz, CH_2NHCO), 1.91 (p, 2 H, J = 6.8 Hz, $CH_2CH_2CH_2$), 1.45 (s, 9 H, 3 CH_3); ^{13}C NMR (75 MHz, CD_2Cl_2) δ 148.79 (CO), 130.54, 124.53, 114.36 (C_6H_3), 80.00 ($C(CH_3)_3$), 41.28 ($NHCH_2$), 38.15 (CH_2NH), 29.86 ($CH_2CH_2CH_2$), 28.44 (3 CH_3); HRMS (EI) *m/z* calcd for $C_{14}H_{20}N_4O_6$ 340.1383, found 340.1376. Anal. calcd for $C_{14}H_{20}N_4O_6$: C, 49.41; H, 5.92; N, 16.46. Found: C, 49.59; H, 5.76; N, 16.17.

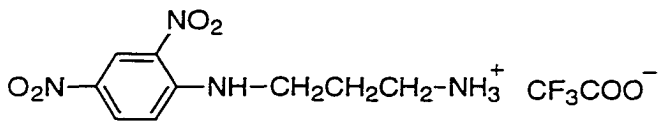


N^1-Boc-N^6-(2,4-Dinitrophenyl)-1,6-diaminohexane 68. Starting with commercially available *N*-Boc-1,6-diaminohexane, **68** was prepared in a similar way to that described for **66**. Thus, a solution of *N*-Boc-1,6-diaminohexane (0.5 g, 2.0 mmol), 2,4-dinitrofluorobenzene (0.36 g, 2.0 mmol) and triethylamine (3.0 mL) in MeCN (30 mL) was stirred at room temperature for 3 h. After the solvent was removed *in vacuo*, the residue was purified on silica gel (solvent: CH_2Cl_2/hexane 3 : 1 containing 1% Et_3N) to give a bright yellow solid **68** (0.76 g, quant.); mp 84-86 °C; IR (microscope) 3348 (s), 1712 (s), 1681 (s), 1620 (s), 1525 (s), 1331 (s), 1308 (s), 704 (m) cm^{-1}; ^1H NMR (360 MHz, CD_2Cl_2) δ 9.08 (d, 1 H, *J* = 2.6 Hz, ArH), 8.54 (br s, 1 H, ArNHCH$_2$), 8.24 (dd, 1 H, *J* = 9.5, 2.6 Hz, ArH), 6.95 (d, 1 H, *J* = 9.5 Hz, ArH), 4.62 (br s, 1 H, NHCO), 3.42 (dt, 2 H, *J* = 7.0, 5.6 Hz, ArNHCH$_2$CH$_2$), 3.08 (dt, 2 H, *J* = 6.5, 6.6 Hz, CH$_2$CH$_2$NHCO), 1.78 (p, 2 H, *J* = 7.0 Hz, NHCH$_2$CH$_2$CH$_2$), 1.52-1.46 (m, 4 H, 2 CH$_2$), 1.42-1.38 (m, 11 H, CH$_2$ and 3 CH$_3$); ^{13}C NMR (75 MHz, CD_2Cl_2) δ 148.86 (CO), 130.55, 124.48, 114.49 (C_6H_3), 79.47 (C(CH$_3$)$_3$), 43.86 (ArNHCH$_2$), 40.69 (CH$_2$NHCO), 30.34 (CH$_2$), 28.97 (CH$_2$), 28.47 (3 CH$_3$), 26.90 (CH$_2$), 26.66 (CH$_2$); FAB MS *m/z* (Cleland) 383.2 (MH^+) and 405.0 (MNa^+).

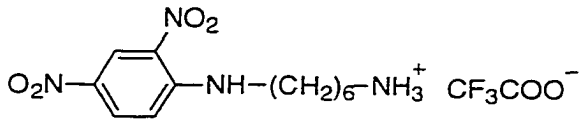


***N*-(2,4-Dinitrophenyl)-diaminoethane trifluoroacetic acid salt 69.** Compound **66** (0.4 g, 1.2 mmol) was stirred in 40% TFA/CH_2Cl_2 (30 mL) at room temperature for 3 h. Evaporation of the solvents gave a light yellow solid **69** (0.32 g, quant.); mp 196 °C

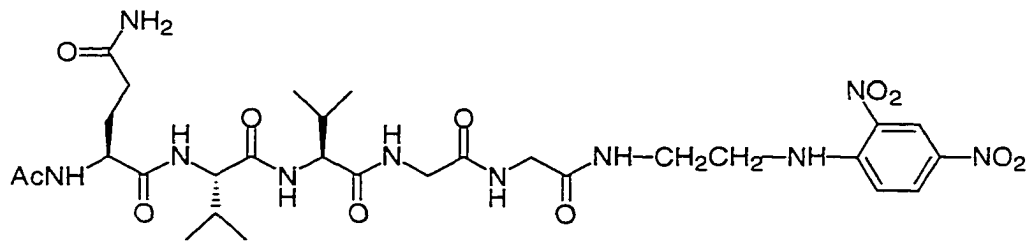
(dec.); IR (microscope) 3349 (s), 3106 (m), 2936 (m), 1671 (s), 1619 (s), 1526 (s), 1344 (s), 724 (m), 842 (m) cm^{-1}; ^1H NMR (360 MHz, D_2O) δ 8.92 (d, 1 H, J = 2.7 Hz, ArH), 8.20 (dd, 1 H, J = 9.5, 2.7 Hz, ArH), 7.05 (d, 1 H, J = 9.5 Hz, ArH), 3.60 (t, 2 H, J = 6.1 Hz, CH_2), 3.22 (t, 2 H, J = 6.1 Hz, CH_2); ^{13}C NMR (75 MHz, DMSO-D_6) δ 148.00, 135.18, 130.71, 129.85, 123.39, 115.04 (C_6H_3), 40.18 (CH_2), 37.25 (CH_2); HRMS (EI) *m/z* calcd for $C_8H_{11}N_4O_4$ (MH^+) 227.0781, found 227.0745. Anal. calcd for $C_{10}H_{11}N_4O_6F_3$: C, 35.30; H, 3.26; N, 16.47. Found: C, 35.43; H, 3.15; N, 16.04.



***N*-(2,4-Dinitrophenyl)-1,3-diaminopropane trifluoroacetic acid salt 70.** Compound **67** (0.4 g, 1.2 mmol) was stirred in 40% TFA/CH_2Cl_2 (30 mL) at room temperature for 3 h. Evaporation of the solvents gave a bright yellow solid **70** (0.5 g, quant.); mp 138-140 °C; IR ($CHCl_3$ cast) 3359 (m), 3113 (b, m), 1676 (s), 1621 (s), 1585 (s), 1339 (s), 834 (m), 722 (m) cm^{-1}; ^1H NMR (360 MHz, D_2O) δ 8.72 (d, 1 H, J = 2.6 Hz, ArH), 8.12 (dd, 1 H, J = 9.6, 2.6 Hz, ArH), 7.04 (d, 1 H, J = 9.6 Hz, ArH), 3.58 (t, 2 H, J = 7.0 Hz, ArNHCH_2), 3.18 (t, 2 H, J = 7.6 Hz, C$H_2NH_3^+$), 2.18-2.06 (m, 2 H, $CH_2CH_2CH_2$); ^{13}C NMR (75 MHz, DMSO-D_6) δ 148.05, 134.82, 130.02, 129.82, 123.58, 115.21 (C_6H_3), 39.65 (ArNHCH_2), 36.43 ($CH_2NH_3^+$), 25.99 ($CH_2CH_2CH_2$); HRMS (EI) *m/z* calcd for $C_9H_{13}N_4O_4$ (MH^+) 241.0937, found 241.0926. Anal. calcd for $C_{11}H_{13}N_4O_6F_3$: C, 37.30; H, 3.70; N, 15.81. Found: C, 37.25; H, 3.62; N, 15.68.

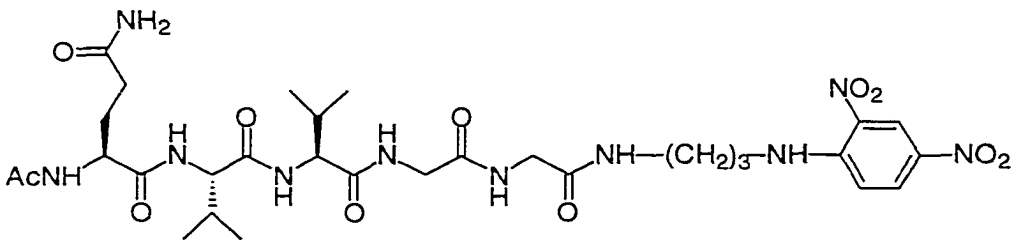


***N*-(2,4-Dinitrophenyl)-1,6-diaminohexane trifluoroacetic acid salt 71.** Compound **68** (0.4 g, 1 mmol) was stirred in 40% TFA/CH_2Cl_2 (30 mL) at room temperature for 3 h. Evaporation of the solvents gave a bright yellow solid **71** (0.4 g, quant.); mp 112-115 °C; IR ($CHCl_3$ cast) 3362 (s), 2931 (m), 1621 (s), 1588 (s), 1335 (s), 744 (m) cm^{-1}; ^1H NMR (360 MHz, CD_3OD) δ 8.90 (d, 1 H, J = 2.6 Hz, ArH), 8.18 (dd, 1 H, J = 9.5, 2.6 Hz, ArH), 7.05 (d, 1 H, J = 9.5 Hz, ArH), 3.49 (t, 2 H, J = 7.0 Hz, $ArNHCH_2$), 2.90 (t, 2 H, J = 7.00 Hz, $CH_2CH_2NH_3^+$), 1.76 (q, 2 H, J = 7.0 Hz, $ArNHCH_2CH_2CH_2$), 1.68-1.62 (m, 2 H, $CH_2CH_2NH_3^+$), 1.52-1.44 (m, 4 H, 2 CH_2); ^{13}C NMR (75 MHz, CD_3OD) δ 148.11, 134.62, 129.89, 129.55, 123.57, 115.19 (C_6H_3), 42.72 (CH_2NH), 39.51 ($NHCH_2$), 28.89 (CH_2), 27.94 (CH_2), 25.86 (CH_2), 25.64 (CH_2); HRMS (EI) *m/z* calcd for $C_{12}H_{18}N_4O_4$ 282.1296, found 282.1283.



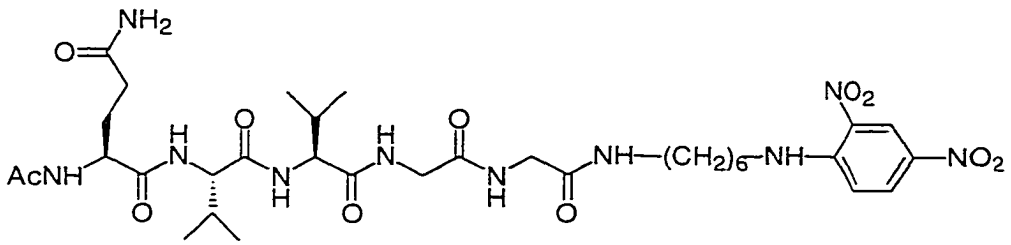
***N*^{1}-(Ac-Gln-Val-Val-Gly-Gly)-*N*^{2}-(2,4-dinitrophenyl)-ethylenediamino amide 72.** Compound **65** (20 mg, 0.04 mmol) was activated with HBTU (20 mg, 0.05 mmol) in dry DMF (5 mL) for 10 min at room temperature, and **69** (25 mg, 0.11 mmol) and Et_3N (37 μL) were then added. The above mixture was stirred at room temperature for 3 h. The solvent was evaporated under reduced pressure and the residue was purified on RP-HPLC (same chromatographic conditions as used for purification of **65**) to give **72**

(18 mg, 64%): IR (microscope) 3284 (s), 3084 (m), 1666 (s), 1631 (s), 1341 (s), 801 (m), 744 (m) cm^{-1}; ^1H NMR (600 MHz, DMSO-D_6) δ 8.91 (t, 1 H, J = 6.0 Hz, $CH_2CH_2N\underline{H}Ar$), 8.84 (d, 1 H, J = 2.8 Hz, Ar$\underline{H}$), 8.26 (dd, 1 H, J = 10.2, 2.8 Hz, Ar$\underline{H}$), 8.22 (t, 1 H, J = 5.5 Hz, N$\underline{H}$-Gly), 8.11-8.03 (m, 3 H, N$\underline{H}$-Gly, CON$\underline{H}CH_2CH_2$, $CH_3CON\underline{H}$), 7.81 (d, 1 H, J = 8.8 Hz, N$\underline{H}$-Val), 7.78 (d, 1 H, J = 8.0 Hz, N$\underline{H}$-Val), 7.28 (d, 1 H, J = 10.2 Hz, one of Ar$\underline{H}$), 7.24 (br s, 1 H, one of CON$\underline{H}_2$), 6.72 (br s, 1 H, one of CON$\underline{H}_2$), 4.26-4.22 (m, 1 H, α-C$\underline{H}$-Gln), 4.18 (dd, 1 H, J = 8.8, 6.8 Hz, α-C$\underline{H}$-Val), 4.12 (dd, 1 H, J = 8.0, 7.0 Hz, α-C$\underline{H}$-Val), 3.78-3.70 (m, 2 H, C$\underline{H}_2$-Gly), 3.67-3.63 (m, 2 H, C$\underline{H}_2$-Gly), 3.54 (dd, 2H, J = 12.3, 6.4 Hz, CONHC$\underline{H}_2CH_2$), 3.37-3.31 (m, 2H, C$\underline{H}_2$NHAr), 2.11-2.04 (m, 2 H, γ-C$\underline{H}_2$-Gln), 1.99-1.93 (m, 2 H, 2 β-C$\underline{H}$-Val), 1.86-1.80 (m, 1 H, one of β-C$\underline{H}_2$-Gln), 1.83 (s, 3H, COC$\underline{H}_3$), 1.70-1.62 (m, 1 H, one of β-C$\underline{H}_2$-Gln), 0.85 (d, 6 H, J = 6.8 Hz, 2 C$\underline{H}_3$-Val), 0.82 (d, 3 H, J = 6.8 Hz, C$\underline{H}_3$-Val), 0.78 (d, 3 H, J = 6.7 Hz, C$\underline{H}_3$-Val); ^{13}C NMR (75 MHz, DMSO-D_6) δ 173.82, 171.57, 171.53, 171.11, 169.36, 169.25, 169.05, 148.42, 134.91, 130.03, 129.93, 123.61, 115.23, 58.10, 57.73, 52.37, 42.57, 42.06, 37.57, 31.50, 30.40, 30.32, 27.78, 22.48, 22.24, 19.21, 19.10, 18.19, 18.05; FAB MS *m/z* (relative intensity) (Cleland) 731.3 (MNa^+, 16), 709.2 (MH^+, 10), 154.8 (100).



***N*^{1}-(Ac-Gln-Val-Val-Gly-Gly)-*N*^{3}-(2,4-Dinitrophenyl) diamino propane amide 73.** Method for the preparation of **72** was used to make **73** from **70** and **65**.

Thus, compound **65** (35 mg, 0.07 mmol) was activated with HBTU (34 mg, 0.09 mmol) in dry DMF (10 mL) for 10 min at room temperature, and **70** (37 mg, 0.11 mmol) and Et_3N (37 μL, 0.27 mmol) were then added. The above mixture was stirred at room temperature for 3 h. The solvent was evaporated under reduced pressure and the residue was purified on RP-HPLC (same chromatographic conditions as used for purification of **65**) to give a bright yellow solid **73** (21 mg, 42%); IR (microscope) 3281 (s), 3085 (m), 1663 (s), 1624 (s), 1338 (s), 708 (m) cm^{-1}; ^1H NMR (360 MHz, DMSO-D_6) δ 8.94 (t, 1 H, J = 5.9 Hz, ArN<u>H</u>CH_2), 8.86 (d, 1 H, J = 2.6 Hz, Ar<u>H</u>), 8.30-8.24 (m, 2 H, N<u>H</u>-Gly, Ar<u>H</u>), 8.15 (t, 1 H, J = 5.9 Hz, N<u>H</u>-Gly), 8.10-8.03 (m, 1 H, CH_3CON<u>H</u>), 7.88 (t, 1 H, J = 5.9 Hz, CON<u>H</u>CH_2CH_2), 7.86-7.78 (m, 2 H, 2 N<u>H</u>-Val), 7.25 (br s, 1 H, one of CON<u>H_2</u>), 7.22 (d, 1 H, J = 9.7 Hz, Ar<u>H</u>), 6.74 (br s, 1 H, one of CON<u>H_2</u>), 4.26-4.22 (m, 1 H, α-C<u>H</u>-Gln), 4.18 (dd, 1 H, J = 8.8, 6.8 Hz, α-C<u>H</u>-Val), 4.12 (dd, 1 H, J = 8.0, 7.0 Hz, α-C<u>H</u>-Val), 3.75-3.70 (m, 2 H, C<u>H_2</u>-Gly), 3.68-3.62 (m, 2 H, C<u>H_2</u>-Gly), 3.57-3.52 (m, 2H, C<u>H_2</u>NHAr), 3.18-3.12 (m, 2H, CONHC<u>H_2</u>), 2.12-2.05 (m, 2 H, γ-C<u>H_2</u>-Gln), 1.98-1.92 (m, 2 H, 2 β-C<u>H</u>-Val), 1.85-1.80 (m, 1 H, one of β-C<u>H_2</u>-Gln), 1.83 (s, 3 H, COC<u>H_3</u>), 1.74-1.66 (m, 3 H, $CH_2CH_2CH_2$, one of β-C<u>H_2</u>-Gln), 0.86-0.78 (m, 12 H, 4 C<u>H_3</u>-Val) (600 MHz, ^1H-^1H COSY confirmed the assignment); ^{13}C NMR (125 MHz, DMSO-D_6), δ 171.43, 169.27, 169.08, 168.97, 148.08, 134.63, 129.93, 129.74, 123.65, 115.17, 58.07, 57.59, 52.24, 45.48, 42.19, 40.24, 35.78, 30.17, 28.19, 22.41, 19.15, 18.18, 17.96; FAB MS *m/z* (relative intensity) (Cleland) 745.2 (MNa^+, 7) and 723.2 (MH^+, 13).



N^1-(Ac-Gln-Val-Val-Gly-Gly)-N^6-(2,4-Dinitrophenyl) diamino hexyl amide 74. Compound **74** was prepared in the same way as that decribed for **72.** Thus, compound **65** (20 mg, 0.04 mmol) was activated with HBTU (20 mg, 0.05 mmol) in dry DMF (5 mL) for 10 min at room temperature, and **69** (13 mg, 0.05 mmol) and Et_3N (37 μL) were then added. The above mixture was stirred at room temperature for 3 h. The solvent was evaporated under reduced pressure and the residue was purified on RP-HPLC (same chromatographic conditions as used for purification of **65**) to give a bright yellow solid **74** (18 mg, 60%); IR (microscope) 3272 (s), 3084 (m), 1625 (s), 1337 (s) cm^{-1}; ^1H NMR (600 MHz, DMSO-D_6) δ 8.85 (d, 1 H, J = 2.7 Hz, ArH), 8.83 (t, 1 H, J = 5.8 Hz, ArNHCH$_2$), 8.26-8.21 (m, 2 H, NH-Gly and one of ArH), 8.06-8.02 (m, 2 H, NH-Gly, CH$_3$CONH), 7.80 (t, 2 H, J = 9.3 Hz, 2 NH-Val), 7.70 (t, 1 H, J = 5.7 Hz, CONHCH$_2$CH$_2$), 7.24 (br s, 1 H, one of CONH$_2$), 7.22 (d, 1 H, J = 9.7 Hz, one of ArH), 6.72 (br s, 1 H, one of CONH$_2$), 4.27-4.22 (m, 1 H, α-CH-Gln), 4.18 (dd, 1 H, J = 8.8, 6.8 Hz, α-CH-Val), 4.10 (dd, 1 H, J = 7.7, 7.0 Hz, α-CH-Val), 3.70 (dd, 2 H, J = 6.0, 2.4 Hz, CH$_2$-Gly), 3.63 (dd, 2 H, J = 6.0, 2.4 Hz, CH$_2$-Gly), 3.48-3.43 (m, 2H, CH$_2$NHAr), 3.06-3.02 (m, 2H, NHCH$_2$), 2.11-2.04 (m, 2 H, γ-CH$_2$-Gln), 1.98-1.93 (m, 2 H, 2 β-CH-Val), 1.86-1.80 (m, 1 H, one of β-CH$_2$-Gln), 1.83 (s, 3 H, COCH$_3$), 1.71-1.64 (m, 1 H, one of β-CH$_2$-Gln), 1.61 (p, 2 H, J = 7.5 Hz, CH$_2$CH$_2$CH$_2$NHAr), 1.42-1.36 (m, 2 H, CONHCH$_2$CH$_2$), 1.36-1.32 (m, 2 H, CH$_2$CH$_2$CH$_2$NHAr), 1.32-1.27 (m, 2 H, NHCH$_2$CH$_2$CH$_2$), 0.84 (d, 6 H, J = 6.8 Hz, 2 CH$_3$-Val), 0.82 (d, 3 H, J = 6.8 Hz,

$C\underline{H}_3$-Val), 0.80 (d, 3 H, J = 6.8 Hz, $C\underline{H}_3$-Val); ^{13}C NMR (100 MHz, APT, DMSO-D_6) δ 173.72, 171.49, 171.44, 171.04, 169.26, 168.89, 168.35, 148.17, 134.61, 129.95, 129.60, 123.69, 115.22, 58.01, 57.58, 52.26, 42.70, 42.05, 38.41, 31.45, 30.32, 30.18, 28.91, 27.98, 27.71, 25.90, 22.40, 21.01, 19.16, 19.10, 18.18, 17.94; FAB MS *m/z* (Cleland) 787.1 (MNa^+) and 765.3 (MH^+).

Preliminary characterization of subtilosin A

1. Mass spectrometry, amino acid analysis and sequencing. A sample of subtilosin A **75** (800 μg total) was provided by our collaborator Professor Peter Zuber at Oregon Graduate Institute of Science and Technology, Portland, OR. The sample was further purified by RP-HPLC with a VYDAC C_8 column (4.6 x 250 mm, 10-μm particle size, 300-Å pore size; flow rate 2.5 mL/min) using a gradient from 40 to 72% MeCN in 0.1% aqueous TFA over a period of 40 min. Major fractions were analyzed by electrospray mass spectrometry and amino acid analysis. Subtilosin A had a retention time of 16.21 min, and accounted for 90% of the detected components based on the peak integration.

The molecular weight was determined to be 3401.40 +/- 0.30. Amino acid analysis of the purified subtilosin A indicated a composition of Asx (3), Thr (1), Ser (1), Glx (1), Pro (2), Gly (7), Ala (5), Cys (3), Val (1), Ile (3), Leu (3), Lys (1), Trp (3-5). The analysis was done at the Alberta Peptide Institute (University of Alberta). Normally the peptide sample was fully hydrolysized in 6 M HCl with 0.1% phenol at 160 °C for 1 h. In this case, cysteine and tryptophan residues are destroyed. Cysteine residues were measured as cysteic acid. Cysteine was oxidized to cysteic acid by addition of 5% (v/v) DMSO in the hydrolysis solution (6 M HCl, 160 °C, 1 h). Tryptophan residues were measured using 4 M methanesulfonic acid in the presence of 0.2% tryptamine (110 °C, 22 h), using

norleucine as an internal standard. Hydrolized amino acids were derivatized with phenylisothiocyanate on an Applied Biosystems (model 420A) derivatizer and separated by HPLC (Applied Biosystems model 130A; monitored at 570 nm and 440 nm). *N*-Terminal amino acid sequencing was also performed for the same sample.

2. Trypsin digestion. To 20 μg of subtilosin A, was added 30 μL of aqueous digestion buffer (8 M urea, 0.4 M ammonium bicarbonate, pH 8.0). After brief vortexing, 5 μL of 45 mM aqueous DTT was added and the solution was incubated at 50 °C for 15 min. The sample was left to cool to room temperature, which was followed by addition of 5 μL of 100 mM iodoacetic acid aqueous solution and incubation at at room temperature for another 15 min. The sample was then diluted with 80 μL of Milli-Q water (final concentrations: urea 2 M; NH_4HCO_3 0.1 M) and 8 μL of trypsin stock solution (0.1 μg/μL in 1 mM HCl) was added. The mixture was incubated at 37 °C for 24 h and the digested products were isolated by RP-HPLC with a VYDAC C_8 column (4.6 x 250 mm, 5-μm particle size, 300-Å pore size; flow rate 1.0 mL/min) using a gradient from 24 to 64% MeCN in 0.1% aqueous TFA over a period of 40 min. Two controls were also performed, one of which the trypsin enzyme was absent and the other subtilosin A was absent. The molecular weights of the resulting products are listed in Table 29.

Table 29. Molecular weights of trypsin digestion products of subtilosin A

RT (min)	25.01	25.93	26.95	27.87	29.08	33.25	34.12
MW	3357.70	3357.00	3357.80	3357.50	3357.80	3400.80	3401.40
(Da)	+/- 0.10	+/- 0.30	+/- 0.40	+/- 0.10	+/- 0.10	+/- 0.60	+/- 0.30

N-Terminal amino acid sequencing on the major components failed. Amino acid analysis of the fraction eluted at 29 min revealed an amino acid composition of Asx (3), Thr (1), Ser (1), Glx (1), Pro (2), Gly (7), Ala (5), Cys (3), Val (1), Ile (3), Leu (3), Lys (1).

3. Partial hydrolysis with hydrochloric acid. To 50 μg subtilosin A was added 100 μL of concentrated hydrochloric acid. The sample was incubated at 37 °C for 17 h. The hydrolyzed mixture was separated by RP-HPLC with a VYDAC C_8 column (4.6 x 250 mm, 5-μm particle size, 300-Å pore size; flow rate 1.0 mL/min) using a gradient from 0 to 48% MeCN in 0.1% aqueous TFA over a period of 40 min. Molecular weights of all detected fractions were determined by electrospray mass spectrometry and sequencing of some fractions were undertaken.

The molecular weights of the hydrolyzed fragments are listed in Table 30. Partial amino acid sequence of the fraction at 31.88 min was: AAXLVDGP and at 32.54 min was: AXLVDGPI. There was no obvious signal in the third sequencing cycle of the first fragment, which implies X is probably cysteine or a modified cysteine residue.

Table 30. Molecular weights of HCl hydrolyzed fragments of subtilosin A

RT (min)	4.55	14.09	29.08	31.88	32.54	33.35	34.43
MW (Da)	1020.40	1317.70	637.30	820.00	1510.70	720.30	1369.40
			770.30			948.70	

4. Partial hydrolysis with thermolysin. A sample of unmodified subtilosin A (100 μg) was dissolved in 500 μL of 0.1 M of ammonium bicarbonate buffer, pH 6.3 containing 10 mM $CaCl_2$. A solution of thermolysin (10 μL, 1 μg/μL) was added and the

sample was incubated at 60 °C for 24 h. The digested products were purified by RP-HPLC under the same conditions as isolation of trypsin digestion products. Molecular weights of the purified peptide fragments were determined by electrospray mass spectrometry and automatic amino acid sequencing was done for the major fragments.

One major fraction was obtained after 24 h thermolysin digestion. Its molecular weight was determined to be 3419.27 Da, 18 units more than the original peptide (MW 3401.40). *N*-Terminal amino acid sequencing of this fraction revealed the first six residues of GPIPDD. The signal intensity dropped significantly after the fifth cycle.

5. Attempts at reduction and modification of the cysteine residues. Subtilosin A (20 μg) was dissolved in 200 μL of buffer containing 6 M guanidinium HCl, 0.6 M Tris-HCl, pH 8.6. The solution was split into two equal portions. One sample was treated with 80 μL of 580 mM aqueous iodoacetamide solution and incubated at 37 °C for 1 h. The other sample was first incubated with 10 μL of 280 mM aqueous DTT at 37 °C for 15 min followed by incubation with iodoacetamide solution as before. The reaction was quenched by direct injection onto RP-HPLC with a VYDAC C_8 column (4.6 x 250 mm, 5-μm particle size, 300-Å pore size; flow rate 1.0 mL/min) using a gradient from 24 to 48% MeCN in 0.1% aqueous TFA over a period of 40 min. Mass spectrometry indicated that modification of the cysteine residues was unsuccessful, regardless of whether the sample was teated with DTT or not. Starting material (MW 3401.6 with DTT reduction; MW 3400.4 without DTT reduction) was recovered in both cases.

6. Production and purification of subtilosin A. Subtilosin A was purified to homogeneity from the fermentation supernatant of *Bacillus subtilis* JH642 (provided by Professor Peter Zuber) through a three-step purification procedure.[185, 187] Thus, 500 mL of

glucose-yeast extract broth (5.0 g yeast extract, 20.0 g glucose and 1.0 mL of trace elements solution per liter) in a 2 liter flask were autoclaved for 15 min. The trace elements solution was made of 2.2 g $ZnSO_4 \cdot 7H_2O$, 1.1 g H_3BO_3, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.16 g $CoCl_2 \cdot 5H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, 0.11 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 5.0 g Na_2EDTA per 100 mL of aqueous solution. The medium was allowed to cool to room temperature, and each flask was inoculated with a single colony of *B. Subtilis* JH642, which had been growing overnight on an agar plate. The medium of the agar plate contains 8.0 g nutrient broth, 10.0 mL of 1.2% Mg SO_4, 10.0 mL of 10% KCl, 0.6 mL of 1.0 N NaOH and 12.0 g agar per liter. After the medium was autoclaved and left to cool briefly, sterilized 1.0 mL of 1.0 M $Ca(NO_3)_2$, 1.0 mL of 10 mM $MnCl_2$, 1.0 mL of 1 mM $FeSO_4$ were added. The inoculated culture was incubated at 37 °C with shaking (200 rpm) for 22 h. At this stage, the culture reached full growth with $OD_{600\ nm}$ *ca.* 0.9.

The culture supernatant was collected by centrifugation at 10,000 rpm for 10 min and ammonium sulfate was added to 80% of saturation (*ca.* 613 g per liter medium). The mixture was kept at 4 °C overnight with stirring. The protein precipitate was pelleted with centrifugation at 10,000 rpm for 30 min and resuspended in 150 mL of methanol. This methanol solution was shaken vigorously and allowed to stand overnight at room temperature with magnetic stirring. The methanol supernatant was collected and concentrated to *ca.* 8 mL by evaporation *in vacuo*. The concentrated methanol solution was loaded onto a Sephadex LH-20 colum (25 x 400 mm). Elution was performed with methanol at a rate of 2 mL/min and fractions were collected at 2 min interval. The protein elution was monitored by UV at 280 nm. Fractions showing inhibitory activity were pooled and dried by evaporation *in vacuo*. The residue was resuspended in 20% aqueous MeCN (0.1% TFA) and subjected to further purification by RP-HPLC with a VYDAC C_8 column (10 x 250 mm, 10-μm particle size, 300 Å pore size; flow rate 2.5 mL/min) using a

gradient from 24 to 64% MeCN in 0.1% aqueous TFA over a period of 40 min. Subtilosin A was eluted at 56% MeCN with a retention time of 38 min. On an analytical column (4.6 x 250 nm, 5-μm particle size, 300 Å pore size; flow rate 1.0 mL/min) subtilosin A was eluted at 33 min with the same mobile phases and gradient.

The purified peptide was lyophilized and stored at –20 °C. The molecular weight of the purified peptide was 3401.44 +/- 0.49 and 2.3 mg of subtilosin A was isolated from one liter of culture. Spot-on-lawn technique was used to detect the activity of subtilosin A using *Listeria monocytogenes* LI0504 as an indicator.

The yield of subtilosin A could be significantly improved by a slight modification of the production and purification method reported by Babasaki *et al.*[187] Thus, one and half milliliters of logarithmically growing pre-culture cells of *B. subtilis* JH642 were transferred into a 2 liter Erlenmeyer flask containing 500 mL of double strength pre-warmed NSM (nutrient sporulation medium, per liter contains 8.0 g nutrient broth (Difco), 0.25 g $MgSO_4 \cdot 7H_2O$ and 1.0 g KCl; in addition, the medium contained 1.0 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2$, and 1.0 μM $FeSO_4$.[190] It should be noted that no glucose was used. The culture was incubated at 37 °C on a New Brunswick rotatory shaker with vigorous shaking (400 rpm) for 7 h, after which time one-quarter volume of *n*-butanol was added to the culture (no separation of supernatant and cells). The mixture was emulsified vigorously for 1 h on the same shaker at the same speed. The mixture was then allowed to stand overnight at room temperature. The butanol layer was isolated by two-phase separation and concentrated *in vacuo*. The residue was extracted twice with 4 mL of methanol and mixed with an equal amount of water containing 0.1% TFA before being further purified by RP-HPLC as described in previous method.

The active fractions from HPLC were concentrated and lyophilized. Ten milligrams of pure subtilosin A was produced from one liter culture with this method. The purity of

the peptide was confirmed by electrospray mass spectrometry, activity test and RP-HPLC. The molecular weight was 3401.39 +/- 0.12 and it showed activity towards *L. monocytogenes* LI0504.

Bacteriocin UAL26 purification. An overnight culture of *Carnobacterium piscicola* UAL26 (plasmidless) was inoculated (2%) into 3 liters of CAA medium[49] including 0.2% Tween 80. The pH of the culture medium was maintained at 6.4 by addition of 1.0 N sodium hydroxide (controlled by Chem-Cadet®, Cole-Parmer, Chicago, IL). The culture was incubated with gentle stirring at 25 °C for 16 to 20 h. The semidefined CAA medium used for the production contained: 15.0 g casamino acids, 5.0 g yeast extract, 20.0 g D-glucose, 2.0 g dipotassium phosphate, 2.0 g diammonium citrate, 0.1 g magnesium sulfate and 0.05 g manganous sulfate per liter.[49]

The supernatant was collected by centrifugation at 10,000 rpm for 20 min and heated at 65 to 70 °C for 30 min. Then the supernatant was loaded onto an Amberlite XAD-8 column (4.5 x 50 cm) pre-equilibrated with 0.1% aqueous TFA, and sequentially eluted with increasing concentrations of ethanol (2 liters each of 0, 30, 60 and 80% of ethanol). The most active fraction (60% ethanol) was concentrated *in vacuo* at 30 °C to *ca.* 10 mL. The concentrated sample was then applied onto a Sephadex G-50 (Sigma) column (2.5 x 80 cm) pre-equilibrated with 0.1% TFA in water. The active fractions from the size exclusion column were concentrated and further purified on a Sepharose SP fastflow cation exchange column at pH 5.0. The column was washed sequentially with increased concentrations of sodium chloride in 20 mM sodium acetate buffer, pH 5.0. The active fractions were concentrated and desalted on a C_{18} Sep-PAK cartridge. The sample was eluted from the Sep-PAK with 60% MeCN and was purified to homogeneity by RP-HPLC with a C_8 VYDAC column (4.6 x 250 mm, 5-μm particle size, 300 Å pore size; Scientific Products and Equipment, Concord, Ontario, Canada; flow rate 1 mL/min, monitored at 218 nm.). The gradient used was 20% to 60% of MeCN in 40 min. Two main fractions were

found with activity, with retention times of 35.46 min and 38.51 min. The molecular weights were determined to be 1844.66 +/- 0.31 and 1828.92 +/- 0.34, respectively. *N*-Terminal amino acid sequencing was performed on the two fractions. *C. divergens* LV13 (UAL9) was used as an indicator to trace the activity of UAL26 bacteriocin.

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Appendix

^{1}H NMR chemical shift assignments,[a] $^3J_{HNH\alpha}$ coupling constants and HN temperature coefficients for CbnB2 in 90% (v/v) aqueous TFE

Amino Acid	HN (ppm)	Hα (ppm)	Hββ (ppm)	Other H (ppm)	$^3J_{HNH\alpha}$ (Hz)	HN temp. Coeff. (ppb/°C)
1 Val	–	3.68	2.07	γCH$_2$ 0.89, 0.85		
2 Asn	8.14	5.03	2.89, 2.73	γNH$_2$ 7.17, 6.47	6.7	5.0
3 Tyr	8.25	4.60	3.15, 3.05	2, 6 H 7.09, 3, 5 H 6.8	7.0	10.9
4 Gly	8.21	3.95, 3.86				
5 Asn	7.92	4.71	3.03, 2.82	γNH$_2$ 6.98, 6.14	6.5	2.6
6 Gly	8.16	4.10, 3.79				
7 Val	7.76	4.16	2.21	γCH$_2$ 1.00, 0.98	8.0	1.4
8 Ser	8.44	4.20	4.04, 3.82			
9 Cys	8.18	4.32	2.90, 2.75			2.8
10 Ser	8.19	4.56	4.22	3.98		
11 Lys	8.05	4.23	1.99	γ, δ CH 1.52, 1.74, 1.62, εCH 3.00, εNH 7.43	6.5	7.3
12 Thr	7.84	4.49	4.34	γCH$_3$ 1.23	8.0	1.8
13 Lys	7.91	4.42	1.94	γ, δ CH 1.45, 1.71 εCH 3.00, εNH 7.43	7.0	
14 Cys	7.94	4.80	3.68; 3.00			
15 Ser	7.84	4.48	3.92, 3.83			
16 Val	7.49	4.18	1.70	γCH$_2$ 0.84, 0.53	6.3	1.7
17 Asn	8.16	4.75	2.99, 2.74	γNH$_2$ 7.29, 6.37		
18 Trp	7.68	4.44	3.38, 3.25	NH 9.34, 4H 7.52, 7H 7.41, 6H 7.25, 5H 7.16, 2H 7.06	6.5	0.1
19 Gly	8.11	3.95				4.4
20 Gln	7.77	4.16	2.14	γCH$_2$ 2.45, 2.37 δNH$_2$ 6.89, 6.31	7.0	1.0
21 Ala	7.95	4.30	1.51		5.5	4.4
22 Phe	8.39	4.26	3.21	2,6 H 7.15, 3,5 H 7.24	6.3	8.2
23 Gln	8.30	4.03	2.35, 2.28	γCH$_2$ 2.45, 2.37 δNH$_2$ 6.89, 6.31	5.5	1.5

24 Glu	8.53	4.03	2.35, 2.28	γCH_2 2.75, 2.52	5.8	5.0
25 Arg	8.02	4.04	1.95	γCH_2 1.80, 1.69 δCH_2 3.21, 3.13, εNH 6.90	5.5	3.4
26 Tyr	8.77	4.21	2.97, 2.83	2,6 H 7.03, 3,5 H 6.97	4.9	10.2
27 Thr	8.17	4.21	3.91	1.37		3.7
28 Ala	8.27	4.18	1.56		5.2	4.2
29 Gly	8.19	3.98, 3.87				2.4
30 Ile	8.12	3.95	1.94	γCH_2 1.48, 1.45 δCH_2 0.99, 0.75	5.3	1.1
31 Asn	8.45	4.50	2.91, 2.76	γNH_2 7.21, 6.28	5.3	4.4
32 Ser	8.44	4.19	4.04, 3.82		5.3	3.4
33 Phe	8.11	4.38	3.35, 3.30	2,6 H 7.21, 3,5 H 7.27	5.3	2.9
34 Val	8.84	3.54	2.19	γCH_2 1.25, 1.03	5,5	7.4
35 Ser	8.19	4.19	4.05, 3.97			2.9
36 Gly	7.91	3.94, 3.81				1.0
37 Val	7.98	3.66	2.05	γCH_2 0.89, 0.73	5.6	0.4
38 Ala	8.67	4.08	1.48		4.8	6.8
39 Ser	7.95	4.33	4.10, 4.03		5.2	3.6
40 Gly	7.82	4.01				0.1
41 Ala	8.38	4.12	1.46		4.4	5.3
42 Gly	8.20	3.95, 3.85				4.6
43 Ser	7.91	4.41	4.04, 3.92		5.2	2.6
44 Ile	7.63	4.01	1.96	γCH_2 1.64, 1.26 δCH_3 0.98		1.6
45 Gly	8.08	4.00, 3.92				6.3
46 Arg	7.67	4.43	1.94, 1.81	γCH_2 1.67, 1.63 δCH_2 3.20, εNH 6.90	5.7	3.5
47 Arg	7.68	4.47	1.93	γCH_2 1.79, 1.73 δCH_2 3.23, εNH 6.91	5.4	8.5
48 Pro		4.47	2,31, 2.05	γCH_2 2.09 δCH_2 3.78, 3.64		

a. Chemical shifts are reported in ppm relative to protonated TFE at 3.88 ppm downfield from DSS.