Antimicrobial peptides

The unique characteristics of antimicrobial peptides (AMPs) – together with an improved understanding of their universal nature – has prompted renewed interest in the development of this group of antimicrobial agents.

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Antimicrobial peptides (AMPs) are a recently discovered group of antimicrobial agents. They are simple peptides, but are widely distributed in animals and plants, and show activity against a broad range of pathogens. They have a number of characteristics that make them interesting candidates for pharmaceutical development; notably, they are fast-acting, microbicidal (rather than microbiostatic) and are associated with little observed resistance development – a key property in an age of multi-resistant bacteria, as represented by MRSA.

Most AMPs are cationic and amphipathic – features that promote interaction with the negatively charged bacterial and fungal membranes. They work primarily by compromising the membrane of the target organism. When analysed at the molecular level, several different mechanisms of membrane disruption have been shown to exist. However, for most AMPs, the overall outcome is membrane disruption and/or cell lysis.

Selectivity for microbial membranes is mediated by membrane composition, membrane charge, trans-membrane potential and lipid polarity. The outer leaflet of microbial membranes is populated with negatively charged phospholipids, whereas the outer leaflet of plant and animal membranes is composed primarily of neutral lipids. In addition to the differences in polarity, the specific types and ratios of phospholipids in microbes differ from those of higher organisms, allowing for discrimination between cell types. A key feature of AMPs is the inability of bacteria to become resistant. It has proven extremely difficult to induce resistance to AMPs in sensitive target organisms. This is a reflection of the unique nature of the molecular ‘target’ – the antimicrobial effect is essentially receptor-independent.

Novozymes A/S has a strong heritage in the discovery and development of peptidic compounds, using technologies that share common ground with companies in the biotechnology and pharmaceutical fields – such as genetic manipulation, recombinant expression, high throughput screening and protein design. Although Novozymes traditionally has used these technologies in its core businesses – industrial enzyme production – the company is now looking to apply these competencies to biopharmaceutical discovery and development. One lead area is anti-infectives, with particular progress being made in the development of AMPs.

At Novozymes, our focus has been on both the development of a solid technology platform around AMPs and the utilisation of this platform for identifying and developing lead molecules. By applying our unique and proven ability to modify peptides, we believe that our AMP discovery platform holds great potential.

AMP Technology Platform

AMPs are gene-encoded; this is an essential feature that has allowed the many biotechnology tools originally developed for enzymes to be applied to AMPs. At Novozymes, focus has been mainly on three separate areas:

- A Discovery Platform for identifying new, potent and structurally diverse AMPs,
- A Directed Evolution Platform for tailoring specific AMPs towards specific clinical indications, and

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**Discovery Platform**  Novozymes has a large microbial strain collection with more than 25,000 catalogued and well-characterised fungi and bacteria. Both traditional and genomic approaches are used to identify new AMPs from the strain collection. In the traditional approach, we screen bacterial and fungal culture supernatants for peptide-based antimicrobials, and then isolate and characterise the peptides. After characterisation, reverse genetics is used to clone and express the corresponding gene. In one of our powerful genomic approaches, Transposon-Assisted-Signal-Trapping (TAST WO 0177315) has been used with great success (Figure 1). This technology allows us to selectively identify and sequence genes that, in the natural host, are secreted. As most or all AMPs would be expected to be secreted from the host to limit niche competition and aid in host defences, this approach focuses and reduces the effort from sequencing whole genomes or large cDNA libraries down to only the much smaller subset of secreted cDNAs.

A number of structurally different AMPs – including one of our lead AMP classes, Plectasin (WO 03044049) – have been identified using TAST. The design principles of many AMP classes are relatively simple when compared with larger and more structurally complex enzymes. Correspondingly, rational approaches have been used to design novel AMPs. Half of our lead series are novel biosynthetic molecules, which are not naturally-occurring. While it has proven fairly easy to design AMPs with a basal level of antimicrobial activity, it has proven more difficult to optimise other features such as potency, antimicrobial spectrum, haemolytic activity, toxicity and systemic stability. Structural modelling and various other computational analyses have – when combined with directed evolution and high throughput screening (HTS) – proven valuable tools to overcome these limitations.

**Directed Evolution Platform and High Throughput Screening**  The AMPs found in nature have been evolutionarily optimised to function in specific physiological or ecological niches – such as tears, saliva, the mucosal barrier or the phagosomes of macrophages – in response to specific microbial challenges. However, AMPs have not been optimised by evolution to function optimally in complex therapeutic conditions such as burn wounds, chronic ulcers or cystic fibrosis. To overcome these limitations, a number of proprietary technologies for generating and screening molecular diversity through HTS have been developed. These screening systems – with the capacity to analyse millions of different AMPs – have been designed to avoid or, in some special cases, directly take advantage of the obvious dilemma of expressing potent and broadly active antimicrobials in microorganisms.

The platform technologies include *cis*-acting screening systems such as the Suicide Expression System (SES – WO 0073433) (Figure 2), where the antimicrobial effect is directed towards the producing cell. In this screening system, potent AMPs can be selected on the basis of their ability to inhibit or kill the host itself. The most severely inhibited host cells, expressing the most potent AMP, can be identified and the amino acid sequence of the specific AMP determined by DNA sequencing of the corresponding gene. In yet other *trans*-acting screening systems (for example, Trans-Active-Peptide-system – patent pending), the AMPs are secreted and interact with target cells different from the producing cells. This more traditional microtiter- or agar plate-compatible format allows the screening of large libraries against a range of desired pathogens, or

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*Figure 1. Schematic outline of Transposon-Assisted Signal Trapping (TAST).*

![Diagram of TAST process]

1. **Construct cDNA**
2. **Insert lactamase/transposase in vitro**
3. **Select clones with signal sequences on ampicillin**
4. **Sequence & analyze**
5. **Donor**
We have discovered that different structural classes of AMPs require different expression hosts and host-cell protection strategies. Accordingly, we have categorised our various AMPs according to structure and bioactivity, and have constructed complementary expression systems for most of the lead classes.

Recombinant Production Platform Being the world-leader in the production of industrial enzymes, Novozymes has large recombinant production capabilities. More than 500,000 metric tonnes of enzymes are produced each year at production plants located in Denmark, Switzerland, the US, Brazil and China. Recently, a fully operational cGMP production facility in Sweden was acquired in order to handle contract production of pharmaceutical peptides.

To achieve the highest possible production yields, both fungal and bacterial strains have been optimised for the production of proteins and peptides. We have discovered that different structural classes of AMPs require different expression hosts and host-cell protection strategies. Accordingly, we have categorised our various AMPs according to structure and bioactivity, and have constructed complementary expression systems for most of the lead classes.

AMP Portfolio Currently, Novozymes has more than ten different classes of AMPs (see Figure 3). These AMPs range in size from 15 to more than 50 amino acids, and they display a variety of antimicrobial activities. Some lead series (such as the NZ1000-, NZ5000- and NZ10000-series) are broadly active against micro-organisms including Gram-positive and Gram-negative bacteria, fungi and yeasts, while others are strictly antifungal (NZ3000) or antibacterial with specificity for either Gram-positive or Gram-negative organisms (for example, NZ2000 and NZ4000). The major pre-clinical focus has been on two backbone-series: Novispirin and Plectasin.

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**Figure 2.** Schematic flow-chart of Suicide Expression System (SES).

**Figure 3.** Development status of Novozymes' AMPs.
A few AMPs – bacitracin, polymyxins and gramicidins – have been used for decades as efficient topical antibiotics. However, the pharmaceutical exploitation of gene-encoded AMPs has been slow so far.

Novospirins (NZ1000-series) The NZ1000-series (US 6492328), recently developed by Robert Lehrer and co-workers at UCLA, are small alpha-helical peptides with broad antimicrobial activity against both Gram-positive and Gram-negative bacteria, as well as certain yeasts and fungi. Despite this broad activity, very little haemolysis or toxicity has been observed. A lead peptide from this series, NZ1001, has been tested in several topical skin and pulmonary animal models. For instance, in a wound model, a fast and dramatic 4-log decline in the number of bacteria was observed. This in vivo bactericidal activity against clinical isolates of both *P. aeruginosa* and *S. aureus* was exerted in a matter of minutes. In a rat pulmonary infection model, NZ1001 significantly reduced the number of bacteria and macro- as well as microscopic pulmonary pathologies when compared with placebo.

These pre-clinical observations indicate that the Novospirins are potent alternatives to current antibiotic therapies directed against wounds, acne and other topical infections. The potency, antimicrobial spectrum, lack of spontaneous resistance and speed of bacterial elimination makes this class of antibiotics an ideal candidate for further clinical development.

Plectasin (NZ2000-series) The second lead series, the Plectasins or NZ2000-series, has been isolated from the saprophytic ascomycete fungus, *Pseudoplectania nigrella*. Plectasin consists of 40 amino acids and belongs to the Defensin-family of AMPs. Sequence analysis and homology alignment indicates that the molecule has homology to the arthropod and mollusc defensin family of AMPs. The characteristic Cys-pattern is conserved, as well as several other regions in the peptide, and the 3-dimensional structure resembles that of other defensins (Figure 4).

Plectasin is selective in its antimicrobial spectrum and displays potent bactericidal activity against Gram-positive bacteria, with MICs and MBCs as low as 0.4 µg/ml against clinical isolates of *S. pneumonia*. Analysis of the killing kinetics revealed that Plectasin eliminates >99.9% of the bacteria in less than 3 hours, with no haemolytic activity detected at concentrations up to 400 µg/ml. The Plectasin cDNA has been cloned, and the fully matured and correctly folded peptide has been successfully expressed in several industrial expression systems. In systemic sepsis and pulmonary animal models, the potency and efficacy of Plectasin were comparable with those of conventional antibiotics. For example, one IV dose of Plectasin an hour after microbial challenge eliminated all bacteria in a mouse peritonitis model. This indicates that Plectasin, which can be recombinantly expressed on a commercial scale, is a promising candidate for further development as a novel therapeutic peptide against Gram-positive bacterial infections.

**Perspective**

A few AMPs – bacitracin, polymyxins and gramicidins – have been used for decades as efficient topical antibiotics. However, the pharmaceutical exploitation of gene-encoded AMPs has been slow so far. There are several reasons for this including a lack of oral availability and systemic potential for peptides, high production costs and a lack of serious commitment from ‘big pharma’ – the latter probably being due to a reliance on traditional approaches and a hesitance to consider lead research candidates that act on cell membranes. This has been compounded by the emergence of several small biotechnology companies in this field, developing a single lead against clinical indications with ill-defined clinical end-points, and with limited financial resources.

Nevertheless, the emergence of multi-resistant pathogenic bacteria and a corresponding demand for antibiotics with novel modes of action, combined with an improved understanding of the universal nature of AMPs, has prompted renewed interest in this unique group of antimicrobial agents. At this point, more than 14 companies have – or have had – an active clinical development programme for AMPs; hopefully, one or more of these companies will succeed and secure further pharmaceutical confidence in these fascinating compounds.

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_Debbie Yaver received a PhD in microbiology from the University of California at Davis (USA). She has been with Novozymes for 12 years. Research in her department includes expression of fungal enzymes, development of fungal expression hosts, and discovery and development of antimicrobial compounds. Dr Yaver is currently leading the preclinical development of a novel antimicrobial peptide._

**Figure 4.** 3D-structure of Plectasin. Courtesy of Svend Ludvigsen, Novo Nordisk.