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Abstract

Stilbenoids are a class of secondary metabolites with a stilbene backbone that can be produced by peanut (*Arachis hypogaea*) as defence metabolites. Six monomeric prenylated stilbenoids, including the compound arachidin-6 (**4**), were isolated from extracts of fungus-elicited peanuts (*Arachis hypogaea*) using preparative liquid chromatography. Their structures were confirmed by MS^n, HRMS and NMR spectroscopy and their antibacterial activity was evaluated against methicillin-resistant *Staphylococcus aureus* (MRSA). Similarly to other phenolic compounds, prenylated derivatives of stilbenoids were more active than their non-prenylated precursors piceatannol, resveratrol, and pinosylvin. Chiricanine A (**6**), a chain-prenylated pinosylvin derivative, was the most potent compound tested, with a minimum inhibitory concentration (MIC) of 12.5 µg mL^{-1}. Arachidin-6 (**4**), a ring-prenylated piceatannol derivative, had moderate potency (MIC 50-75 µg mL^{-1}). In conclusion, prenylated stilbenoids represent a group of potential natural antibacterials which show promising activity against MRSA.

Highlights

- New stilbenoid: arachidin-6, a ring-prenylated piceatannol derivative.
- Antibacterial activity of purified prenylated stilbenoids assessed against MRSA.
- Chiricanine A (MIC 12.5 µg mL^{-1}) and other stilbenoids are active against MRSA.

Keywords

Leguminosae, natural product, secondary metabolite, stilbene, prenylation, antimicrobial

1. Introduction

Stilbenoids, a class of secondary metabolites with a stilbene backbone (**Figure 1A**), can be produced by peanut (*Arachis hypogaea*) as defence metabolites (Sobolev, 2013). Analogous to phenolic metabolites of other members of the *Leguminosae* family, e.g. soy bean and mung bean (Aisyah et al., 2016; Simons et al., 2011), the production of stilbenoids and, in particular, their prenylated derivatives can be stimulated by fungal elicitation of germinating peanut seeds (Aisyah et al., 2015; Sobolev et al., 2016; Sobolev et al., 2010). Prenylation refers to the attachment of a prenyl-moiety (i.e. 3,3-dimethylallyl) by a prenyltransferase and in the case of peanut stilbenoids occurs mainly at the 4-position (Yang et al., 2016) as in, for example, arachidin-2 (**Figure 1B, compound 2**) (Sobolev et al., 2016). Prenylation of phenolic compounds has been shown to increase their antibacterial activity, which is exemplified by the minimum inhibitory concentrations (MICs) of genistein (MIC >128 µg mL^{-1}), 6-prenyl-genistein (MIC 32 µg mL^{-1}), and 6,8-diprenyl-genistein (MIC 8 µg mL^{-1}) against methicillin-resistant *Staphylococcus aureus* (MRSA) (Hatano et al., 2000). More generally, prenylated (iso)flavonoids have been shown to possess antibacterial activity against antibiotic-resistant strains of *S. aureus* and other pathogenic gram-positive bacteria (Araya-Cloutier et al., 2017; Araya-Cloutier et al., 2018a; Gibbons, 2004). An extract from fungus (*Rhizopus*) elicited peanut seedlings, enriched in prenylated stilbenoids, already showed promising antibacterial activity against *E. coli*, *L. monocytogenes* and MRSA (Araya-Cloutier et al., 2017; Araya-Cloutier et al., 2018b). In this study, we have isolated and characterized several prenylated compounds with different stilbenoid precursors and prenyl configurations from an extract of *Rhizopus*-elicited peanut seedlings and assessed their antibacterial activity against MRSA. In analogy with other phenolic compounds, we hypothesize that prenylation of stilbenoids will enhance their antibacterial activity.

2. Results and discussion

2.1 Sample Clean-up, Pre-purification and Purification by Preparative RP-HPLC

The crude extract of *Rhizopus*-elicited peanut seedlings showed a chromatographic profile on RP-UHPLC comparable to what was described previously (Aisyah et al., 2015). The clean-up with ethyl acetate effectively removed the majority of polar impurities in the extract, yielding the cleaned extract which contained mainly prenylated stilbenoids (**Figure S1**, supplementary data, 6-19 min) and apolar impurities (**Figure S1**, 19-28 min) which, based on LC-MS analysis, were mostly lipids like oxylipins and free fatty acids (Murphy, 2014). After pre-purification by Flash chromatography, most of the apolar impurities were removed and several pools were obtained enriched in mixtures of prenylated stilbenoids and some oxylipins (**Figure S1**). After subjecting these pools to preparative RP-HPLC separation, six purified compounds were obtained.

2.2 Structure Elucidation of the Prenylated Stilbenoids

The six compounds that were isolated were first analysed by UHPLC-PDA-ESI-IT-MS, the corresponding spectrometric and spectroscopic data of which is shown in **Table 1**. Based on the comparison of this data to literature (Aisyah et al., 2015; Sobolev et al., 2009; Sobolev et al., 2006), the compounds were tentatively annotated. For most purified compounds, the *trans* isomer was most abundant but the *cis* isomer was also present. The two isomers were distinguished by their λ_{max}, and the peak with the higher λ_{max} was assigned as the *trans* isomer, in accordance with previously reported data (Trela and Waterhouse, 1996). Based on UV_{310} area compound **1** was approximately 60% *trans*, compound **3** was approximately 88% *trans*, and compounds **2**, **4**, **5** and **6** were all more than 97% *trans*. Spectrometric and spectroscopic data provided is based on the *trans* isomer.

In ESI-IT-MS (**Table 1**), the most abundant fragments observed for compounds **1**-**3** and **6** in negative ionisation mode were those with a neutral loss (NL) of 56 u (C_4H_8) and NL of 69 or

70 u, corresponding to complete loss of the prenyl chain (as C_5H_8 or C_5H_9). In positive ionisation mode the main fragments were also related to the prenyl-moiety, resulting in a NL of 56 u (C_4H_8), as described previously for chain-prenylated (iso)flavonoids (Simons et al., 2009). For compounds **4** and **5** the main fragment observed in positive ionisation mode, *m/z* 201, corresponded to loss of the catechol (NL 110 u, $C_6H_4O_2$) or phenol (NL 94 u, C_6H_4O) moiety, respectively. Fragments corresponding to neutral losses of 56 u and 42 u were also observed. For compound **4** the fragment at *m/z* 269 (NL 42 u, rel. abundance 25) was more intense than the fragment at *m/z* 255 (NL 56 u, rel. abundance 13, not shown in **Table 1**). The same was observed for compound **5** with the fragments at *m/z* 253 (NL 42 u, rel. abundance 51) and *m/z* 239 (NL 56 u, rel. abundance 23). The abundance ratio of NL 42:56 u was > 1 for both compounds, indicating the presence of a ring prenyl rather than a chain prenyl (Simons et al., 2011). In negative ionisation mode, the prenyl-moiety of compounds **4** and **5** did not readily fragment, instead the unfragmented parent ion, radical fragments and small neutral losses like $\bullet CH_3$ (15 u), H_2O (18 u), CO (28 u), and CO_2 (44 u) were observed.

High resolution mass spectrometric data, as determined by UHPLC-ESI-FTMS, confirmed the expected molecular formulae of all six compounds (**Table 1**). To confirm the tentative annotations of the structures, ^{1}H NMR spectra of compounds **1**-**6** were acquired. The structure of compounds **1**-**3**, **5** and **6** was confirmed by comparison of their ^{1}H NMR spectra to published data (Chang et al., 2006; Park et al., 2011; Royer et al., 2010; Sobolev et al., 2009). Compound **4** ($C_{19}H_{18}O_4$ based on FTMS), however, which was previously tentatively annotated as 4-isopentadienyl-3,5,3′,4′-tetrahydroxystilbene (IPP) based on UHPLC-PDA-ESI-IT-MS (Aisyah et al., 2015), did not match this compound's expected ^{1}H NMR spectrum. HMBC and HMQC were performed in order to elucidate the structure of compound **4** (see **Table 2** for the ^{1}H and ^{13}C NMR spectroscopic data). The ^{13}C NMR spectrum showed signals identical to those described for the catechol moiety of arachidin-1 (Chang et al., 2006). These signals were

thereby assigned as aromatic carbons C-1′ to C-6′. Based on the HMBC and HMQC cross peaks of these carbons, the three ^{1}H NMR signals at δ_H 6.974 (d, J = 2.0 Hz, H-2′), 6.737 (d, J = 8.2 Hz, H-5′), and 6.835 (dd, J = 8.2 and 2.0 Hz, H-6′) were assigned as the corresponding protons. The olefin carbons C-α (δ_C 126.75) and C-α′ (δ_C 129.69) showed HMQC cross peaks with two doublet proton signals at δ_H 6.721 (J = 16.2 Hz, H-α) and 6.889 (J = 16.2 Hz, H-α′), whose coupling constants confirmed the *trans*-olefin. Both of these protons showed cross peaks with aromatic carbons C-1′ and C-1 (δ_C 140.23). Proton H-α also showed HMBC cross peaks with ^{13}C NMR signals δ_C 106.47 (C-6) and 106.79 (C-2). The ^{1}H NMR signals δ_H 6.418 (bs, H-2) and 6.475 (d, J = 1.3 Hz, H-6) were assigned by their HMQC cross peaks to C-2 and C-6, respectively. These chemical shifts and their HMBC cross peaks were comparable to those described for arahypin-5 (Sobolev et al., 2009). Analogous to arahypin-5 and arachidin-1 the carbon signal δ_C 110.23 (C-4) showed HMBC cross peaks with H-2 and H-6 and did not have any HMQC cross peaks, indicating that the prenyl was attached at C-4. The remaining ^{13}C NMR signals were consistent with spectroscopic data of C-3 (δ_C 155.26), C-5 (δ_C 154.42) and the five carbons of the prenyl group reported for arahypin-5 (Sobolev et al., 2009). The signal of C-3″ (δ_C 76.77) was downfield compared to arachidin-1, which indicated that the prenyl was cyclised to a pyran as in arahypin-5. The remaining ^{1}H NMR signals and their HMBC cross peaks corresponded to those described for arahypin-5 (Sobolev et al., 2009). To the best of our knowledge the elucidated structure, a ring-prenylated piceatannol derivative, has not been previously reported. Consequently, compound **4** was identified as the new compound**,** 7-[(*E*)-2-(3,4-dihydroxyphenyl)ethenyl]-2,2-dimethylchromen-5-ol, herein named arachidin-6. The structures of compounds **1**-**6** are shown in **Figure 1B**.

Prenyl chains in (iso)flavonoids and chalcones have been described to be liable to cyclisation under acidic conditions (Popłoński et al., 2018; Singhal et al., 1980). Thus, it might be argued compound **4** might be an artefact formed due to the addition of 1% (*v/v*) formic acid in the

preparative chromatography eluent. Acid-catalysed cyclisation of a prenyl chain, however, typically yields a dihydropyran (Popłoński et al., 2018; Singhal et al., 1980), whereas the prenyl in compound **4** is present as a pyran as evidenced by the structure elucidation. It was, therefore, concluded that the compound as such was present in the elicited peanut seedlings.

2.3 Stability and Antibacterial Activity of Prenylated Stilbenoids

During the purification process, the prenylated stilbenoids were prone to both *trans-cis* isomerisation, as was previously reported (Trela and Waterhouse, 1996), and dimerization. The compounds' reactivity could potentially be of influence on their antimicrobial activity. Therefore, the stability of the compounds during the antibacterial assay was assessed by incubating them under normal assay conditions. Under these conditions, no further *trans* to *cis* isomerisation or dimerization was observed for compounds **2**-**6** (**Figure S2**, supplementary data). Arachidin-1 (**1**) was not included due to low amounts of available material. However, we assumed its stability during the assay would be similar to that of the other five compounds. Thus, we did not expect any influence of isomerization or dimerization on the antibacterial assay results.

The antibacterial activity of arachidin-1 (**1**), arachdin-2 (**2**), arachidin-3 (**3**), arachidin-6 (**4**), arahypin-5 (**5**), and chiricanine A (**6**) against MRSA was assessed. For reference, non-prenylated stilbenoids piceatannol (precursor of arachidin-1 (**1**) and -6 (**4**)), resveratrol (precursor of arachidin-2 (**2**) and -3 (**3**) and arahypin-5 (**5**)), and pinosylvin (precursor of chiricanine A (**6**)) were also tested. The resulting MIC and TTD values are shown in **Table 3**, together with some structural properties of these molecules. For resveratrol and piceatannol, no MIC was found below 200 µg mL^{-1}. Therefore, they were considered not to be active antibacterials. The attachment of a prenyl-moiety to these precursors increased the resulting molecule's antibacterial activity, as expected. For example, resveratrol had an MIC of > 200 µg mL^{-1} (> 877 µM) and its ring-prenylated derivative arahypin-5 (**5**) had an MIC of 25-50 µg

144 mL^{-1} (85-170 µM), thus the prenyl group enhanced activity by up to ten fold in this case. No
145 MIC was found below 50 µg mL^{-1} for arachidin-1 (**1**), arachidin-2 (**2**), or arachidin-3 (**3**). Based
146 on the TTD_{50} (time-to-detection at 50 µg mL^{-1}) values of these compounds, arachidin-2 (**2**) was
147 the most active of the three. In addition, the TTD_{50} and TTD_{25} values obtained for these
148 compounds indicated that they were less active than compounds **4**-**6** and, especially arachidin-
149 1 (**1**) and arachidin-3 (**3**), were only marginally more active than non-prenylated resveratrol and
150 piceatannol. The new compound, arachidin-6 (**4**), showed moderate activity with MIC at 50-75
151 µg mL^{-1}. The prenylated pinosylvin derivative, chiricanine A (**6**), was the most active
152 compound tested in this work with an observed MIC of 12.5 µg mL^{-1} (44 µM) against MRSA,
153 which is quite promising and in range of that of some traditional antibiotics (Braga et al., 2005).

154 **2.4 Structure-Activity Relationships of Prenylated Stilbenoids against MRSA**

155 The antimicrobial activity of prenylated phenolics has been related to the increased
156 hydrophobicity conferred by the addition of the prenyl-group (Botta et al., 2005). Chiricanine
157 A (**6**) possessed the highest $LogD_{7.2}$ of the six purified compounds. The anti-MRSA activity of
158 compounds **1**-**6** (expressed as TTD_{25}, using 24 h if TTD > 24 h) was, however, not correlated
159 to their $LogD_{7.2}$ ($r = 0.002$). The lack of a correlation between activity and hydrophobicity has
160 been previously described for prenylated phenolic compounds (Araya-Cloutier et al., 2018b).
161 The configuration of the prenyl-moiety (chain or ring) seems to have an effect on the observed
162 antibacterial activity. Ring prenylation was found to be more effective than chain prenylation,
163 as demonstrated when comparing molecules with the same stilbenoid precursor: arachidin-1 (**1**)
164 (chain, TTD_{25} 6.1 h) vs. arachidin-6 (**4**) (ring, TTD_{25} 11.5 h) and arachidin-2 (**2**) or -3 (**3**) (chain,
165 TTD_{25} 7.4 or 6.6 h, respectively) vs. arahypin-5 (**5**) (ring, TTD_{25} 9.6 – >24 h) (**Table 3**). This
166 was in contrast with previous results which indicated that chain-prenylated (iso)flavonoids were
167 generally more active than ring-prenylated ones (Araya-Cloutier et al., 2018a; Araya-Cloutier
168 et al., 2018b). The effect of a 3-methyl-1-butene (arachidin-1 (**1**) and -3 (**3**)) or a 3-methyl-2-

butene (arachidin-2 (**2**)) type prenyl chain was not completely clear but the higher TTD_{50} of arachidin-2 indicated that a 3-methyl-2-butene chain might result in better activity than a 3-methyl-1-butene chain.

The stilbenoid precursor of the prenylated compounds might also affect their activity. So far, our results indicated that the order of activity for the three tested precursors is pinosylvin > resveratrol > piceatannol. Within the group of compounds **1**-**6**, the number of hydrogen bond donors is negatively correlated with TTD_{25} ($r = -0.89$), i.e. having less hydrogen bond donors seems to result in better anti-MRSA activity. This matches previously described results for pterostilbene (i.e. 3,5-dimethyl-resveratrol, MIC 78 µM) which was found to be up to 16 times more active against MRSA than resveratrol in that study (MIC 1,250 µM) (Yang et al., 2017).

The structure-antibacterial activity relationships described above indicate that prenylated stilbenoids from the pinosylvin precursor seem to be most potent. In addition, ring prenylation seems to confer more antibacterial potential than chain prenylation. A candidate anti-MRSA molecule reported in literature would be arahypin-13 (ring-prenylated pinosylvin) (Sobolev et al., 2016). This molecule might show similar or even more potent activity than chiricanine A. To establish quantitative structure-activity relationships, evaluation of a larger set of compounds will be necessary.

3. Conclusion

Similarly to other phenolic compounds, prenylation of stilbenoids enhances their antibacterial activity. Prenylated stilbenoids represent a group of natural potential antibacterials with activity against the antibiotic-resistant gram-positive bacterium MRSA. The newly discovered prenylated stilbenoid, arachidin-6, was moderately potent with an MIC of 50-75 μg mL^{-1} against MRSA, whereas the activity of chiricanine A, with its MIC of 12.5 μg mL^{-1}, is in range with some traditional antibiotics. Within the set of stilbenoids assessed in this work prenylation enhanced antimicrobial activity. Hydrophobicity was not correlated with antimicrobial activity, whereas ring-prenylation seems to convey a larger increase in activity than chain-prenylation.

4. Experimental

4.1 General Experimental Procedures

NMR Spectra were recorded on a Bruker Avance-III-600 spectrometer, equipped with a cryo-probe. Compounds **1**, **2**, **4**, and **5** were dissolved in 0.5 mL methanol-d_4 (99.9 atom%, Isotec); compounds **3** and **6** were dissolved in 0.5 mL chloroform-d (99.9 atom%, Isotec). ^{1}H and ^{13}C NMR spectra were recorded at a probe temperature of 300 K. Chemical shifts are expressed in ppm relative to internal TMS at 0.00 ppm, but were actually measured to the residual solvent signals of methanol (δC = 49.00 ppm, δH = 3.31 ppm) or chloroform (δC = 77.16 ppm, δH = 7.26 ppm). For all compounds, 1D ^{1}H spectra were acquired. For compound **4**, additional 1D ^{13}C and 2D COSY, HMBC, and HMQC spectra were acquired. ESI-IT-MS^n spectra were acquired on an LTQ Velos Pro linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a heated ESI probe coupled *in-line* to the Accela RP-UHPLC system (Thermo Scientific). The Accela UHPLC system was used in the same configuration as previously described (de Bruijn et al., 2016). The flow rate was 300 µL min^{-1} at a column temperature of 35 °C. Eluents used were water (A) and MeOH (B), both with 0.1% (*v/v*) formic acid. The elution profile can be found in the supplementary data. Detection wavelengths for UV−Vis were set to the range of 200-600 nm and data were recorded at 20 Hz. High resolution mass data were acquired on a Thermo Q Exactive Focus hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific) equipped with a heated ESI probe (ESI-FTMS) coupled *in-line* to the Vanquish RP-UHPLC system. The Vanquish UHPLC system (Thermo Scientific) in the same configuration and with the same column as described previously (de Bruijn et al., 2016). The samples were eluted with water (A) and ACN (B), both with 0.1% (*v/v*) formic acid with a flow rate of 400 µL min^{-1} at 45 °C. The elution profile can be found in the supplementary data. A Waters Acquity BEH C18 2.1 × 150 mm, 1.7 µm particle size column with a Waters VanGuard 2.1 × 5 mm guard column of the same material was used for all analytical RP-

UHPLC separations. Flash chromatography was performed on a Reveleris Flash chromatography system (Grace, Columbia, MD, USA). A Reveleris C18 RP 80 g cartridge (particle size 40 µm) was eluted with water (A) and MeOH (B), both with 1% (*v/v*) formic acid, at room temperature at a flow rate of 60 mL min^{-1}. Preparative chromatography was performed on a Waters preparative RP-HPLC-MS system (Waters, Milford, MA, USA) as previously described (van de Schans et al., 2016). The column used was a Waters XBridge Prep C18 OBD column (19 × 250 mm, 5 µm particle size) and was eluted with water (A) and ACN (B), both with 1% (*v/v*) formic acid, at room temperature at a flow rate of 17 mL min^{-1}.

4.2 Chemicals

Sodium hypochlorite 47/50% (*w/v*) solution (~13% active Cl) was obtained from Chem-Lab (Zedelgem, Belgium). Technical grade *n*-hexane 98% (*v/v*) was obtained from VWR International (Radnor, PA, USA). UHPLC-MS grade solvents and HPLC grade ACN (for preparative chromatography) were purchased from Biosolve (Valkenswaard, The Netherlands). *trans*-Piceatannol >98% (*w/w*) and *trans*-resveratrol ≥98% (*w/w*) were purchased from Cayman Chemical (Ann Arbor, MI, USA). *trans*-Pinosylvin ≥97% (*w/w*) and *tert*-butanol ≥98% (*w/w*) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water (MQ) for other purposes than UHPLC was prepared using a Milli-Q water purification system (Merck Millipore, Billerica, MA, USA). Ethanol absolute ≥99.9% (*v/v*) was purchased from Merck Millipore.

4.3 Plant and Fungal Material

Peeled peanuts (*Arachis hypogaea*) with skin for feed purposes were purchased locally. Dried *Rhizopus* culture was purchased as tempeh starter culture from TopCultures (Zoersel, Belgium).

241 **4.4 Peanut Germination and Elicitation with *Rhizopus***

242 Peanuts were surface-sterilized by soaking in a 1% (*w/v*) hypochlorite solution (5 L kg^{-1} seeds)
243 for 15 min at room temperature and were then rinsed with demineralized water. Surface-
244 sterilized peanuts were germinated in the dark in a pilot-scale two-tank steep germinator
245 (Custom Laboratory Products, Keith, UK). The cleaning procedure, trays, and setup of the
246 germinator were the same as described previously (de Bruijn et al., 2016). In total 3 kg of
247 peanuts were germinated in two identical experiments. Approximately 300 g peanuts were
248 placed in each of the five tray compartments that were lined with disinfected cellulose filter
249 paper (Whatman 595 ½, folded, 320 mm). The program set in the germinator was as follows:
250 soaking for 16 h at 25 °C (aeration 1 min every 10 min), followed by germination for 48 h at 25
251 °C. The germinating peanuts were then inoculated by pouring the *Rhizopus* starter culture (0.2
252 L kg^{-1} peanuts equalling approximately 2×10^5 CFU g^{-1} peanuts) over them. Prior to
253 application, the starter culture was rehydrated with peptone physiological salt solution (Tritium
254 Microbiologie, Eindhoven, The Netherlands) (100 mg mL^{-1}) and incubated for 1 h at 37 °C.
255 After fungal inoculation, peanuts were incubated for 72 h at 30 °C. During the entire process,
256 relative humidity (RH) was controlled by periodical aeration with humidified air, RH of the air
257 supplied to the tanks was maintained between 60-83% (monitored by germinator), resulting in
258 RH of 82-95% inside the tanks (measured externally). The described conditions proved to be
259 effective to ensure germination of the seeds as well as growth of the fungus. At the end of the
260 experiment, the peanuts were frozen and stored at -20 °C until further processing.

261 **4.5 Extraction and Isolation**

262 Peanut seedlings were extracted according to a method adapted from Sobolev and co-
263 workers.(Sobolev et al., 2009) In short, the elicited peanut seedlings were defatted by
264 continuous Soxhlet extraction with *n*-hexane for 5 h. The defatted peanut seedlings were then
265 extracted with MeOH in a blender for 1 min (1 L MeOH per 200 g seedlings). The suspension

was filtered over a paper filter under reduced pressure and the retentate peanut pulp was subjected to one more identical extraction. The methanolic extracts were combined and defatted once more by liquid-liquid partitioning with *n*-hexane (hexane:MeOH, 1:3). The defatted methanolic extract was filtered over cellulose filter paper (Whatman 595 ½, folded, 320 mm) and the MeOH was evaporated under reduced pressure to yield the crude extract. Exposure to light was avoided where possible during further sample preparation and purification. In order to remove polar impurities, the dried crude extract was then suspended in ethyl acetate (33 mg mL^{-1}), subjected to an ultrasonic bath for 10 min, and centrifuged (10 min, 4,000 × *g*). The supernatant, containing the prenylated stilbenoids, was collected while the pellet, containing mostly polar impurities, was discarded. The supernatant was evaporated to dryness under reduced pressure, recollected using *tert*-butanol and lyophilised to yield the cleaned extract. Cleaned extracts were pre-purified using Flash chromatography. The sample was solubilised in MeOH acidified with 1% (*v/v*) FA (final sample concentration 300-500 mg mL^{-1}) and was manually injected (1.0-2.5 g per run). The elution profile used can be found in the supplementary data. The collected fractions were analysed by RP-UHPLC-PDA-ESI-IT-MS^n and those containing similar compounds were pooled. The MeOH was removed under reduced pressure and the remaining water was removed by lyophilisation.

The pools obtained from Flash chromatography were further purified using a Waters preparative RP-HPLC system (Waters, Milford, MA, USA) as previously described (van de Schans et al., 2016). Pools were solubilised at 2 mg mL^{-1} in 50% (*v/v*) aqueous MeOH and injected (2.0-2.5 mL) on a Waters XBridge Prep C18 OBD column (19 × 250 mm, 5 µm particle size) and were eluted with water (A) and ACN (B), both with 1% (*v/v*) formic acid, at room temperature at a flow rate of 17 mL min^{-1}. The elution profile used for each compound can be found in the supplementary data. Data was acquired and analysed by MassLynx (version 4.1, Waters). The collected fractions were analysed by RP-UHPLC-PDA-ESI-IT-MS^n. Based on

the analysis, fractions containing the same compound were pooled and the ACN was evaporated under a stream of N_2. The remaining water phase was immediately frozen and lyophilised.

4.6 Micro-Broth Dilution Assay

Compounds **1**-**6** were tested for their antibacterial activity against the gram-positive bacterium methicillin-resistant *Staphylococcus aureus* (MRSA) (18HN, spa type t034; RIVM, Bilthoven, The Netherlands), according to a previously described method (Araya-Cloutier et al., 2017). The final inoculum size used was 4.2 ± 0.2 $\log_{10}$ CFU mL^{-1}. Final concentrations tested ranged from 6.25 to 200 µg mL^{-1}, depending on the amount of material available, with a maximum of 2.1% (*v/v*) ethanol in the final solution. Stock solutions of the tested compounds were prepared in 70% (*v/v*) aqueous ethanol. Prior to the micro-broth dilution assay, the stocks were diluted in tryptone soy broth (TSB) (Oxoid, Basingstoke, UK). Equal volumes (100 µL) of the diluted compound and of inoculum were transferred to each well of a 96-well plate. Inoculum with vancomycin (VWR International) (final concentration of 4 µg mL^{-1}) was used as a positive control. Inoculum with sterile TSB containing 2.1% (*v/v*) ethanol was used as a negative control. All compounds were tested in at least two independent biological replicates. The 96-well plate was covered with a gas-permeable imaging seal (4Titude, Wotton, UK) and incubated in a SpectraMax M2e (Molecular Devices, Sunnyvale, CA, USA) at 37 °C for 24 h with constant linear shaking. The optical density at 600 nm (OD_{600}) was measured every 5 min. Time-to-detection (TTD) was defined as the time (h) to reach a difference of 0.05 units from the initial OD_{600} (Araya-Cloutier et al., 2017; Araya-Cloutier et al., 2018b). If no measurable increase in OD_{600} was observed, the TTD was defined as >24 h and the tested concentration was considered to be inhibitory. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of each compound that was found to be inhibitory. The TTD of the negative control with 2.1% (*v/v*) ethanol was 5.6 h (standard deviation ± 0.1 h) (average of four

315 independent biological replicates, each with triplicates). For comparison of the compounds,

316 TTD at concentrations of 25 and 50 μg mL^{-1}, respectively TTD_{25} and TTD_{50}, were determined.

Supplementary data

Elution profiles used for preparative and analytical liquid chromatography. UHPLC-ESI-IT-MS chromatograms of cleaned peanut extract and Flash pools (**Figure S1**). UHPLC-PDA chromatogram stability assessment of compounds **2**-**6** under antibacterial assay conditions (**Figure S2**).

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Figures

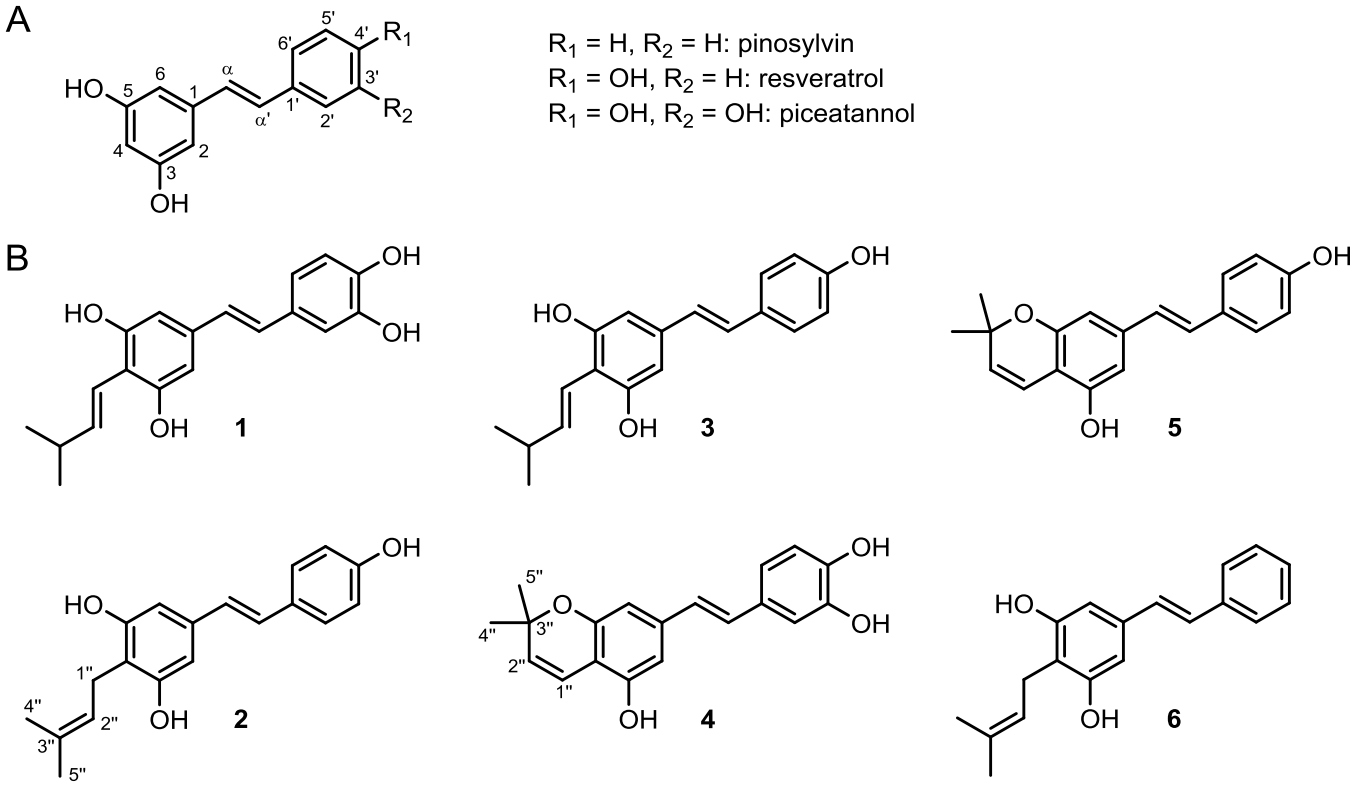

Figure 1. A, natural stilbenoids with a stilbene backbone. B, prenylated stilbenoids isolated in this work: **1**, arachidin-1; **2**, arachidin-2; **3**, arachidin-3; **4**, arachidin-6; **5**, arahypin-5; and **6**, chiricanine A.

Table 1. Spectrometric and spectroscopic data of purified compounds as determined by UHPLC-PDA coupled to ESI-IT-MS and ESI-FTMS.

			IT-MS				FTMS	
compound [a]	**λ_{max} (nm)**	**ionisation**	***m/z* precursor**	**MS^2 product ions (relative abundance)** [b]	**molecular formula**	***m/z* calc.**	***m/z* obs.** [c]	**error (ppm)**
1	339	$[M-H]^-$	311	241, 242 (93), 312 (76), 255 (46), 267 (45), 311 (31), 293 (25), 224 (20), 172 (16)	$C_{19}H_{20}O_4$	311.12888	311.12907	0.60
		$[M+H]^+$	313	257				
2	322	$[M-H]^-$	295	239, 296 (55), 240 (43), 226 (42), 295 (38)	$C_{19}H_{20}O_3$	295.13397	295.13388	-0.30
		$[M+H]^+$	297	241				
3	338	$[M-H]^-$	295	239, 240 (48), 226 (31), 295 (26), 227 (25), 251 (18)	$C_{19}H_{20}O_3$	295.13397	295.13402	0.18
		$[M+H]^+$	297	241				
4	342	$[M-H]^-$	309	309, 310 (63), 265 (60), 291 (24), 294 (21), 281 (18)	$C_{19}H_{18}O_4$	309.11323	309.11334	0.35
		$[M+H]^+$	311	201, 283 (55), 135 (50), 187 (40), 177 (30), 293 (29), 123 (28), 269 (25), 175 (22), 183 (20), 173 (19), 265 (17), 189 (16), 202 (16)				
5	339	$[M-H]^-$	293	293, 278 (47), 294 (35)	$C_{19}H_{18}O_3$	293.11832	293.11847	0.52
		$[M+H]^+$	295	201, 267 (93), 253 (51), 175 (37), 107 (37), 277 (32), 183 (28), 225 (24), 239 (23), 173 (19), 159 (18), 119 (18), 249 (15)				
6	312	$[M-H]^-$	279	224, 223 (85), 279 (66), 280 (50), 211 (15)	$C_{19}H_{20}O_2$	279.13905	279.13914	0.31
		$[M+H]^+$	281	225				

[a] Data provided based on the *trans* isomer.

[b] Most abundant fragment is underlined, only fragment ions with a relative abundance of at least 15 are shown.

[c] Based on the average of 5 spectra in negative ionisation mode.

Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Spectroscopic data of arachidin-6 (**4**) recorded in methanol-d_4 at 300K (δ in ppm, *J* in Hz).

arachidin-6 **(4)**		
position	δ_C, type	δ_H, mult. (*J* in Hz) [a]
1	140.23, C	
2	106.79, CH	6.418, bs
3	155.26, C	
4	110.23, C	
5	154.42, C	
6	106.47, CH	6.475, d (1.3)
α	126.75, CH	6.721, d (16.2)
α′	129.69, CH	6.889, d (16.2)
1′	131.02, C	
2′	113.83, CH	6.974, d (2.0)
3′	146.51, C	
4′	146.60, C	
5′	116.43, CH	6.737, d (8.2)
6′	120.27, CH	6.835, dd (8.2, 2.0)
1″	118.23, CH	6.626, d (9.9)
2″	129.10, CH	5.568, d (9.8)
3″	76.77, C	
4″	28.01, CH_3	1.391, s
5″	28.01, CH_3	1.391, s

[a] bs, broad singlet; d, doublet; dd, doublet of doublets; s, singlet.

Table 3. Structural characteristics, purity, and antibacterial activity of piceatannol, resveratrol, pinosylvin and the purified prenylated stilbenoids against MRSA.

compound	precursor	prenyl configuration, type	no. of H-bond donors	$LogD_{7.2}$ [a]	purity (%) [b]			MIC (µg mL^{-1})	TTD_{25} (± StDev) (h) [c]	TTD_{50} (± StDev) (h) [c]
					UV_{310}	MS (NI)	^1H NMR			
Piceatannol	n.a.	n.a.	n.a.	3.06	99	99	n.d.	>200	5.4 (± 0.1)	6.3 (± 0.3)
Resveratrol	n.a.	n.a.	n.a.	3.38	≥99	98	n.d.	>200	6.0 (± 0.1)	6.9 (± 0.7)
Pinosylvin	n.a.	n.a.	n.a.	3.69	≥99	≥99	n.d.	≤100 [d]	n.d. [d]	n.d. [d]
Arachidin-1	pice	chain, 3m1b	4	4.93	87	88	80	>50	6.1 (± 0.6)	8.4 (± 0.5)
Arachidin-2	resv	chain, 3m2b	3	5.10	98	89	95	>50	7.4 (± 0.5)	17.1 (± 3.0)
Arachidin-3	resv	chain, 3m1b	3	5.23	80	63	60	>50	6.6 (± 1.2)	8.6 (± 1.8)
Arachidin-6	pice	ring	3	4.27	96	96	n.d.	50-75 [e]	11.5 (± 2.2)	21.5 (± 0.5) – >24 [e]
Arahypin-5	resv	ring	2	4.58	99	94	80	25-50 [e]	9.6 (± 4.2) – >24 [e]	>24 (± n.a.)
Chiricanine A	pino	chain, 3m2b	2	5.40	99	98	99	12.5 [e]	>24 (± n.a.)	>24 (± n.a.)

n.a., not applicable; n.d., not determined; resv., resveratrol; pice, piceatannol; pino, pinosylvin; 3m1b, 3-methyl-1-butene, 3m2b; 3-methyl-2-butene.

[a] $LogD_{7.2}$, calculated octanol-water partitioning coefficient at pH 7.2, as calculated using MarvinSketch 17.2.27 with default settings.

[b] Combined purity of the *trans* and *cis* isomers of the purified compounds. UV_{310}, based on total peak area in UHPLC-PDA at 310 nm; MS (NI), based on total peak area in UHPLC-ESI-MS negative ionisation mode (*m/z* range 200-1500); ^1H NMR, based on the aromatic region in proton NMR spectroscopy.

[c] TTD_{25}, time-to-detection at 25 µg mL^{-1}; TTD_{50}, time-to-detection at 50 µg mL^{-1}.

[d] Pinosylvin was only tested in the concentration range 100-400 µg mL^{-1} (one biological experiment, triplicate measurement).

[e] Determined in four independent biological replicates, ranges indicate that MIC and TTD varied between experiments.

Supplementary data with "Antibacterial activity of prenylated stilbenoids from peanut" by de Bruijn, Araya-Cloutier, Bijlsma, de Swart, Sanders, de Waard, Gruppen, and Vincken

Elution profiles reversed-phase chromatography

Pre-purification by RP-Flash Chromatography

For Flash chromatography, samples were injected on a Reveleris C18 RP 80 g cartridge (particle size 40 µm) and eluents used were water (A) and MeOH (B), both with 1% (*v/v*) formic acid. Elution program: Isocratic at 48% B for 2.2 min, linear gradient to 80% B from 2.2-74.8 min, linear gradient to 100% B from 74.8-77.0 min, isocratic at 100% B from 77.0-88.0 min.

Preparative RP-HPLC-ESI-MS

For preparative HPLC, samples were injected on a Waters XBridge Prep C18 OBD column (19 × 250 mm, 5 µm particle size) (Waters, Milford, MA, USA) and eluents used were water (A) and ACN (HPLC-R grade) (B), both with 1% (*v/v*) formic acid. The elution programs for the different compounds were as follows:

Compound **1**: Isocratic at 37% B for 3.68 min, linear gradient to 47% B from 3.68-38.68 min, linear gradient to 100% B from 38.68-42.18 min, isocratic at 100%B from 42.18-59.69 min, linear gradient to 37% B from 59.69-63.19 min, isocratic at 37% B from 63.19-80.96 min.

Compounds **2**, **3**, **4** and **5**: Isocratic at 38% B for 3.68 min, linear gradient to 48% B from 3.68-38.68 min, linear gradient to 100% B from 38.68-42.18 min, isocratic at 100% B from 42.18-59.69 min, linear gradient to 38% B from 59.69-63.19 min, isocratic at 38% B from 63.19-80.96 min.

Compound **6**: Isocratic at 42% B for 3.68 min, linear gradient to 52% B from 3.68-38.68 min, linear gradient to 100% B from 38.68-42.18 min, isocratic at 100% B from 42.18-59.69 min, linear gradient to 52% B from 59.69-63.19 min, isocratic at 52% B from 63.19-80.96 min.

Supplementary data with "Antibacterial activity of prenylated stilbenoids from peanut" by de Bruijn, Araya-Cloutier, Bijlsma, de Swart, Sanders, de Waard, Gruppen, and Vincken

Analytical RP-UHPLC-PDA-ESI-IT-MS^n

For analytical UHPLC coupled to ESI-IT-MS^n, samples were injected on a Waters Acquity BEH C18 2.1 × 150 mm, 1.7 μm particle size column with a Waters VanGuard 2.1 × 5 mm guard column of the same material. Eluents used were water (A) and MeOH (B), both with 0.1% (*v/v*) formic acid. Elution program: Isocratic at 48% B for 1.45 min, linear gradient to 80% B from 1.45-16.96 min, linear gradient to 100% B from 16.96-18.42 min, isocratic at 100% B from 18.42-25.96 min. The eluent was adjusted to its starting composition in 1.18 min, followed by equilibration for 7.27 min.

Analytical RP-UHPLC-PDA-ESI-FTMS

For analytical UHPLC coupled to ESI-FTMS, samples were injected on a Waters Acquity BEH C18 2.1 × 150 mm, 1.7 μm particle size column with a Waters VanGuard 2.1 × 5 mm guard column of the same material. eluents used were water (A) and ACN (B), both with 0.1% (*v/v*) formic acid. Elution program: Isocratic at 20% B for 0.58 min, linear gradient to 100% B from 0.58-47.18 min, isocratic at 100% B from 47.18-50.10 min.

Supplementary data with "Antibacterial activity of prenylated stilbenoids from peanut" by de Bruijn, Araya-Cloutier, Bijlsma, de Swart, Sanders, de Waard, Gruppen, and Vincken

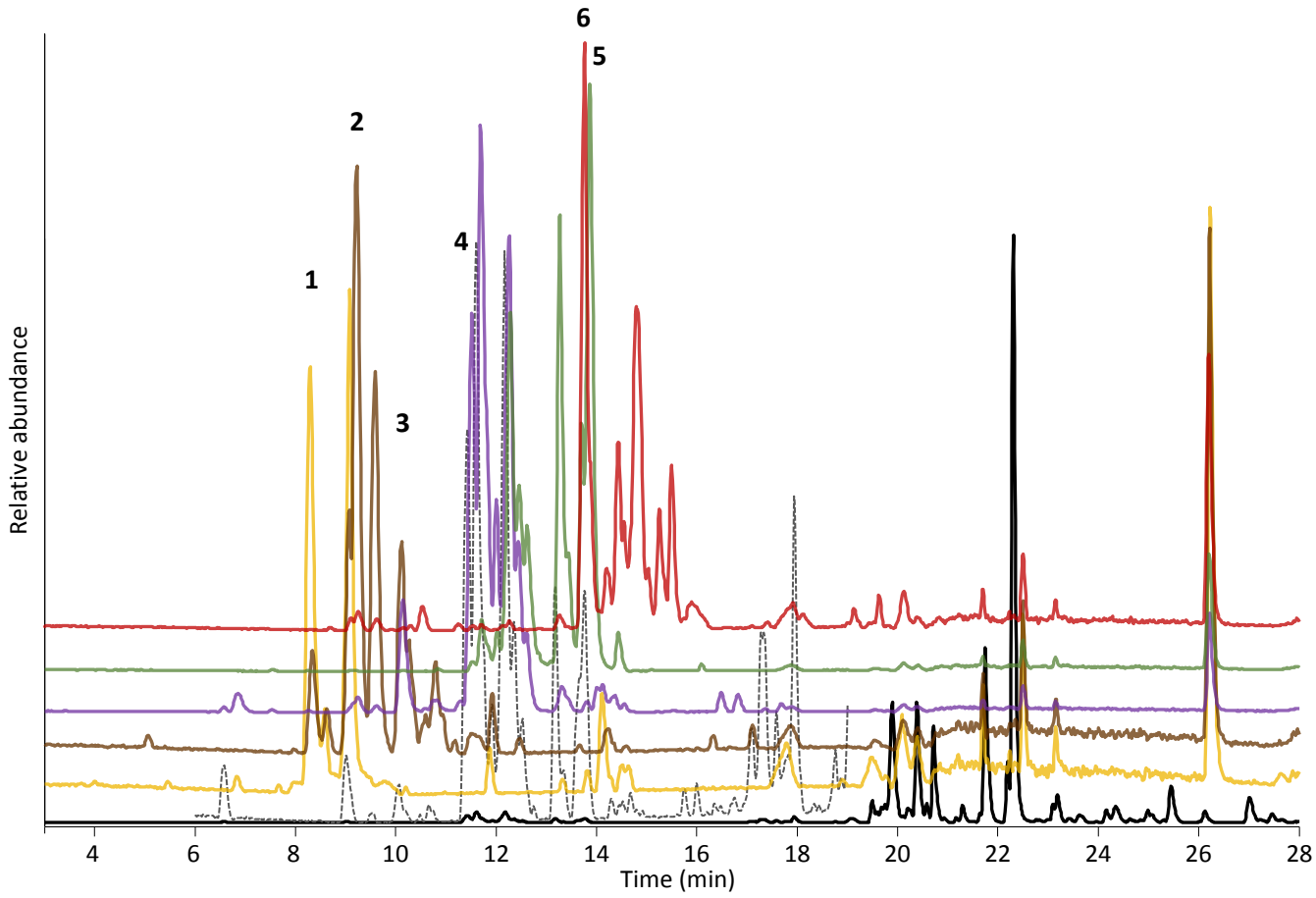

Figure S1. UHPLC-ESI-IT-MS negative mode (*m/z* 250-1000) chromatograms of cleaned peanut extract (black, full chromatogram; grey-dashed, zoom of 6-19 min) and pools obtained after Flash chromatography. Yellow, pool with compound **1**; brown, pool with compounds **2** and **3**; purple, pool with compound **4**; green, pool with compound **5**; and red, pool with compound **6**.

Supplementary data with "Antibacterial activity of prenylated stilbenoids from peanut" by de Bruijn, Araya-Cloutier, Bijlsma, de Swart, Sanders, de Waard, Gruppen, and Vincken

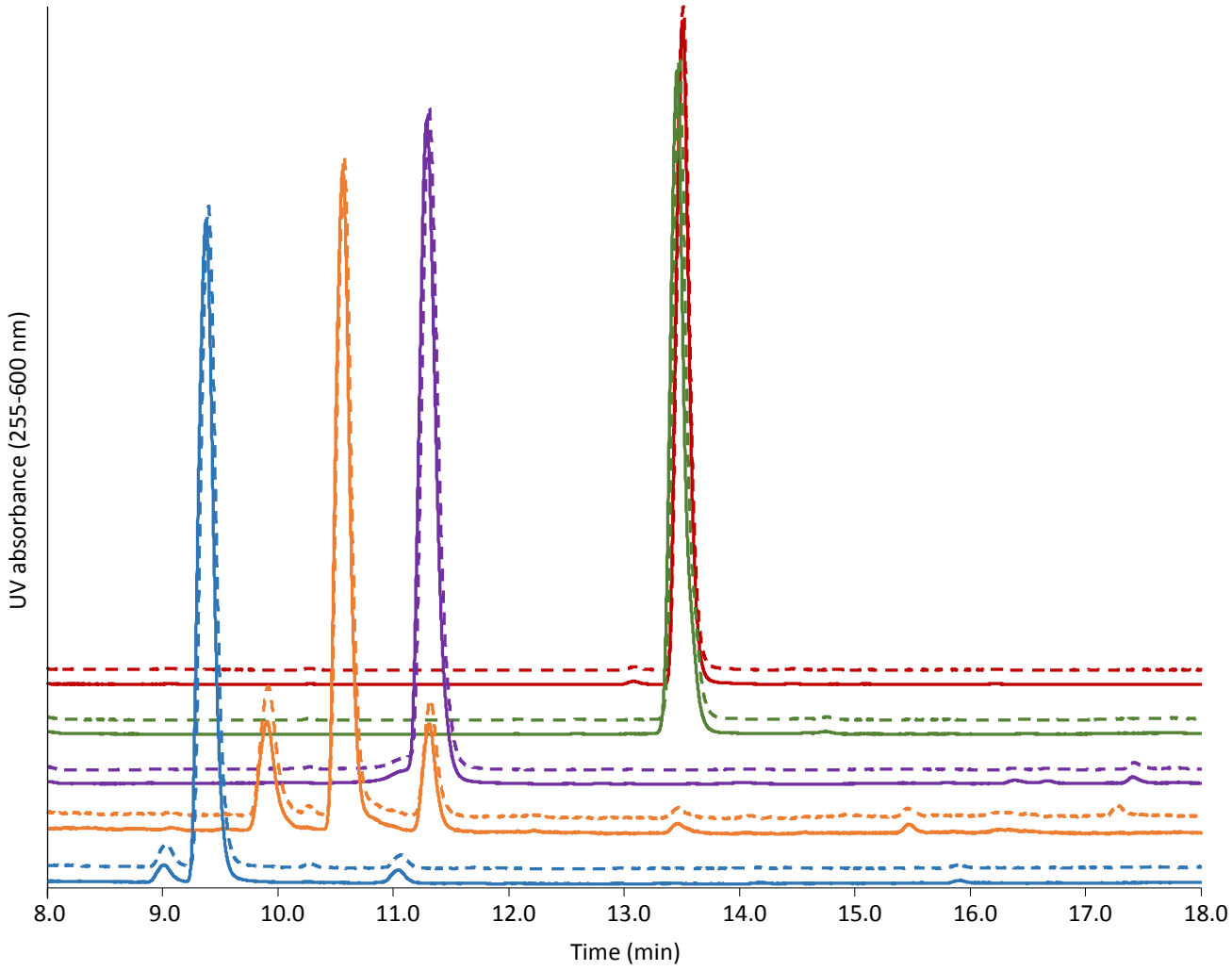

Figure S2. UHPLC-PDA (255-600 nm) chromatograms of compounds **2**, **3**, **4**, **5** and **6** before (solid line) and after (dashed line) incubation for 24 h under antimicrobial assay conditions. Blue, arachidin-2 (**2**); orange, arachidin-3 (**3**); purple, arachidin-6 (**4**); green, arahypin-5 (**5**); and red, chiricanine A (**6**).