Prenylated stilbenoids and (iso)flavonoids

Synthesis, characterization, and antimicrobial activity against MRSA 18HN

The increasing prevalence of MRSA and its bacterial resistance towards existing antibiotics because of intensive use, overuse and unfinished treatments leads to increased mortality and morbidity in patients with difficult to treat MRSA infections and has a significant impact on a socio-economic level. Natural product research regained prominence as it could provide novel antimicrobial lead compounds with a large chemical diversity and novel mechanisms of actions. For example, prenylated stilbenoids and (iso)flavonoids, found in plants are promising candidates. Prenylation refers to a C5 isoprenoid moiety attached either to an OH functional group or CH backbone to generate *O*- and *C*-prenylated analogues respectively. These compounds have been shown to be potent antimicrobials against Gram-positive and Gram-negative bacteria and yeast. Chemical synthesis of prenylated stilbenoids and (iso)flavonoids allows the preparation of unique prenylated stilbenoids and (iso)flavonoids that are normally rare in nature and difficult to obtain. Also, chemical synthesis has advantages over plant extraction that is normally used to isolate these compounds. In this report, *O*- and *C*-prenylated stilbenoids and (iso)flavonoids were prepared synthetically, characterized, and examined for their anti-MRSA activity *in-vitro* against a clinical isolate of MRSA 18HN in a broth microdilution assay. Subsequently, the same prenylated stilbenoids and (iso)flavonoids were predicted for their anti-MRSA activity using an already existing QSAR model developed on MRSA. Both *in-vitro* and *in-silico* data were then compared. The *in-silico* model was able to predict MIC values that were comparable to those obtained *in-vitro* for prenylated flavanones; 8-prenylnaringenin (24 µg/mL compared to 12.5-25 µg/mL), 6-prenylnaringenin (64 µg/mL compared to 12.5-25 µg/mL) and 7-*O*-prenylnaringenin (17 µg/mL compared to 12.5-25 µg/mL). Unfortunately, *in-vitro* MIC values for both mono *O*- and *C*-prenylated flavones were not obtained due to solubility issues occurring at concentrations from 6.25-50 µg/mL. The prenylated stilbenoid 2-prenylresveratrol exhibited minor anti-MRSA activity *in-vitro* (50 µg/mL). Surprisingly, 7-*O*-prenylnaringenin is the first documented *O*-prenylated flavanone that is active against MRSA 18HN *in-vitro* (12.5-25 µg/mL) and is similar in antimicrobial activity compared to all the synthesized *C*-prenylated flavanones described in this thesis and sparks interest in further examining these *O*-prenylated compounds as novel antimicrobial agents against other Gram-positive bacteria. Hopefully, in the future, structurally various prenylated stilbenoids and (iso)flavonoids can be synthesized and examined for their activity against MRSA and other Gram-positive and potentially Gram-negative bacteria that result in new and more effective therapies.

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Master Drug Innovation: Minor Research Project Natural Antimicrobials

Date: 20-10-2021

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List of abbreviations:

4CL	4-coumarate:CoA ligase
APCI	Atmosphere pressure chemical ionization
C4H	Cinnamic acid 4-hydroxylase
CFU	Final inoculum concentration
CID	Colission-induced ionization
DCM	Dichloromethane/methylenechloride
d	Doublet
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DMAPP	Dimethylallyl phosphate
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
ESI	Electrospray ionization
EtOAc	Ethyl acetate
HPLC	High performance liquid chromatography
Hz	Hertz
J	Joule
LC	Liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
m	Multiplet
MBC	Minimum bactericidal concentration
MEP	2-Methyl-D-erythritol-4-phosphate
MIC	Minimum inhibitory concentration
MOE	Molecular modelling program
MRSA	Methicillin-resistant *Staphylococcus Aureus*
MS	Mass spectrometry
MVA	Mevalonic acid
NI	Negative ionization
NMR	Nuclear magnetic resonance
OD	Optical density
PAL	Phenylalanine ammonia lyase
PE	Petroleum ether (40-60%)
PI	Positive ionization
QSAR	Quantitative structure-activity relationships
RDA	Retro-Diels Alder
s	Singlet
SAR	Structure-activity relationships
TLC	Thin layer chromatography
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TSB	Tryptone soya broth
TTD	Time-to-detection

1 Introduction

The intensive use and/or overuse of antimicrobial agents and unfinished treatments in humans and animals inevitably result in bacterial resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) towards existing antimicrobial agents. As a result, the current situation is recognized a public health problem worldwide. Methicillin-resistance has occurred in *S. aureus* through a mutation of penicillin-binding protein, is transferred between *S. aureus* organisms by bacteriophages and the only medically relevant examples of chromosome-mediated drug resistance by phage transduction[1]. The resulting various MRSA strains have further acquired resistance to most of the beta-lactams class of antibiotics due to the presence of the mecA gene sequence, which is known to generate transpeptidase PB2a that lowers the affinity of the organism to beta-lactam antibiotics[2]. MRSA is a high priority pathogen according to the WHO and is responsible for a wide range of organ-specific infections, the most common being the skin and subcutaneous tissues, followed by invasive infections like meningitis, lung abscess, pneumonia and osteomyelitis all having the possibility of increasing the time of patient hospitalizations and mortality[3]. Treatment of these infections depends on the type of the disease and local *S. aureus* side effect profile but relies heavily on administration of oral antibiotics such as trimethoprim/sulfamethoxazole or intravenous infusion with vancomycin or daptomycin or last-resort therapy like linezolid and tedizolid[4,5]. However, since development of new antibiotics is limited and a costly process, access to new lead compounds is imperative. Natural product research regained prominence in an attempt to provide novel antimicrobial agents[6,7].

1.1 (Iso)flavonoids and stilbenoids

Phytochemicals are natural products and produced by plants through primary or secondary metabolism and play a role in plant growth and defense against predators, pathogens and competitors[8]. They are further divided in subsets including carotenoids and phenolic compounds of which the latter consist of phenolic acids, stilbenoids and flavonoids including their structure numbering (figure 1).

Flavonoids consists of subsets of polyphenolic compounds produced by plants like soybean (*Glycine max*), lupin (*Lupinus* spp) and other legumes that are recognized as for their health-promoting properties[9]. Over 7000 unique flavonoids have been identified with most serving important biological roles[10]. The flavanone naringenin[11] **(1a)** is flavorless, colorless and predominantly found in grapefruit and some other fruits and herbs[12,13] while the flavone chrysin[14] **(2a)** is found in honey and has been known for centuries for being an anti-septic. Mainly flavanones have been studied extensively for their bio functionality, estrogenic[15] and anticancer[16] activity and have been shown to have antimicrobial activity[17,18].

Stilbenoids like resveratrol **3a** exist as monomers and oligomers and can be conjugated to glucosides to from complex structures[19]. They are produced *de novo* in plants to protect from fungal infection and toxins and are present in grapes, peanuts, cranberries, and other botanical sources[20–23]. Resveratrol has relevant biological functions including; anti-tumor[24,25], anti-aging[26] and anti-inflammatory[27]. Although the pharmacological potential of resveratrol and resveratrol-derived compounds have been recognized for decades, they suffer from poor bioavailability and pharmacodynamics that remain incompletely understood and therefore hamper their therapeutic applications[28].

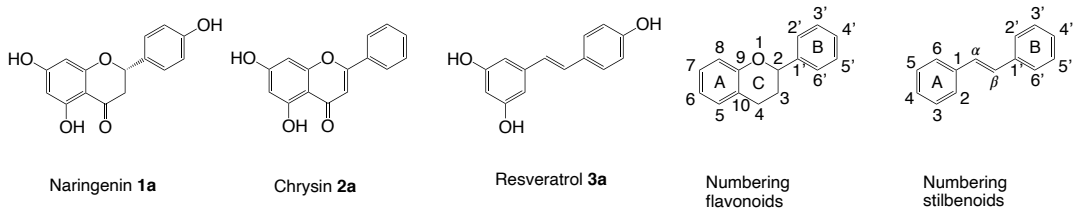


Figure 1: Chemical structures of the flavanone naringenin **1a**, flavanone chrysin **2a**, stillbenoid resveratrol **3a**, universal nomenclature for flavonoids and stillbenoids.

1.2 Structural diversity of prenyl moieties

The chemical diversity of stilbenoids and (iso)flavonoids is increased through the addition of functional groups that include hydroxyl, glycosyl, *O*-methyl and isoprenoid moieties of which the latter is the core subject of this thesis (figure 2). Prenylation is a common modification by which one or more prenyl groups, such as 5-carbon dimethylallyl, 10-carbon geranyl, or 15-carbon farnesyl moieties are attached to stilbenoids and (iso)flavonoids through plant secondary metabolism[29]. *C*-prenylated compounds are found in plants present on both the A- and B-ring of the stilbenoid and (iso)flavonoid backbone[30,31]. Less is known about *O*-

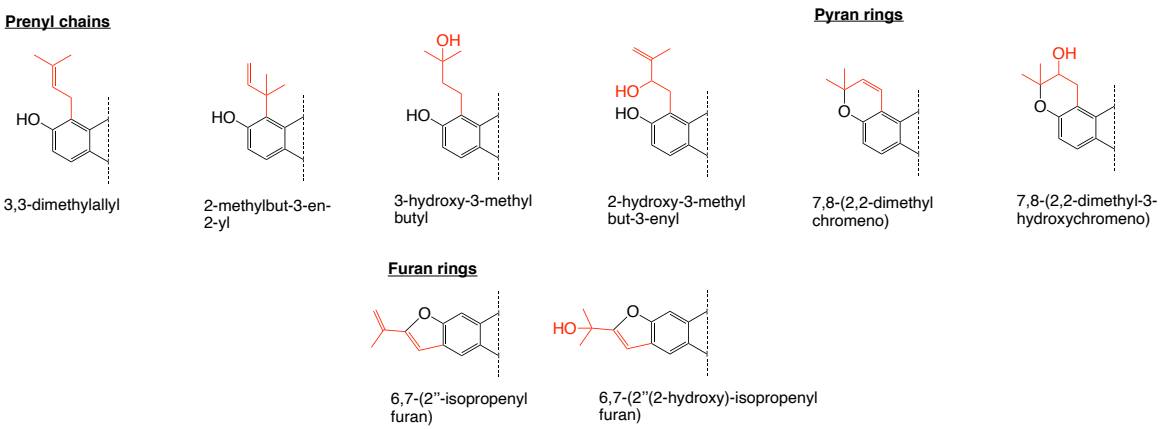


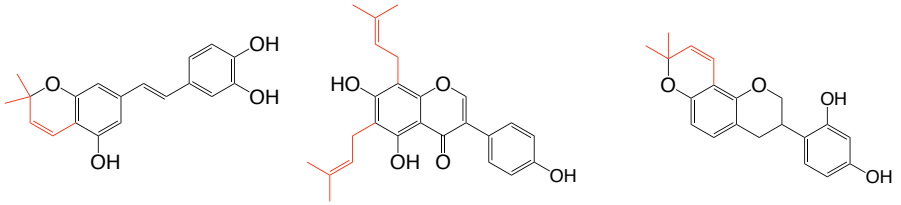
Figure 2: Examples of chain, pyran ring and furan ring prenylation on an aromatic ring with numbering. Hydroxylation gives structurally diverse molecules.

prenyated stilbenoids and (iso)flavonoids and data in the literature is very scarce. Also, it is unknown if they are *O*-prenylated mainly on the A-ring or B-ring and no data is reported regarding their anti-MRSA activity. Finally, *O*-methylation is a common functional group that is present in stilbenoids and (iso)flavonoids but less about their activity against MRSA is known.

Subsequently, varying the prenylation position, length, number and associated modifications like hydroxylation or cyclization creates a large variety of structurally diverse compounds. Prenyl moieties adopt multiple configurations including chain (3-dimethylallyl or 3-methylbut-2-enyl), 5-membered furan ring (2''-isopropenylfuran) or 6-membered pyran ring (2,2-dimethylpyran). Additionally, hydroxylation on prenyl chains (3-hydroxy-3-methylbutyl), pyran (7,8-(2,2-dimethylchromeno))[32] and furan (6,7-(2''-isopropenylfuran))[33] are encountered in nature along with saturated varieties showing the absence of the characteristic prenyl C=C double bond (not shown in figure).

1.3 Prenylated stilbenoids and (iso)flavonoids as natural antimicrobials against Gram-positive bacteria

Prenylated (iso)flavonoids have been studied extensively and show biological[34] and pharmacological activities[35], including anti-inflammatory, anti-microbial[36–39], anti-tumor[40], anti-diabetic[38] and estrogenic[41]. Both prenylated stilbenoids and (iso)flavonoids have varying antimicrobial activities towards Gram-positive bacteria[36]. The position of the prenyl moiety affects the potency of the prenylated stilbenoid as higher activity was observed against Gram-positive bacteria when the prenyl moiety was positioned on the C-4 position of the A-ring instead of the C-3' of the B-ring[42]. For example, ring-prenylated arachidin-6 showed activity against MRSA with minimum inhibitory concentration (MIC) between 50-75 µg/mL but was less potent compared to its chain-prenylated analogue arachidin-1 (MIC: 50 µg/mL)[43] (figure 3). An example of a very potent isoflavone is 6,8-diprenylgenistein (9 µg/mL) having two chain prenyl moieties and the isoflavan glabridin (13 µg/mL) with a single pyran ring prenyl moiety[44]. The latter is used as a positive control in micro broth dilution assays and used to compare the antimicrobial activity of other prenylated compounds.



Arachidin-6
MRSA:
(50-75 ug/ML^{-1})

6,8-diprenylgenistein
MRSA:
(9 ug/ML^{-1})

Glabridin
MRSA:
(13 ug/ML^{-1})

Figure 3: Examples of a ring prenylated stilbenoid arachidin-6, isoflavone 6,8-diprenylgenistein, isoflavan glabridin and their activity against MRSA.

1.4 Structure-activity relationships of prenylated stilbenoids and (iso)flavonoids

Generally, prenylated stilbenoids and (iso)flavonoids have better activity against Gram-positive bacteria and the research presented in this thesis will focus on this[45–48]. Antimicrobial activity data obtained can be used subsequently to determine SAR of prenylated stilbenoids and (iso)flavonoids. Results from SAR-studies against various MRSA strains indicated that ring prenylated compounds have more antibacterial activity against Gram-positive bacteria than chain prenylated compounds and increases the hydrophobicity of the molecule potentially allowing easier diffusion across the bacterial membrane. Interestingly, hydrophobicity was not correlated to antimicrobial activity among the examined monomeric stilbenoids[36]. Additionally, dimers of prenylated stilbenoids such as arahypin-6 could be promising compounds against MRSA[43]. However, to establish quantitative structure-activity relationships evaluation of a larger set of both mono- and dimeric stilbenoid compounds is required.

Interestingly, (iso)flavonoid subclasses do not follow the same SARs. For example, C6-prenylation compared to C8-prenylation is favored for antimicrobial activity for isoflavones whereas the exact opposite is observed for flavanones[49,50]. Similarly, C3'-prenylation is preferred over C6-prenylation in flavanones but the opposite for isoflavones. Unfortunately, data from SARs fail to explain the true correlation between chemical diversity and antimicrobial properties of prenylated (iso)flavonoids. To perform SAR studies, first compounds need to be isolated or synthesized.

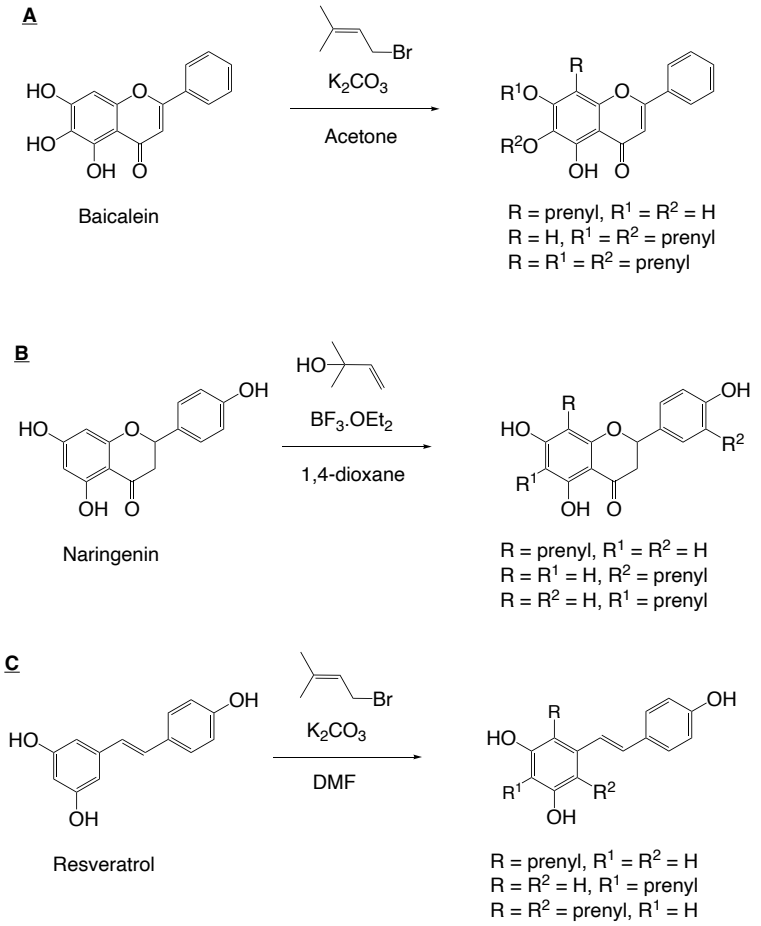
1.5 Chemical synthesis of *O*- and *C*-prenylated stilbenoids and (iso)flavonoids

Prenylated stilbenoids and (iso)flavonoids can be extracted from plant material. However, it requires large amounts of plant material, large amounts of solvent for extractions that are time consuming and laborious and generally the yield of prenylated compounds obtained is very low (several milligrams). Therefore, as an alternative method to obtain a wide variety of prenylated stilbenoids and (iso)flavonoids, chemical synthesis offers a solution to the drawbacks of extraction. Also, using one-pot procedures, multiple compounds can be generated at once. In this section, three one-pot procedures will be described that were found in the literature to generate both *O*- and *C*-prenylated stilbenoids and (iso)flavonoids to give an idea of the possibilities of reactions.

First, described in Neves *et al*., the flavone baicalein was reacted with prenylbromide and K_2CO_3 as weak base in acetone to afford a mixture of mainly *O*- and minor *C*-prenylated baicalein (scheme 1.A)[51]. K_2CO_3 deprotonates the most acidic protons on the aromatic A-ring of baicalein leading to an anion that attacks the electrophilic carbon of prenylbromide to form the *O*-prenylated products; 7-*O*-prenylbaicalein (47% yield), 6,7-bis-*O*-prenylbaicalein (22% yield) and 8-prenyl-6,7-bis-*O*-prenylbaicalein (1% yield).

Next, described in Tahara *et al*., the flavanone naringenin was selectively *C*-prenylated using the Lewis-acid $BF_3.OEt_2$ and 2-methyl-3-buten-2-ol in 1,4-dioxane to afford a mixture of prenylated naringenin derivatives; 6-prenylnaringenin (7% yield), 8-prenylnaringenin (12.8% yield), 3'-prenylnaringenin (1.4% yield) (scheme 1.B)[49]. Compounds were isolated using regular flash chromatography. The mechanism relies on a Friedel-Crafts like mechanism.

Finally, described in Puksasook *et al.* the stilbenoid resveratrol was *C*-prenylated using prenylbromide as reagent and Li_2CO_3 as *ortho*-lithiation base in DMF to afford a mixture of prenylated resveratrol derivatives; 2-prenylresveratrol (62% yield), 4-prenylresveratrol (3%) and 2,6-diprenylresveratrol (5% yield) (scheme 1.C)[52]. Again, compounds were isolated using flash



Scheme 1: Examples of literature for the preparation of a wide variety of *O*- and *C*-prenylated (iso)flavonoids and stilbenoids using chemical synthesis.

chromatography. The above-mentioned reaction conditions to generate both *O*- and *C*-prenylated stilbenoids and (iso)flavonoids are common conditions found in the literature.

1.6 Identification and characterization of prenylated stilbenoids and (iso)flavonoids using MS and NMR

After isolating prenylated stilbenoids and (iso)flavonoids using either plant material extraction or chemical synthesis, the compounds are characterized using liquid chromatography coupled to mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). MS is an analytical technique that coupled to liquid chromatography (LC) is used for the identification of prenylated

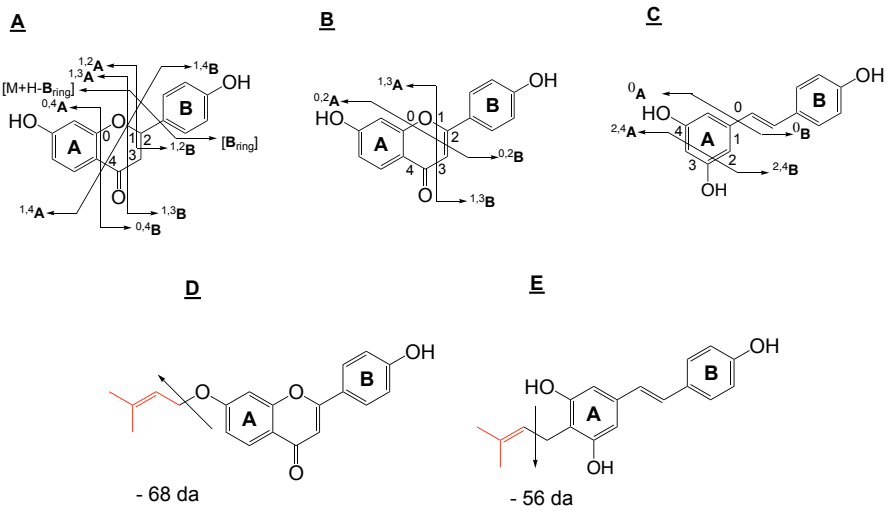


Figure 4: Nomenclature adopted for the various retro Diels-Alder (RDA) fragment possibilities on the unsubstituted backbones of flavanone (A), flavone (B) and stilbenoid (C). Also, the characteristic fragmentation of the *O*-prenyl moiety (D) and *C*-prenyl moiety (E) are presented.

(iso)flavonoids and stilbenoids. Soft ionization techniques like electrospray ionization (ESI) are generally preferred due to their higher sensitivity and are excellent for analyzing prenylated stilbenoids and (iso)flavonoids[53,54]. Soft ionization proceeds with protonation of the analyte in PI (positive ionization) mode leading to a positively charged molecule indicated by $[M+H]^+$ or deprotonation of the analyte in NI (negative ionization) leading to a negatively charged molecule indicated by $[M-H]^-$. The latter is considered more sensitive and selective in flavonoid analysis[55,56]. Further structural information can be acquired by applying collision-induced dissociation (CID) resulting in the fragmentation of the charged analyte. Fragmentation of stilbenoids and (iso)flavonoids are characterized through *retro*-Diels Alder (RDA) mechanisms.

A-ring and B-ring fragments are indicated either $^{i,j}A^+$ and $^{i,j}B^+$ for PI mode and $^{i,j}A^-$ and $^{i,j}B^-$ for NI mode fragmentation. The superscripts ^{i,j} represent the bonds that are cleaved in the C-ring[57,58] (figure 4). The most common cleavage both in PI and NI mode in flavanones and flavones is of the C-ring where the 1/3 bonds are cleaved[59]. However, C-ring cleavage in flavanones and flavones is limited in NI mode[60,61]. The main fragmentation of *O*-prenylated compounds is characterized by the neutral loss of 68 da indicating complete cleavage of the prenyl moiety and for *C*-prenylated compounds a neutral loss of 56 da indicating partial cleavage. For the compounds that are described in this thesis, MS is used to determine whether prenyl moieties are *O*- and/or *C*-prenylated.

Nuclear magnetic resonance (NMR) is a powerful analytical technique used for determining the content and purity of a sample as well as its molecular structure. The principle behind NMR is that many nuclei have spin, and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level (generally a single energy gap). The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency. The signal that matches this transfer is measured in many ways and processed to yield an NMR spectrum for the nucleus concerned. ^{1}H-NMR has characteristic proton signals for the prenyl moiety (figure 5). Mainly the C-1'' of the prenyl moiety is located as a doublet at 4.8 ppm followed by the C-2'' proton as a triplet of triplets between 5.2 to 5.6 ppm. Finally, the two CH_3-groups are present between 1.6 and 1.8 ppm in the aliphatic region[62].

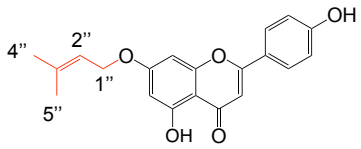


Figure 5: Assigning of characteristic ^{1}H-NMR signals of the prenyl moiety. Flavone taken as example structure.

1.7 Broth microdilution assay to determine antimicrobial activities of prenylated stilbenoids and (iso)flavonoids

After characterization of prenylated stilbenoids and (iso)flavonoids, they can be assessed for their anti-MRSA activity in a broth micro-dilution assay. It is a quantitative measure for antimicrobial activity where minimum inhibitory concentration (MIC) is determined[63]. Wells are filled with target compounds after which target bacteria dissolved in media are added and the growth of these bacteria measured as an increase in optical density (OD) at a wavelength of 600 nm over time using a spectrophotometer. If an increase in compound concentration results in a decrease in bacterial growth than that means the compound has inhibitory properties hence MIC. The lowest concentration of a compound killing 99% of the bacteria is considered a minimum bactericidal concentration (MBC). The advantage of this method is that multiple microorganisms and multiple antimicrobials can be tested at once and is highly accurate and comparable to agar dilution assays. Additionally, time to detection (TTD) of bacterial growth[42] can be determined as well and is generally for compounds that have some kind of activity but not enough to be inhibitory or bactericidal.

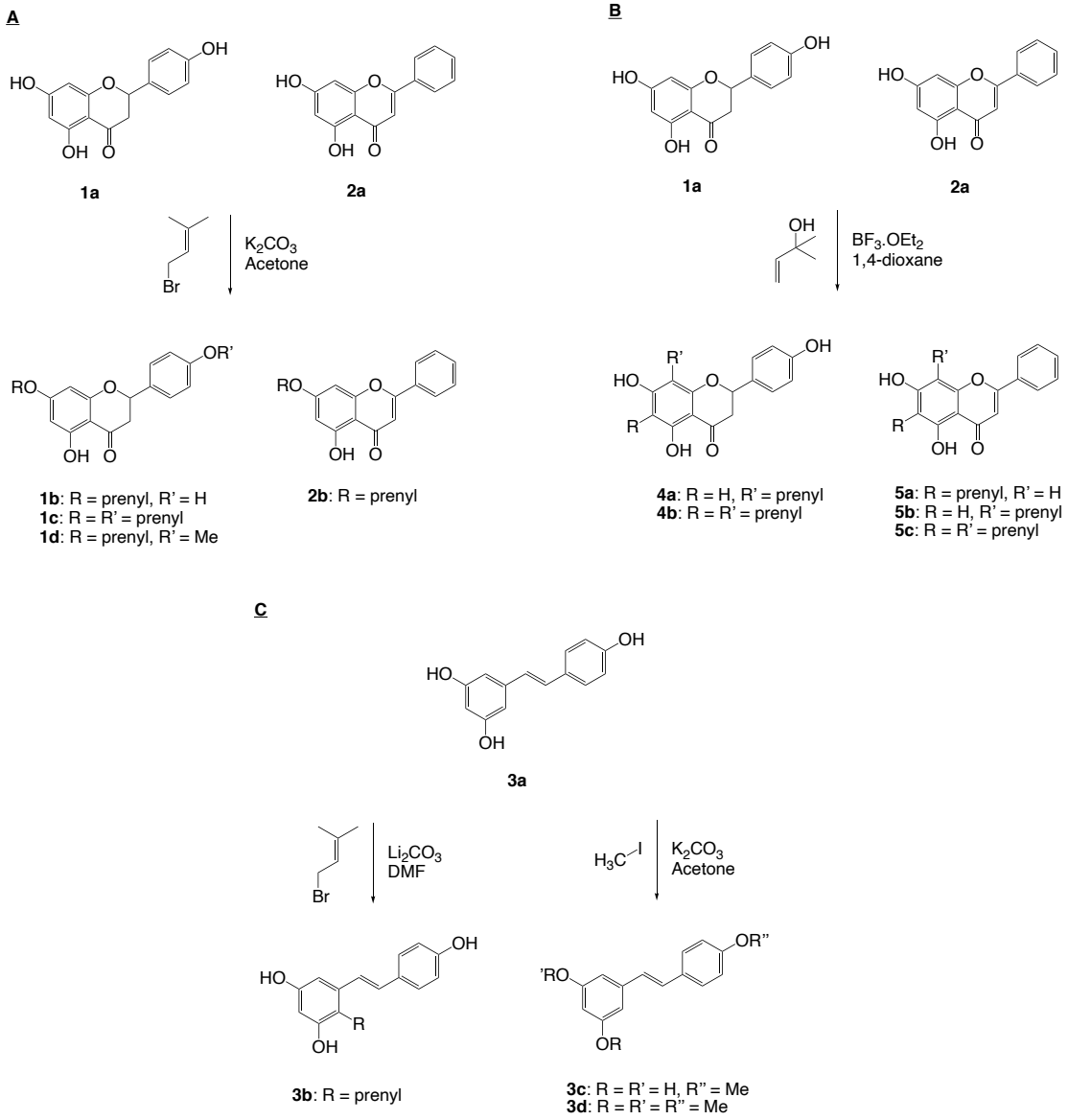
2 Aim of this thesis

The aim of this thesis is the chemical synthetic preparation of *O*- and *C*-prenylated stilbenoids and (iso)flavonoids and to examine their antimicrobial activity against MRSA 18HN in broth microdilution assays. As mentioned earlier, prenylated stilbenoids and (iso)flavonoids have intrinsic antimicrobial activity because of structurally varying prenyl moieties on their backbones. These compounds can be extracted and isolated from a variety of plants such as legumes and grains. However, it requires large amounts of plant materials to isolate these compounds. Furthermore, separating and characterizing them remain challenging. Alternatively, chemically synthesis allows the preparation of prenylated stilbenoids and (iso)flavonoids in larger quantities while cutting down the number of raw materials necessary.

The Laboratory of Food Chemistry already worked on the chemical synthesis of *O*-prenylated compounds but had limited success for *C*-prenylated compounds. In this project, chain prenylated compounds of the flavanone naringenin **1a**, flavone chrysin **2a** and the stilbenoid resveratrol **3a** were synthesized with emphasis on the use of one-pot procedures for the preparation of both *O*- and *C*-prenylated compounds. Generally, one-pot procedures can be time efficient and pragmatic and adopted more readily. The compounds were separated using flash-chromatography and preparative LC using in-house knowledge and optimized procedures. After isolation, these compounds were elucidated using both MS and NMR to determine location of the chain prenyl moieties. To improve readability, NMR spectra of one flavanone, flavone and stilbenoid will be discussed in detail. The obtained prenylated compounds were then examined for their antimicrobial activity (MIC) *in-vitro* against MRSA 18HN. In parallel, an *in-silico* model was used to predict the MIC values of all the *in-vitro* examined compounds as a complementary set of data.

2.1 Synthetic plan of approach

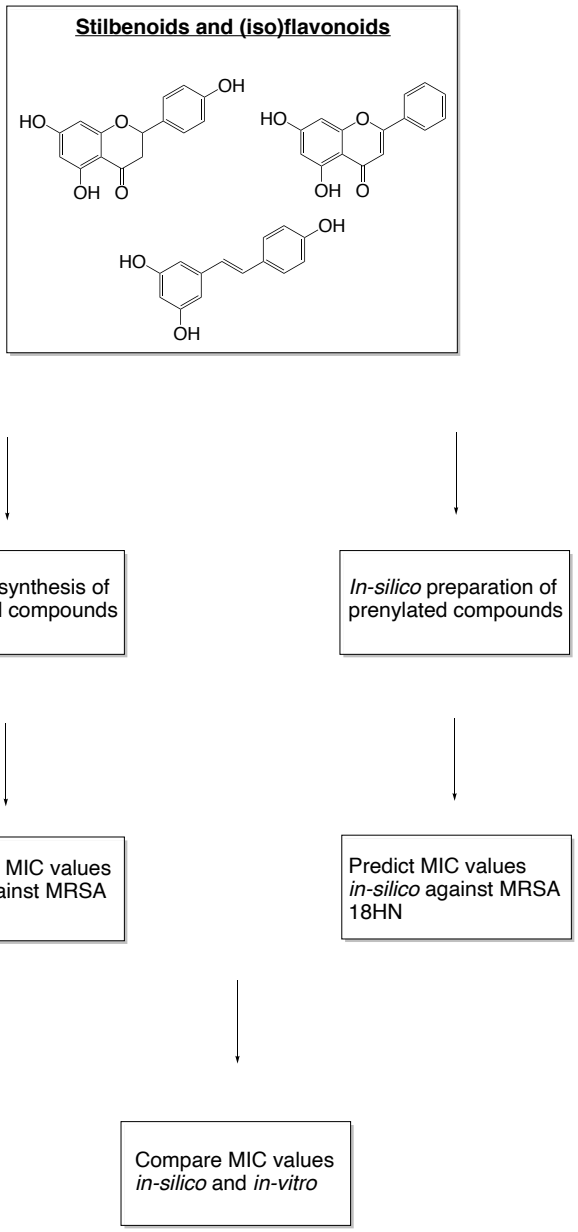
To obtain prenylated stilbenoids and (iso)flavonoids, several organic synthetic techniques prenylation strategies can be applied. Examples of compounds that were prepared in this thesis are presented in scheme 2. *O*-prenylation has been achieved starting from naringenin **1a** and using a combination of weak base such as K_2CO_3 and polar aprotic solvent acetone with prenylbromide to give *O*-prenylated **1b** and **1c** (scheme 2A)[51]. Methylation on the 4'-position of **1c** using MeI and K_2CO_3 in acetone provides **1d**. *O*-



Scheme 2: Examples of prenylation reaction conditions to afford *O*- and *C*-prenylated derivatives of flavanones, flavones and stilbenoids in this thesis.

prenylated compounds are interesting to obtain since some of them are present in nature although there is limited antimicrobial activity data available.

Next, *C*-prenylation without protecting group strategies for free -OH groups remain challenging due to harsher chemistry and strong bases such as hydrides or alkyl lithium reagents[64,65]. Fortunately, milder procedures are found in the literature. Starting from chrysin **2a** and using Lewis-acids such as $BF_3.OEt_2$ and reagent 2-methylbut-3-en-2-ol *C*-prenylated chrysin **5a**-**5c** were prepared[49] (scheme 2B). Finally, *C*-prenylation of the stilbenoid resveratrol **3a** was performed through *ortho*-lithiation using Li_2CO_3 in DMF and prenylbromide[66] to provide **3b** (scheme 2C). Additionally, *O*-methylated resveratrol **3c** and **3d** were prepared to examine if *O*-methylated stilbenoids have antibacterial activity against MRSA 18HN. All the above-mentioned procedures lead to chain-prenylated compounds. The prepared compounds were tested for their antimicrobial activity *in-vitro* in a broth microdilution assay against MRSA 18HN to obtain minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) data (scheme 3 workflow). In parallel, molecular modelling program (MOE) was used with certain molecular descriptors to predict *in-silico* MIC data of the synthesized compounds following a model developed by S. Kalli *et al*[44].



Scheme 3: Chart for the synthesized stilbenoids and (iso)flavonoids presented in this thesis.

In this report, chapter 1 provides an introduction into prenylated stilbenoids and (iso)flavonoids, their biosynthesis, prenyl moieties, antimicrobial activities against MRSA structure-activity relationships and characterization of prenylated stilbenoids and (iso)flavonoids. Next, chapter 2 details the aim of the thesis that mainly focusses on chemical synthetic one-pot procedures to prepare chain-prenylated stilbenoids and (iso)flavonoids. Subsequently, in chapter 3 the results and discussion of the synthesis, isolation and characterization using MS and NMR are described in detail. Also, the antimicrobial activity *in-vitro* data against MRSA 18HN and the *in-silico* generated MIC data are discussed and compared. Finally, chapter 4 provides the conclusion followed by recommendations in chapter 5. The report is finalized with the materials and experimental section, and the appendix with supplementary data.

3 Results and discussion

This chapter describes the chemical synthesis of chain *O*- and *C*-prenylated flavanones, flavones and stilbenoids. All prepared compounds were isolated from reaction mixtures and subsequently characterized using NMR and LC-MS and eventually their structures presented with reaction conditions in reaction schemes. Not all LC-MS and NMR is discussed for every compound to improve readability and the rest is presented in Appendix B as supplementary information, the spectral properties table in Appendix C and calculation of purity using ^{1}H-NMR in Appendix D.

3.1 Synthesis of chain *O*-prenylated flavanones and flavones

Readily available naringenin **1a** and chrysin **2a** were reacted under conditions described in Neves *et al.* using prenylbromide as reagent and K_2CO_3 as weak base in acetone[51]. This afforded, after fractionation and purification through flash chromatography, a mixture of compounds that was structurally elucidated using LC-MS and NMR.

3.1.1 MS characterization of *O*-prenylated naringenin

MS fragmentations were used to characterize the synthesized compounds and understand their fragmentation patterns. In general, the C-ring is cleaved in various places resulting in different retro-Diels-Alder (RDA) fragments with m/z values that can then be elucidated for their structure. The nomenclature used is adopted from Ma *et al*[57].

Purified samples of *O*-prenylated naringenin were subjected to LC-MS analysis. Fragment ions m/z 341, 409 and 355 respectively were detected in MS^1 (full MS) in PI (positive ionization) mode. Next, fragment ions m/z 273 (naringenin **1a)**, 341 (mono *O*-prenylnaringenin) and 287 (4'-OMe naringenin) respectively all had neutral losses of $[M+H-68]^+$ or $[M+H-C_5H_8]^+$ indicating complete loss of the prenyl moiety and proved that the isolated compounds corresponded to the structures of **1b**, **1c** and **1d** (Appendix A.1). Finally, NI (negative ionization) mode showed identical fragmentation patterns for **1b**, **1c** and **1d** in which a loss of fragment ion $[M-H-69]^-$ was observed both in-source or after MS^2.

Subsequent MS^2 of fragment ion m/z 273 and 287 gave identical fragment ion $^{1,3}A^+$ as m/z $[153]^+$ that was abundant in both **1b** and **1d** and is formed through cleavage of the bonds 1/3. The structure of ion $^{1,3}B^+$ was determined for **1b** as m/z $[120]^+$ or $[C_8H_8O]^+$ and for **1d** as m/z $[134]^+$ or $[C_9H_{11}O]^+$. The difference of 14 Da between the two compounds suggests the presence of an additional methyl group on the 4'-position of compound **1d** and explains why the $^{1,3}A^+$ ion was identical for both compounds[67,68].

3.1.2 ^{1}H-NMR characterization of 7-*O*-prenylnaringenin

Figure 6 provides the ^{1}H-NMR spectra of 7-*O*-prenylnaringenin **1b**. The spectra show the characteristic C=C double bond proton located between 5.4 to 5.6 ppm on C-2'' of the prenyl moiety that is present as a triplet of triplets but in this case overlaps with the C-2 proton of the naringenin backbone. Furthermore, at ppm 1.8, the C-4''/C-5'' protons indicate the two CH_3 groups as two singlets are derived from the prenyl moiety. Finally, to prove that the prenyl moiety is indeed *O*-prenylated, the presence of both C-6 and C-8 protons as one singlet with an integral of two protons indicate that the prenyl group must be on one of the free -OH groups either on position C5 or C7. Because the C7 -OH-group is the most acidic it is the most reactive and therefore the position that is prenylated. Also, a small amount of this compound **1b** was further methylated to **1d** and it was shown in the previous section explaining MS fragmentation of **1d** that the 4'-position was indeed methylated. Additionally, if the prenyl moiety was *C*-prenylated than this would mean either one C-6 or C-8 proton as a single integral in the NMR spectra is present which is clearly not the case. An alternative to accurately determine the location of the prenyl moiety would be a NOESY experiment were possibly the C-1'' and C-6 proton would give a signal.

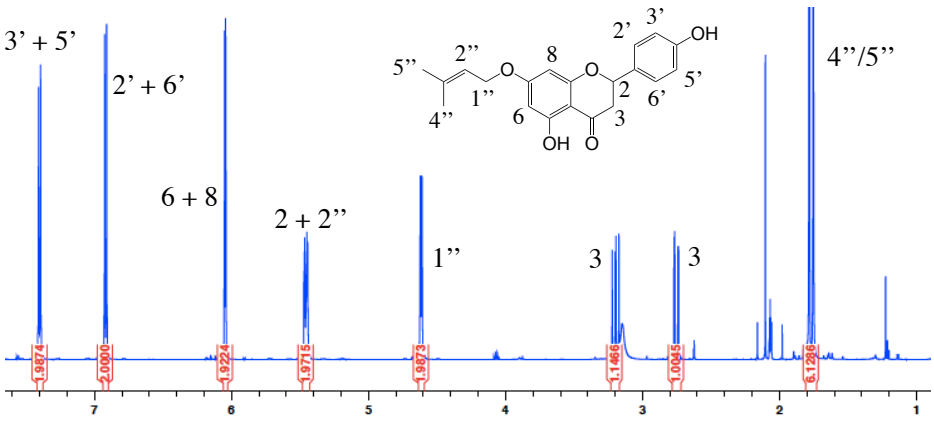
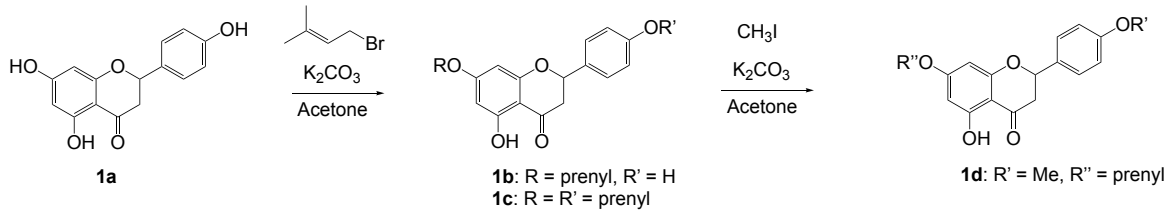


Figure 6: ^{1}H-NMR spectra of 7-*O*-prenylnaringenin **1b**. The characteristic prenyl moiety protons are present as C-2'' and two C-4''/C-5'' methyl groups.

Following the reaction scheme as described in scheme 4, 7- and 7,4'-*O*-prenylated naringenin derivatives, **1b** and **1c** respectively were elucidated and obtained in low yield (20% and 3.5% respectively) but excellent purity (>95%) according to ^{1}H-NMR and LC-MS. Next, compound **1b** was further reacted and methylated on the remaining free 4'-OH position using CH_3I as reagent K_2CO_3 as weak base in acetone to afford **1d** in low yield (6%) but excellent purity (>95%). The *O*-methylated derivative **1d** was prepared to examine whether this would influence the antibacterial activity when the B-ring 4'-OH is functionalized. As the reaction conditions were adapted from Neves *et al*. in which the flavone 8-prenyl baicalein was isolated (1% yield) as a side product, some 8-prenyl naringenin was expected in our case. However, this was not the case in our reaction. It is possible that it is formed during the reaction but since the overall yield of the reaction was 6% the 8-prenylnaringenin might be formed in such low quantity that it was not possible to isolate it after flash chromatography.



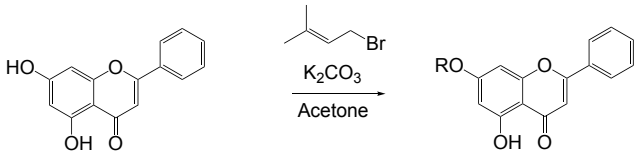
1a **1b**: R = prenyl, R' = H **1c**: R = R' = prenyl **1d**: R' = Me, R'' = prenyl

Scheme 4: Chemical synthesis of *O*-prenylated naringenin **1b** and **1c** and further diversification of the former through *O*-methylation to **1d**.

3.1.3 MS characterization of *O*-prenylated chrysin

Proceeding to *O*-prenylated chrysin, a sample was examined with LC-MS where initially an m/z of 323 was expected (Appendix A.2). However, fragment ion m/z $[255]^+$ (chrysin **2a** backbone) was found as a result of a neutral loss of fragment ion $[M+H-68]^+$ or $[M+H-C_5H_8]^+$ in PI mode indicating in-source cleavage of the prenyl moiety. This data showed that **2b** was the only product of the reaction. Subsequent MS^2 led to fragmentation of the chrysin showed an abundant fragment ion of m/z $[153]^+$ similarly to **1b** and **1d**. Indeed, since the A- and C-ring of flavanone **1b** and **1d** and flavone **2a** are identical apart from the C2-C3 double bond this fragment was formed through identical cleavage of the bonds 1/3 to give fragments $^{1,3}A^+$ and $^{1,3}B^+$. The structure of ion $^{1,3}B$ was determined as $[M+H-102]^+$ or $[M+H-C_8H_8]^+$ clearly as a highly conjugated and stable fragment (Appendix A.2). Finally, NI (negative ionization) mode showed a loss of a dominant fragment ion $[M-H-69]^-$ which is identical to earlier discussed *O*-prenylated naringenin derivatives **1b-1d**. ^{1}H-NMR characterization is not described in this section for **2b** as it is like that of 7-*O*-prenylnaringenin **1b** and therefore the data is showed in appendix A.2.

Following the reaction sequence as outlined in scheme 5, commercially available chrysin **2a** was reacted under conditions described in Neves *et al.* using prenylbromide as reagent and K_2CO_3 as weak base in acetone. This afforded compound **2b** as the sole product that was isolated after crystallization from EtOAc in low yield (4.5%) but excellent purity according to ^{1}H-NMR and LC-MS (>95%). Again, the formation of some 8-prenylchrysin was expected as was discussed for naringenin in the previous section. However, no 8-prenylchrysin was isolated as a side product possibly due to the fact it is formed as a minor product during the reaction and not isolatable after flash chromatography.



2a **2b**: R = prenyl

Scheme 5: Chemical synthesis of *O*-prenylated chrysin.

3.2 Challenging preparation of *C*-prenylated naringenin and chrysin

With *O*-prenylated naringenin and chrysin in hand, focus was directed towards the preparation of *C*-prenylated naringenin and chrysin derivatives. The reaction conditions was adapted from Tahara *et al*. Naringenin **1a** was reacted with $BF_3.OEt_2$ as Lewis-acid and 2-methyl-3-buten-2-ol as reagent in 1,4-dioxane[49]. In parallel, chrysin **2a** was reacted with $ZnCl_2$ as Lewis-acid and prenol as reagent in EtOAc[69]. In both cases this afforded, after fractionation and purification using flash chromatography, a mixture of compounds that was structurally elucidated using LC-MS and NMR.

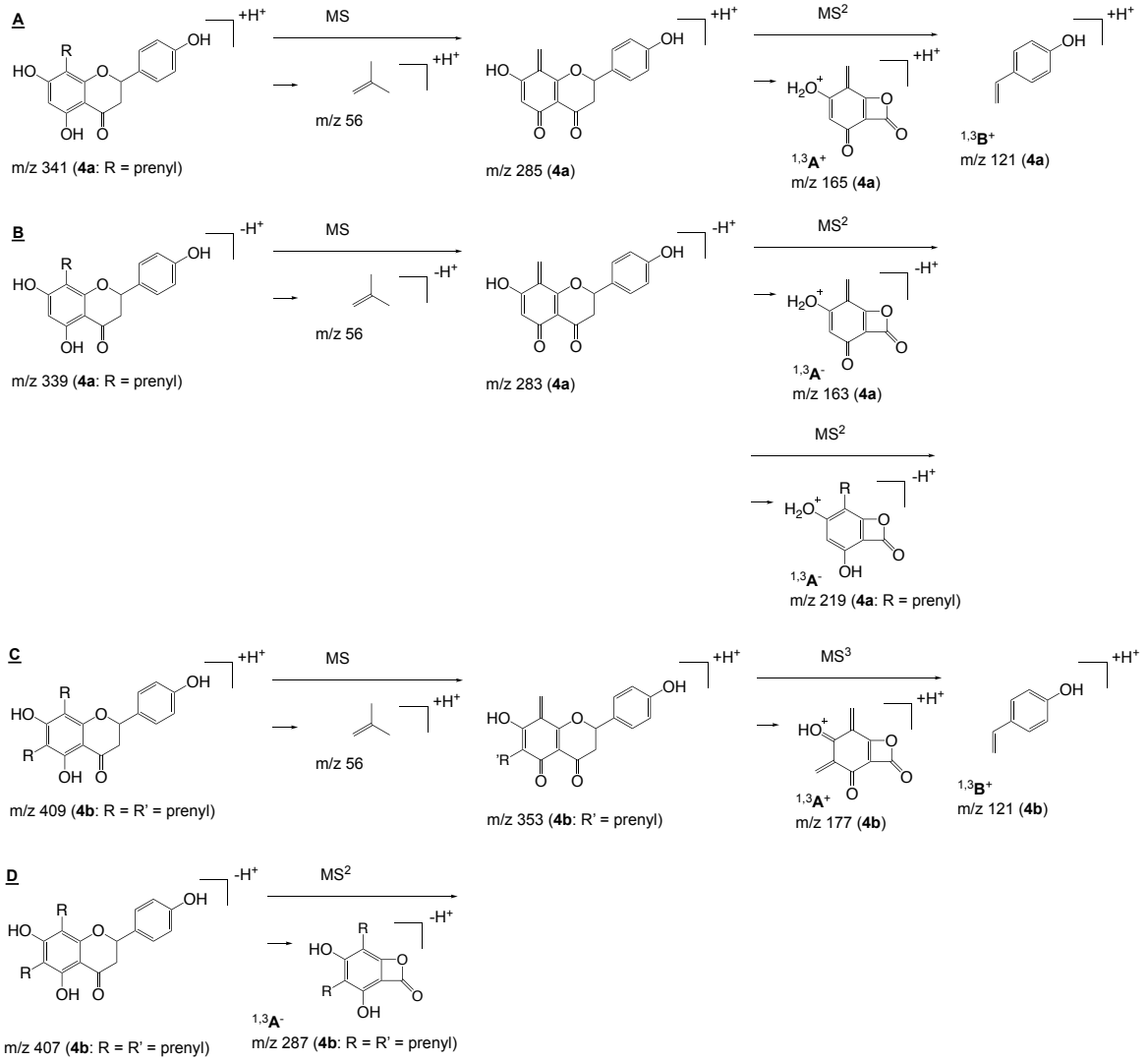
3.2.1 Elucidation of *C*-prenylated naringenin derivatives using MS

Starting with **4a** and subjecting it to MS analysis revealed a fragmentation pattern that was in line with data described in the literature[70]. A parent ion of m/z 341 was observed that after a neutral loss of 56 da $[M+H-C_4H_8]^+$ indicated the loss of a prenyl chain in PI mode (scheme 6A). The resulting ion fragment $[285]^+$ was further fragmented at bonds 1 and 3 to give ion fragment $^{1,3}B^+$ m/z $[121]^+$ or $[C_8H_8O]^+$ and $^{1,3}A^+$ m/z $[165]^+$ of which both are highly conjugated and resonance stabilized fragments. Interestingly, the MS data was compared to that of published literature and proved that compound **4a** is 8-prenylnaringenin[71].

Similar fragmentation occurred in NI mode (scheme 6B) starting from parent ion m/z 339 that after a neutral loss of 56 da $[M-H-C_4H_8]^-$ led to fragment m/z 283, losing a B-ring fragment m/z $[121]^-$ or $[C_8H_8O]^-$ that resulted in fragment ion $^{1,3}A^-$ m/z $[163]^-$. Finally, an abundant fragment $^{1,3}A^-$ m/z $[219]^-$ was observed that was formed through cleavage of bonds 1 and 3 where the C-8 prenyl moiety remains intact.

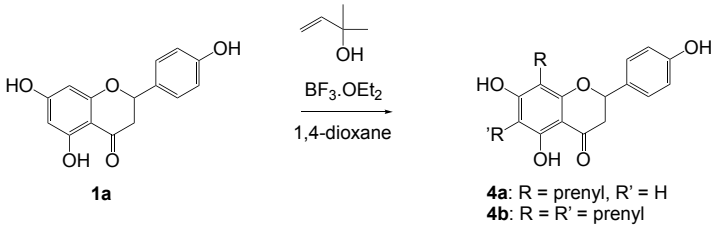
Next, for compound **4b** a parent ion of m/z 409 was observed in PI mode, indicating di-prenylated product. In-source fragmentation gave two fragment ions m/z 353 and 297 respectively representing neutral losses of 56 da $[M+H-C_4H_8]^+$ and 118 da $[M+H-C_8H_{16}]^+$, indicating single and double degradation of chain prenyl moieties (scheme 6C). Finally, MS^3 fragmentation of the abundant m/z 353 fragment ion led to cleavage of bonds 1 and 3 resulting in ion fragment $^{1,3}B^+$ m/z $[121]^+$ or $[C_8H_8O]^+$ and $^{1,3}A^+$ m/z $[177]^+$. In NI mode, fragmentation of parent ion m/z 407 gave fragment ion $^{1,3}A^-$ m/z [287] that has both C-6 and C-8 prenyl moieties intact (scheme 6D).

^{1}H-NMR data for prenylated naringenin derivatives **4a** and **4b** were not elaborated and are presented in Appendix B.



Scheme 6: MS fragmentation patterns for *C*-prenylated compounds **4a** and **4b**. Data obtained both in PI and NI mode.

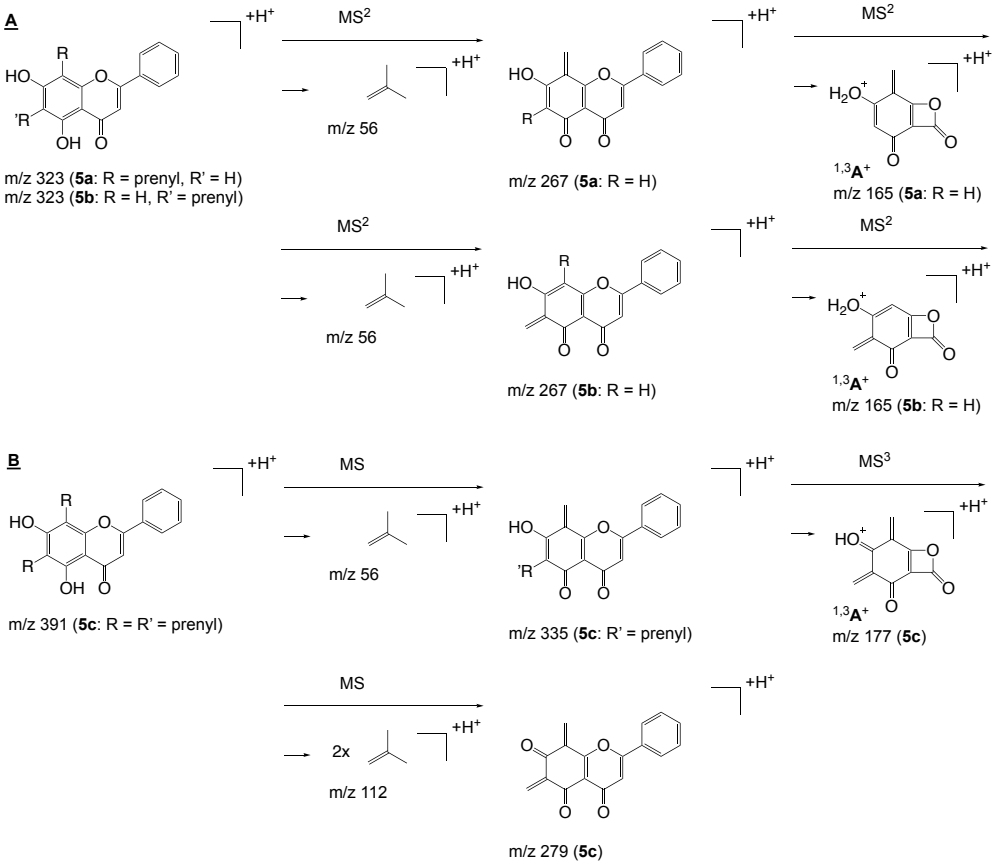
Following the reaction conditions as outlined in scheme 7, naringenin **1a** was reacted under conditions described in Tahara *et al*. using $BF_3.OEt_2$ as Lewis acid and 2-methyl-3-buten-2-ol as reagent in 1,4-dioxane[49]. This proved to be successful as it was possible to obtain mono prenylated **4a** although in very low yield (1%) but excellent purity (>95%) according to ^{1}H-NMR and LC-MS. Additionally, 6,8-diprenylnaringenin **4b** was obtained albeit its low yield (0.2%) but good purity (90%).



Scheme 7: Preparation of mono- and di- prenylated naringenin.

3.2.2 Characterization of *C*-prenylated chrysin structures using MS

Next, a mixture of mono prenylated **5a** and **5b** was subjected to LC-MS analysis (scheme 8A). As both compounds have identical m/z of 341, the fragmentation patterns are also identical. Indeed in both PI and NI mode, fragmentation of the prenyl moietey occurs leading to a neutral loss of 56 da to give fragment ion $[267]^+$ that upon further cleavage of bonds 1 and 3 gives fragment $^{1,3}A^+$ m/z $[165]^+$. The mass of this fragment ion proves the position of the prenyl chain is indeed on the A-ring of the chrysin core.



Scheme 8: MS fragmentation patterns for *C*-prenylated compounds **4b**. Data obtained both in PI and NI mode.

Next, for di-prenylated **5c** a m/z 391 was observed that after further fragmentation led to neutral losses of 56 da $[M+H-C_4H_8]^+$ and 118 da $[M+H-C_8H_{16}]^+$ for fragment ions 335 and 297 respectively (scheme 8B). Subsequent fragmentation of the m/z 335 peak gave the characteristic $^{1,3}A^+$ $[177]^+$ as a stable conjugated fragment that is identical for the earlier discussed 6,8-diprenylnaringenin where both prenyl moieties were present on the aromatic A-ring. Finally, NI mode fragmentation was identical to fragmentation of 6,8-diprenylnaringen and therefore not discussed separately for 6,8-diprenylchrysin.

3.2.3 ^{1}H-NMR characterization of 6,8-diprenylchrysin

Figure 7 provides the NMR spectra of di-prenylated chrysin **5c** where the characteristic C-2 and C-3 C=C double bond of chrysin is visible as a singlet at 6.8 ppm. Also, overlapping C-2'' and C-2''' protons as a broad triplet of triplets at 5.3 ppm clearly prove the presence of two prenyl moieties. Additionally, all aromatic signals are assigned for the B-ring as 5 integrals total. Finally, to prove that both prenyl moieties are positioned on the C-6 and C-8 position of the A-ring, deducing shows that the absence of both C-6 and C-8 protons as singlets normally present in the range of 6 to 7 ppm mean that both prenyl moieties are on those positions.

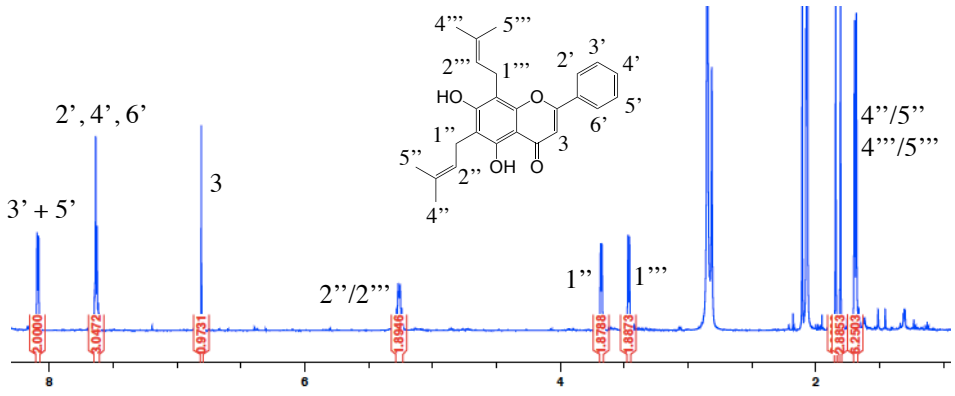
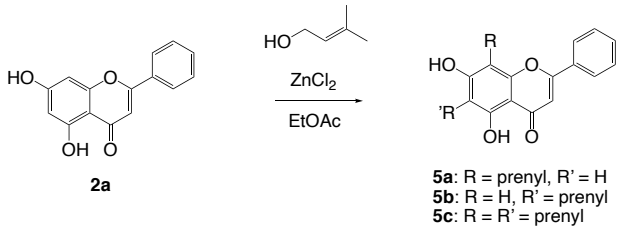


Figure 7: ^{1}H-NMR spectra of 6,8-diprenylchrysin **5c** with characteristic prenyl moiety C-2'' proton at 5.3 ppm overlapping with C-2.

Following the reaction conditions as outlined in scheme 9, chrysin **2a** was reacted with $ZnCl_2$ was used as Lewis-acid and prenol as reagent in EtOAc[69] to afford compounds **5a** and **5b** as an inseparable mixture (0.7% yield) and **5c** as a separate product (0.9% yield), the latter in very low yield but excellent purity (>95%) according to LC-MS and ^{1}H-NMR.



Scheme 9: Preparation of 8- and 6-prenylchrysin **5a**/**5b** and 6,8-diprenylchrysin **5c**.

3.3 Challenging synthesis of prenylated resveratrol

Synthesis of *C*-prenylated resveratrol commenced using a one-pot procedure developed by Puksasook et al[52] where a combination of Li_2CO_3 in DMF is used as reaction conditions. After a difficult purification by flash chromatography, one compound was isolated that was characterized first using NMR followed by LC-MS.

3.3.1 ^{1}H-NMR characterization of 2-prenylresveratrol

Figure 8 shows the NMR spectra of 2-prenylresveratrol. Characteristics are the alpha/beta protons clearly visible as a doublet of doublets that are 1 ppm apart from each other. Furthermore, the presence of the two CH_3 groups and the C-2'' triplet of triplets at 5.3 ppm indicates the presence of a prenyl moiety. The location of the prenyl moiety can be determined by looking at the protons of the aromatic A-ring. If the prenyl moiety was positioned on C-4 that would mean that C-2 and C-6 are equal in splitting pattern and theoretically be presented as a singlet with an integral of two as they are symmetrical. However, the fact that both singlets are positions at least 1 ppm apart indicates that the prenyl moiety is either on C-2 or C-6 position and the C-4 is free. Since C-2 and C-6 are the same carbons and the C-1 bond can rotate freely, it does not matter which position it is present because they are the same.

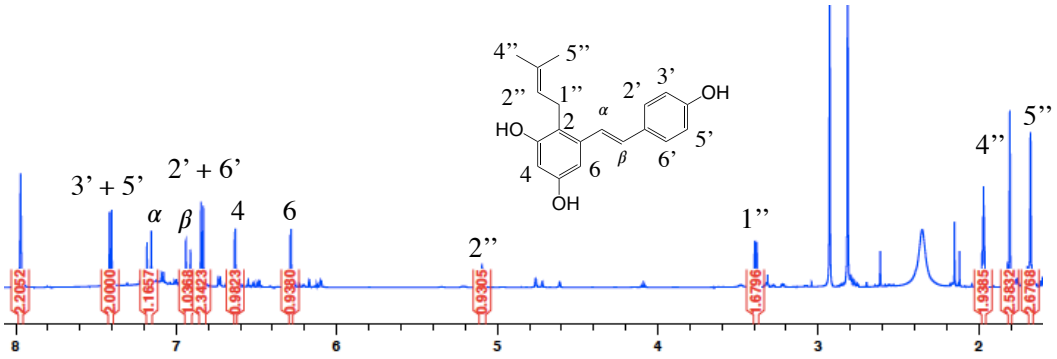
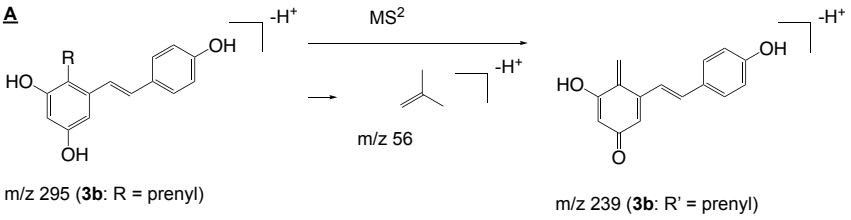


Figure 8: ^{1}H-NMR spectra of 2-prenylresveratrol **3b**.

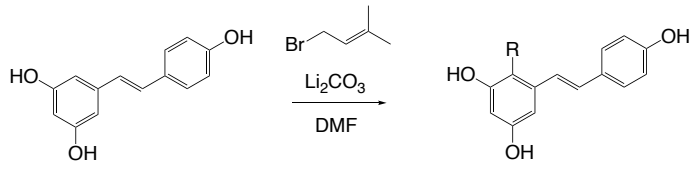
3.3.2 MS fragmentation of prenylated stilbenoid

Compound **3b** was subjected to MS analysis and a parent ion of m/z 295 was observed that after a neutral loss of 56 da $[M+H-C_4H_8]^+$ indicated the degradation of a prenyl chain in NI mode which is in line with previous discussed *C*-prenylated compounds (scheme 10). This 56 da neutral loss proves that the prenyl moiety is indeed *C*-prenylated. It was only possible to determine fragmentation patterns in NI mode as no reliable data could be obtained for PI mode. Interestingly, this is the exact opposite as was seen for prenylated flavanones and flavones. Since fragmentation of stilbenoids is complex and to many varying fragmentation patters are reported in the literature no further elucidation was examined as the most important fragmentation pattern is the degradation of the prenyl moiety[52].



Scheme 10: MS fragmentation patterns for *C*-prenylated compounds **3b**. Data obtained in NI mode.

In our case, it was impossible to obtain both 4-prenylresveratrol and 2,6-di-prenylresveratrol as was discussed in the article and obtained at high yields. Moreover, the isolation and purification of the reaction mixture was tedious and, in the end, required both normal phase and C18 reverse phase chromatography. A small amount of **3b** in low yield (6.1% yield) was obtained with moderate purity (70-80%) according to both ^{1}H-NMR and LC-MS. However, further purifying this compound was not pursued at the scale obtained.

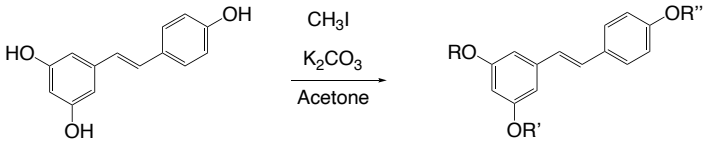


3a **3b**: R = prenyl

Scheme 11: Prenylation of resveratrol through *ortho*-lithiation leading to 2-prenylresveratrol **3b**.

3.3.3 Preparation of *O*-methylated resveratrol for structure-activity relationships

Finally, *O*-methylated resveratrol derivatives **3c** and **3d** were prepared to examine their anti-MRSA with the synthesized prenylated stilbenoids and (iso)flavonoids. Following reaction conditions described in Puksasook *et al* (scheme 12). A one-pot *O*-methylation of resveratrol using methyliodide as reagent and K_2CO_3 as weak base in acetone gave after normal-phase flash chromatography the corresponding tri-methylated and mono-methylated products **3c** and **3d** respectively in low yield but excellent purity according to NMR and LC-MS (>95%). No MS fragmentation and NMR data are discussed for these compounds as they have been confirmed using data described in the literature[52].



3a **3c**: R = R' = R" = Me
3d: R = R' = H, R" = Me

Scheme 12: *O*-methylation of resveratrol leading to methoxylated resveratrol **3c** and **3d**.

3.4 Experimental activity *in-vitro* against MRSA 18HN

The antibacterial potency of prenylated flavonoids against MRSA 18HN are shown in table 1 and structures of the compounds presented in figure 9. Active compounds were considered with MIC values < or equal to 25 µg/mL, moderately active between 25 and 100 µg/mL and inactive above 100 µg/mL[72].

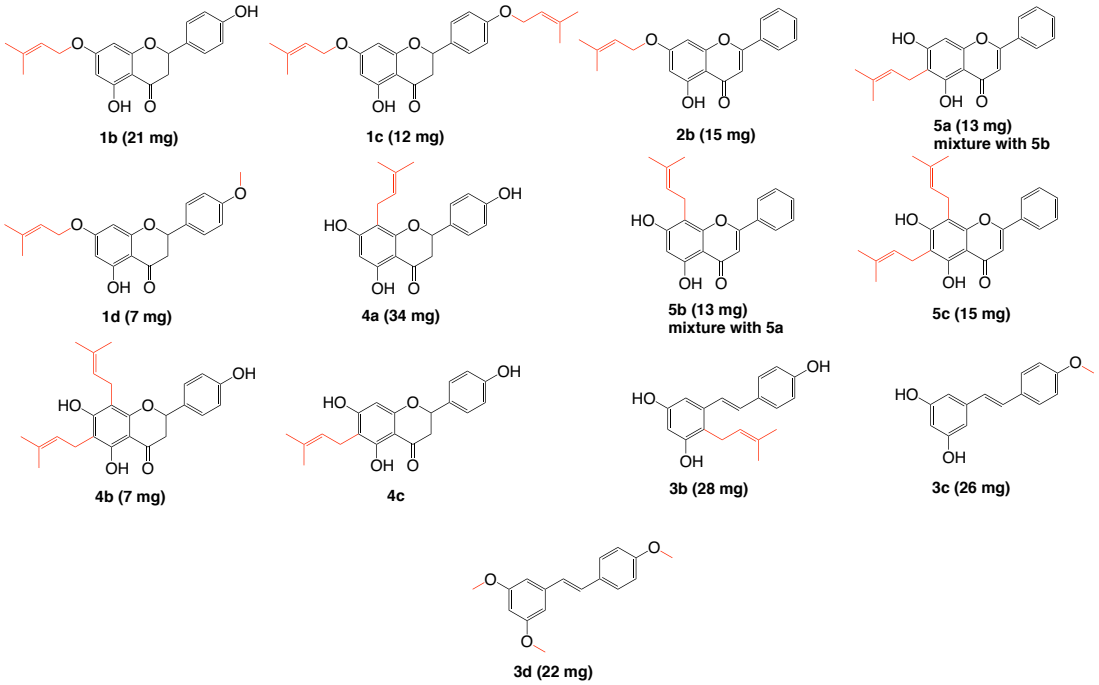


Figure 9: Overview of synthesized compounds with amount in milligrams.

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of prenylated flavonoids tested in this study against MRSA 18HN.

Subclass	Name	MIC (µg/mL) [µM]	MBC (µg/mL) [µM]	Predicted MIC (µg/mL) [µM]	Within AD[g]
Flavanones	7-*O*-prenylnaringenin (**1b**)	12.5-25 [36-73]	25 [73]	17.6 [51]	Yes
	7,4'-di-*O*-prenylnaringenin (**1c**)[a]	n.d.[d]	n.d.[d]	3.5 [8]	No[h]
	7-*O*-prenyl-4'-OMenaringenin (**1d**)[a]	n.d.[d]	n.d.[d]	28.7 [81]	No[h]
	8-prenylnaringenin (**4a**)	12.5-25 [36-73]	25 [73]	24.2 [71]	Yes
	6,8-diprenylnaringenin (**4b**)	12.5-25 [30-61]	25 [61]	41.5 [101]	No[h]
	6-prenylnaringenin (**4c**)	12.5-25 [36-73]	25 [73]	64.8 [190]	Yes
Flavones	7-*O*-prenylchrysin (**2b**)[a]	n.d.[d]	n.d.[d]	8.4 [25]	No[h]
	8-prenylchrysin (**5a**)[b,c]	n.d.[d]	n.d.[d]	28.1 [87]	No[h]
	6,8-diprenylchrysin (**5c**)	n.a.[f]	n.a.[f]	8.9 [27]	No[i]
	6-prenylchrysin (**5b**)[b,c]	n.d.[d]	n.d[d]	13.5 [34]	Yes
Stilbenoids	2-prenylresveratrol (**3b**)[a]	25-50 [84-169]	n.d.[e]	6.6 [22]	No[h]
	4'-*O*-Me-resveratrol (**3c**)	n.a.[f]	n.a.[f]	16.8 [69]	Yes
	3,5,4'-tri-*O*-Me-resveratrol (**3d**)[a]	n.a.[f]	n.a.[f]	11.2 [41]	No[i]

[a] MIC value obtained based on one biological replicate.

[b] Tested as a mixture of compounds (3:1 ratio based on NMR).

[c] Ratio not conclusive for which isomer is the most abundant.

[d] Not determined due to solubility issues.

[e] MBC larger than >50 µg/mL.

[f] Not active at all concentrations (3.125 to 50 µg/mL).

[g] Applicability domain.

[h] Exceeds > 10%.

[i] Between 0-10%.

Positive controls glabridin and the antibiotic vancomycin were MBC at 18.75 µg/mL and 1.25 µg/mL respectively against MRSA, in line with earlier described activity[44,73]. 4'-OMe resveratrol **3c** and 3,5,4'-trimethoxyresveratrol **3d** showed no inhibitory action at 50 µg/mL but only a time-to-detection (TTD) of 4 h increasing with an increase in concentration.

7-*O*-prenylnaringenin **1b** is active at MIC 12.5-25 µg/mL and MBC at 25 µg/mL comparable to the positive control glabridin. Interestingly, **1b** is structurally like a 7-*O*-butyl derivative of naringenin that is a very potent antimicrobial against MRSA (1.9 µg/mL)[74]. However, the potency of **1b** is at least 5-10-fold lower compared to the 7-*O*-butyl derivative. This is interesting as the butyl and prenyl moiety do not differ that much in structure. This observation leads to different questions regarding the prenyl moiety itself. For example, maybe the C=C double bond of the prenyl moiety is not that important in antibacterial activity and just present because of the biosynthetic pathway it follows and not necessarily serving any antibacterial role.

Unfortunately, 7,4'-di-*O*-prenyl naringenin **1c** failed to show any activity due to solubility issues occurring at all concentrations from 6.25-50 µg/mL. The same was observed for 7-*O*-prenyl-4'-*O*-Me naringenin **1d** at concentrations from 12.5 to 50 µg/mL.

Next, both for 8-prenylnaringenin **4a** and 6-prenylnaringenin **4c** MIC values between 25 and 12.5 µg/mL were observed. Also, both compounds were MBC at 25 µg/mL in line with previously reported data[45]. Interestingly, at 50 µg/mL the viable count showed that 8-prenylnaringenin had a factor 10 less colonies present indicating that it is more potent to its 6-position analogue. Subsequently, 6,8-diprenylnaringenin **4b** showed MIC between 25 and 12.5 µg/mL and is MBC at 25 µg/mL. Unfortunately, this result was less in line with previously reported data, as some di-prenylated compounds are more active against Gram-positive bacteria but not all. It is difficult to explain this finding in our case, but it could be attributed to solubility of the compound in the TSB media prior to adding it. Even though the product is dissolved, some aggregation might occur over time decreasing the anti-MRSA activity of the compound.

The *O*-prenylated flavone **2b** showed solubility issues in the concentrations (25-50 µg/mL) and gave no MIC values across all concentrations tested. Next, the mixture of 6- and 8-prenylchrysin **5a**/**5b** at the (25-100 µg/mL) concentration showed different results across all three biological replicates but in all cases gave no reliable MIC values. Turbidity was observed possibly because of the more hydrophobic nature of the compounds as the lack of a 4'-OH group on the external B-ring compared to naringenin. This greatly decreases the solubilizing capacity in aqueous media. It is also possible that only one isomer of the **5a**/**5b** mixture is insoluble and crashes out of solution explaining the turbidity. Finally, for 6,8-diprenylchrysin **5c** a clear solution was observed when dissolving the compound prior to the assay. However, a complete lack of activity was observed.

Subsequently, 2-prenylresveratrol **3b** was moderately active with MIC at the concentration (25-50 µg/mL, one biological replicate) and no MBC was established at this the maximum concentration tested (50 µg/mL). Interestingly, using ^{1}H-NMR it was clear that the compound was far from pure (70-80% purity estimate) meaning that if the compound was completely pure it would probably have a lower MIC value and be more effective.

For the compounds where the MIC value was determined based on a single biological replicate, at least two additional biological replicates should be performed in the future to accurately determine the MIC values of these compounds.

3.4.1 Prediction of MIC *in-silico* against MRSA 18HN

Table 1 provides the *in-silico* predicted MIC values of prenylated stilbenoids and (iso)flavonoids. Some compounds fall outside the applicability domain of the model and will therefore not be discussed as their data is irrelevant. The compounds that stand out are 7-*O*-prenylated naringenin **1b** with a MIC of 17.6 µg/mL. 8-prenylnaringenin **4a** and 6-prenylnaringenin **4c** had MIC values of 24.2 µg/mL and 64.8 µg/mL respectively. Clearly, **4a** is considered more potent.

Of the prenylated chrysin derivatives only 6-prenylchrysin fell inside the applicability domain with a MIC of 13.5 µg/mL which is almost 2-fold more potent compared to 8-prenylnaringenin possibly since the hydrophobic volume descriptor (*vsurf_D4*) of the model is the most important and results in lower predicted MIC values for more lipophilic compounds.

Finally, the stilbenoid 4'-OMeresveratrol **3c** had a MIC value of 16.8 µg/mL which is unexpectedly potent. This is an indication that hydrophobic volume is not the only predictor for antimicrobial activity even though the structural similarities of the A-ring between these molecules are the same. Furthermore, the lack of the C-ring in stilbenoid **3b** allows for an increase of the degrees of freedom of the C1-C2 double bond and allows for different structural conformations of the molecule and as a result a potential better fit in the active site to elicit antimicrobial activity.

3.4.2 Comparison of *in-vitro* and *in-silico* data of prenylated and methylated compounds.

Comparing *in-vitro* and *in-silico* data and starting from 7-*O*-prenylnaringenin **1b** both data match and show it as a potent compound. Also, the minimum bactericidal concentration (MBC) is the same as glabridin. Furthermore, 7-*O*-prenylnaringenin is the first documented *O*-prenylated flavanone that is active against MRSA with good activity.

Next, 8-prenylnaringenin **4a** is more potent compared to 6-prenylnaringenin **4c** as is the case when the MIC was predicted but, in the model, it is more evident. Next, although 6-prenylchrysin **5b** gave low predicted MIC *in-silico*, as a mixture with 8-prenylchrysin **5a**, it failed to show anti-MRSA activity *in-vitro*. Unfortunately, at this stage it becomes clear that the choice for chrysin as starting material to prepare prenylated derivatives and subsequently test them *in-vitro* does not give adequate results.

Finally, **3c** was potent *in-silico* but failed to give anti-MRSA activity *in-vitro*. Also, the model was developed for, and the test set based around (iso)flavonoids that have rigid C-rings and limited degree of freedom. Therefore, stilbenoids fall outside the scope of the model and therefore the predicted *in-silico* MIC values are not accurate.

4 Conclusion

MRSA continues to be a dangerous pathogen that is becoming increasingly resistant to existing antimicrobial agents. As a result, new and more effective treatments are vital. Natural compounds such as prenylated stilbenoids and (iso)flavonoids can be active antimicrobials. In this report is presented the chemical synthesis of chain *O*- and *C*-prenylated stilbenoids, flavanones and flavones. The aim of this thesis was to start from naringenin, chrysin and resveratrol and in one-pot procedures produce chain prenylated derivatives. In the end, a wide variety of derivatives were prepared, characterized, and subsequently tested *in-vitro* against MRSA 18HN. In general, yields were lower than reported in the literature that was used for the synthesis of prenylated stilbenoids and (iso)flavonoids that limited the possibility to generate larger quantities of chain prenylated compounds and continue diversification to ring prenylated stilbenoids and (iso)flavonoids.

Next, antimicrobial activity was examined of all the synthesized compounds. Especially, the flavanone 7-*O*-prenylnaringenin was shown to be an active compound against MRSA with comparable activity to 8-prenyl, 6-prenyl and 6,8-diprenylnaringenin and is the first documented *O*-prenylated flavanone that is active against MRSA. This sparks interest in further examining *O*-prenylated stilbenoids and (iso)flavonoids as potential anti-MRSA agents. Furthermore, 2-prenylresveratrol was moderately active against MRSA and generating other structural isomers of prenylated resveratrol might result in a derivative that is more active against MRSA than 2-prenylresveratrol. Finally, both the *O*- and *C*-prenylated chrysin derivatives were inactive *in-vitro* against MRSA.

This can be explained partially because of solubility issues that were encountered. However, clearly the lack of a 4'-OH moiety on the B-ring of chrysin derivatives suggests that this is a major contributor to a lack of anti-MRSA activity. Therefore, for further experiments, selecting a flavone with a 4'-OH moiety present followed by generating prenylated derivatives and examine their activity *in-vitro* against MRSA would be interesting as likely solubility issues will be eliminated and activity will be observed.

The preparation of this set of prenylated flavones and studying their anti-MRSA activity will shed more light on the anti-MRSA activity of prenylated stilbenoids and (iso)flavonoids in general and hopefully in the future lead to the development of new and more effective therapies to halt the progression of even the most resistant strains of MRSA and other Gram-positive and Gram-negative bacteria.

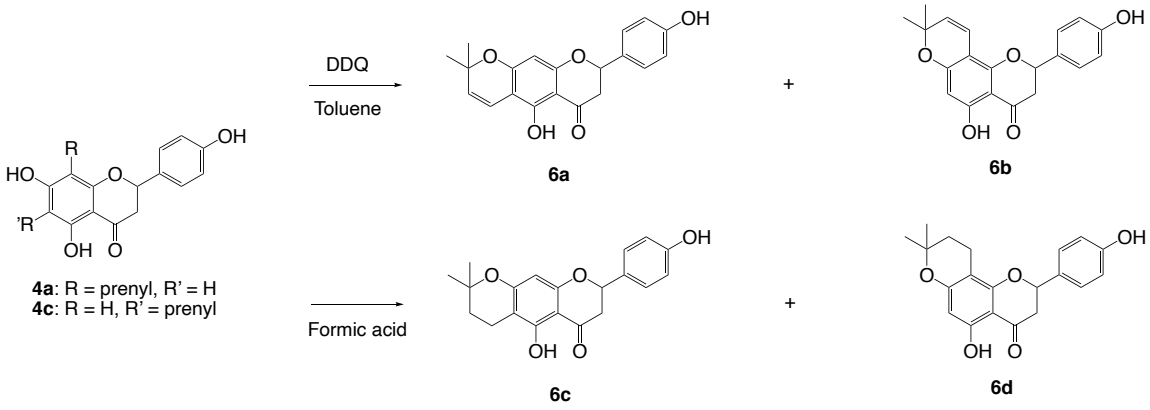
5 Recommendations

Recommendations for this thesis are mostly focused on the synthetic part as this has the potential to increase the number of prenylated compounds that can be used to further study the effects of prenyl moieties on the antimicrobial activity against both Gram-positive and Gram-negative bacteria.

5.1 Alternative ring prenylated and geranylated stilbenoids and (iso)flavonoids

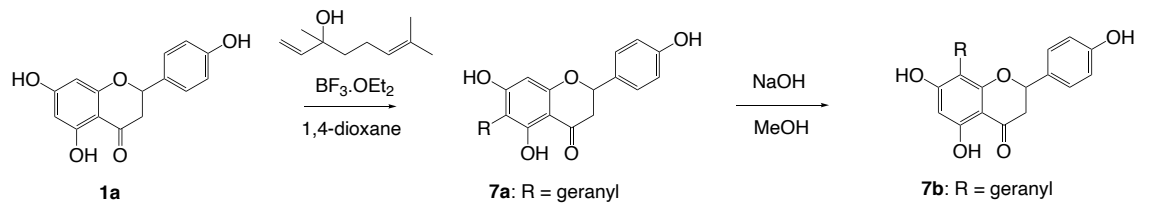
The prenylated compounds synthesized in this report can be complemented by the addition of other unique and interesting analogues as will be described in this section.

Yields of synthesized prenylated compounds as shown in this thesis are low. As a result, this limits the amount of material that can be used to prepare pyran prenylated analogues from chain prenylated compounds under certain conditions. For example, DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) and 8- or 6-prenylnaringenin **4a**/**4c** can be reacted in an oxidative cyclization reaction to afford 6-prenyl pyran **6a** and 8-prenyl pyran **6b** products[75,76] (scheme 13). However, since DDQ is a strong oxidant, alternatives such as chloranil (tetrachloro-1,4-benzoquinone) or other milder oxidative reagents can be used to examine whether side products are formed.



Scheme 13: DDQ-mediated oxidative cyclization to afford pyran, and formic acid-mediated cyclization to afford saturated pyran prenylated compounds.

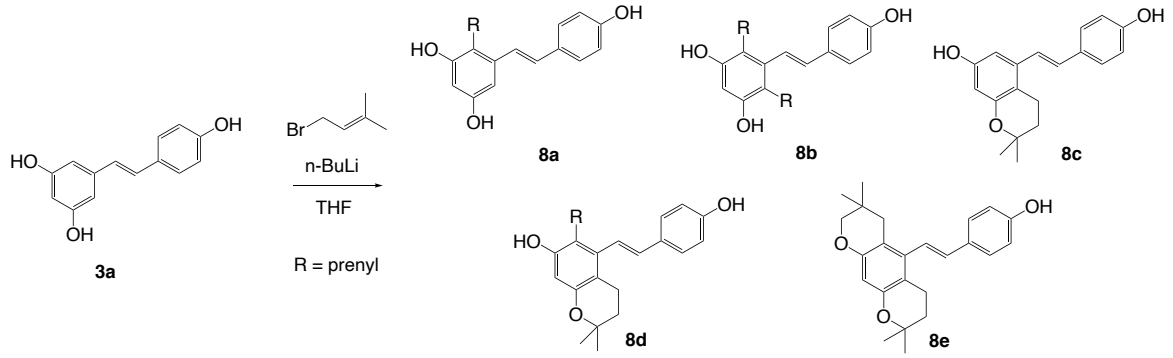
Additionally, formic acid can be used to prepare saturated pyran prenyl derivatives **6c** and **6d** respectively of naringenin following the above-mentioned procedure. The saturated pyran analogues can be tested for their antimicrobial activity against MRSA 18HN alongside pyran prenylated compounds to compare MIC-values and could shed light into why the prenyl C=C double bond is present in the first place and if it is essential in the antimicrobial mechanism of action or completely redundant.



Scheme 14: *C*-geranylation of naringenin to afford 6- and 8-geranylnaringenin as structural isomers.

Next, to extend the scope of tested compounds and obtain more antimicrobial activity data, an extended ten carbon isoprenoid geranyl moiety can be installed to generate a new subset of *O*- and *C*-geranylated analogues following the same procedure for the preparation of *O*- and *C*-prenylated derivatives[77]. Geranyl is a second prenyl added to the terminal carbon of the first prenyl attached to a flavonoid backbone. Geranylated stilbenoids and (iso)flavonoids have been described in nature but are less studied and limited data is available regarding their antimicrobial activity. For example, as is shown in scheme 14, naringenin can be selectively geranylated on the 6-position using $BF_3.OEt_2$ as Lewis-acid and linalool as reagent to afford compound **7a** as a single product. Finally, under strong basic conditions the 6-geranyl can be migrated to provide 8-geranylnaringenin **7b**.

Cyclization of chain prenylated resveratrol derivatives to the corresponding pyran prenyl analogues is more challenging due to the inherent reactivity of the C1-C2 double bond of the resveratrol backbone that is reactive under light, increased temperature and mild to strong oxidative conditions and has the tendency to oligomerize for example to (+)-viniferin and ampelopsin B, both dimers of resveratrol[78,79] (not shown in figure). Alternatively, following a procedure described in a patent, it is possible to prepare a mixture of chain prenylated **8a**/**8b** and saturated pyran prenyl **8c**-**8e** resveratrol derivatives[80]. Using a combination of the alkyllithium reagent *n*-BuLi and prenylbromide/geranylbromide gives 5 compounds in one procedure in sufficient yields (scheme 15).



Scheme 15: Simultaneous chain prenyl and saturated pyran prenylated resveratrol provides five different analogues.

6 Materials

6-prenylnaringenin (6-PN) and vancomycin were purchased from Sigma Aldrich (St. Louis, MO, USA). Glabridin was purchased from Wako (Osaka, Japan). Tryptone soya broth (TSB) was used as growing medium for MRSA. Tryptone soya broth (TSB) and bacteriological agar from Oxoid Ltd (Basingstoke, UK), and peptone physiological salt solution (PPS) from Tritium Microbiologie (Eindhoven, The Netherlands). Dimethylsulfoxide (DMSO) was purchased from Biosolve (Valkenswaard, The Netherlands).

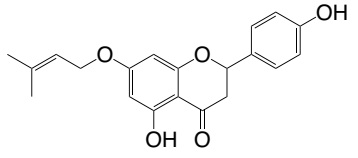
7 Experimental

General methods chemical synthesis: Nuclear magnetic resonance spectra (^{1}H-NMR) were recorded using a Bruker® Avance Ultrashield™ Plus (^{1}H-NMR, 600 MHz; ^{13}C-NMR, 150 MHz respectively) spectrometer with samples dissolved in acetone$_{D6}$, acetonitrile$_{D3}$ and methanol$_{D4}$ with tetramethylsilane (TMS) as internal standard. Chemical shifts are reported in (δ) ppm downfield from TMS. NMR data are presented as follows: Chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublet, m = multiplet and/or multiple resonances); coupling constants (*J*) are reported in Hertz (Hz). All NMR signals were assigned based on ^{1}H-NMR, COSY, HSQC, and ^{13}C-experiments. TLC was conducted on both Silicagel 60 F254 (Merck) with detection by UV light (254 nm) where applicable, and by charring with 10% H_2SO_4 in EtOH. Unless otherwise stated, reactions were carried out at room temperature (RT) in glassware with magnetic stirring. Compounds were dried under high vacuum. All reagents used were obtained from commercial sources and all solvents were of analytical grade.

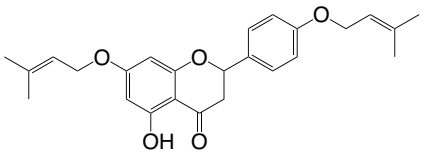
General methods LC-MS procedures: ESI-IT-MS^n spectra were acquired on a LTQ Velos Pro linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a heated ESI probe coupled *in-line* to a Vanquish UHPLC system (Thermo Scientific). The flow rate was 300 uL/min at a column temperature of 45 °C. Eluents used were water (A) and acetonitrile (B), both with 0.1% (*v/v*) formic acid. Detection wavelengths for UV-Vis were set to the range of 200-600 nm and data were recorded at 20 Hz. A Waters Acquity BEH C18 2.1 x 150 mm, 1.7 um particle size column with a Waters VanGuard 2.1 x 5 mm guard column of the same material was used for all analytical RP-UHPLC separations. Unfortunately, elution profiles were not recorded.

General methods flash chromatography: Flash chromatography was performed on a Buchi FlashPrep system (Buchi Labortechnik GmbH) using silica gel cartridges for normal phase separation and C18 particles for reverse-phase separation.

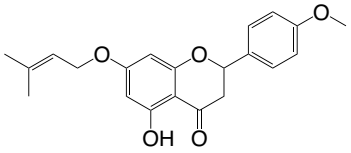
5,7-dihydroxy-2-(4-((3-methylbut-2-en-1-yl)oxy)phenyl)chroman-4-one, 4'-*O*-prenylnaringenin (1b). To a solution of naringenin **1a** (1 g, 3.7 mmol) in acetone (70 ml) was added K_2CO_3 (1 g, 7.4 mmol, 2 eq) and prenylbromide (520 uL, 4.4 mmol, 1.2 eq) before the resulting suspension was heated to reflux for 24 h. After completion of the reaction, as was determined by TLC (DCM/MeOH (20:1), 10% H_2SO_4/EtOH stain), the reaction was cooled, and the solids filtered off. The filtrate was washed with acetone (30 ml), organics pooled, evaporated *in vacuo* and the orange oily residue purified by silica gel flash chromatography using (PE/EtOAc (1:0) increasing to (4:1), (1:1) and finally (0:1)) to afford the title compound **1b** as a yellow oil (23 mg, 0.8% yield). ^{1}H-NMR (600 MHz, $MeOD_6$) δ 7.41-7.38 (d, *J* = 8.46 Hz, 2H, H-2', H-6'), 6.93- 6.90 (d, *J* = 8.60 Hz, 2H, H-3', H-5'), 6.05-6.03 (dd, *J* = 8.10, 3.54, 2H, H-6, H-8), 5.47-5.43 (m, 2H, H-2, H-2''), 4.62-4.60 (d, *J* = 6.70 Hz, 2H, H-1''), 3.22-3.16 (dd, *J* = 17.1, 12.9 Hz, 1H, H-3α), 2.77-2.73 (dd, *J* = 17.1, 3.02 Hz, 1H, H-3β), 1.78 (s, 3H, H-4''), 1.75 (s, 3H, H-5''). ^{13}C-NMR (150 MHz, $MeOD_6$) δ 196.1 (C=O), 164.9 (C_q-7), 164.3 (C_q-9), 163.2 (C_q-5), 159.3 (C_q-4'), 138.6 (C_{Alkene}-3''), 130.1 (C_q-1'), 127.7 (C_{Ar}-2',6'), 119.3 (C_{Alkene}-2''), 114.9 (C_{Ar}-3',5'), 103.1 (C_q-10), 96.7 (C_{Ar}-8), 95.5 (C_{Ar}-6), 79.0 (C-2), 64.9 (C-1''), 43.1 (C-3), 25.8 (C-5''), 18.2 (C-4'').



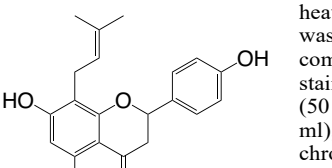
5-hydroxy-2,7-bis((3-methylbut-2-en-1-yl)oxy)-phenyl)chroman-4-one, 7,4'-*O*-diprenylnaringenin (1c). The title compound **1c** (12 mg, 0.4% yield) was isolated as a separate product from the previous reaction. ^{1}H-NMR (600 MHz, Me_2CO_{D6}) δ 7.48 (d, *J* = 8.64 Hz, 2H, H-2', H-6'), 7.00 (d, *J* = 8.64 Hz, 2H, H-3', H-5'), 6.05 (dd, *J* = 8.52, 2.19 Hz, 2H, H-6, H-8), 5.53-5.44 (m, 3H, H-2, H-2'', H-2'''), 4.64-4.58 (dd, *J* = 15.33, 6.74 Hz, 4H, H-1'', H-1'''), 3.24-3.18 (dd, *J* = 16.93, 12.91 Hz, 1H, H-3α), 2.81-2.76 (dd, *J* = 17.90, 2.90 Hz, 1H, H-3β), 1.80-1.74 (m, 12H, H-4'', H-4''', H-5'', H-5'''). ^{13}C-NMR (150 MHz, Me_2CO_{D6}) δ 196.8 (C=O), 167.3 (C_q-7), 163.4 (C_q-5), 163.2 (C_q-9), 157.0 (C_q-4'), 138.4 (C_{Alkene}-3'', C_{Alkene}-3'''), 130.6 (C_q-1'), 127.0 (C_{Ar}-2',6'), 119.7 (C_{Ar}-3',5'), 119.6 (C_{Alkene}-2'', C_{Alkene}-2'''), 102.5 (C_q-10), 94.8 (C_{Ar}-6), 94.0 (C_{Ar}-8), 82.5 (C-2), 64.9 (C-1'', C-1'''), 43.0 (C-3), 24.6 (C-5'', C-5'''), 18.6 (C-4'', C-4''').



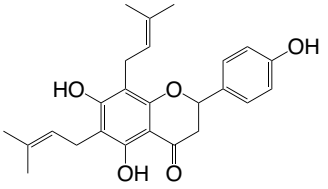
5-hydroxy-7-methoxy-2-(4-((3-methylbut-2-en-1-yl)oxy)phenyl)chroman-4-one, 7-*O*-methoxy-4'-*O*-prenylnaringenin (1d). To a solution of **1c** (97 mg, 0.28 mmol) in acetone (4 ml) was added K_2CO_3 (78 mg, 0.57 mmol, 2 eq) and CH_3I (53 uL, 0.85 mmol, 3 eq) before the suspension was refluxed for 3 h. After completion of the reaction, as was determined by TLC (PE/EtOAc (3:1), 10% H_2SO_4/EtOH stain), the solids were filtered off and the filtrate washed with acetone (15 ml), organics pooled, evaporated *in vacuo* and the residue purified by silica gel flash chromatography using (PE/EtOAc (1:0), increasing to (5:1), to (4:1) and finally (1:1) to afford the title compound **1d** (7 mg, 6% yield) as a yellow solid. ^{1}H-NMR (600 MHz, Me_2CO_{D6}) δ 7.51 (d, *J* = 8.90 Hz, 2H, H-2', H-6'), 7.01 (d, *J* = 8.43 Hz, 2H, H-3', H-5'), 6.06 (dd, *J* = 10.05, 1.97 Hz, 2H, H-6, H-8), 5.53 (dd, *J* = 12.80, 2.95 Hz, 1H, H-2), 5.46 (bt, 1H, H-2''), 4.63 (d, *J* = 6.61 Hz, 2H, H-1''), 3.84 (s, 3H, OMe-7), 3.23 (dd, *J* = 17.45, 13.22 Hz, 1H, H-3α), 2.97 (dd, 17.45, 2.91 Hz, 1H, H-3β), 1.79 (s, 3H, H-4''), 1.76 (s, 3H, H-5''). ^{13}C-NMR (150 MHz, Me_2CO_{D6}) δ 196.8 (C=O), 165.8 (C_q-7), 164.3 (C_q-9), 163.2 (C_q-5), 157.0 (C_q-4'), 138.4 (C_{Alkene}-3''), 130.6 (C_q-1'), 127.0 (C_{Ar}-2',6'), 119.7 (C_{Alkene}-2'', C_{Ar}-3',5'), 102.5 (C_q-10), 94.8 (C_{Ar}-6), 94.0 (C_{Ar}-8), 82.5 (C-2), 64.9 (C-1''), 55.8 (7-OCH_3), 43.0 (C-3), 24.6 (C-5''), 18.6 (C-4'').



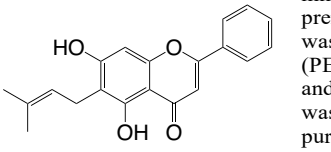
5,7-dihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-en-1-yl)chroman-4-one, 8-prenylnaringenin (4a). To a solution of naringenin **1a** (2.5 g, 9.15 mmol) in dioxane (7.5 ml) was added BF_3.OEt_2 (0.6 ml, 3.7 mmol, 0.4 eq) after which the mixture was heated to 50 °C. Subsequently, a solution of 2-methyl-3-buten-2-ol (1.1 ml, 10.7 mmol, 1.16 eq) was added dropwise over 30 minutes and the reaction continuously stirred for 40 minutes. After completion of the reaction, as was determined by TLC (DCM/MeOH (20:1), 10% H_2SO_4/EtOH stain), the reaction was quenched with satd, $NaHCO_3$ (25 ml) and the solvent evaporated. Water (50 ml) was added and extracted with EtOAc (3x25 ml), organics pooled, washed with brine (50 ml), dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified using reverse-phase flash chromatography using (ACN/H_2O + 0.1% FA (1:0) increasing to (35:65) and finally (0:1) to afford the title compound **4a** as a yellow solid (34 mg, 1% yield). ^{1}H-NMR (600 MHz, Me_2CO_{D6}) δ 7.42 (d, *J* = 8.56 Hz, 2H, H-2', H-6'), 6.91 (d, *J* = 8.56 Hz, 2H, H-3', H-5'), 6.05 (s, 1H, H-6), 5.45 (dd, *J* = 12.95, 2.95 Hz, 1H, H-2), 5.20 (bt, 1H, H-2''), 3.23 (d, *J* = 7.27 Hz, 2H, H-1''), 3.14 (dd, *J* = 17.70, 12.67 Hz, 1H, H-3α), 2.77 (dd, *J* = 17.70, 12.67 Hz, 1H, H-3β), 1.62 (s, 3H, H-4''), 1.61 (s, 3H, H-5''). ^{13}C-NMR (150 MHz, Me_2CO_{D6}) δ 196.8 (C=O), 162.5 (C_q-7), 161.0 (C_q-9), 160.8 (C_q-5), 157.4 (C_q-4'), 131.8 (C_{Alkene}-3''), 130.9 (C_q-1'), 127.4 (C_{Ar}-2',6'), 123.1 (C_{Alkene}-2''), 116.1 (C_{Ar}-3',5'), 107.2 (C_q-8), 102.7 (C_q-4), 95.0 (C_{Ar}-6), 82.8 (C-2), 43.0 (C-3), 24.6 (C-5''), 21.9 (C-1''), 18.6 (C-4'').

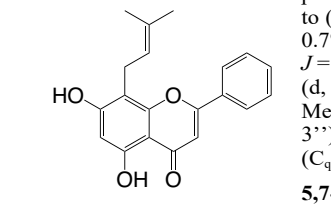


5,7-dihydroxy-6,8-bis(3-methylbut-2-en-1-yl)-2-phenyl-4*H*-chromen-4-one, 6,8-diprenylnaringenin (4b). The title compound **4b** was isolated as a separate product from the previous reaction (7 mg, 0.2% yield). ^{1}H-NMR (600 MHz, Me_2CO_{D6}) δ 7.36 (d, *J* = 8.60 Hz, 2H, H-2', H-6'), 6.89 (d, *J* = 8.60 Hz, 2H, H-3', H-5'), 5.40 (dd, *J* = 12.58, 2.92 Hz, 1H, H-2), 5.15 (m, 2H, H-2'', H-2'''), 3.30 (d, *J* = 7.33 Hz, 2H, H-1''), 3.26 (d, *J* = 7.79 Hz, 2H, H-1'''), 3.13 (dd, *J* = 17.70, 12.67 Hz, 1H, H-3α), 2.78 (dd, *J* = 17.70, 12.67 Hz, 1H, H-3β), 1.77 (s, 3H, H-5'''), 1.67 (s, 3H, H-4'''), 1.62 (s, 3H, H-4''), 1.61 (s, 3H, H-5''). ^{13}C-NMR (150 MHz, Me_2CO_{D6}) δ 196.8 (C=O), 163.2 (C_q-7), 159.4 (C_q-5), 158.0 (C_q-9), 157.4 (C_q-4'), 131.8 (C_{Alkene}-3'', C_{Alkene}-3'''), 130.9 (C_q-1'), 127.4 (C_{Ar}-2',6'), 123.1 (C_{Alkene}-2'', C_{Alkene}-2'''), 116.1 (C_{Ar}-3',5'), 108.6 (C_q-8), 107.6 (C_q-6), 102.6 (C_q-10), 82.8 (C-2), 43.0 (C-3), 24.6 (C-5'', C-5'''), 21.9 (C-1'', C-1'''), 18.6 (C-4'', C-4''').

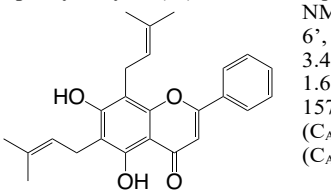


5,7-dihydroxy-6-(3-methylbut-2-en-1-yl)-2-phenyl-4*H*-chromen-4-one, 6-prenylchrysin (5a) / 5,7-dihydroxy-8-(3-methylbut-2-en-1-yl)-2-phenyl-4*H*-chromen-4-one, 8-prenylchrysin (5b). A suspension of chrysin 2a (1 g, 3.9 mmol) and $ZnCl_2$ (1.05 g, 15.6 mmol, 4 eq) in EtOAc (50 ml) was heated to 40 °C. Subsequently, prenol (760 uL, 15.6 mmol, 4 eq) was added dropwise over a period of 1 h after which the reaction was stirred heated to reflux for 4 h. After completion of the reaction, as was determined by TLC (PE/EtOAc (3:1), 10% H_2SO_4/EtOH), the reaction was quenched with pH 1 HCl solution (100 ml) and the layers separated. The aqueous layer was extracted with EtOAc (3x25 ml), organics pooled, washed with brine (50 ml), dried (Na_2SO_4), filtered and concentrated *in vacuo*. The residue was purified through reverse-phase flash chromatography using (ACN/H_2O + 0.1% FA (1:0) increasing to (1:1) and finally (0:1) to afford the title compounds 5a and 5b as a mixture of isomers (13 mg, 0.7% yield). ^{1}H-NMR (600 MHz, Me_2CO_{D6}) δ 8.09 (d, *J* = 7.57 Hz, 2H, H-3', H-5'), 7.67-7.58 (d, *J* = 8.56 Hz, 3H, H-2', H-6', H-4'), 6.78 (s, 1H, H-6), 6.39 (s, 1H, H-3), 5.31 (bt, 1H, H-2''), 3.59 (d, *J* = 7.27 Hz, 2H, H-1''), 1.83 (s, 3H, H-5''), 1.68 (s, 3H, H-4''). ^{13}C-NMR (150 MHz, Me_2CO_{D6}) δ 182.1 (C=O), 164.0 (C_q-7), 163.6 (C_q-2), 158.8 (C_q-5), 156.0 (C_q-9), 131.8 (C_{Alkene}-3''), 130.2 (C_q-1'), 128.6 (C_{Ar}-3',5'), 127.9 (C_{Ar}-2',4',6'), 123.1 (C_{Alkene}-2''), 105.9 (C_q-8), 105.4 (C_q-10), 104.5 (C_q-3), 98.2 (C_{Ar}-6), 24.6 (C-5''), 22.0 (C-1''), 18.6 (C-4'').

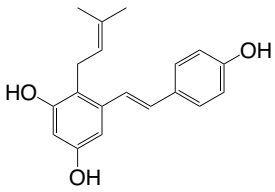




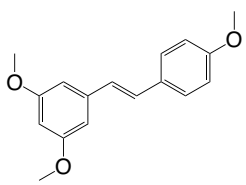
5,7-dihydroxy-6,8-bis(3-methylbut-2-en-1-yl)-2-phenyl-4*H*-chromen-4-one, 6,8-diprenylchrysin (5c) The title compound **5c** was isolated as a separate product from the previous reaction (15 mg, 0.9% yield). ^{1}H-NMR (600 MHz, Me_2CO_{D6}) δ 8.08 (d, *J* = 8.00 Hz, 2H, H-3', H-5'), 7.65-7.60 (m, 3H, H-2', H-6', H-4'), 6.80 (s, 1H, H-3), 5.28-5.23 (bt, 2H, H-2'', H-2'''), 3.67 (d, *J* = 6.54 Hz, 2H, H-1''), 3.46 (d, *J* = 7.09 Hz, 2H, H-1'''), 1.84 (s, 3H, H-5''), 1.80 (s, 3H, H-5'''), 1.69 (s, 3H, H-4''), 1.68 (s, 3H, H-4'''). ^{13}C-NMR (150 MHz, Me_2CO_{D6}) δ 182.1 (C=O), 163.6 (C_q-2), 159.6 (C_q-7), 157.1 (C_q-5), 153.0 (C_q-9), 131.8 (C_{Alkene}-3'', C_{Alkene}-3'''), 130.2 (C_q-1'), 128.6 (C_{Ar}-3',5'), 127.9 (C_{Ar}-2',4',6'), 123.1 (C_{Alkene}-2'', C_{Alkene}2-'''), 110.5 (C_q-8), 105.3 (C_q-10), 104.5 (C_q-3), 102.1 (C_{Ar}-6), 25.3 (C-1'''), 24.6 (C-5'', C-5'''), 21.9 (C-1''), 18.6 (C-4'', C-4''').



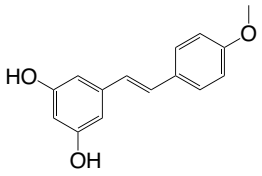
5-hydroxy-7-((3-methylbut-2-en-1-yl)oxy)-2-phenyl-4*H*-chromen-4-one (2b). To a solution of chrysin **2a** (250 mg, 0.98 mmol) in acetone (20 ml) was added K_2CO_3 (270 mg, 1.96 mmol, 2 eq) and prenylbromide (136 uL, 1.17 mmol, 1.2 eq) before the resulting suspension was refluxed for 1 h. After completion of the reaction, as was determined by TLC (PE/EtOAc (4:1), 10% H_2SO_4/EtOH), the reaction mixture was cooled, and the solids filtered off. The filtrate was washed with acetone (30 ml), organics pooled and evaporated *in vacuo*. The residue was crystallized from EtOAc to afford the title compound **2b** as a yellow solid (15 mg, 4.5% yield). ^{1}H-NMR (600 MHz, Me_2CO_{D6}) δ 8.06 (d, *J* = 7.58 Hz, 2H, H-2',H-6'), 7.64-7.57 (m, 3H, H-3',H-5',H-4'), 6.79 (s, 1H, H-3$_{Alkene}$), 6.70 (d, *J* = 2.23 Hz, 1H, H-6), 6.33 (d, *J* = 2.13 Hz, 1H, H-8), 5.50 (tt, *J* = 13.25, 6.25 Hz, 1H, H-2''), 4.70 (d, *J* = 7.40 Hz, 2H, H-1''), 1.80 (d, *J* = 7.70 Hz, 6H, H-4'', H-5''). ^{13}C-NMR (150 MHz, Me_2CO_{D6}) δ 182.1 (C=O), 167.0 (C-7), 163.6 (C_q-2), 161.4 (C-5), 158.4 (C_q-9), 138.4 (C_q-3''), 130.3 (C_q-1'), 128.6 (C-3', C-5'), 127.9 (C-2', C-6', C-4'), 119.6 (C-2''), 104.5 (C-3), 104.1 (C_q-10), 98.0 (C-6), 92.8 (C-8), 64.9 (C-1''), 24.6 (C-4''), 18.6 (C-5'').



***(E)*-5-(4-hydroxystyryl)-4-(3-methylbut-2-en-1-yl)benzene-1,3-diol (3b).** To a solution of resveratrol **3a** (0.5 g, 2.19 mmol) was added dry Li_2CO_3 (325 mg, 4.38 mmol, 2 eq) and the dropwise addition of prenylbromide (760 uL, 6.6 mmol, 3 eq) before the reaction mixture was heated to reflux for 3 h. After completion of the reaction, as was determined by TLC (DCM/MeOH, H_2SO_4/EtOH stain), the reaction was cooled, diluted with water (100 ml) and extracted with EtOAc (3x50 ml). The organics were pooled, washed with brine (50 ml), dried (Na_2SO_4), filtered and concentrated *in vacuo*. The residue was purified by silica gel flash chromatography using (PE/EtOAc (1:0), increasing to (3:1), (1:1) and finally (0:1)) to give the title compound **3b** as a yellow solid (21 mg, 6.1% yield). ^{1}H-NMR (600 MHz, Me_2CO_{D6}) δ 7.40 (d, *J* = 9.28 Hz, 2H, H-2',H-6'), 7.16 (d, *J* = 16.24 Hz, 1H, H-α), 6.92 (d, *J* = 16.24 Hz, 1H, H-β), 6.83 (d, *J* = 8.81 Hz, 2H, H-3', H-5'), 6.63 (d, *J* = 2.60 Hz, 1H, H-6), 6.28 (d, *J* = 2.36 Hz, 1H, H-4), 5.09 (t, *J* = 7.51 Hz, 1H, H-2''), 3.38 (d, *J* = 6.94 Hz, 2H, H-1''), 1.80 (s, 3H, H-4''), 1.67 (s, 3H, H-5''). ^{13}C-NMR (150 MHz, Me_2CO_{D6}) δ 157.7 (C_q-4'), 157.4 (C_q-3), 156.8 (C_q-5), 139.0 (C_q-1), 131.8 (C_{Alkene}-3''), 130.6 (C-2',6'), 130.1 (C-1'), 127.4 (C_{Alkene}-β), 125.5 (C_q-2, C_{Alkene}-α), 123.1 (C_{Alkene}-2''), 115.8 (C-3',5'), 104.6 (C-6), 102.7 (C-4), 25.7 (C-1''), 24.6 (C-4''), 18.6 (C-5'').



***(E)*-1,3-dimethoxy-5-(4-methoxystyryl)benzene, 3,5,4'-trimethoxyresveratrol (3d).** To a suspension of resveratrol **3a** (1 g, 4.38 mmol) and Li_2CO_3 (650 mg, 8.76 mmol, 2 eq) in DMF (60 ml) was added the dropwise addition of prenylbromide (1.52 ml, 13.2 mmol, 3 eq) after which the reaction was refluxed for 3 h. After consumption of the starting material, as was determined by TLC (DCM/MeOH (20:1), 10% H_2SO_4/EtOH stain), the reaction mixture was diluted with water (100 ml), extracted with EtOAc (3x50 ml), organics pooled, dried (Na_2SO_4), filtered and concentrated *in vacuo*. The residue was purified by silica gel flash chromatography using (PE/EtOAc (1:0) increasing to (3:1), (1:1) and finally (0:1)) to afford the title compound **3d** as a white solid (26 mg, 1.3% yield). ^{1}H-NMR (600 MHz, Me_2CO_{D6}) δ 7.55-7.52 (d, *J* = 8.80 Hz, 2H, H-2',H-6'), 7.23-7.19 (d, *J* = 16.02 Hz, 1H, H-α), 7.06-7.02 (d, *J* = 16.43 Hz, 1H, H-β), 6.97-6.93 (d, *J* = 8.62 Hz, 2H, H-3', H-5'), 6.76 (d, *J* = 2.25 Hz, 2H, H-2, H-6), 6.40 (t, *J* = 2.26 Hz, 1H, H-4), 3.82 (s, 9H, 3x OCH_3). ^{13}C-NMR (150 MHz, Me_2CO_{D6}) δ 161.5 (C-3,5), 159.8 (C_q-4'), 140.5 (C_q-1), 130.2 (C-2',6'), 129.8 (C_q-1'), 127.4 (C_{Alkene}-α, β), 114.2 (C-3',5'), 105.5 (C-2,6), 99.6 (C-4), 55.8 (3x C_{Me}).



***(E)*-5-(4-methoxystyryl)benzene-1,3-diol, 4'-methoxyresveratrol (3c).** The title compound **3c** (46 mg, 2.2% yield) was isolated as a separate product from the previous reaction. ^{1}H-NMR (600 MHz, Me_2CO_{D6}) δ 7.51 (d, *J* = 8.72 Hz, 2H, H-2', H-6'), 7.06 (d, *J* = 16.31 Hz, 1H, H-α), 6.94 (d, *J* = 15.91 Hz, 1H, H-β), 6.93 (d, *J* = 8.72 Hz, 2H, H-3', H-5'), 6.58 (d, 2H, H-2, H-6), 6.31 (m, 1H, H-4), 3.81 (s, 3H, OCH_3). ^{13}C-NMR (150 MHz, $MeOD_6$) δ 159.8 (C_q-3, C_q-5, C_q-4'), 141.3 (C_q-1), 130.2 (C-2', C-6'), 129.8 (C-1'), 127.4 (C_{Alkene}-α, β), 114.2 (C-3', C-5'), 104.7 (C-2, C-6), 102.8 (C-4), 55.8 (C_{Me}).

7.1 Procedure for the broth microdilution assay against MRSA 18HN

The synthesized prenylated stilbenoids and (iso)flavonoids and methylated stilbenoids were tested against methicillin-resistant staphylococcus aureus (MRSA 18HN) (strain provided by RIVM, Bilthoven, The Netherlands). From a -80 °C glycerol stock, bacteria were streaked onto a TSB agar plate and incubated for 24 h at 37 °C. Next, one colony was transferred to TSB broth (10 ml) and further incubated for 18 h at 37 °C. The overnight cultures were diluted with TSB (final inoculum concentration 4 log CFU/ml). Stock solutions of the different prenylated and non-prenylated compounds were prepared in DMSO and subsequently diluted with TSB. Series of concentrations ranging from 3.125 to 100 ug/mL of prenylated stilbenoids and (iso)flavonoids and methylated stilbenoids were tested. Equal volumes (100 µL) of prenylated stilbenoids and (iso)flavonoids and methylated stilbenoids in TSB and bacteria inoculum (100 µL) were mixed into a 96-well plate and the plate incubated at 37 °C for 24 h in a SpectraMax M2e (Molecular Devices, Sunnyvale, CA, USA) with constant linear shaking and kinetic measurement. The optical density (OD) at 600 nm was measured every 2 min for 24 h.

Positive controls (vancomycin, 1.25 to 2.5 µg/mL) and (glabridin 3.125 to 50 µg/mL), negative control (TSB suspension of bacteria with 2.5% (v/v) max. solvent), (4'-*O*-methylresveratrol 3.125 to 50 µg/mL) (3,5,4'-tri-*O*-methylresveratrol 3.125 to 50 µg/mL) and blanks (TSB medium with no bacteria) were considered for optical comparison and sterility control. Inhibition of growth was assessed by measuring the time to detection (TTD), i.e., the time to reach a change in OD of 0.05 units. When no change in OD (i.e., deltaOD <0.05) was observed after 24 h of incubation, cell viability was verified by plate counting. Briefly, 100 µL of the well with no change in OD was decimally diluted in PPS solution and 100 µL of each dilution was spread onto TSB agar plates. Subsequently, plates were incubated at 37 °C for 24 h and colonies were counted. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of compound that resulted in a bacterial count equal or lower than that of the initial inoculum. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of compound that resulted in > 99% bacterial inactivation from the initial bacterial inoculum. Prenylated stilbenoids and (iso)flavonoids and methylated stilbenoids were tested in three independent biological reproductions, each performed in duplicate.

7.2 Predicting antimicrobial activity of synthesized prenylated and non-prenylated compounds

The QSAR model was developed using a training set of compounds. Based on that set an applicability domain was developed to determine whether new compounds used to predict new MIC values fall within that domain and show reliability of the model. The applicability domain starts with standardizing the data for each compound for all the four molecular descriptors: (*vsurf_D4*), (*h_emd_C*), (*E_vdw*) and (*vsurf_IW7*). Following standardization, the mean and standard deviation (SD) of each standardized data

set were calculated and the compounds examined for fitting within the model. If the mean plus SD exceeds the value of 3xSD than the compound does not fall within the applicability domain and the MIC value prediction of the model is not accurate.

A QSAR model was used to predict *in-silico* the MIC values of the synthesized compounds and compare them to the *in-vitro* data of the compounds in the broth microdilution assay. This model contained four molecular descriptors that complied with the threshold for the validation parameters of the QSAR model. The four-descriptor model was chosen as the best, since it balances well statistical validity, predictivity of the training (Q^2_{LOO} 0.57 and average prediction error of 4%) and the test set (Q^2_{test} 0.75 and average prediction error of 5%) and models interpretability.

Using Molecular Operating Environment (MOE), compounds were sketched before being energy minimized and a conformational search performed to determine the conformation with the lowest energy state. Subsequently, the four descriptors were calculated for each compound. The descriptor (*vsurf_D4*) is the most significant descriptor as it shows the hydrophobic volume at the 4th energetic level (-0.8 kcal/mol) followed by the sum of hydrogen bond donor strengths of carbon atoms (*h_emd_C*). Also, the van der Waals interaction energy (*E_vdw*) and the unbalance between the center of mass of a molecule and the position of the hydrophilic regions around it (*vsurf_IW7*) were calculated. The data obtained for each descriptor for each compound (Appendix C) was inserted into an equation (Appendix E) that was determined to be the best fitting GA-MLR model for predicting the antimicrobial activity in MIC of prenylated (iso)flavonoids against MRSA and is abstracted from Kalli *et al*. The pMIC values obtained from the data of the four molecular descriptors were converted to µg/mL.

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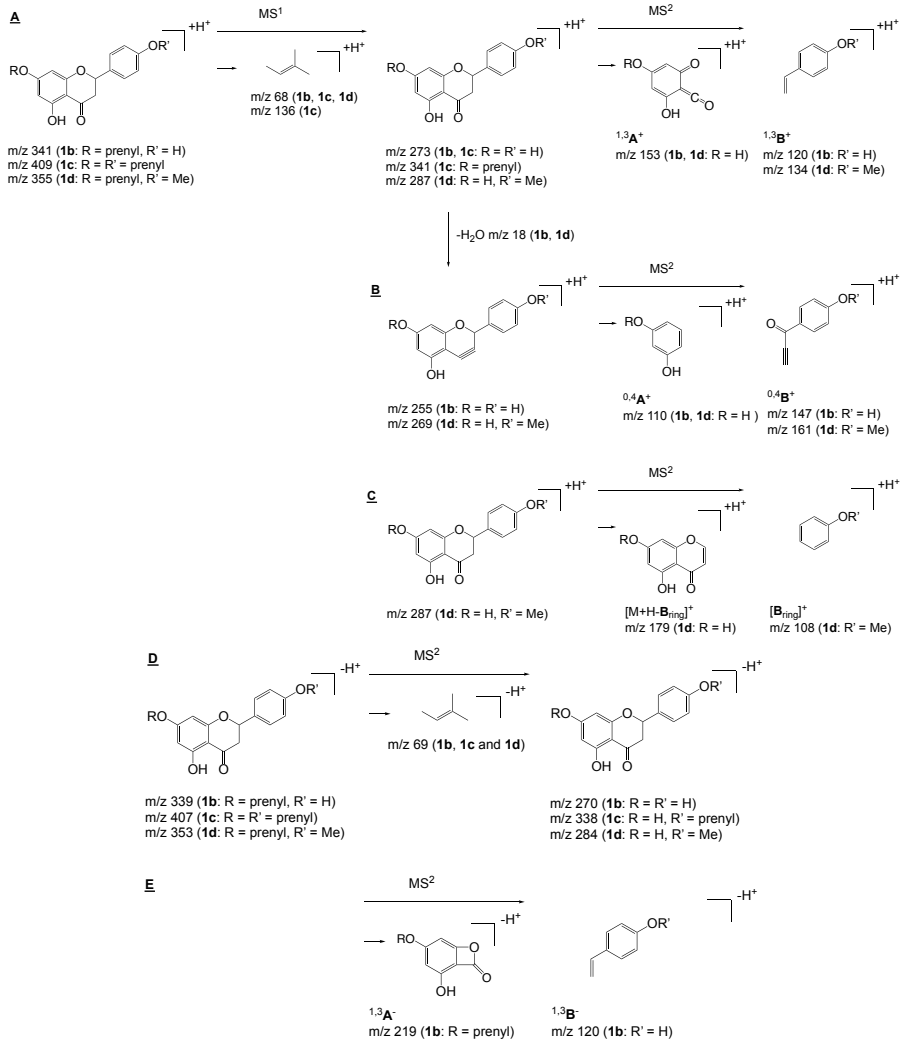
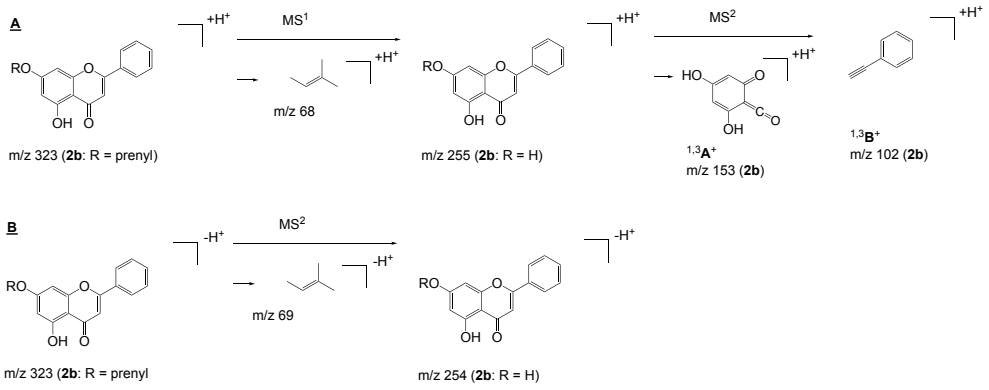
Appendix

Appendix A

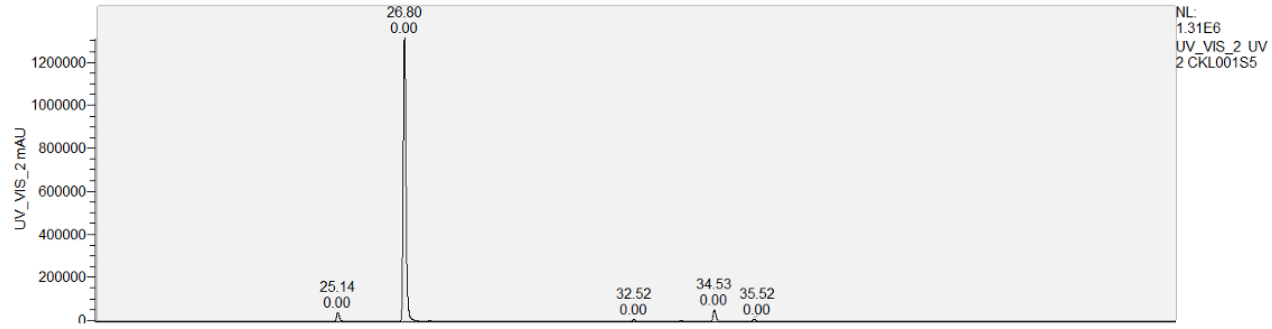


Appendix A.1: MS fragmentation patterns for *O*-prenylated compounds **1b**-**1d**. Data obtained both in PI and NI mode.

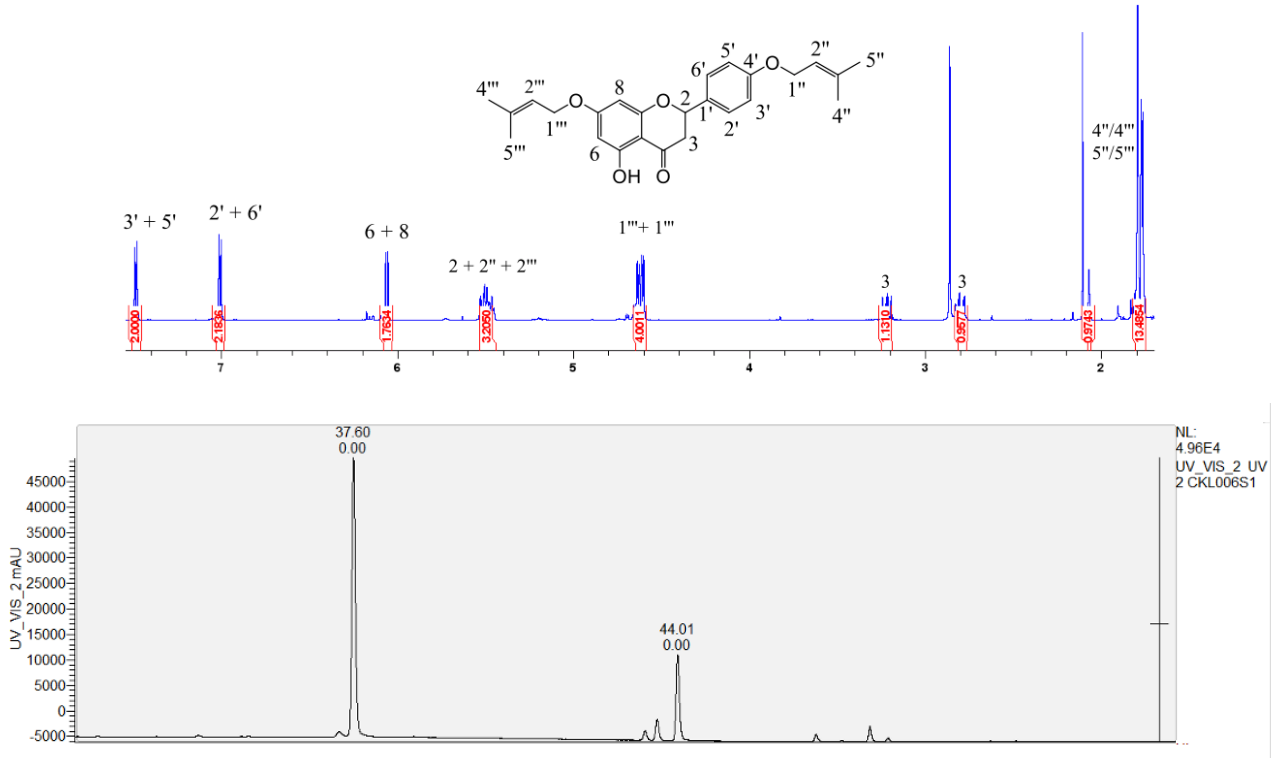


Appendix A.2: MS fragmentation patterns for *O*-prenylated chrysin **2b**. Data obtained in PI mode.

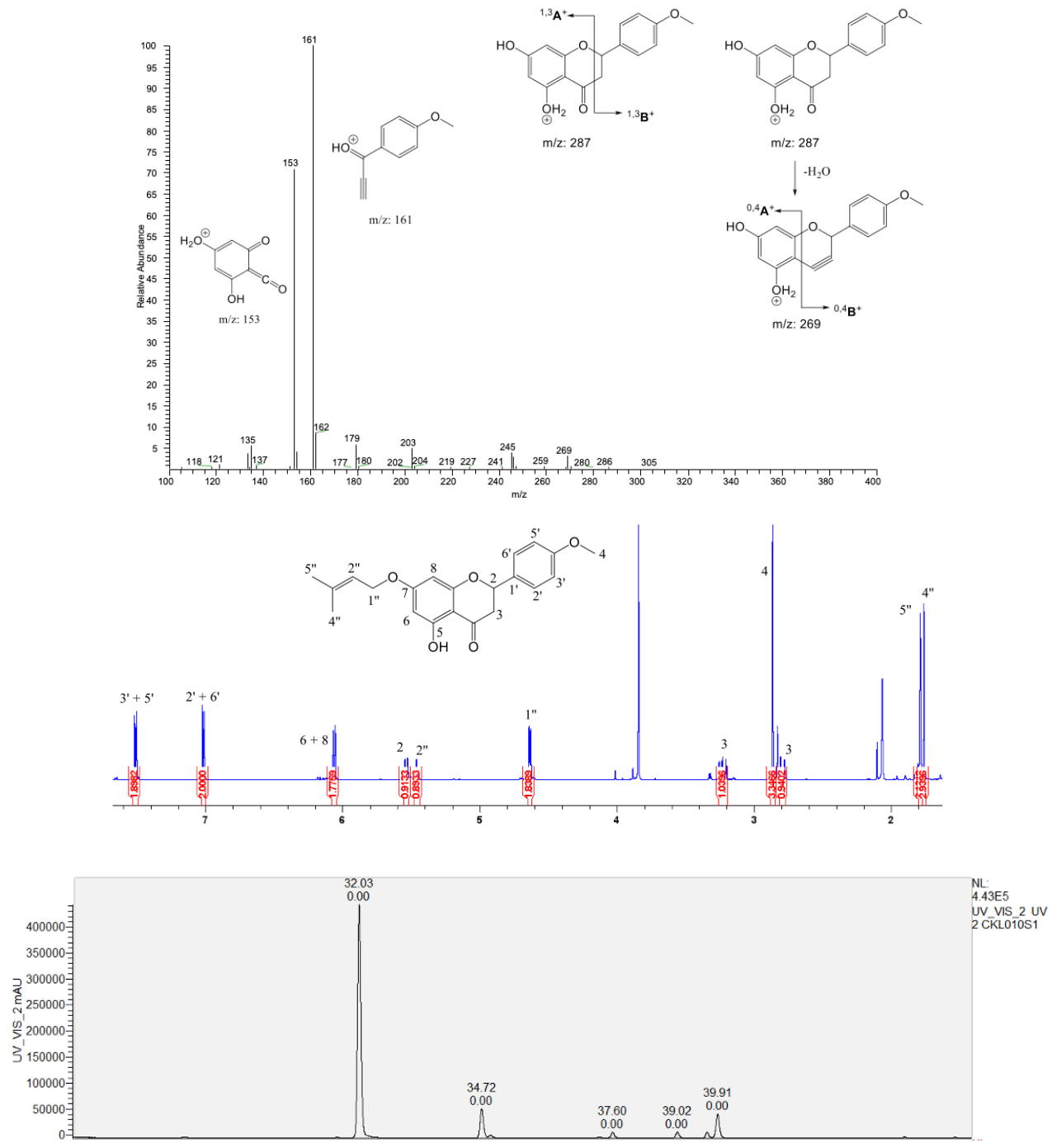
Appendix B



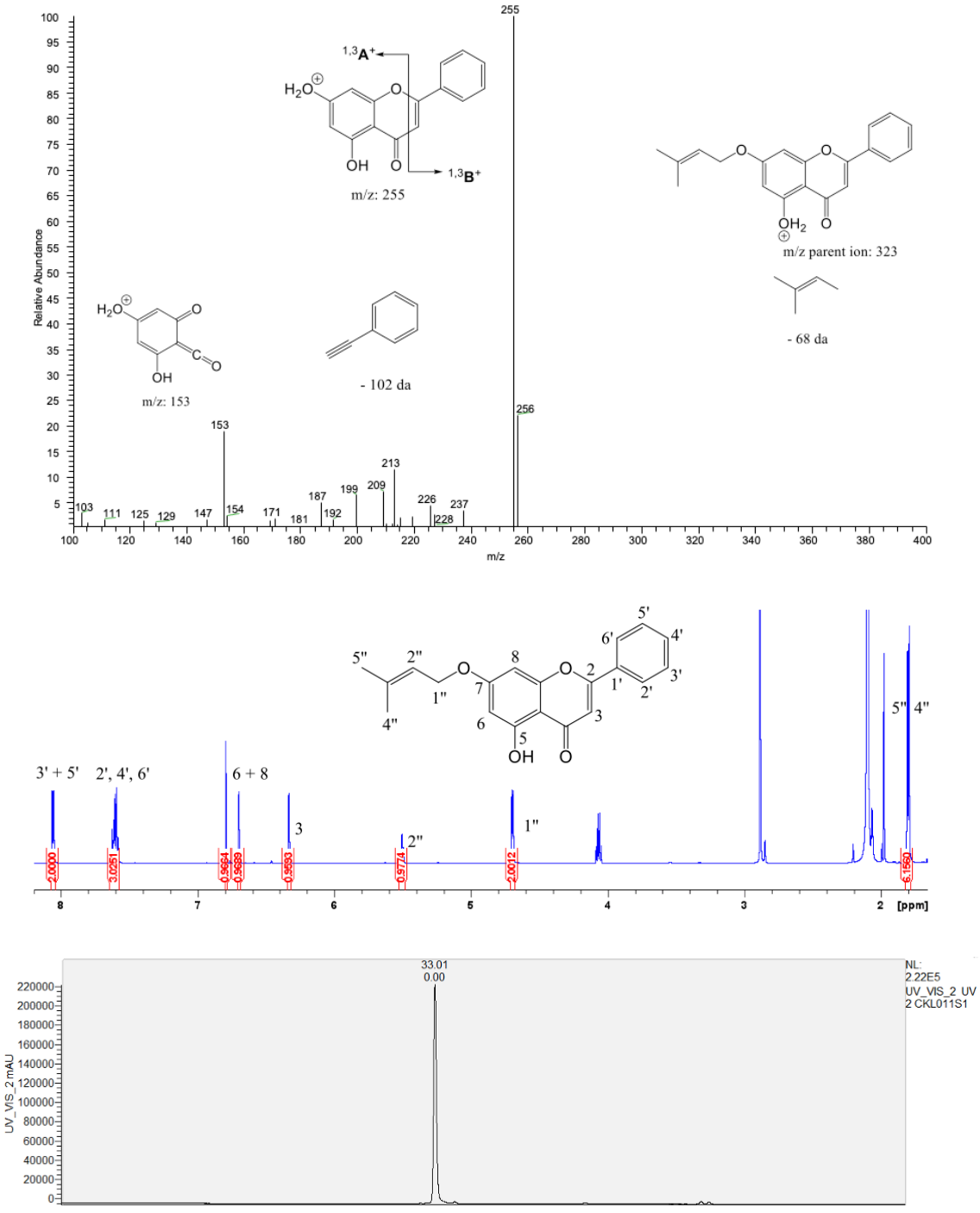
Appendix B.1: LC data of 7-*O*-prenylnaringenin **1b**.



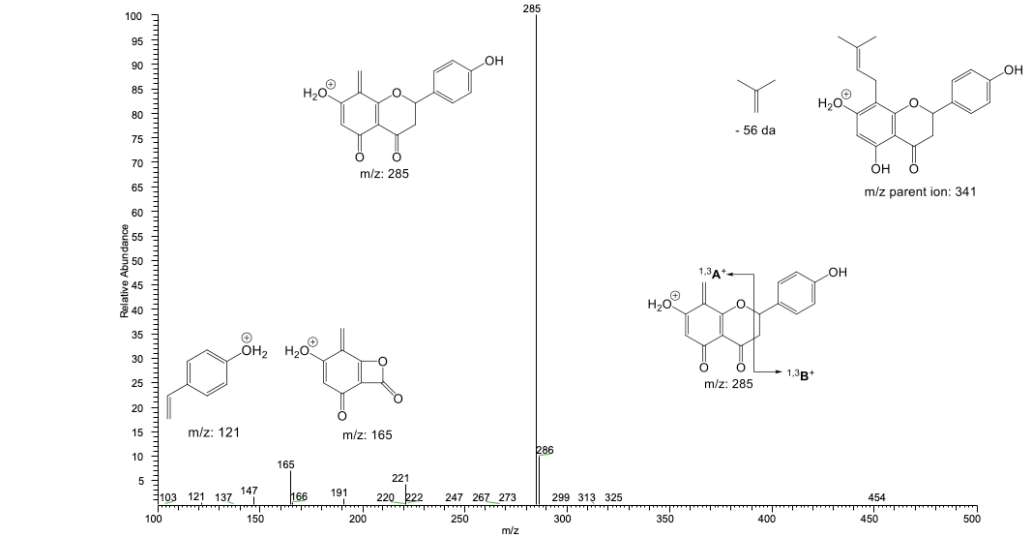
Appendix B.2: NMR and LC data of 7,4'-di-*O*-prenylnaringenin **1c**.

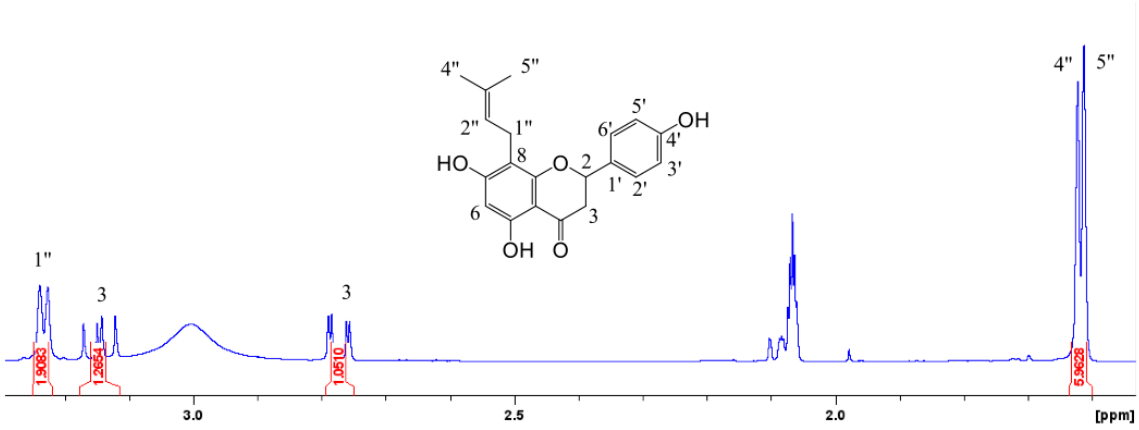


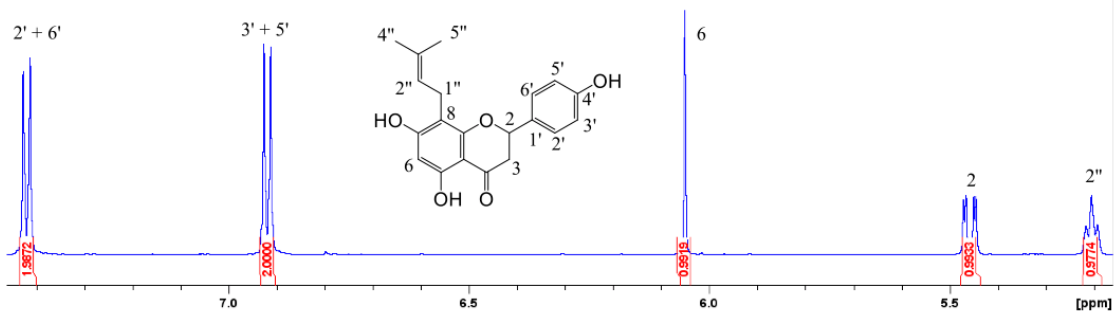
Appendix B.3: MS, NMR and LC data of 7-*O*-prenyl-4'-*O*-methylnaringenin **1d**.

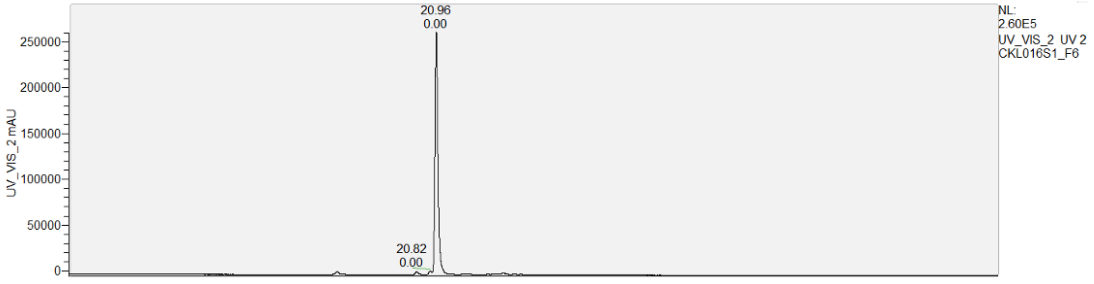


Appendix B.4: MS, NMR and LC data of 7-*O*-prenylchrysin **2b**.

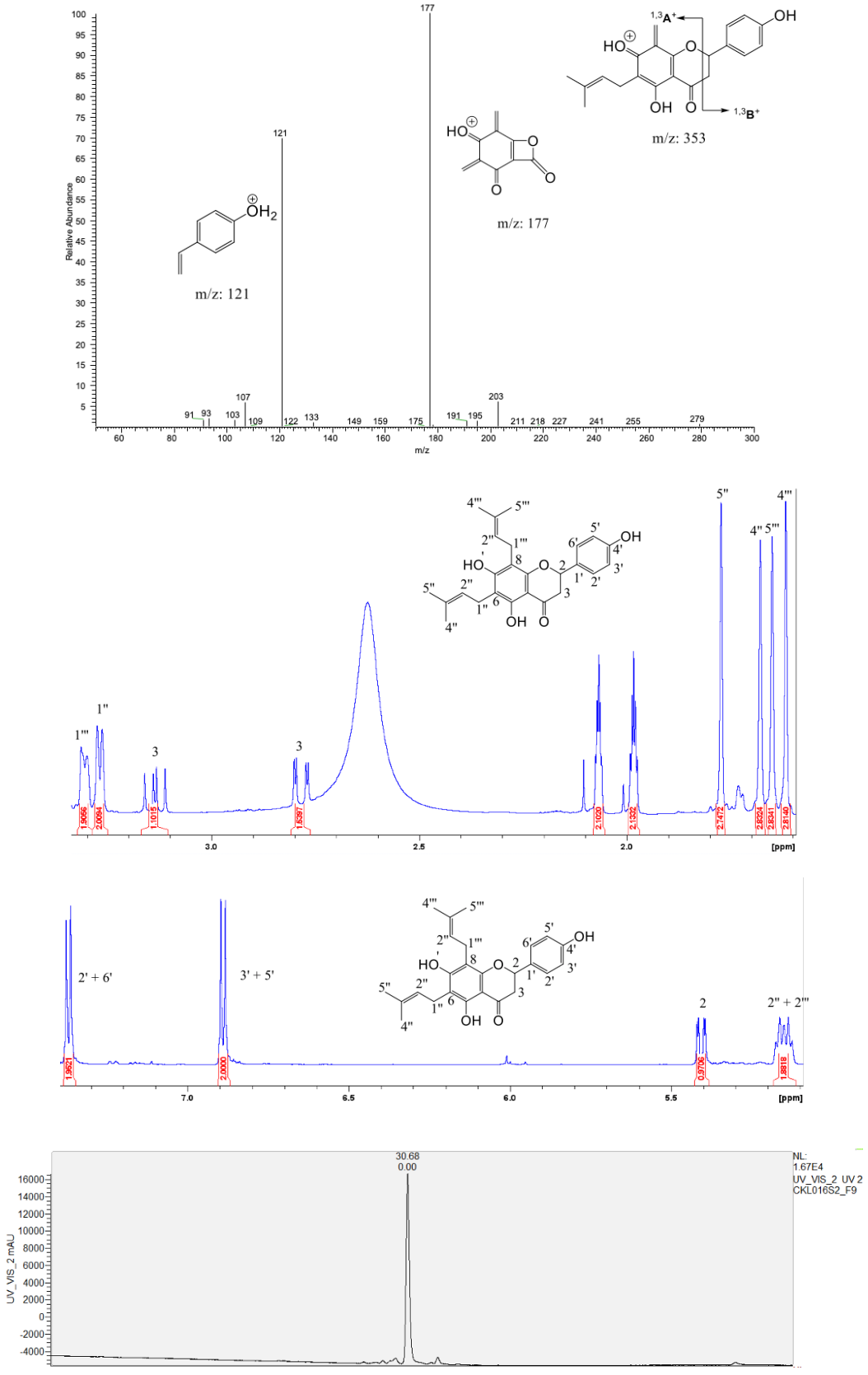




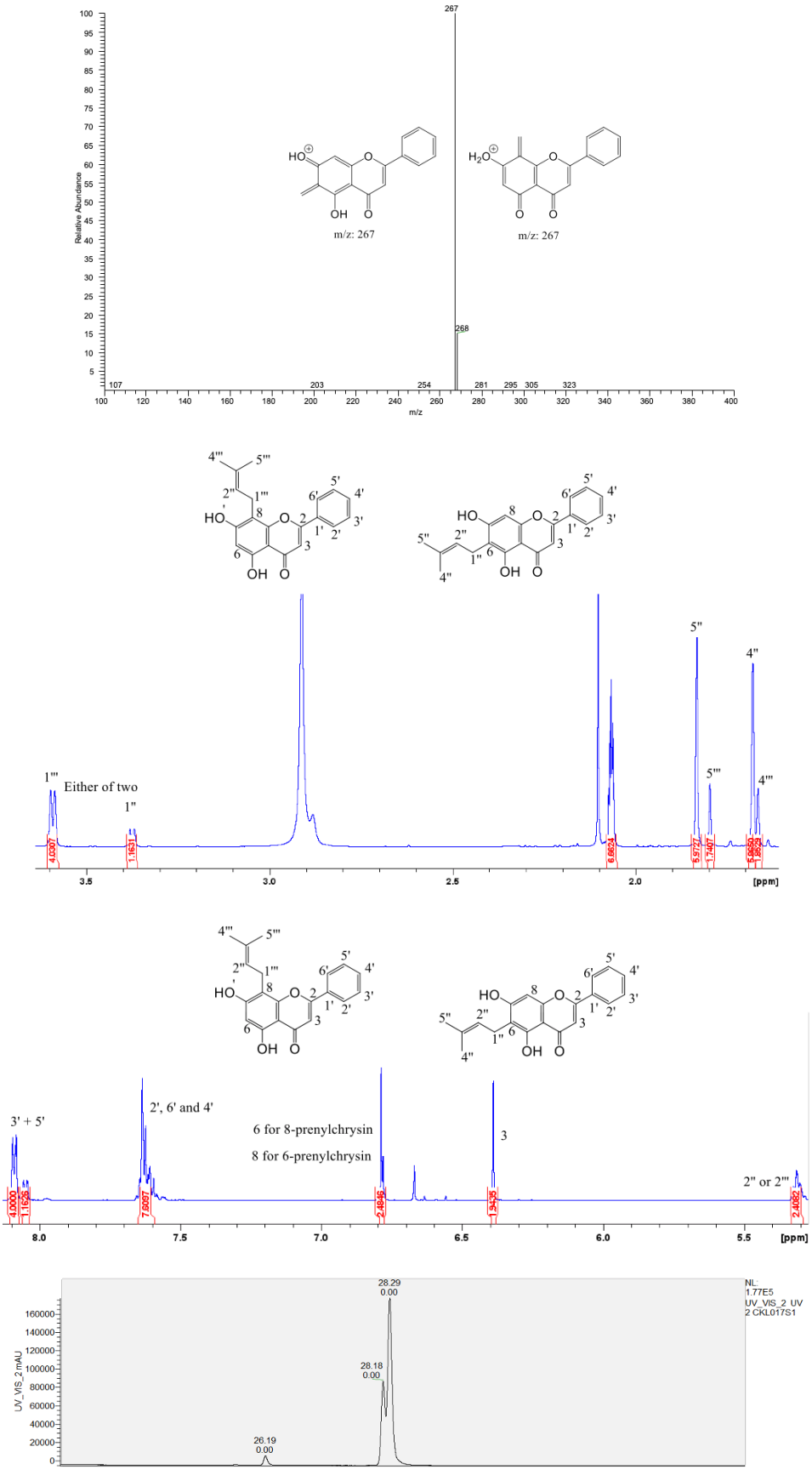




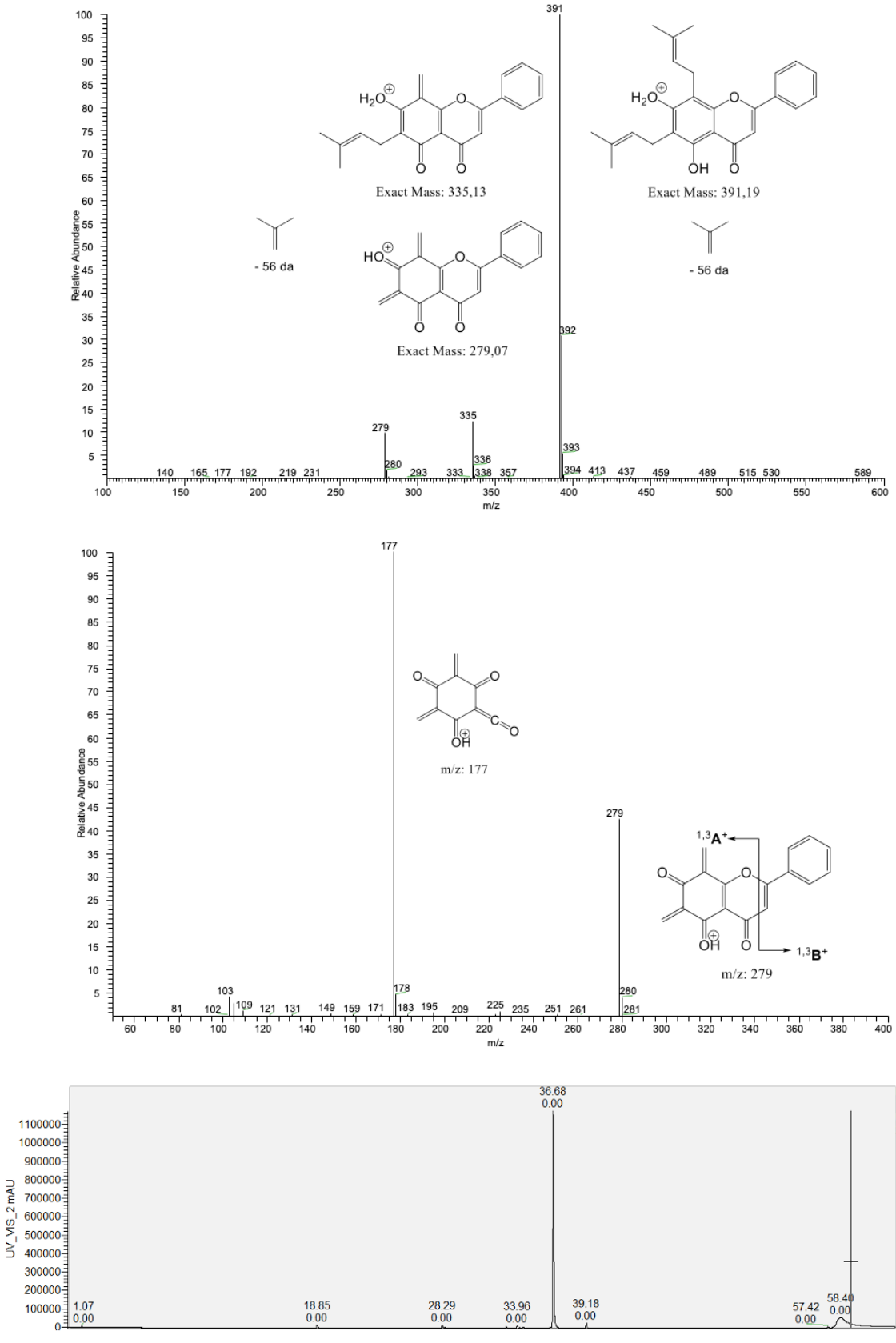
Appendix B.5: MS, NMR and LC data of 8-prenylnaringenin **4a**.



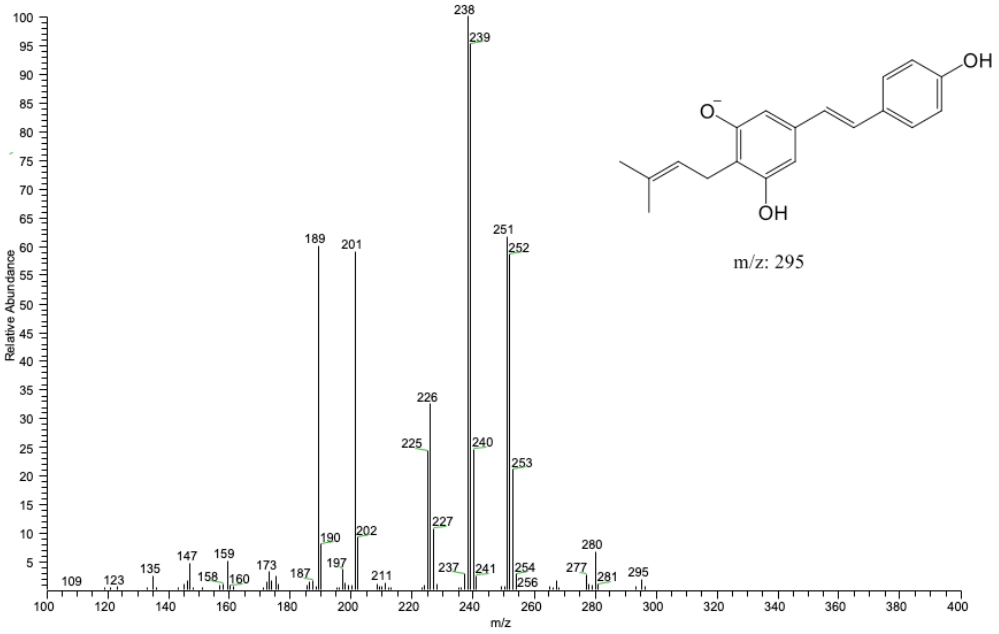
Appendix B.6: MS, NMR and LC data of 6,8-diprenylnaringenin **4b**.

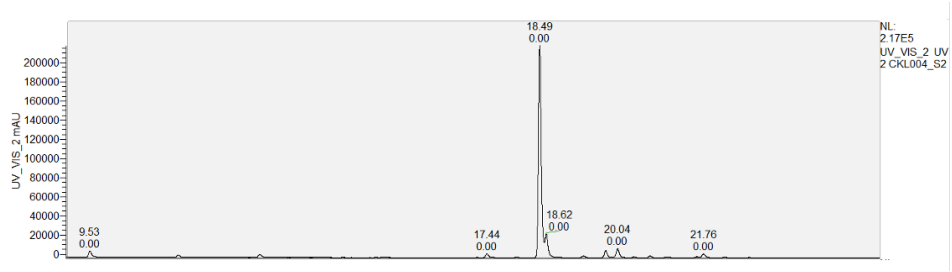


Appendix B.7: MS, NMR and LC data of mixture of 6-/8-prenylchrysin **5a/5b**.



Appendix B.8: MS and NMR data of 6,8-diprenylchrysin **5c**.





Appendix B.9: MS, NMR and LC data of 2-prenylresveratrol **3b**.

Appendix C

No.	UV_{max} (nm)	Identification	$[M-H]^-$	MS^2 product ions (relative intensity)	MS3 product ions (relative intensity)	$[M+H]^+$	MS^2 product ions (relative intensity)	MS3 product ions (relative intensity)	Purity[a]
1	238, 290	7-*O*-prenylnaringenin	339	119(3), 164(12), 193(20), 219(55, 270(100)		341, 273(100)[b]	147(71), 153(100)		95
2	234, 290	7,4'-di-*O*-prenylnaringenin	407	338(100)		409	273(5), 341(100)		95
3	234, 290	7-*O*-prenyl-4'-*O*-methylnaringenin	353	219(5), 256(5), 283(5), 284(100), 309(66), 311(8), 339(7)		355 287(95)[b]	135(12), 153(97), 161(100), 179(14), 203(5), 245(5)		95
4	234, 294	8-prenylnaringenin	339	219 (85), 283 (100)		341	285(100), 290(32)		95
5	234, 294	6,8-diprenylnaringenin	407			409 353[b]	353(100) 121(1), 177(2), 233(35), 297(100)	121(70), 177(100) 77(4), 123(5), 173(100)	90
6	234, 266	7-*O*-prenylchrysin	321			323, 255(100)[b]	147(2), 153(19), 199(5), 213(11), 226(5), 255(100)		95
7	234, 274	6-prenylchrysin/8-prenylchrysin	321	266(100)		323	267(100)	137(32), 123(100)	N.d.[d]
8	237, 278	6,8-diprenylchrysin	389	291(25), 334(100)	235(10), 251(100)	391	279(10), 335(100)	279(45), 177(100)	95
9	234, 306	2-prenylresveratrol	295	226(34), 201(58), 251(60), 189(61), 238(100)					70-80

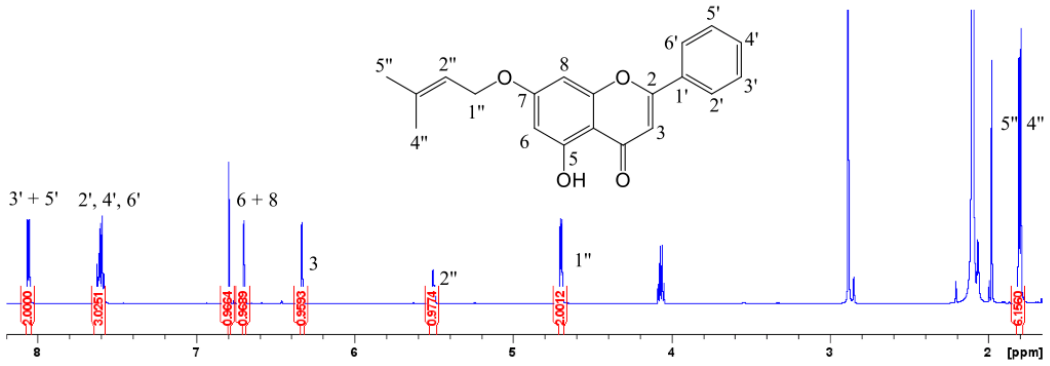
[a] Based on ^1H proton NMR spectra and LC-MS data.

[b] In-source fragmentation.

[c] Mixture of inseparable *C*-prenyl isomers.

[d] N.d. = not determined

Appendix D



Appendix D.1: Determining purity using 7-*O*-prenylchrysin **2b** as example. First, all protons in the spectrum are assigned at the locations in ppm where they are expected. All protons are given a number a well as integrals that correspond to the protons at each carbon. If the integrals of for example the aromatic B-ring (5 protons = 2+3.0251 integrals total 5.0251) match to other signals for example the C-3 proton (1 proton = 0.9593 integrals total) (ratio 5:1) than the compound is pure based on the ratio. However, solvent signals can have very high integrals and should not be assigned. A list of solvent peak residues can be found in the literature. If peaks remain that are neither solvent or peaks of the compound then they are impurities. The integrals of such impurity can be calculated. For example, if the impurity is a singlet with an integral of 0.03 and comparing that to a singlet with integral 0.98 than dividing 0.03 to 0.98 gives around 0.0306 which is 3.06% impurity. Meaning that the compound of interest is 97% pure.

Appendix E

Name	Subclass	h_emd_C	vsurf_D4	vsurf_IW7	E_vdw	pMIC values	MIC in M	mg/mmoles	ug/ml
8-prenylnaringenin	Flavanone	4,8983	319,3750	0,0000	14,2754	4,15	0,000071074531	340.000	24,2
6-prenylnaringenin	Flavanone	4,8982	305,2500	4,5162	15,5030	3,72	0,000190605751	340.000	64,8
6,8-diprenylnaringenin	Flavanone	5,6501	342,8750	3,7791	15,5986	3,99	0,000101660678	408.000	41,5
7-O-prenylnaringenin	Flavanone	5,9308	337,2500	0,0000	11,3929	4,29	0,000051842866	340.000	17,6
7,4'-di-O-prenylnaringenin	Flavanone	7,6280	451,5000	0,0000	12,5433	5,06	0,000008614003	408.000	3,5
7-O-prenyl-4'-O-methylnaringeni	Flavanone	7,1732	375,5000	6,4451	12,0140	4,09	0,000081120571	354.000	28,7
7-O-prenylchrysin	Flavone	5,1422	361,8750	0,0000	13,1411	4,59	0,000025935881	322.000	8,4
6-prenylchrysin	Flavone	4,1009	330,3750	4,1012	17,0281	4,06	0,000087272416	322.000	28,1
6,8-diprenylchrysin	Flavone	4,8301	387,2500	4,3325	17,4974	4,46	0,000034547212	390.000	13,5
8-prenylchrysin	Flavone	4,0826	352,5000	0,0000	15,6631	4,56	0,000027734670	322.000	8,9
2-prenylresveratrol	Stilbenoid	3,5334	339,8750	0,0000	13,3245	4,65	0,000022567684	293.000	6,6
4'-O-methylresveratrol	Stilbenoid	4,0348	282,5000	0,0000	9,8225	4,16	0,000069564713	242.000	16,8
3,5,4'-tri-O-methylresveratrol	Stilbenoid	6,5419	356,1250	0,0000	11,0497	4,38	0,000041569710	269.000	11,2

Appendix E.1: Excel data *in-silico* model MIC prediction of compounds against MRSA. Four descriptors present.

Appendix F

$$Y = 2.55\text{-}0.18 * h\_emd\_C + 0.01 * vsurf\_D4 - 0.05 * vsurf\_IW7 - 0.05\ E\_vdq$$

Appendix F.1: Equation for the MIC prediction of *in-silico* generated compounds.