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Rapid membrane permeabilization of *Listeria monocytogenes* and *Escherichia coli* induced by antibacterial prenylated phenolic compounds from legumes

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ABSTRACT

Prenylated phenolics from the Fabaceae are promising lead compounds for new antibacterials. Pools enriched in prenylated phenolics were made from lupine, peanut and soybean seedlings. One pool was rich in chain prenylated isoflavones (*c*Isf), one in chain prenylated stilbenoids (*c*Sti), one in chain prenylated (*c*Pta) and one in ring-closed prenylated pterocarpans (*r*Pta), as characterized by RP-UHPLC-UV-MS. Antibacterial activity of the pools and membrane permeabilization was investigated. Pools showed high antibacterial activity against *Listeria monocytogenes*: *c*Isf pool had a minimum inhibitory concentration of 10 μg/ml prenylated compounds, followed by *c*Pta pool (25 μg/ml) and *c*Sti pool (35 μg/ml). Activity against *E. coli* was found only when the pools were co-administered with an efflux pump inhibitor. The pool enriched in chain prenylated isoflavones permeabilized the bacterial membrane within minutes of exposure, whereas ampicillin did not. Bent conformation and chain prenylation, were molecular features of main prenylated phenolics found in pools with high antibacterial activity.

1. Introduction

In recent years there has been a significant increase in bacterial resistance to common antimicrobials and a lack of antimicrobial discovery, which represents a threat to public health (WHO., 2014). Historically, natural products were the source of virtually all medicinal preparations and have been the single most productive source of leads for the development of antimicrobials (Harvey, Edrada-Ebel, & Quinn, 2015). Natural products, either as pure compounds or as standardized extracts, provide unlimited opportunities for control of microbial growth owing to their chemical diversity (Negi, 2012).

The Fabaceae (Leguminosae) is one of the largest medicinal plant families in the world and legume seeds are known to produce a large number of potential antimicrobial phytochemicals (Gao et al., 2010). Under (a)biotic stress this plant family produces, in particular, prenylated phenolic compounds as a defense mechanism (Veitch, 2007). These prenylated phenolic compounds have shown very good antibacterial activity, especially against Gram positive pathogens. Low minimum inhibitory concentrations (<15 μg/ml) have been found for prenylated phenolic compounds against Gram positives, including strains resistant to current antibiotics (Botta et al., 2009). In contrast, it is known that phytochemicals are less effective against Gram negative bacteria. The intrinsic resistance of Gram negatives is attributed to the interplay between reduced influx and more effective efflux of antimicrobials compared to Gram positives. Gram negatives have an outer membrane composed of a rigid leaflet consisting of lipopolysaccharide with low permeability and narrow porins facilitating the penetration of hydrophilic solutes up to a certain size exclusion limit (Fernández & Hancock, 2012). Active efflux of antimicrobials in Gram negatives is done by double-membrane-spanning efflux systems with unusually broad substrate specificity, allowing extrusion of substrates across the entire cell envelope (Blair, Webber, Baylay, Ogbolu, & Piddock, 2015).

It is accepted that prenylation increases antibacterial activity (partially) by increasing the hydrophobicity of the molecule. As a result, the interaction with biological targets, such as bacterial membranes increases (Yazaki, Sasaki, & Tsurumaru, 2009). However, other characteristics, such as molecular geometry or charge, can contribute to the affinity of a molecule to a bacterial target site and, consequently, affect the antibacterial activity (Aptula et al., 2003). Prenylated phenolic compounds have large structural vari-

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ety derived from the number and arrangement of the (aromatic) rings defining the main skeleton (i.e. flavonoids, isoflavonoids, stilbenoids, phenolic acids and chromones). Additionally, the number, type and position of substituents attached to the skeleton will increase the structural variety (Yazaki et al., 2009). Substituents, like prenyl groups, can be attached to the main skeleton at different positions and in different configurations (i.e. chain or ring-closed prenylation). All these structural differences possibly affect the antibacterial properties of these prenylated phenolic compounds.

We previously investigated the antibacterial properties of crude extracts from (fungus-elicited) legume seedlings against Gram positive pathogens (Araya-Cloutier, den Besten, Aisyah, Gruppen, & Vincken, 2017). Despite the complexity of these extracts, it was found that the content of prenylated aglyconic phenolic compounds correlated with the antibacterial activity of the extracts and that the position of the prenyl group also influenced the activity. Prenylated stilbenoids from peanut, prenylated isoflavones from lupine and prenylated 6a-hydroxy-pterocarpans from soybean were among the antibacterials showing potential against Gram positive pathogens (Araya-Cloutier et al., 2017).

Due to the complexity of plant extracts, it is still difficult to understand the effect of the configuration of prenylation (chain or ring-closed) or the skeleton of the phenolic (sub)class on the antibacterial properties of prenylated phenolic compounds. Therefore in this study, extracts from fungus-elicited legume seedlings were fractionated using flash chromatography and pools enriched in different types of prenylated compounds were made. Structure-antibacterial activity relationships were proposed by using *Listeria monocytogenes* (Gram positive) and *Escherichia coli* (Gram negative) as target bacteria. The effects on the permeability of the bacterial membrane were studied, as well as the role of the efflux pump systems on *E. coli*'s intrinsic resistance.

2. Materials and methods

2.1. Materials

Lupine (*Lupinus angustifolius*) and peanut (*Arachis hypogaea*) seeds were obtained from Vreeken's Zaaden (Dordrecht, The Netherlands). Soybeans (*Glycine* max) were provided by Frutarom (Londerzeel, Belgium). Acetonitrile (ACN; ULC/MS grade), water acidified with 0.1% (v/v) formic acid (FA; ULC/MS grade), methanol (MeOH; ULC/MS grade) and silica gel (60Å, 70–230 mesh) were purchased from Biosolve (Valkenswaard, The Netherlands). Ethanol absolute (EtOH) and *n*-hexane were purchased from VWR International (Radnor, PA, USA). Water for purposes other than UHPLC was prepared using a Milli-Q water purification system (Millipore, Molsheim, France). Growth media, including Bacto brain heart infusion (BHI) broth were purchased from BD (Franklin Lakes, NJ, USA), tryptone soya broth (TSB) and bacteriological agar from Oxoid Ltd (Basingstoke, UK), and peptone physiological salt solution (PPS) from Tritium Microbiologie (Eindhoven, The Netherlands). Pure prenylated isoflavones 2,3-dehydrokievitone, licoisoflavone A, luteone and wighteone were purchased from Plantech UK (Reading, UK). All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA).

2.2. Elicitation and extraction of legume seedlings

Legume seeds were germinated and elicited with the fungus *Rhizopus oryzae* (LU581) as described elsewhere (Aisyah, Gruppen, Andini, Bettonvil, Severing, & Vincken, 2016; Aisyah, Vincken, Andini, Mardiah, & Gruppen, 2016; Simons et al., 2011). In short, the process consisted of three main steps: soaking in water for 1 day at room temperature, germination for 2 days at 22–25 °C, 90–100% relative humidity (RH) and fungal exposure for 5–7 days at 25–30 °C, 70–90% RH. After this, the elicited seedlings were freeze-dried, milled (Ultra Centrifugal Mill ZM 200, Retsch, Haan, Germany) and extracted, under sonication (40 °C/30 min), with hexane followed by aqueous EtOH (80% v/v) (Aisyah, Vincken et al., 2016; Simons et al., 2011). EtOH was removed under reduced pressure and the remaining extracts were freeze-dried.

2.3. Preparative enrichment of prenylated phenolic compounds

A Reveleris® flash chromatography system (Grace Davison Discovery Science, Columbia, MD, USA) was used to obtain pools enriched in prenylated phenolics from extracts of the elicited legume seedlings. The crude seedling extracts (250–400 mg) were mixed thoroughly with 3 g of silica gel, and dry-loaded in 5 g cartridges (Grace) on the flash system with a solid loader plunger. The fractionation was performed at room temperature using a 12 g Reveleris C18 RP column (particle size 38.6 μm, Grace). Water acidified with 0.1% (v/v) FA, eluent A, and ACN (soybean and lupine) or MeOH (peanut) acidified with 0.1% (v/v) FA, eluent B, were used as eluents. The flow rate was 30 ml/min and UV detection was set at 280 nm (soybean and lupine) and 315 nm (peanut). For soybean the following elution profile was performed: 0–10 min, linear gradient from 0–10% (v/v) B; 10–20 min, linear gradient from 10–40% B; 20–24 min, linear gradient from 40–50%; 24–28 min, isocratic on 50% B; 28–33 min, linear gradient from 50–60% B; 33–34 min, linear gradient from 60–70% B; 34–37 min, linear gradient from 70–80% B; 37–38 min, linear gradient from 80–100% B. For lupine the following elution profile was performed: 0–2 min, isocratic at 0% (v/v) B; 2–24 min, linear gradient from 0–100% B. For peanut the following elution gradient was performed: 0–2 min, isocratic at 0% (v/v) B; 2–16 min, linear gradient from 0–100% B.

Flash fractions (10–15 ml) were collected in glass tubes and subsequently analysed by RP-UHPLC-UV-MS. Fractions containing similar prenylated compounds were pooled. Fractionation of crude extracts and pooling was performed several times until enough material was collected for further experiments. Pools enriched in prenylated phenolics were evaporated under vacuum, solubilized in *tert*-butanol and freeze-dried. Stock solutions in aqueous EtOH (70% v/v) were made for compositional analysis.

2.4. RP-UHPLC-UV-MS analysis

Phenolic compound analysis was performed on an Accela ultra high performance liquid chromatography (RP-UHPLC) system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, autosampler and photodiode array (PDA) detector.

Pools enriched in prenylated phenolics (1 μl, 2–5 mg/ml) were injected onto an Acquity UPLC BEH RP18 column (2.1 mm i.d. × 150 mm, 1.7 μm particle size) with an Acquity UPLC RP18 Vanguard guard-column (2.1 mm i.d. × 5 mm, 1.7 μm particle size; Waters, Milford, MA, USA). Water containing 0.1% (v/v) FA and 1% (v/v) ACN, eluent A, and ACN containing 0.1% (v/v) FA, eluent B, were used as solvents at a flow rate of 300 μl/min. Column temperature was set at 35 °C and the PDA detector was set to measure from 200–600 nm.

For the lupine and soybean pools the following elution profile was used: 0–1 min, isocratic on 9% (v/v) B; 1–3 min, linear gradient from 9–25% B; 3–10 min, linear gradient from 25–50% B; 10–13 min, isocratic on 50% B; 13–23 min, linear gradient from 50–100% B. For the peanut pool the following elution gradient was used: 0–1 min, isocratic on 10% (v/v) B; 1–10 min, linear gradient from 10–34% B; 10–11 min, linear gradient from 34–42% B; 11–