The flavanone naringenin reduces the production of quorum sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1

Olivier M. Vandeputte,^{1,2†} Martin Kiendrebeogo,^{1,3†} Tsiry Rasamiravaka,⁴ Caroline Stévigny,⁵ Pierre Duez,⁵ Sanda Rajaonson,^{1,4} Billo Diallo,^{1,2} Adeline Mol,¹ Marie Baucher¹ and Mondher El Jaziri¹

Correspondence

Olivier M. Vandeputte
ovdeputt@ulb.ac.be

¹Laboratoire de Biotechnologie Végétale, Université Libre de Bruxelles, rue des Professeurs Jeener et Brachet 12, B-6041 Gosselies, Belgium

²Plant Biotechnology Unit, BioVallée, rue Adrienne Bolland 8, B-6041 Gosselies, Belgium

³Laboratoire de Biochimie et de Chimie Appliquées, Université de Ouagadougou, 09 BP 848 Ouagadougou 09, Burkina Faso

⁴Laboratoire de Physiologie Végétale, Université d'Antananarivo, BP 906 Antananarivo 101, Madagascar

⁵Laboratoire de Pharmacognosie, de Bromatologie et de Nutrition Humaine, Université Libre de Bruxelles, CP 205/9, Boulevard du Triomphe, B-1050 Brussels, Belgium

Preliminary screening of the Malagasy plant *Combretum albiflorum* for compounds attenuating the production of quorum sensing (QS)-controlled virulence factors in bacteria led to the identification of active fractions containing flavonoids. In the present study, several flavonoids belonging to the flavone, flavanone, flavonol and chalcone structural groups were screened for their capacity to reduce the production of QS-controlled factors in the opportunistic pathogen *Pseudomonas aeruginosa* (strain PAO1). Flavanones (i.e. naringenin, eriodictyol and taxifolin) significantly reduced the production of pyocyanin and elastase in *P. aeruginosa* without affecting bacterial growth. Consistently, naringenin and taxifolin reduced the expression of several QS-controlled genes (i.e. *lasI*, *lasR*, *rhlI*, *rhlR*, *lasA*, *lasB*, *phzA1* and *rhlA*) in *P. aeruginosa* PAO1. Naringenin also dramatically reduced the production of the acylhomoserine lactones *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL), which is driven by the *lasI* and *rhlI* gene products, respectively. In addition, using mutant strains deficient for autoinduction (Δ *lasI* and Δ *rhlI*) and LasR- and RhlR-based biosensors, it was shown that QS inhibition by naringenin not only is the consequence of a reduced production of autoinduction compounds but also results from a defect in the proper functioning of the RhlR–C4-HSL complex. Widely distributed in the plant kingdom, flavonoids are known for their numerous and determinant roles in plant physiology, plant development and in the success of plant–rhizobia interactions, but, as shown here, some of them also have a role as inhibitors of the virulence of pathogenic bacteria by interfering with QS mechanisms.

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INTRODUCTION

In many pathogenic bacteria the production of virulence factors is triggered in a population density-dependent manner through quorum sensing (QS), a cell-to-cell communication mechanism that enables bacteria to

coordinate virulence factor production by means of the synthesis, release and perception of small diffusible molecules called autoinducers (Antunes *et al.*, 2010; Bjarnsholt *et al.*, 2010; Case *et al.*, 2008; Ng & Bassler, 2009). For instance, in the plant and mammal opportunistic pathogen *Pseudomonas aeruginosa*, two main QS systems (*lasI/R* and *rhlI/R*), responsible for the synthesis and perception of the acylhomoserine lactones (AHLs) *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL), respectively (Pesci & Iglewski, 1997), control the expression

†These authors contributed equally to this work.

Abbreviations: AHL, acylhomoserine lactone; C4-HSL, *N*-butanoyl-L-homoserine lactone; ESI-MS, electrospray ionization-MS; HHL, *N*-hexanoyl-L-homoserine lactone; 3-oxo-C12-HSL, *N*-(3-oxododecanoyl)-L-homoserine lactone; PI, propidium iodide; QS, quorum sensing.

of an arsenal of virulence factors. The transcription factors LasR and RhlR interact with and are activated by 3-oxo-C12-HSL and C4-HSL, respectively, triggering the production of biofilms, LasB elastase, LasA protease, Apr alkaline protease, exotoxin A, rhamnolipids, pyocyanin, hydrogen cyanide and cytotoxic lectins, among other virulence factors (Davies *et al.*, 1998; Gambello & Iglewski, 1991; Gambello *et al.*, 1993; Pesci *et al.*, 1997; Seed *et al.*, 1995; Toder *et al.*, 1991, 1994). In conjunction with a third QS system, using quinolone signalling, the *las* and *rhl* systems are tightly co-regulated in a hierarchical manner (Pesci & Iglewski, 1997; Pesci *et al.*, 1999; Wade *et al.*, 2005).

Considering its central role in regulating the production of virulence factors by pathogenic bacteria, QS is considered an attractive target for the development of strategies to attenuate the virulence of these bacteria (Bjarnsholt *et al.*, 2010; González & Keshavan, 2006; McDougald *et al.*, 2007; Njoroge & Sperandio, 2009). In that context, plants have been widely screened for compounds that interfere with QS mechanisms (González & Keshavan, 2006; Janssens *et al.*, 2008; Teplitski *et al.*, 2011). Recently, we have shown that the Malagasy plant *Combretum albiflorum* (Tul.) Jongkind contains phenolic compounds that affect the production of QS-regulated factors in *P. aeruginosa* PAO1 (Vandeputte *et al.*, 2010). The characterization of one of the active chromatographic fractions led to the identification of several flavonoid-related structures and to the identification of catechin as one of the active compounds (Vandeputte *et al.*, 2010). Flavonoids are widely produced by plants and have important physiological functions, acting as signals in the legume–rhizobia symbiosis; they present a large structural diversity and display many pharmacological activities (Buer *et al.*, 2010; Dixon & Steele, 1999; Dixon & Pasinetti, 2010; Mandal *et al.*, 2010). Based on the screening performed on *Combretum albiflorum* (Vandeputte *et al.*, 2010), several commercially available flavonoids belonging to the flavone, flavanone, flavonol and chalcone groups have been selected to assess their capacity to interfere with QS mechanisms in *P. aeruginosa* PAO1 and *Chromobacterium violaceum* CV026. These structurally related compounds differ in their structures by the oxidation and hydroxylation of their central C-ring and by the substitution pattern of their A- and B-rings (Buer *et al.*, 2010; Dixon & Steele, 1999; Dixon & Pasinetti, 2010) (Fig. 1). Among the tested flavonoids, those of the flavanone group (naringenin, eriodictyol and taxifolin) are shown to dramatically interfere with QS mechanisms with limited/or no effect on bacterial growth.

METHODS

Bacterial strains, plasmids and culture conditions. *C. violaceum* CV026 was grown in liquid Luria–Bertani (LB) medium at 28 °C (McClean *et al.*, 1997) and tested as described previously (Vandeputte *et al.*, 2010). *P. aeruginosa* PAO1 wild-type strain was obtained from the *Pseudomonas* Genetic Stock Center (strain PAO0001; <http://www.pseudomonas.med.ecu.edu/>). *P. aeruginosa* PAO1 mutant strains were obtained from the Transposon Mutant Collection (Department

of Genome Sciences, University of Washington; <http://www.gs.washington.edu/labs/manoil/libraryindex.htm>) and include mutants 11174 (Δ PA1432, Δ lasI), 17281 (Δ PA1430, Δ lasR), 32454 (Δ PA3476, Δ rhlI) and 3452 (Δ PA3477, Δ rhlR) (Jacobs *et al.*, 2003). Plasmids p β 01 and p β 02 (Ishida *et al.*, 2007), pPCS223 and pLPRI (Van Delden *et al.*, 1998), pPCS1001 and pPCS1002 (Pesci *et al.*, 1997) and pTB4124 (Kretzschmar *et al.*, 2008) (see Table 1) were introduced into *P. aeruginosa* PAO1 as described by Smith & Iglewski (1989). All *P. aeruginosa* strains were grown at 37 °C with agitation (175 r.p.m.) for 18 h in liquid LB (5 ml) supplemented with 50 mM MOPS at pH 7.0 and with the appropriate antibiotics (tetracycline at 60 μ g ml^{−1} and carbenicillin at 300 μ g ml^{−1}) (Vandeputte *et al.*, 2010). All strains were grown and incubated with the different flavonoids as previously described (Vandeputte *et al.*, 2010).

Escherichia coli JLD271 biosensor strains harbouring LasR- and RhlR-based plasmids pAL105 and pAL101 and control plasmids pAL106 (LasR[−]) and pAL102 (RhlR[−]) (see Table 1) were grown in LB medium supplemented with tetracycline (10 μ g ml^{−1}) and chloramphenicol (25 μ g ml^{−1}) as previously described (Lindsay & Ahmer, 2005; Vandeputte *et al.*, 2010). When required, the medium was supplemented with 0, 1, 10 or 100 μ M (final) 3-oxo-C12-HSL or C4-HSL.

Chemicals. All flavonoids tested in this study were purchased from Sigma-Aldrich, dissolved in 100% DMSO and used at 4 mM (final concentration, resulting in 1% DMSO). These flavonoids included: apigenin (4',5,7-trihydroxyflavone), eriodictyol (3',4',5,7-tetrahydroxyflavanone), kaempferol (3,4',5,7-tetrahydroxyflavone), luteolin (3',4',5,7-tetrahydroxyflavone), myricetin (3,3',4',5,5',7-hexahydroxyflavone), naringenin (4',5,7-trihydroxyflavanone), naringin (4',5,7-trihydroxyflavanone 7-rhamnoglucoside), quercetin (3,3',4',5,7-pentahydroxyflavone), taxifolin (3,3',4',5,7-pentahydroxyflavanone) and chalcone (*trans*-benzylideneacetophenone). The AHLs *N*-hexanoyl-L-homoserine lactone (HHL), 3-oxo-C12-HSL and C4-HSL were purchased from Sigma-Aldrich and dissolved in 100% DMSO.

β -Galactosidase measurements. β -Galactosidase measurements were performed as previously described (Vandeputte *et al.*, 2010). After growth in liquid LB-MOPS-carbenicillin at 37 °C and 175 r.p.m. for 18 h, PAO1 reporter strains were washed twice in fresh LB medium and resuspended in liquid LB-MOPS-carbenicillin as described previously (Vandeputte *et al.*, 2010). Fifty microlitres of washed PAO1 reporter strains were added to 1 ml LB-MOPS-carbenicillin in order to obtain a starting OD₆₀₀ of 0.020–0.025, and 10 μ l of the flavonoid to be tested (4 mM final) or 10 μ l DMSO was added before incubation for 8 or 18 h at 37 °C with agitation (175 r.p.m.). After incubation, cell density was assessed by spectrophotometry (OD₆₀₀) and the β -galactosidase activity was measured using ONPG, as described elsewhere (Zhang & Bremer, 1995). All experiments were performed in triplicate and repeated in three independent experiments. The data were statistically analysed by Student's *t* test (i.e. each test was compared with the DMSO condition) and $P \leq 0.01$ was considered significant.

Quantitative analysis of violacein production in *C. violaceum* CV026. Inhibition of violacein production in *C. violaceum* CV026 by authentic flavonoid standards was tested by using a liquid assay according to reported protocols (Blosser & Gray, 2000; McClean *et al.*, 1997). Violacein production was induced in *C. violaceum* CV026 by adding HHL (3 μ M final). After 18 h and assessment of bacterial growth (OD₆₀₀), violacein contents were quantified according to Blosser & Gray (2000). Violacein absorbance was measured at 575 nm and violacein production was calculated as the ratio between A₅₇₅ and OD₆₀₀. The experiments were performed in triplicate and the data were statistically analysed by Student's *t* test (i.e. each test was compared with the DMSO condition) and $P \leq 0.01$ was considered significant.

Fig. 1. Structures of the flavonoids used in this study. The flavonoids used belong to (a) the flavones (apigenin and luteolin), (b) the flavonols (kaempferol, quercetin and myricetin), (c) the flavanones (naringenin, naringin, eriodictyol and taxifolin) and (d) the chalcones (*trans*-benzylideneacetophenone).

Quantitative analysis of pyocyanin and elastase production in *P. aeruginosa* PAO1 and *P. aeruginosa* mutants. Inhibition of pyocyanin and elastase production was assessed according to

described procedures (Ishida *et al.*, 2007; Müh *et al.*, 2006). *P. aeruginosa* PAO1 wild-type or mutant strains were grown for 18 h in liquid LB-MOPS supplemented with 60 µg tetracycline ml⁻¹ (mutant

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Strains		
<i>C. violaceum</i> CV026	Violacein-negative, <i>cviI</i> mini-Tn5 mutant of <i>C. violaceum</i>	McClellan <i>et al.</i> (1997)
<i>P. aeruginosa</i> PAO1	Wild-type (strain PAO0001; http://www.pseudomonas.med.ecu.edu/)	
<i>P. aeruginosa</i> ΔPA1430	<i>P. aeruginosa</i> transposon mutant ID 17281; <i>lasR</i> ::ISlacZ/hah; Tet ^R	Jacobs <i>et al.</i> (2003)
<i>P. aeruginosa</i> ΔPA1432	<i>P. aeruginosa</i> transposon mutant ID 11174; <i>lasI</i> ::ISlacZ/hah; Tet ^R	Jacobs <i>et al.</i> (2003)
<i>P. aeruginosa</i> ΔPA3476	<i>P. aeruginosa</i> transposon mutant ID 32454; <i>rhlI</i> ::ISphoA/hah; Tet ^R	Jacobs <i>et al.</i> (2003)
<i>P. aeruginosa</i> ΔPA3477	<i>P. aeruginosa</i> transposon mutant ID 3452; <i>rhlR</i> ::ISlacZ/hah; Tet ^R	Jacobs <i>et al.</i> (2003)
<i>E. coli</i> JLD271	<i>E. coli</i> K-12 Δ <i>lacX74 sdiA271</i> ::Cam	Lindsay & Ahmer (2005)
Plasmids		
pβ01	pQF50 derivative containing P _{lasB} - <i>lacZ</i> transcriptional fusion	Ishida <i>et al.</i> (2007)
pβ02	pQF50 derivative containing P _{rhlA} - <i>lacZ</i> transcriptional fusion	Ishida <i>et al.</i> (2007)
pPCS223	pLP170 derivative containing P _{lasI} - <i>lacZ</i> transcriptional fusion	Van Delden <i>et al.</i> (1998)
pPCS1001	pLP170 derivative containing P _{lasR} - <i>lacZ</i> transcriptional fusion	Pesci <i>et al.</i> (1997)
pLPR1	pLP170 derivative containing P _{rhlR} - <i>lacZ</i> transcriptional fusion	Van Delden <i>et al.</i> (1998)
pPCS1002	pLP170 derivative containing P _{rhlR} - <i>lacZ</i> transcriptional fusion	Pesci <i>et al.</i> (1997)
pAL101	pSB401 derivative containing <i>rhlR</i> ⁺ <i>rhlI</i> :: <i>luxCDABE</i> ; Tet ^R	Lindsay & Ahmer (2005)
pAL102	pSB401 derivative containing <i>rhlI</i> :: <i>luxCDABE</i> ; Tet ^R	Lindsay & Ahmer (2005)
pAL105	pSB401 derivative containing <i>lasR</i> ⁺ <i>lasI</i> :: <i>luxCDABE</i> ; Tet ^R	Lindsay & Ahmer (2005)
pAL106	pSB401 derivative containing <i>lasI</i> :: <i>luxCDABE</i> ; Tet ^R	Lindsay & Ahmer (2005)
pTB4124	pQF50 derivative containing P _{aceA} - <i>lacZ</i> transcriptional fusion	Kretzschmar <i>et al.</i> (2008)

strains) when appropriate at 37 °C and 175 r.p.m. *P. aeruginosa* cells were washed twice in fresh LB-MOPS medium and the cell suspension (50 µl), diluted appropriately, was added to 1 ml LB-MOPS (starting OD₆₀₀ 0.020–0.025) supplemented with 10 µl of authentic flavonoid standards (4 mM final) or DMSO. After 8 h (pyocyanin) or 18 h (elastase) of growth with or without flavonoids, samples were taken to assess bacterial growth (OD₆₀₀), to extract and quantify pyocyanin through absorbance measurements at 380 nm (Ishida *et al.*, 2007; Müh *et al.*, 2006), and to assess elastase production through the measurement of elastase activity using elastin–Congo red (Ishida *et al.*, 2007; Müh *et al.*, 2006). The experiments were performed in four replicates and the data were statistically analysed by Student's *t* test (i.e. each test was compared with the DMSO condition) or one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, and $P \leq 0.01$ was considered significant.

Homoserine lactone quantification. AHLs (C4-HSL and 3-oxo-C12-HSL) were extracted from PAO1 cultures and quantified by direct infusion in an electrospray mass spectrometer (electrospray ionization-MS; ESI-MS) as described by Makemson *et al.* (2006). *P. aeruginosa* PAO1 was grown at 37 °C with agitation at 175 r.p.m. for 8 and 18 h in 5 ml LB-MOPS medium supplemented with naringenin or naringenin (4 mM final concentration) or DMSO (0.1%, v/v). Bacterial cultures were centrifuged (3220 g, room temperature, 5 min) and supernatants (4 ml) were acidified with 80 µl glacial acetic acid prior to being extracted three times with ethyl acetate (4 ml). Ethyl acetate extracts were combined, evaporated to dryness and dissolved in 1 ml acidified ethyl acetate (0.1%, v/v, glacial acetic acid). For background measurement, supernatants (4 ml) were alkalized with 80 µl 4 M NaOH before ethyl acetate extraction, hydrolysed lactone rings being too polar to be extracted.

ESI-MS quantification was done by direct injection into a Finnigan LCQ DUO mass spectrometer under soft ionization conditions (positive ionization mode; nebulizer tip set at 250 °C and 4.52 kV; cone voltage set at 5 kV; sheath gas (nitrogen) flow rate: 30 arbitrary units) that did not fragment the AHLs. Scans were averaged over 1 min. The peak intensities for C4-HSL (pseudomolecular ion m/z =172; ammonium adduct m/z =189; sodium adduct m/z =194; solvent adduct m/z =260) and 3-oxo-C12-HSL (pseudomolecular ion m/z =298; ammonium adduct m/z =315; sodium adduct m/z =320; solvent adduct m/z =386) were combined and converted to concentrations by using a standard curve generated from the pure compounds. Background readings from hydrolysed samples extracted with ethyl acetate were subtracted from those of the acid-extracted bacterial cultures before conversion. The data ($n=6$) were statistically analysed by Student's *t* test (i.e. each test was compared with the DMSO condition) and $P \leq 0.01$ was considered significant.

Gene expression analysis by RT-PCR. After incubation with naringenin (4 mM final) as described above, *P. aeruginosa* PAO1 cells were frozen in liquid nitrogen, disrupted with acid-washed glass beads (425–600 µm) from Sigma using a MagNA Lyser instrument (Roche Diagnostics) and homogenized in TRI Reagent (Sigma). After UV quantification, DNA contamination was eliminated by 1 h DNase treatment (Amplification Grade DNase I from Sigma) according to the manufacturer's instructions. RNA quality and quantity were assessed at DNASion (<http://www.dnasion.com>) with a Bioanalyzer 2100 (Agilent).

cDNAs were synthesized from 250 ng total RNA using the Reverse Transcription System (Promega) and random hexamers according to Promega's instructions. Primers (5 µM each) were designed using Primer3 (<http://frodo.wi.mit.edu/primer3/>) (Rozen & Skaletsky, 2000) and are as follows: *16S*-left, 5'-CTGAGAGGATGATCAGT-CACACTGG-3'; *16S*-right, 5'-GATTTTCACATCCAACCTTGCTGA-ACC-3'; *lasB*-left, 5'-GTAGGTGTACTTGCCGATCTTCTGG-3';

lasB-right, 5'-AATGACAAAGTGGAACTGGTGATCC-3'; *lasA*-left, 5'-AAGAGCGAATACCTGGAGCACTGG-3'; *lasA*-right, 5'-TGGTATTCTCTCGAAACCGTAGTAGC-3'; *rhlA*-left, 5'-AGACCAGGTGAT-TGACCTCGAAGC-3'; *rhlA*-right, 5'-AGTCTGTTGGTATCGGTT-TGCAAGG-3'; *phzA1*-left, 5'-CGAACCATTCTGGGTCGAGTGC-3'; *phzA1*-right, 5'-GGGAATACCGTCACGTTTATTTCG-3'.

Intensities of PCR products after SYBR Gold (Invitrogen) staining were quantified using the ImageQuant TL software (GE Healthcare Life Sciences) and normalized against the values obtained for the 16S rRNA amplification. Normalized values were used to calculate the gene expression ratios between expression in DMSO-treated and naringenin-treated *P. aeruginosa* PAO1. The data ($n=3$) were statistically analysed by Student's *t* test (i.e. each test was compared with the DMSO condition) and $P \leq 0.01$ was considered significant.

Assessment of *P. aeruginosa* PAO1 viability and growth kinetics. *P. aeruginosa* PAO1 cells were incubated with DMSO or naringenin (4 mM final) as described above for 8 and 18 h and stained with SYTO-9 (Molecular Probes, Invitrogen) and propidium iodide (PI) according to the Molecular Probes instructions. Briefly, *P. aeruginosa* PAO1 cultures were diluted to an OD₆₀₀ 0.10–0.15, and 100 µl of a mix of SYTO-9 and PI was added to 100 µl diluted bacteria. Cells were transferred to a 96-well OptiPlate-96 F microplate (PerkinElmer) and fluorescence intensities were determined [excitation, 485 nm; emission, 530 nm (SYTO-9) and 630 nm (PI) using a SpectraMax M2 device (Molecular Devices). Ratios between the green and the red fluorescence (530 nm/630 nm) were compared to assess the cytotoxicity of naringenin. DMSO-treated cultures were used as controls. The data ($n=6$) were statistically analysed by Student's *t* test and $P \leq 0.01$ was considered significant. The effect of naringenin on the growth kinetics of *P. aeruginosa* PAO1 was evaluated by measuring the OD₆₀₀ of PAO1 cells grown under the same conditions over a 24 h period.

RESULTS

Flavonoids affect the production of QS-dependent factors in *P. aeruginosa* PAO1 and *C. violaceum* CV026

Several commercially available flavonoids belonging to the structural classes flavones (apigenin and luteolin), flavonols (kaempferol, quercetin and myricetin), flavanones (naringenin, naringin, eriodictyol and taxifolin) and chalcones (*trans*-benzylideneacetophenone) were screened for their capacity to interfere with QS in *P. aeruginosa* PAO1 and *C. violaceum* CV026. These flavonoids (Fig. 1) were tested at a final concentration of 4 mM, since, at this concentration, catechin has been shown to inhibit the production of QS-dependent factors by both species (Vandeputte *et al.*, 2010). As shown in Fig. 2, several of the flavonoids had an effect on the production of pyocyanin and violacein. Indeed, with the exception of naringin, all tested flavonoids inhibited the production of pyocyanin (Fig. 2a). Moreover, as shown in Fig. 2(b), the negative impact of the flavones (apigenin and luteolin) and the flavonols (kaempferol, quercetin and myricetin) on pyocyanin production was correlated with a significantly reduced cell density of *P. aeruginosa* PAO1. Notably, the flavanones (i.e. naringenin, eriodictyol and taxifolin) that had a limited impact on PAO1 final cell density led to significant reductions in

Fig. 2. Effect of flavonoids on (a) pyocyanin production by *P. aeruginosa* PAO1, (b) the final cell density of *P. aeruginosa* PAO1 and (c) violacein production by *C. violaceum* CV026. The cell density of the bacteria was assessed at 600 nm, and violacein and pyocyanin were extracted as described in Methods and quantified by absorbance measurements at 380 nm (pyocyanin) and 575 nm (violacein). Violacein production was calculated as the ratio between A_{575} and OD_{600} . All flavonoids were used at 4 mM. DMSO-treated cultures were used as controls and the statistical significance of each test ($n=4$) was evaluated by conducting Student's *t* test (i.e. each test was compared with the DMSO condition), and a *P* value of ≤ 0.01 was considered significant. Abbreviations: Api, apigenin; Lut, luteolin; Kae, kaempferol; Que, quercetin; Myr, myricetin; Nar, naringenin; Nin, naringin; Eri, eriodictyol; Tax, taxifolin; Ben, *trans*-benzylideneacetophenone. Asterisks indicate data that are statistically different ($P \leq 0.01$). I, flavones; II, flavonols; III, flavanones.

pyocyanin production by $86.8 \pm 1.4\%$, $73.2 \pm 5.2\%$ and $55.8 \pm 8.1\%$, respectively ($n=4$; Fig. 2a). Naringenin, which is a 7-*O*-rhamnoglucoside derivative of naringenin (Fig. 1), did not reduce the production of pyocyanin (Fig. 2a), suggesting that steric effects or the presence of particular chemical functions are important factors in the capacity of naringenin to reduce pyocyanin production. An open chain flavonoid (i.e. *trans*-benzylideneacetophenone) had a negative impact on PAO1 pyocyanin production [$68.3 \pm 3.7\%$ reduction ($n=4$); Fig. 2a], with no effect on the final bacterial density (Fig. 2b). QS mechanisms are also involved in the production of a purple pigment (violacein) in *C. violaceum* (McClellan *et al.*, 1997), a bacterium with a low incidence in human disease but with severe consequences upon infection (Teoh *et al.*, 2006) that is widely used as a reporter strain in QS screening (Chu *et al.*, 2011). Assays were carried out using the *C. violaceum* CV026 reporter strain, which is mutated in the AHL synthase gene *cviI*, and consequently this strain is able to produce violacein only when the natural inducer HHL is supplied in the growth medium (McClellan *et al.*, 1997). As shown in Fig. 2(c), several of the selected flavonoids had a negative effect on violacein production, although the flavones and the flavonols had an effect on CV026 growth (data not shown). The tested flavanones (naringenin, eriodictyol and taxifolin) reduced violacein production by 69 ± 2 , 78 ± 1 and $67 \pm 10\%$ ($n=4$), while naringenin and *trans*-benzylideneacetophenone had no significant effect on violacein production (Fig. 2c). Based on these preliminary screenings, flavones and flavonols were not further considered, and the effects of flavanones (which had no effect on the cell density of either *P. aeruginosa* PAO1 or *C. violaceum* CV026) on QS regulation in *P. aeruginosa* PAO1 were further analysed.

Flavanones inhibit the production of elastase by *P. aeruginosa* PAO1

In order to assess the effect of flavanones on elastase production, PAO1 cells were incubated overnight in the presence of naringenin, naringin, eriodictyol and taxifolin (4 mM final). Elastase production was quantified by an elastolysis assay (Ishida *et al.*, 2007; Müh *et al.*, 2006), and as shown in Fig. 3, naringenin, eriodictyol and taxifolin reduced elastase production by 46 ± 1 , 62 ± 6 and $47 \pm 2\%$, respectively ($n=4$), although, as for pyocyanin production, naringin had no effect. The open chain flavonoid *trans*-benzylideneacetophenone was also tested and reduced elastase production by the same amount ($51 \pm 5\%$), further supporting the supposition that this particular flavonoid might also interfere with QS mechanisms.

Flavanones reduce the expression of QS genes in *P. aeruginosa*

If flavanones interfere with QS mechanisms, this should be reflected in the transcription level of QS-controlling and QS-regulated genes. The effect of flavanones on QS systems was therefore characterized by measuring the expression of

Fig. 3. Effect of the flavanones (eriodictyol, naringenin, naringin and taxifolin) and *trans*-benzylideneacetophenone on elastase production by *P. aeruginosa* PAO1 assessed via an elastolysis assay as described in Methods. As shown in Fig. 2, no difference in final cell density was observed between the treatments. All flavonoids were used at 4 mM. DMSO-treated cultures were used as controls and the statistical significance of each test ($n=4$) was evaluated by Student's *t* test, and a *P* value of ≤ 0.01 was considered significant. Abbreviations: Tax, taxifolin; Eri, eriodictyol; Nar, naringenin; Nin, naringin; Ben, *trans*-benzylideneacetophenone. Asterisks indicate data that are statistically different ($P \leq 0.01$).

the AHL synthetase genes *lasI* and *rhlI* and the QS regulator genes *lasR* and *rhlR* in *P. aeruginosa* PAO1 grown with or without these flavanones. Due to its limited commercial availability, eriodictyol was not included in these further experiments. As shown in Table 2, after 8 h, naringenin significantly reduced the expression of both QS systems (*lasRI* and *rhlRI*) and downstream genes (*lasB* and *rhlA*), while naringin did not reduce the expression of any of the selected QS genes. This effect was more marked after 18 h, although naringin also had a negative effect on *lasR* and *rhlI*, possibly through partial hydrolysis of the rhamnoglucoside into the naringenin aglycone. After 8 h, taxifolin significantly reduced the expression of the synthase genes *lasI* and *rhlI*, although none of the other QS-related genes (i.e. *lasB*, *lasR*, *rhlR* and *rhlA*) seemed affected (Table 2). After 18 h, taxifolin significantly reduced the expression of all QS-genes with the exception of *rhlA*. *trans*-Benzylideneacetophenone reduced the expression of all QS-related (except *lasI*) genes after 8 h but had a limited impact after 18 h. Indeed, only the *lasR*, *lasB* and *rhlA* genes were affected, while the others had an expression equivalent to that of DMSO-treated PAO1 cells. To verify that the drop in β -galactosidase activity was indeed associated with a reduction in QS-related gene expression and was not due to a general effect on transcription/translation mechanisms, the activity of the *aceA* promoter (regulating the expression of the isocitrate lyase gene PA2634) from *P. aeruginosa* (Kretzschmar *et al.*, 2008) was studied in strain PAO1 grown in the presence or absence of the selected flavanones. As shown in Table 2, the addition of the flavonoids did not have a negative impact on the transcription of the *aceA* gene (although naringin had a significant positive impact), indicating that they affect the

expression of QS-related genes without affecting the transcription machinery of *P. aeruginosa* PAO1.

Though these flavanones have closely related structures, they apparently have different effects on the expression of genes involved in QS, and naringenin seems to be the molecule with the widest and most persistent action on QS systems in *P. aeruginosa* PAO1. Therefore, and in order to provide more evidence that QS mechanisms in PAO1 are affected by naringenin, we analysed the expression of four QS-related genes by semiquantitative RT-PCR: those involved in the production of LasA protease (*lasA*), LasB elastase (*lasB*), pyocyanin (*phzA1*) and rhamnolipids (*rhlA*). PAO1 cells were incubated in triplicate for 18 h with or without naringenin (4 mM final). As shown in Fig. 4, the expression of these genes was downregulated by the application of naringenin, further confirming the observed reduction of pyocyanin and elastase production and the decreased expression of QS-related genes (Figs 2 and 3, Table 2). As shown in Fig. 4(c), none of the effects of naringenin could be attributed to a diminution of PAO1 growth or viability.

Naringenin, but not naringin, inhibits the production of AHLs in *P. aeruginosa* PAO1

To determine whether the QS inhibitory effect of naringenin was linked to a defect in 3-oxo-C12-HSL or C4-HSL synthesis, concentrations of 3-oxo-C12-HSL and C4-HSL were determined in the growth media of PAO1 cells grown for 8 or 18 h in the presence of DMSO, naringenin (4 mM final) or naringin (4 mM final). As shown in Fig. 5, naringenin significantly reduced the concentration of both autoinducers after 8 h (Fig. 5a, c) and 18 h (Fig. 5b, d). After 8 h, concentrations of 3-oxo-C12-HSL and C4-HSL were reduced three- and 10-fold, respectively. Eighteen hours after the addition of naringenin (Fig. 5c, d), two- and fourfold reductions in autoinducer concentrations were observed, respectively. Naringin, in contrast, had no significant effect on the production of either AHL, at 8 or 18 h.

Exogenously supplied AHLs do not compensate the effect of naringenin on pyocyanin production by *P. aeruginosa* PAO1

Since AHL production is impaired by naringenin, 3-oxo-C12-HSL and C4-HSL were added exogenously to naringenin-treated PAO1 cells. As shown in Fig. 6, the addition of 10 μ M 3-oxo-C12-HSL or C4-HSL did not restore the production of pyocyanin in naringenin-treated wild-type PAO1 cells (Fig. 6a), while, as already observed, naringin had no effect (Fig. 6b). The same experiment was performed with the Δ PA1432 and Δ PA3476 mutant strains (which lack functional *lasI* and *rhlI* synthetase genes, respectively). As shown in Fig. 6(c), the exogenous addition of 3-oxo-C12-HSL to the Δ PA1432 strain significantly increased pyocyanin production, but adding naringenin reduced this production (Fig. 6c; + Nar), indicating that the exogenous supply of AHLs is not sufficient to compensate

Table 2. Effect of flavanones that inhibit pyocyanin and elastase production in *P. aeruginosa* PAO1 on the expression of QS-regulated genes after 8 and 18 h of incubation

Culture condition	Gene expression*						
	<i>lasI</i>	<i>lasR</i>	<i>lasB</i>	<i>rhII</i>	<i>rhIR</i>	<i>rhIA</i>	<i>aceA</i> †
8 h							
DMSO	532 ± 12	4621 ± 283	3248 ± 436	9075 ± 835	4154 ± 94	2848 ± 74	2615 ± 418
Naringenin	464 ± 14‡	3392 ± 326‡	2440 ± 124‡	4424 ± 503‡	2771 ± 250‡	2260 ± 107‡	2679 ± 129
Naringin	1602 ± 139‡	4579 ± 586	3607 ± 101	11 826 ± 898‡	4701 ± 332	3414 ± 281‡	3480 ± 421‡
Taxifolin	207 ± 19‡	5413 ± 650	2859 ± 39	5890 ± 746‡	3610 ± 526	2845 ± 201	2592 ± 161
<i>trans</i> -Benzylideneacetophenone	558 ± 36	3026 ± 436‡	2676 ± 69‡	11583 ± 428‡	2305 ± 265‡	2085 ± 252‡	2841 ± 161
18 h							
DMSO	968 ± 98	4814 ± 577	1116 ± 121	4046 ± 373	2206 ± 492	8455 ± 330	2424 ± 84
Naringenin	392 ± 62‡	1675 ± 103‡	592 ± 32‡	1675 ± 405‡	1261 ± 316‡	5274 ± 532‡	2178 ± 177
Naringin	1363 ± 674	2993 ± 271‡	848 ± 395	3107 ± 581	3174 ± 736	6742 ± 1192	2609 ± 350
Taxifolin	329 ± 49‡	1951 ± 660‡	885 ± 76‡	938 ± 305‡	1692 ± 227‡	9134 ± 628	2845 ± 294
<i>trans</i> -Benzylideneacetophenone	1168 ± 122	3457 ± 555‡	655 ± 191‡	3387 ± 477	3421 ± 409	5189 ± 98‡	2477 ± 206

*Gene expression was measured as the β -galactosidase activity of the *lacZ* gene fusions expressed in Miller units.

†Expression of *aceA* (isocitrate lyase) was used as a QS-independent control.

‡Significant at $P < 0.01$.

the effect of naringenin. As shown in Fig. 6(e), the Δ PA3476 mutant was unable to produce pyocyanin unless C4-HSL was exogenously supplied (while 3-oxo-C12-HSL had no effect). Similarly to strain Δ PA1432, when naringenin was added to C4-HSL-induced Δ PA3476 cells, the level of pyocyanin production was lower than that observed when C4-HSL alone was added (Fig. 6e; + Nar). Adding naringin to both strains had no effect on their capacity to produce pyocyanin when the appropriate autoinducer was added to the growth medium (Fig. 6d, f; + Nin), further highlighting that a steric non-hindrance of the A-ring is important for the activity of naringenin. For comparison, pyocyanin production in the Δ *lasR* (Δ PA1430) and Δ *rhIR* (Δ PA3477) mutants grown under the various conditions tested was also quantified, and only basal pyocyanin levels were detected (data not shown). As shown in Fig. 6(a, c, e), adding naringenin to PAO1 cells reduced pyocyanin production to levels between those detected in the Δ *lasI* and Δ *rhII* mutant strains, demonstrating the potency of naringenin as an efficient QS inhibitor. In addition, pyocyanin and elastase production (Figs 2 and 3) was reduced to a greater extent by naringenin (80–90 and 50 % reduction, respectively) than by catechin (50 and 30 % reduction, respectively) (Vandeputte *et al.*, 2010). The stronger effect of naringenin was also obvious in the expression of QS-related genes (Table 2). Indeed, naringenin clearly had a greater effect on these genes after 18 h than catechin or salicylic acid, while all molecules affected QS gene expression to the same extent after 8 h (Vandeputte *et al.*, 2010).

Naringenin interferes with the perception of AHLs by *P. aeruginosa* PAO1

Since the addition of AHLs to naringenin-treated *P. aeruginosa* PAO1 did not completely restore its capacity

to produce pyocyanin (Fig. 6), we determined whether the LasR and RhIR transcription factors were impaired in their capacity to perceive their respective QS signals (3-oxo-C12-HSL or C4-HSL). This was achieved by using *E. coli* biosensors carrying the pAL plasmids (Lindsay & Ahmer, 2005). These constructs allow the determination of whether the perception of 3-oxo-C12-HSL by LasR (pAL105) or C4-HSL by RhIR (pAL101) is affected. These biosensor strains were grown overnight and induced by adding the appropriate molecule directly to the cultures at various concentrations (0, 1, 10 and 100 μ M). As shown in Fig. 7, adding increasing concentrations of C4-HSL to the pAL101 biosensor strain induced the expression of the *lux* operon and the consequent production of luminescence (while only background levels of luminescence were detected in the control pAL102 strain, which lacks the *rhIR* gene; data not shown). Naringenin and naringin were added to these biosensor strains at a concentration of 2 mM (final), a concentration lower than that used for PAO1 cells because of the toxicity of naringenin towards *E. coli* (data not shown). As shown in Fig. 7, the pAL101 biosensor strain produced less luminescence when naringenin was added to the growth medium as compared with DMSO- and naringin-treated biosensor cells, indicating that the functioning of the RhIR protein is impaired. No significant difference was observed with the pAL105 biosensor strain, suggesting that the LasR protein is less or not affected (data not shown).

DISCUSSION

In order to cope with bacterial infections, the search for QS quenchers or inhibitors is an emerging strategy aimed at developing new compounds to attenuate or abolish the

Fig. 4. Analysis of the expression of the QS-regulated genes *lasA* (protease), *lasB* (elastase), *phzA1* (pyocyanin) and *rhlA* (rhamnolipids) in *P. aeruginosa* PAO1 treated with DMSO or 4 mM naringenin. (a) Agarose gels of RT-PCRs stained with SYBR Gold. (b) Relative expression of the *lasA*, *lasB*, *phzA1* and *rhlA* genes under control conditions (DMSO) and following addition of naringenin (Nar). Intensities of PCR products after SYBR Gold (Invitrogen) staining were quantified using ImageQuant TL software and normalized against the values obtained for 16S rRNA amplification. Normalized values were used to calculate the gene expression ratios between expression in DMSO-treated *P. aeruginosa* PAO1 and naringenin-treated *P. aeruginosa* PAO1. (c) Growth kinetics of *P. aeruginosa* PAO1 in the presence of DMSO (×) or 4 mM naringenin (○). PAO1 cell viability was assessed after 18 h using SYTO-9 and PI. All flavonoids were used at 4 mM. The statistical significance of each test ($n=3$) was evaluated by Student's *t* test (i.e. each test was compared with the DMSO condition), and a *P* value of ≤ 0.01 was considered significant.

production of virulence factors by pathogenic bacteria (Bjarnsholt *et al.*, 2010). Various types of screening have been carried out to find candidate molecules, for example: (i) in libraries resulting from combinatorial chemistry or containing AHL analogues (Ishida *et al.*, 2007; Janssens *et al.*, 2008; Müh *et al.*, 2006); (ii) via virtual screening based on the structure of the LuxR homologues bound to their cognate signal molecules (Persson *et al.*, 2005; Taha *et al.*, 2006; Yang *et al.*, 2009; Zeng *et al.*, 2008); or (iii) in eukaryotes, including marine organisms (Givskov *et al.*, 1996; Hentzer *et al.*, 2002; Skindersoe *et al.*, 2008) and plants (Adonizio *et al.*, 2008; Bjarnsholt *et al.*, 2005; Gao *et al.*, 2003; Keshavan *et al.*, 2005; Rasmussen *et al.*, 2005; Teplitski *et al.*, 2011).

We recently showed that extracts from *Combretum albiflorum*, a plant native to Madagascar and belonging to the family *Combretaceae*, negatively affect QS mechanisms in *C. violaceum* and *P. aeruginosa* PAO1 (Vandeputte *et al.*, 2010). Catechin was identified as one of the flavonoids present in *Combretum* bark extract that reduces the production of QS-controlled virulence factors in *P. aeruginosa* PAO1 but, based on spectral characteristics, other active fractions were shown to contain flavonoid-like structures. Consequently, several commercially available flavonoids were screened here for their capacity to interfere with QS mechanisms in *P. aeruginosa* PAO1. Flavonoids derive from the phenylpropanoid pathway and are found ubiquitously in plants, where they fulfil different ecological and physiological functions (Buer *et al.*, 2010; Dixon & Steele, 1999; Dixon & Pasinetti, 2010). Flavonoids are also known for their useful medicinal properties, and have been shown to possess anti-inflammatory, cytotoxic antitumour, antiallergic, antioxidant and antimicrobial activities, among others (Crespo *et al.*, 2008; Cushnie & Lamb, 2005; Lee *et al.*, 2009; Singh *et al.*, 2008; Williams *et al.*, 2004). As antimicrobials, some flavonoids have been shown to inhibit gyrase activity, nucleic acid synthesis, type-IV topoisomerase, cytoplasmic membrane function and energy metabolism (reviewed by Cushnie & Lamb, 2005). Flavonoids are also known for their implication in cell-to-cell communication mechanisms involved in the establishment of the symbiosis between rhizobia bacteria and their respective legume hosts (Buer *et al.*, 2010; Subramanian *et al.*, 2007; Zhang *et al.*, 2009).

From the data presented here and elsewhere (Vikram *et al.*, 2010; Zeng *et al.*, 2008), an additional function might be ascribed to certain groups of flavonoids as inhibitors of QS mechanisms. Indeed, the flavone baicalein has been shown to inhibit biofilm formation, which is QS dependent (Davies *et al.*, 1998), in *P. aeruginosa* PAO1 (at micromolar concentrations) as well as to promote the proteolysis of the *Agrobacterium tumefaciens* QS-signal receptor TraR in *E. coli* cells at millimolar concentrations (Zeng *et al.*, 2008). More recently, several flavonoids commonly found in *Citrus* plants were screened for their ability to interfere with QS-dependent bioluminescence mechanisms and biofilm formation (Vikram *et al.*, 2010). Those authors

Fig. 5. Naringenin, but not naringin, inhibits the production of the AHLs 3-oxo-C12-HSL and C4-HSL by *P. aeruginosa* PAO1. (a, b) Quantification of 3-oxo-C12-HSL (3OC12-HSL) produced by PAO1 cells in the presence of DMSO, naringenin (Nar) or naringin (Nin) after 8 h (a) and 18 h (b) of growth. (c, d) Quantification of C4-HSL produced by PAO1 cells in the presence of DMSO, naringenin (Nar) or naringin (Nin) after 8 h (c) and 18 h (d) of growth. AHLs were extracted and quantified by MS as described in Methods. All flavanones were used at 4 mM. DMSO-treated cultures were used as controls and the statistical significance of each test ($n=6$) was evaluated by Student's *t* test (i.e. each test was compared with the DMSO condition), and a *P* value of ≤ 0.01 was considered significant. Asterisks indicate data that are statistically different.

showed that naringenin, among other flavonoids, reduces the induction of bioluminescence by the QS signals HAI-1 and AI-2 in *Vibrio harveyi* reporter strains as well as the production of biofilm by *V. harveyi* BB120 and *E. coli* 0157:H7. It was also shown that the expression of three type III secretion system genes, suggested to be controlled by cell-to-cell signalling, is downregulated by naringenin (Vikram *et al.*, 2010); the mechanism(s) by which naringenin affects QS in these biological systems was, however, not investigated. From the present screening, naringenin also emerges as a potential inhibitor of QS mechanisms in *P. aeruginosa* PAO1, and we set out to better understand the effect of naringenin on the regulation of these mechanisms. The data collected here suggest that the action of naringenin most probably results from a combination of the reduction of the production of both AHL molecules (which could be the major effect, and which is corroborated by the down-regulation of the expression of the *lasI* and *rhlI* genes) and of the capacity of the LuxR-type transcription factors to perceive their cognate molecules, with a consequent reduction of the expression of QS-related genes. It is widely known that *lasI* and *rhlI* mutants deficient in AHL synthesis are indeed impaired in their capacity to express a wide range of so-called QS genes, among which are *lasB* (encoding LasB elastase), *rhlA* (encoding the first protein involved in the production of rhamnolipids) and the *phz* operon involved in the production of pyocyanin (Brint & Ohman, 1995; Gambello & Iglewski, 1991; Gambello *et al.*, 1993; Latifi *et al.*, 1995; Pearson *et al.*, 1997; Pesci *et al.*, 1997; Schuster *et al.*, 2003; Toder *et al.*, 1994; Wagner *et al.*, 2004).

Flavonoids are a large class of phenylpropanoid-derived plant metabolites that are classified according to the degree of oxidation of their C-ring and whose structural diversity results from substitutions of their carbon skeleton through hydroxylation, glycosylation, methylation, acylation and prenylation (Buer *et al.*, 2010; Dixon & Steele, 1999; Dixon & Pasinetti, 2010). Although all these molecules are

structurally similar, the selected flavanones (eriodictyol, naringenin, taxifolin) demonstrated a strong QS-inhibition activity, while, at the same concentration, the three flavonols (kaempferol, myricetin, quercetin) and the two flavones (apigenin, luteolin) showed no QS modulation, but a bactericidal or bacteriostatic activity, as already reported for other bacteria (Ulanowska *et al.*, 2006; Xu & Lee, 2001). This highlights the possible involvement of the hydroxyl substitutions and/or the double bond in the central pyran C-ring in the opposing activities (bacteriostatic/bactericidal versus anti-QS) of these flavonoids. In the case of naringenin, the hydroxyl in position 7 seems important for the anti-QS activity, since naringin (a glycoside of naringenin in which the hydroxyl group at position 7 is substituted by an O-rhamnoglucoside) had no anti-QS activity, inducing no reduction in the production of violacein, pyocyanin and elastase, the expression of QS genes, or the production of C4-HSL and 3-oxo-C12-HSL. It is also possible that the O-rhamnoglucoside increases the steric hindrance of naringin, precluding its ability to interact with its target in the same way as naringenin. Consistently, baicalein (a flavone) has been found to have a high TraR (a LuxR homologue of *A. tumefaciens*) docking score and to affect TraR stability, while baicalin (the 7-glucuronide of baicalein) has been found to have a low docking score and no effect on the stability of the TraR protein (Zeng *et al.*, 2008), suggesting that the steric hindrance of glycosylated flavonoid moieties might indeed affect their capacity to interact with their targets. Modifying the hydroxylation, methoxylation and glycosylation status of flavonoids is well known to affect many of their biological activities, underlining the importance of the relationship between their precise structure and their biological properties (Halbwirth, 2010; Jiang *et al.*, 2010; Liu *et al.*, 2010; Pourcel *et al.*, 2007; Shimada *et al.*, 2010).

Finally, a more detailed investigation of the importance of the various radicals and rings required for the anti-QS activity of flavanones via quantitative structure–activity

Fig. 6. Effect of naringenin and naringin on the production of pyocyanin after exogenous supply of AHLs to wild-type and mutant strains of *P. aeruginosa*. Production of pyocyanin by the wild-type strain PAO1 (a, b), the $\Delta lasI$ (Δ PA1432, mutant ID 11174) (c, d) and the $\Delta rhII$ (Δ PA3476, mutant ID 32454) (e, f) mutant strains was quantified as described in Methods and is expressed as the ratio between A₃₈₀ and OD₆₀₀. In each case, bacteria were incubated with DMSO, naringenin (Nar), naringin (Nin) or the appropriate AHL. Bacteria were also induced with the appropriate AHL and simultaneously treated with naringenin (+Nar) or naringin (+Nin). C4-HSL and 3-oxo-C12-HSL (3OC12-HSL) were added at 10 μ M final concentration. DMSO-treated cultures were used as controls, the statistical significance of each test ($n=4$) was evaluated by conducting one-way ANOVA with Tukey's multiple comparison tests, and a P value of ≤ 0.01 was considered significant. All flavanones were used at 4 mM. Different letters (a, b, c, ab) above the bars indicate data that are statistically different from each other according to the one-way ANOVA with Tukey's multiple comparison test ($P \leq 0.01$).

relationship (QSAR) analysis might shed light on the chemical positions that can be modified in flavonoids that have anti-QS activities at high concentrations only, to confer anti-QS activity upon antimicrobial flavonoids or to

modify promising QS inhibitors to obtain more potent compounds. This QSAR analysis could also serve for the rational design of anti-QS compounds through combinatorial chemistry. In addition, by using their natural

Fig. 7. Naringenin affects the functioning of the RhlR–C4-HSL complex. The effect of naringenin and naringin on the pAL101 *E. coli* biosensor was assessed by adding increasing concentrations of C4-HSL (0, 1, 10 and 100 µM) plus DMSO, naringenin (Nar) or naringin (Nin) to overnight cultures for 2 h, and samples were taken to record the luminescence of the culture in relative light units (RLU) and measure OD₆₀₀. The pAL102 *E. coli* biosensor was used for background measurements. All flavonoids were used at 2 mM. DMSO-treated cultures were used as controls, the statistical significance of each test ($n=6$) was evaluated by Student's *t* test (i.e. each test was compared with the DMSO condition), and a *P* value of ≤ 0.01 was considered significant. Asterisks indicate data that are statistically different.

chemical diversity and versatility, flavonoids and flavanones in particular might become important lead compounds for the development of anti-QS compounds that could be used at lower concentrations.

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