Antimicrobial compounds as side products from the agricultural processing industry

Sumthong, P.

Chapter 2

Developing antimicrobial compounds from natural sources

Pattarawadee Sumthong and Robert Verpoorte

Division of Pharmacognosy, Section of Metabolomics,
Institute of Biology, Leiden University,
Einsteinweg 55, P.O. Box 9502, 2300 RA Leiden, The Netherlands

Natural products from plants are of interest for the discovery of antimicrobial compounds (see general introduction). Assays used in the identification of antimicrobial compounds are reviewed in this chapter. The measurement of growth inhibition of microorganisms by diffusion or dilution assays is used for screening antimicrobial compounds and plant extracts. For drug discovery, microbial growth inhibition is not sufficient. Additional studies are required on the mode of action in pathogenic microorganisms such as effects on bacterial cell membranes, fungal cell wall synthesis, DNA replication and repair, ribosome binding, protein synthesis and metabolic enzymes. It is therefore important to study the mode of action of plant antimicrobial compounds after positive screening for microbial growth inhibition. This chapter discusses first the most common mode of action of antibiotics followed by an overview of possible assays which can be used as tools to find antimicrobial compounds and discover novel leads for drug development.

2.1 Mode of action

From the discovery of penicillin in 1928 and during the four decades after World Wall II, many advances were made in antimicrobial therapy. Today, the pace of antimicrobial discovery has slowed. During the 20-year period from 1983 to 2002, the FDA's (Food and Drug Administration) approval of new antibacterial agents decreased by 56%. Between 2004 and 2006, only three new antibacterial agents have been approved [Mukhopadhyay and Peterson, 2006]. Most antimicrobial agents used for the treatment of bacterial infections may be categorized according to their principle mode of action. The most common modes of action are interference with the cell membrane and cell wall, interference with nucleic acids, and enzyme interactions [Lambert and O'Grady, 1992; Hugo and Russell, 1992; Neu, 1992; Tenover, 2006].

2.1.1 Cell membrane and cell wall interactions

Disruption of the bacterial membrane structure by antimicrobial compounds has not yet been well characterized in term of the mode of action. It is postulated that polymyxins exert their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial cell contents. Lipopeptides consist of a linear or cyclic peptide sequence, with either a net positive or negative charge, to which a fatty acid moiety is covalently attached to its N-terminus. They are a class of antibiotics which are highly active against multidrug resistant bacteria. Some lipopeptides also display anti-fungal activity. In the anionic lipopeptide class, the first naturally occurring compound discovered was amphomycin over fifty years ago. Additional members of this class of compounds include crystallomycin, aspartocin, glumamycin, laspartomycin, tsushimycin, and, by far the most studied, daptomycin. They neither inhibit cell wall synthesis by interacting with ribosome subunits nor do they inhibit protein synthesis. Rather, they are thought to target and bind to the bacterial membrane directly, and cause rapid depolarization of the antibacterial membrane potential as well as eventually death of the bacterium [Storm et al., 1977; Carpenter and Chambers, 2004; Straus and Hancock, 2006].

The fungal cell wall is a unique structure that is essential for the survivor of fungi. It differs from the mammalian cells and consequently presents an attractive target for new antifungals. The fungal cell wall is a vital and complex structure containing mannoproteins, chitins and glucans. Chitin and glucan components of the cell wall should be good drug targets because they are unique and essential to fungi [Georgopapadakou and Tkacz, 1995]. Any disruption in cell wall integrity should affect growth. The echinocandins are cyclic hexapeptides, members of a new class of antifungal agents. They appear to inhibit the synthesis of 1,3-β-D-glucan, a major cell wall component which provides structural integrity and osmotic stability in

most pathogenic fungi [González et al., 2001]. Caspofungin is a noncompetitive inhibitor of the enzyme β-(1,3)-glucan synthase, which catalyzes the polymerization of uridine diphosphate-glucose (UDP-glucose) into β-(1,3)-glucan, a structural component of the fungal cell wall responsible for maintaining integrity and rigidity. When β-(1,3)-glucan synthesis is inhibited, ballooning out of the weakened cell wall occurs as a result of the high osmotic pressure of the protoplast and causes cell lysis [Stone et al., 2002].

More recently, studies focused on the search for water-soluble inhibitors of fungal 1,3-β-D-glucan synthase, an enzyme critical for the synthesis of 1,3-β-D-glucan, a major component of the cell wall of a number of key pathogenic fungi. Aerothricin lipopeptidolactones and Sankyo lipopeptides have been identified as novel members of liposaccharide glucan synthesis inhibitors. Aerothricins, like natural product molecules, act as antifungal drugs that inhibit the formation of the β-1,3-D-glucan component of the cell wall, but they are less water soluble than the related semi-synthetic molecules. The semi-synthetic molecules contain various basic amino acids and a large series of aminoalkyl groups [Schwartz, 2001].

Bacterial cell walls have only a single layer of peptidoglycan. A single unit of peptidoglycan is a combination of alternatively β-(1→4) linked disaccharides of *N*-acetylglucosamine (NAG) and *N*-acetyl muramic acid (NAM) and four amino acids such as L-alanine, D-isoglutamic acid, L-lysine and D-alanine attached through peptide bonds at the NAM residue [Rai et al., 2003]. Antibacterial drugs that work by inhibiting bacterial cell wall synthesis are the β-lactams (e.g. penicillins, cephalosporins), carbapenems, monobactams, glycopeptides, vancomycin and teicoplanin. β-Lactams inhibit synthesis of bacterial cell walls by interfering with the enzymes required for the synthesis of the peptidoglycan layer. Vancomycin and teicoplanin bind to the terminal D-alanine residues of the nascent peptidoglycan chain, thereby preventing the cross-linking steps required for stable cell wall synthesis [McManus, 1997].

2.1.2 Nucleic acid interactions

Fluoroquinolones exert their antibacterial effects by disturbing DNA synthesis and causing lethal double-strand DNA breaks during DNA replication [Drlica and Zhoa, 1997; Yao and Moellering, 2003; Petri, 2006]. The 4-quinolones are antibacterial agents that have two essential bacterial enzymes, DNA gyrase and DNA topoisomerase IV, as targets. DNA gyrase controls DNA supercoiling and relieves topological stress arising from the translocation of transcription and replication complexes along DNA; topoisomerase IV is an enzyme that resolves interlinked daughter chromosomes following DNA replication. Both enzymes are

required for cell growth and division. It is thus not surprising that the quinolones are bactericidal. However, these compounds do not simply eliminate topoisomerase function: trapping of gyrase and topoisomerase IV on DNA probably leads to the lethal release of double-strand DNA breaks. For three decades, the quinolones have been used for a variety of physiological studies, serving as convenient inhibitors of DNA synthesis and as probes for the study of topoisomerase-DNA interactions [Drlica and Zhao, 1997]. Chloramphenicol has an inhibitory effect on DNA synthesis [Chen et al., 1996]. The common antibacterial drug combination of TMP (a folic acid analogue) with sulfamethoxazole (SMX, a sulfonamide), inhibits two steps in the enzymatic pathway for bacterial folate synthesis [Petri, 2006; Tenover, 2006].

Bacterial ribosomes differ in structure from their counterparts in eukaryotic cells. These differences can be used to selectively inhibit bacterial growth. Aminoglycosides, a large family of water-soluble polycationic amino sugars, are used as broad spectrum antibacterial agents. Aminoglycosides target the microbial ribosome by direct interaction with ribosomal RNA, and they affect protein synthesis by inducing codon misreading and by inhibiting translocation of the tRNA-mRNA complex [Hobbie et al., 2006; Neu, 1992; McManus, 1997]. Antibacterial agents like aminoglycosides, macrolides and tetracyclines bind to the 30S subunit of the ribosome, whereas chloramphenicol binds to the 50S subunit [Tenover, 2006].

2.1.3 Enzyme interactions

There are many possible target enzymes in microorganisms. The gram-negative bacterium *Pseudomonas aeruginosa* is an important pathogen of plants and animals. Given the high prevalence of antibiotic resistant strains of *P. aeruginosa*, it is desirable to design new chemotherapeutic agents against this opportunistic pathogen, which is a growing human health problem because of the susceptibility to infection in the increasing number of immuno-suppressed people. Betaine aldehyde dehydrogenase (BADH) is a target enzyme for inhibition of *P. aeruginosa* growth. Glycine betaine, the product of the BADH catalyzed reaction, is an effective osmoprotectant and most likely acts as such in bacterial cells growing in the hyperosmotic environment of infected tissues. It has been found that *P. aeruginosa* is able to thrive under osmotic stress if glycine betaine, choline, or choline precursors are present. Indeed, the virulence of this bacterium has been correlated with its ability to adapt to osmotic stress and to express phospholipase C, the first enzyme in the pathway from phosphatidylcholine to glycine betaine. BADH from *P. aeruginosa* therefore might be a key enzyme for the survival of the pathogen and thus a potential target for chemotherapeutic agents. Velasco-García et al. [2006] suggested that the growth inhibition is due to the accumulation of the BADH substrate, betaine

aldehyde, which is highly toxic. However, they found that disulfiram destabilized the quaternary structure of BADH and promoted irreversible aggregation of this enzyme. Inhibition of glutamate dehydrogenase and 2-ketoglutarate reductase, the first enzymes in the 2-ketoglutarate pathway of glutamate catabolism by *Fusobacterium nucleatum*, the oral anaerobes, were assayed. Benzimidazoles and lansoprazole were found to be antimicrobial against *F. nucleatum* by inhibition of those enzymes [Sheng et al., 2006].

Most clinically useful antifungal agents inhibit the biosynthesis of ergosterol or interact directly with ergosterol in membranes. Ergosterol is the principle sterol in yeast and fungi, except the Oomycete genera *Pythium* and *Phytophthora*, which do not synthesize any sterol. Beuchet et al. [1998] reported that the synthetic compound 6-β-aminocholestanol inhibits the biosynthesis of ergosterol. The azole antifungal agents, such as fluconazole, itraconazole and azolylmethyloxolane derivatives with modified sterol side-chain structures, inhibit cytochrome P450 14α-demethylase (14DM) and Δ^{24}-sterol methyltransferase (24-SMT) which are the key enzymes involved in fungal ergosterol biosynthesis [Chung, et al., 1998; Chung et al., 2000]. Amorolfine inhibits Δ^{14} reductase and $\Delta^{7,8}$ isomerase which are part of the ergosterol biosynthesis pathway [Polak-Wyss, 1995]. The α-bisabolol in chamomile interfered with zymosterol and prevents the formation of fecosterol from zymosterol which is the first fungal specific step in ergosterol biosynthesis [Pauli, 2006].

2.2 General screening

There are many different assays for screening antimicrobial activity. Many publications report the antimicrobial activity of plants using general screening assays for microbial growth inhibition which are *in vitro*. The standard general screening assays are diffusion assays, dilution assays and bioautographic assays.

2.2.1 Diffusion assays

2.2.1.1 Disc diffusion assay

Paper disc diffusion assays are generally used for screening of antibacterial and antifungal activities from natural extracts and compounds [Quiroga, et al., 2001; Ahmad et al., 2005; Pyun and Shin, 2005]. However, the diffusion method is not appropriate for testing non-polar samples or samples that do not easily diffuse into agar if the inhibition diameter has to be measured [Cos et al., 2006]. Plant extracts are dissolved in organic solvents such as ethanol, methanol or ethyl acetate [Moreno et al., 1999; Pyun and Shin, 2005; Eldeen et al., 2005]. The concentration of bacterial or fungal inoculum used for the tests is between 10^4-10^8 CFU (Colony

Forming Units) /mL. The inoculi are spread on the agar surface or mix into the agar media [Pyun and Shin, 2005; Eldeen et al., 2005]. Sterile filter paper discs, Whatman No.4 or No.1, 5 mm or 8 mm diameter,r are the most often used [Moreno, et al., 1999; Quiroga, et al., 2001; Ahmad et al., 2005; Pyun and Shin, 2005].

2.2.1.2 Well diffusion assay

The well diffusion assay is suitable for aqueous extracts because they are difficult to dry on paper discs [Vlietinck, et al., 1995; Fazeli et al., 2007; Magaldi, et al., 2004; Tadeg, et al., 2005]. However, the leaking of sample under the agar layer must be considered. Wells with 8 mm diameter are cut in the agar plate using a cork borer and 100 µL of sample is loaded into the well [Fazeli et al., 2007; Patton et al., 2006]. Microbial cell suspension is used in a similar way to the disc diffusion assay and the inhibition diameter is measured after incubation.

2.2.2 Dilution assays

Dilution assays are standard methods used to compare the inhibition efficiency of antimicrobial agents. The test extracts or compounds are mixed with suitable media that has been inoculated with the test microorganism. It can be carried out in liquid media (broth dilution assay) or in solid media (agar dilution assay). Growth inhibition is expressed as Minimal Inhibitory Concentration (MIC) which is defined as the lowest concentration able to inhibit any visible microbial growth. The Minimal Bactericidal or Fungicidal Concentration (MBC or MFC) is determined by plating-out samples of completely inhibited dilution cultures and assessing growth after incubation [Cos et al., 2006; Yin and Tsao et al., 1999; Salie et al., 1996]. The inoculate concentrations of bacterial or fungal cultures are between 10^4-10^8 CFU/mL [Camporese et al., 2003; Karaman et al., 2003]. In the agar plate dilution assay, the microbial cell suspension is spread over the surface of the agar plate [Verástegui et al., 1996], inoculated on the center of the agar surface [Sato et al., 2000; Quiroga, et al., 2001], by the streak method [Kumar et al., 2006] or mixed with the media as in the broth dilution assay [Navarro and Delgado, 1999; Cos et al., 2002; Pyun and Shin, 2005].

2.2.3 Bioautographic assays

There are three different approaches for bioautography to localize antimicrobial activity on a TLC chromatogram [Cos et al., 2006]. In direct bioautography, the microorganism grows directly on the thin-layer chromatographic (TLC) plate [García, et al., 1997; Yff et al., 2002]. In contact bioautography (biogram assay), the antimicrobial compounds are transferred from the

TLC plate to an inoculated agar plate through direct contact. In the agar overlay bioautography, agar media is applied directly onto the TLC plate [Silva et al., 1996; Chomnawang et al., 2005; Schmourlo et al., 2005]. Those assays supply a quick screen for new antimicrobial compounds through bioassay-guided isolation. The concentrations of bacterial or fungal inoculates are 10^6 CFU/mL [Moreno et al., 1999].

2.3 Advanced screening on modes of action

To discover antimicrobial compounds with multiple applications, the mode of action in a microorganism must be considered as the drug target. The advanced screening on mode of action can be divided into two groups: assays on microbial cells *in vivo* and assays on molecular targets *in vitro*.

2.3.1 Assays on microbial cells

2.3.1.1 Viability of cells

The fluorescent viability test uses fluorescein diacetate (FDA) and ethidium bromide (EB) which show a strong contrast between living and dead cells. The living cells show a green fluorescence as fluorescein diacetate can pass through the membrane into the cell where it is hydrolyzed into fluorescein and acetate by esterases. Due to their polarity, intact fluoresceins cannot traverse the cell membranes. Dead cells show a bright red fluorescence due to ethidium bromide penetration into the dead cells in which esterases were inactive. The fluorescence can be observed under a fluorescent microscope [Aquino, et al., 2005].

2.3.1.2 Microbial cell membrane and cell wall targets

Electron microscopy was used to investigate the mechanism of action of biocides in pathogenic microorganisms. Scanning and transmission electron microscopy (SEM and TEM) were used to observe membrane damage and leakage of intracellular materials in *Aspergillus fumigatus*, *Candida albicans*, *P. aeruginosa*, *Serratia marcescens* and *Staphylococcus aureus* after treatment with polyquaternium-1 (PQ-1) and myristamidopropyl dimethylamine (MAPD) [Codling et al., 2005].

Yang et al. [2006] studied the mode of action of antimicrobial compounds on the bacterial membrane using a membrane depolarization assay. *Staphylococcus aureus* and *Escherichia coli* were grown and incubated with the inhibitors. The collapse of the cytoplasmic membrane potential was monitored using a spectrofluorometer at 622 nm excitation wavelength and 670 nm emission wavelength.

The bacterial cell membrane integrity can be examined by determination of the release of material absorbing at 260 nm, which is monitored by UV spectrometry. Outer membrane permeabilization is determined by the NPN (1-*N*-phenyl-naphthylamine) assay, in which fluorescence of NPN is recorded using a fluorescence spectrophotometer. Enhanced fluorescence is due to NPN uptake by *E. coli*. The inner membrane permeabilization assay is measuring the release of cytoplasmic *β*-galactosidase from *E. coli* into the culture medium using *O*-nitrophenyl-*β*-D-galactoside (ONPG) as the substrate. The production of *O*-nitrophenol over time is determined by monitoring the change in absorbance (420 nm) using a spectrophotometer [Je and Kim, 2006].

The depolarization of the cytoplasmic membrane of yeast and *S. aureus* by antimicrobial peptides is determined using the membrane potential sensitive cyanine dye $DiSC_3$-5 (3,3'- dipropyl-2,2'-thiadicarbocyanine iodide). Fluorescence is monitored by a fluorescence spectrometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Membrane depolarization is determined by an increase in fluorescence units as a function of antimicrobial peptide concentration [Friedrich, et al., 2000; Zhu, et al., 2006].

A commercially available Live/Dead Bacterial Viability Kit (Molecular probes, Inc., Eugene, Oregon, USA) is rapid test for distinguishing membrane-active antibacterial agents. This method utilizes two fluorescent nucleic acid stains, SYTO9 (stains all cells green) and propidium iodide (stains cells with damaged membrane red) for the drug-treated bacterial cells. The cells are then either examined visually by fluorescence microscopy or their fluorescence emissions are recorded using a multi-label plate reader set to measure emissions at two different wavelengths [Singh, 2006].

Straus and Hancock [2006] determined the interaction of an inhibitor with bacterial membranes using differential scanning calorimetry in model membranes of calorimetry lipid films, DiPoPE (dipalmitoleoyl phosphatidylethanolamine). The interaction between the inhibitor and the bacteria was detected by NMR analysis of Ca^+ level which is involved in bacterial membrane damaged.

Aspergillus fumigatus was incubated with wheat germ agglutinin fluorescein isothiocyanate (WGA-FITC). An intense fluorescence all along the hyphal wall was observed for the negative control. The labeling was detected when the fungi was grown in the presence of caledonixanthone E, an antifungal compound. WGA-FITC recognizes chitin, a structural polysaccharide of the fungal cell wall, and the reduction of the chitin content in hyphae after exposure to caledonixanthone E was observed under a fluorescence microscope [Larcher, et al., 2004].

2.3.2 Assays on molecular targets

2.3.2.1 Nucleic acid targets

DNA replication is a well known target for screening of antibiotics. *Escherichia coli* DnaG primase is a single-stranded DNA-dependent RNA polymerase. The primase catalyzes synthesis of a short RNA primer to initiate DNA replication at the origin and to initiate Okazaki fragment synthesis for the lagging strand. *Escherichia coli* DnaG and DnaB, which overexpressed primase and helicase, respectively are used. The SPA primase assay is monitored using the topcount instrument which is assessed by comparison to a filter-binding method. DnaB helicase activity is monitored by a FRET method in which the fluorescence of a double-stranded, forked DNA substrate, labeled on the 5′ ends with the fluorochrome Texas Red, is internally quenched by a Dabcyl moiety located on the complementary strand [Zhang et al., 2002].

DNA microarray assays can be used to study gene expression profiles of *Saccharomyces cerevisiae* treated with ergosterol biosynthesis inhibitors. It leads to the identification of a subset of genes that are up- or down-regulated in response to these compounds, and to the determination of the mode of action of an unknown compound based on the similarity of its gene expression profile to those of an ergosterol biosynthesis inhibitor [Bammert and Fostel, 2000 *In* Kagen et al., 2005].

Mycobacterium smegmatis, a non-pathogenic microorganism, carries two rRNA operons, rrnA and rrnB, which allow for mutagenesis of its ribosomal nucleic acids. One of the two chromosomal rrn operons is usually inactivated by insertion-deletion mutagenesis, which results in cells carrying homogenous populations of mutant ribosomes. This model has provided an important tool in the investigation of drug-target activity of ribosomal inhibitors [Hobbie, et al., 2006].

2.3.2.2 Enzyme targets

Transgenic *S. cerevisiae* strains are used to determine the inhibition of ergosterol biosynthesis. The specific target enzymes in ergosterol biosynthesis, lanosterol C-14 demethylase, C-14 reductase, Δ^{8}- Δ^{7}-isomerase, C-3 ketoreductase or squalene epoxidase are encoded by ERG11, ERG24, ERG2, ERG27 and ERG1 gene of *S. cerevisiae*, respectively [Daum et at., 1998; Bammert and Fostel, 2000; Gachotte et al., 1999; Mercer, 1991 *In* Kagen et al., 2005]. The crude extract of *S. cervisiae* is used to determine the inhibition of Δ^{14}-reductase and Δ^{8}- Δ^{7}-isomerase, two enzymes in the pathway of ergosterol biosynthesis by IC_{50} values [Polak-Wyss, 1995].

Barrett [2002] reported an assay for the inhibition of the enzyme 1,3-*β*-D-glucan synthase from *C. albicans* 6406. Membrane and cell wall targets in *A. fumigatus* were studied using transgenic *A. fumigatus* which overexpress the *β*-(1,3)-glucanosyltransferase (GEL) gene [Beauvais and Latgé, 2001].

Betaine aldehyde dehydrogenase (PaBADH) is a target enzyme for inhibition of *P. aeruginosa*, a plants and animals pathogen. *Escherichia coli* strains were transformed with a mutant plasmid to express PaBADH. Enzyme activity was assessed by spectrophotometer [Velasco-García et al., 2006].

Glutamate dehydrogenase and 2-ketoglutarate reductase, the first 2 enzymes in the 2-ketoglutarate pathway of glutamate catabolism by *F. neucleatum* were assayed for screening of antimicrobial activity of bacteria cell extracts. Enzyme activities were assayed following the procedure described by Fujimura and Makamura [1987] with use of L-X-glutamyl-*p*-nitroanilide as a substrate [Sheng, et al., 2006].

2.4 Methods used in this thesis

In this thesis both general and advanced screening assays were used in order to find antimicrobial compounds for application in anti-wood rot preparations. The disc diffusion assay was the general method used to select the active crude extract and compounds. The agar plate dilution assay was used to evaluate the MIC and MFC values of anti-wood rot compounds. The broth dilution assay was used to determine MIC value of active compounds against *Aspergillus niger* for further study on the mode of action in fungi. The contact bioautographic assay (biogram assay) was used for fast screening after separation of crude extracts.

Two additional advanced screening assays were used to further investigate the mode of action of natural products in microorganisms. Anthranilate synthase, one of the enzymes in tryptophan biosynthesis pathway, is a new enzyme target for inhibition due to its present in microorganisms, plants and some parasites, but not in mammals. 1,3-*α*-D-glucan is the prominent component in the cell wall of many fungal species, and was used as a target in a study of the induction of fungal cell wall stress in transgenic *A. niger*.