At a Supra-Physiological Concentration, Human Sexual Hormones Act as Quorum-Sensing Inhibitors

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Introduction

Bacterial populations synthesize and exchange chemical signals which coordinate and synchronize gene expression in a cell-density dependent manner. Such regulatory pathways are called quorum-sensing (QS) and involve diverse QS-signals, including N-acylhomoserine lactones (AHLs) [1]. The canonical proteins required for the synthesis of AHLs belong to the LuxI family, and those for AHL-sensing to the LuxR family [2]. The AHL-mediated QS is widespread among Proteobacteria, controlling - for instance - the expression of genes involved in bacterial virulence in animal and plant hosts, horizontal gene transfer by plasmid conjugation, as well as bacterial competitiveness in the environment through production of antibiotics [1–2].

Natural and synthetic compounds which alter QS signalling and thereby disrupt QS-regulated gene expression are called QS inhibitors (QSI). Considering the central role played by QS in the expression of virulence genes in pathogenic bacteria, the search for QSI has driven many efforts [3]. Over the past several years, numerous QSI's with diverse structures have been identified using different approaches such as the synthesis of structural analogues, experimental and virtual screening of chemo-libraries and purification of natural QSIs from diverse organisms, especially plants [3–5]. The natural QSIs contribute to host defense against bacteria and both natural and synthetic QSIs have been proposed as promising molecules because they may act synergistically with antibiotics to limit bacterial infection [6–8].

In this work, we screened a chemo-library for the presence of QSI's and validated the QSI activity of the identified compounds using two bacterial species, the plant pathogen Agrobacterium tumefaciens in which QS regulates the horizontal transfer of the tumor-inducing (Ti) plasmid, and the opportunistic pathogen Pseudomonas aeruginosa, in which QS controls the expression of virulence factors. This paper reports the identification of novel natural (hordenine) and synthetic (indoline-2-carboxamides) QSIs, and also experimentally demonstrates QSI-activity of three human sexual hormones: estrone, estriol, and estradiol.
Results

Identification of the QSIs

The ICSN chemical library (see materials and methods) was screened with two bacterial AHL-bioindicators, *C. violaceum* CV026 and *A. tumefaciens* NT1(pZLR4) in the presence of the appropriate AHLs. The strains and plasmids used in this study are listed in Table 1. Using appropriate AHLs, the strains and plasmids used in this study are.

Improving the selectivity of the screening, we reduced the concentration of the tested compounds to 5 μM/ml over 150 potential QSI-compounds, as they were already known as antimicrobial agents.

They were consequently removed from this study. Hence, only 15 of the identified hits numbered 14, 15, 283, 729, 937, 1099, 1102, 1248, 1283, 1577, 1868, 1949, 3028, 3492, and 3499 were retained for further analyses (Figure 1). Compounds 14 [9–10], 15 [11], 1102 [12], 1283 [13], 1577 [14–15], 3028 [16] have been previously described, while compounds 283, 729, 1248 and 1949 are described in the experimental section. Compounds 937, 1099, 1868, 3492 and 3499 are commercially available (Sigma Aldrich and SynChem, Inc.). The 15 hits belong to different structural families such as carbazole (i.e. 15), indole (i.e. 1248), pyridoindole (i.e. 3492), steroids (i.e. 1099, 1868) including the plant sexual hormone estrone (i.e. 729), as well as the plant phenylethylamine alkaloid hordenine (i.e. 3499).

Synthesis of each diastereoisomer of the QSI ID1248

Sample ID1248 being a mixture of 4 stereoisomers, each 4 was synthesized separately and unambiguously starting from the commercially available, optically-active precursors to determine which isomer is the most active (Figure 2). Thus, (R)- and (S)-indoline-2-carboxylic acid 1 were each coupled with (R)- and (S)-1-(1-naphthyl)ethylamine 2 using 1-ethyl-3-(3-dimethylaminopropyl)crodiamide (EDCI) and 1-hydroxybenzotriazole (HOBT) in dichloromethane. The carboxamide bond of each compound formed IIIa-d was then reduced to the amine using borane-THF complex and the products (S,S)-1248, (S,R)-1248, (R,S)-1248 and (R,R)-1248 were isolated as their hydrochloride salts.

IC50 values and bacterial toxicity of the QSIs in *A. tumefaciens*

The IC50 values of the chemical library QSIs and commercial compounds (Figure 3), such as the hormones estradiol and estriol, the plant-defense signal jasmonic acid, and the QSI-reference 4-nitropropyridine-N-oxide (4-NPO) already published by Rasmussen et al [17], were measured using the *A. tumefaciens* bioindicator that expresses the trcG-lacZ reporter fusion. According to our procedure, the QSI-reference 4-NPO exhibited an IC50 of 24 μg/ml (Table 2). Compound 1577 exhibited an IC50 value (IC50 = 2.5 μg/ml) lower than that of 4-NPO. The IC50 of 10 compounds ranged between 30 and 90 μg/ml (283, 729, 1099, 1248, (R,S)-1248, (S,R)-1248, 3492, 1102, 1289, (R,S)-1248, (R,R)-1248 and 3499). Notably, (S,R)-1248 exhibited a QSI activity higher than that of the racemic mixture of 1248 and the other diastereoisomers. This stereoselectivity of the inhibitory activity points out the most probable occurrence of a specific interaction of the QSI with the biological target rather than to simple non-specific activity. With the exception of compound 1577, none variation of the cell density (OD600) was observed in the IC50 determination assay. To know more about bactericidal activity of these compounds, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were calculated according to the

Table 1. Bacterial strains and plasmids used in this study.

| Strains and plasmids | Relevant characteristics | Reference or source |
|----------------------|--------------------------|---------------------|
| Agrobacterium NT1(pZLR4) | *A. tumefaciens* C58 derivative expressing traT and traG-lacZ, AHL-bioindicator | [42] |
| Agrobacterium pTi-donor | *A. tumefaciens* C58 derivative with pTiC58-locG::R::traI::Km | [45] |
| A. tumefaciens C58-00 | *A. tumefaciens* C58 derivative, cured of its plasmids, recipient strain | Lab collection, CNRS, Gif-sur-Yvette |
| Chromobacterium violaceum CV026 | *C. violaceum* ATCC 31532 derivative, violacein producer, AHL-bioindicator | [41] |
| Escherichia coli JLD271 | K12 derivative, tolC::lacX74sdA271::Cam | [21] |
| Pseudomonas aeruginosa PAO1 | Wild-type | http://www.pseudomonas.med.ecu.edu/ |
| pJ01 | pQF50-derivative, P<sub>traG</sub>-lacZ | [51] |
| pJ02 | pQF50-derivative, P<sub>traT</sub>-lacZ | [51] |
| pLP101 | pLP170-derivative, P<sub>traI</sub>-lacZ | [52] |
| pPCS223 | pLP170-derivative, P<sub>traG</sub>-lacZ | [52] |
| pPCS1001 | pLP170-derivative, P<sub>traG</sub>-lacZ | [53] |
| pPCS1002 | pLP170-derivative, P<sub>traI</sub>-lacZ | [53] |
| pAL101 | pS8401-derivative, rhlR::Hill::lacCDABE | [21] |
| pAL102 | pS8401-derivative, rhlI::Hill::lacCDABE | [21] |
| pAL105 | pS8402-derivative, lasR::Hill::lacCDABE | [21] |
| pAL106 | pS8401-derivative, lasI::Hill::lacCDABE | [21] |
| pTB4124 | pQF50-derivative, P<sub>trcG</sub>-lacZ | [20] |

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Andrews’ recommendations [18]. The QSI-reference 4-NPO weakly inhibited the growth of *A. tumefaciens* as the MIC value reached 25 μg/ml (Table 2). All the other tested compounds, with the exception of 1577 and 1868 (MIC at 3 and 12.5 μg/ml respectively), exhibited a MIC value equal to or greater than 100 μg/ml, which was the highest concentration tested for determining IC₅₀ value and impact of the compounds on QS-regulated plasmid transfer in *A. tumefaciens*. The MBC test confirmed the weak toxicity of the studied QSI because all the MBC values were equal to or higher than 100 μg/ml, with the exception of that of 1577 (MBC at 25 μg/ml).

QSIs modulate QS-regulated Ti-plasmid transfer in *A. tumefaciens*

In *A. tumefaciens*, QS positively regulates horizontal transfer of the virulence plasmid (called Ti plasmid) from a donor strain to a recipient strain. Recipient strains which have received the plasmid are called transconjugants. In this assay, a Ti-plasmid donor and
recipient strains were mixed in the presence of AHL (OC8-HSL) and QSI. Resulting transconjugants were counted on rich agar medium supplemented with appropriate antibiotics. When the QSI-reference 4-NPO was added at 0.1 mg/ml, the plasmid transfer efficiency decreased by 10-fold (Figure 4A). Five chemical library-QSIs (729, 1099, 1102, 1577 and 3499) and estriol were also able to significantly reduce the plasmid transfer frequency by one order of magnitude. In another experiment (Figure 4B), the four 1248-diastereoisomers were compared. All of them significantly affected horizontal transfer of the Ti plasmid. Noticeably, in conjugation assays, the level of the donor and recipient cells remained at a high level (around 10⁸ CFU/ml, Figure 4), suggesting that the measured decrease in plasmid transfer would not be caused by a toxic effect of the tested compounds.

QSI modulate QS-signal accumulation in P. aeruginosa

Because the assays with the plant pathogen A. tumefaciens highlighted human hormones as QSI (estrone = 729, estradiol, and estriol), their QSI-activity was evaluated with the opportunist pathogen P. aeruginosa.

Growth curves of P. aeruginosa cells were determined in the presence of the QSI-modulated factors (Figure 5). The addition of estradiol, estrone and estriol at 0.5 mg/ml did not affect the growth of P. aeruginosa. Only hordenine and 4-NPO slightly delayed the growth of P. aeruginosa. This was further confirmed by the enumeration of CFU after 8- and 18-hour of incubation. Noticeably, in all cases, the Pseudomonas cells reached the same final cell density at the stationary phase. At 18-hour, the concentrations of the AHLs butyrylhomoserine lactone (C4-HSL) and 3-oxododecanoylhomoserine lactone (OC12-HSL), which are produced by P. aeruginosa, were quantified using mass spectrometry as described by Vandeputte et al. [19]. The presence of the human hormones estradiol, estrone and estriol, as well as that of hordenine and 4-NPO provoked a reduction of the C4-HSL and OC12-HSL concentrations in the cell cultures, suggesting the QSI-signal synthesis and QS-signalling were affected.

QSI modulate QS-regulated genes in P. aeruginosa

The expression of six QSI-regulated genes was measured in P. aeruginosa the AHL-synthetase genes lasI and rhlI, the AHL-sensor genes lasR and rhlR, and downstream genes lasB and rhlB, which are regulated by the las and rhl systems, respectively (Figure 6). The effect of the QSI was compared to that of naringenin, a known QSI in P. aeruginosa [19]. All tested compounds markedly affected the expression of the synthetase genes lasI and rhlI, though estriol and hordenine had a lower impact on rhlI expression than naringenin, estradiol and estrone. Expression of the regulators lasR and rhlR was also affected, with hordenine being the most and estriol the least potent inhibitors. Other QS-regulated genes, lasB and rhlA, were also down-regulated in the presence of the tested QSI. To verify that the drop in β-galactosidase activity of the reporter genes was indeed associated with a reduction in QS-related gene expression and not with a general effect on transcription/translation mechanisms, the activity of the aceA promoter, the expression of which is not regulated by QS [20] was assessed. The addition of the QSI did not modify the transcription of the aceA gene (Figure 6), indicating that these compounds affected the expression of QS-related genes without affecting the transcription machinery of P. aeruginosa PAO1.

QSI modulate activity of the QS-signal sensors LasR and RhlR of P. aeruginosa

We determined whether the AHL-binding transcriptional factors LasR and RhlR were impaired in their capacity to activate expression of the QS-regulated genes in the presence of various QSI. This was achieved by using two E. coli bioindicator strains expressing either LasR or RhlR proteins and harboring an appropriate reporter lux operon for measuring their transcriptional activity in the presence of OC12-HSL at 100 µM and C4-HSL at 10 µM, respectively [21]. Adding C4-HSL to the pAL101-bioindcator or OC12-HSL to the pAL105-bioindcator induced the expression of the reporter lux operon and the consequent production of luminescence. In contrast, only background levels of luminescence were detected in control strains harboring the plasmids pAL102 or pAL106, which lacks the rhlR or lasR gene, respectively.

As shown in Figure 7, both biosensor strains produced less luminescence when naringenin was added to the growth medium as compared with DMSO-treated biosensor cells, indicating that the functioning of the LasR and RhlR proteins was impaired, while the structurally-related compound naringenin had no effect. Estradiol affected LasR functionality but not that of RhlR. The other QSIs, estrone, estradiol and hordenine, had a significant impact on the perception of both OC12-HSL and C4-HSL by LasR and RhlR, respectively. This observation indicated that the inhibition of the expression of the QSI genes in P. aeruginosa PAO1 was likely due to a competition between the endogenous AHLs
Discussion

This article reports the identification of novel QSIIs such as the natural plant compound hordenine and the synthetic indoline-2-carboxamides, and also demonstrates the QSI-activity of the three human sexual hormones that are estrone, estriol and estradiol.

QSIIs have been identified in many organisms, plants being the most frequently investigated source of QSI compounds and algae the providers of the most potent ones [1,3]. Our results revealed the QSI potentiality of alkaloids such as hordenine (ID 283), 1248, and 3492. Hordenin (CAS# 3359-05-9) is a natural alkaloid of the phenethylamine class exhibiting a widespread occurrence in plants (ornamentals, fruits and vegetables), including those that are used for human and animal consumption [22–25]. Following injection, hordenine stimulates the release of norepinephrine in mammals hence acting indirectly as an adrenergic drug [24–25]. In the literature, alkaloid compounds have been less frequently reported as acting as QSI than aromatic or polyaromatic compounds [1]. Indeed, solenopsin A, a venom alkaloid produced by the fire ant Solenopsis invicta, has been shown to inhibit biofilm formation, pyocyanin and elastase production as well as the expression of QS-regulated genes lasB, rhlB and lasI in P. aeruginosa [26]. Peters and co-workers [27] also demonstrated that brominated tryptamine-based alkaloids from Flustrafolicaea, a sea bryozoan, inhibit AHL-regulated gene expression using biosensors P. putida (pKR-C12), P. putida (pAS-C10) and E. coli (pSB405) lasR, cepR and luxR coupled to the promoter of lasB, cep and luxR, respectively.

In this study, the QSI activity of human hormones was supported by complementary features. The pure hormones, especially estriol and estrone, affected expression of the QS-regulated reporter fusion traG-lacZ and QS-dependent horizontal transfer of the virulence Ti-plasmid in A. tumefaciens. They also decreased the expression of six QS-regulated genes lasB, rhlB, rhlR, rhlR, rhlL, and rhlL in P. aeruginosa, but none decreased expression of the QS-independent gene aceI. Because of the effect on las and rhl, the AHL concentration was also affected in the presence of the sexual hormones. In agreement with a previous report comparing the effect of steroid hormones on the growth of several pathogens [20], they did not affect the growth of A. tumefaciens and P. aeruginosa at the concentrations used for describing QSI activity.

The sexual hormones act as QSIIs at a mM-range concentration which is similar to that of the natural polycyclic QSIIs such as catechin and naringenin [19,29], but higher than that of some other natural and synthetic QSIIs which act at a μM range or lower [3,30]. Our work also revealed that pure hormones affected the QS-regulated reporter gene of P. aeruginosa when RhlR or LasR was expressed in E. coli in the presence of the appropriate AHL. Moreover, molecular modeling confirmed the competitive hormone-binding capacity of the two AHL-sensors LasR and TraR, suggesting that the AHL-LasR sensors are targets of the discovered QSIIs. This mechanism of action is frequently encountered among QSIIs [3]. Such a putative cross-talk between QS and hormonal signalling was hypothesized in prospective reviews by Rumbacka [31] and Hughes and Sperandio [32] and in a paper reporting docking-type screening of QSIIs [33], but, to our knowledge, was never experimentally observed in vitro until this report.

Finally, the hypothesis rose about QSI-activity of sexual hormones in vivo because the opportunistic pathogen P. aeruginosa is detectable in several tissues and organs of hospitalized patients and healthy women, and can thus come into contact with sexual hormones [34–36]. A major argument against this hypothesis is that QSI activity of hormones was observed at 2 mM (0.5 mg/ml) while, in serum, concentrations of hormones such as estradiol reach up to 0.4–1.6 nM (100–400 mg/ml) in healthy women and 2–18 nM during fertilizing protocols [37]. However, the debate remains still unclosed because clinical and environmental Pseudomonas isolates are known for their capacity to import, bind and biodegrade human hormones, including estrogens, via proteins and pathways that are still poorly-characterized [30–40]. These hormone-modifying capabilities would contribute to underestimate the QSI-efficiency of hormones in our in vitro assay.
Materials and Methods

Instrumentation

Infrared spectra were recorded on a Perkin Elmer Spectrum BX FT-IR spectrometer. Proton (1H) and carbon (13C) NMR spectra were recorded on Bruker spectrometers: Avance 300 MHz (QNP - 13C- probe or Dual 13C probe) and Avance 500 MHz (BB0 - ATM probe or BBI - ATM probe). Carbon NMR (13C) spectra were recorded at 125 or 75 MHz, using a broadband decoupled mode with the multiplicities obtained using a JMOD or DEPT sequence. NMR experiments were carried out in deuteriochloroform (CDCl3) or dimethyl sulfoxide (D6-DMSO), chemical shifts (δ) are reported in parts per million (ppm) with reference to CDCl3 (1H: 7.24; 13C: 77.23). The following abbreviations are used for the proton spectra multiplicities: s: singlet, bs: broad singlet, d: doublet, t: triplet, q: quartet, hept: heptuplet, m: multiplet, br: broad. Coupling constants (J) are reported in Hertz (Hz). Mass spectra were obtained either with a LCT (Micromass) instrument using electrospray ionization (ES), or from a Time of Flight analyzer (ESI-MS) for the high resolution mass spectra (HRMS). The purity and the exact mass were determined for ID 1949 and ID 283 with a Waters Acquity liquid chromatograph equipped with a Photodiode Array Detector, an Evaporative Light Scattering Detector and a Triple Quadripole Detector. A reverse-phase HSS T3 column, 2.6 mm, 4.6 x 100 mm was used for the UPLC work with a mixture acetonitrile/water as the solvent system.

Elemental analyses were performed on a Perkin Elmer CHN 2400 analyzer with detection by catharometry. Thin-layer chromatography was performed on silica gel 60 F254 on aluminium plates (Merck) and visualized under a UVP Minerva UVLS-28 lamp (254 nm) and with ninhydrin and p-anisaldehyde in ethanol. Flash chromatography was conducted on Merck silica gel 60 (40–63 μm) at medium pressure (300 mbar) or on CombiFlash apparatus (Seralbo Technologies), using standard settings. Reagents and substrates were purchased from Sigma-Aldrich Chemical Company.

Compounds: General Procedures

Procedure A-Preparation of carboxamides IIIa-d: To a solution of the indolinylcarboxylic acid I (1 eq) in dry methylene chloride (0.3M) at 0°C, was added the amine II (1.05 eq), 1-hydroxybenzotriazole (HOBt, 1.05 eq), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 1.05 eq) and triethylamine (1.05 eq). The mixture was stirred at 0°C for 1 h and at room temperature for 1 h. The resulting mixture was then filtered, the filter cake washed with dichloromethane and the combined organic fractions were concentrated in vacuo to a yellow solid.

Table 2. IC50, MIC and MBC values of the tested compounds.

| Source          | Name         | IC50 a | MIC b | MBC b |
|-----------------|--------------|--------|-------|-------|
| QSI-reference   | 4-NPO        | 24     | 25    | >100  |
| Chemical library| 14           | >100 (0%) b | >100 | >100 |
|                 | 15           | >100 (19%) b | >100 | >100 |
|                 | 283          | 73     | >100  | >100  |
|                 | 729          | 75     | >100  | >100  |
|                 | 937          | >100 (15%) b | >100 | >100 |
|                 | 1099         | 35     | 100   | >100  |
|                 | 1102         | >100 (0%) b | >100 | >100 |
|                 | 1248         | 63     | >100  | >100  |
|                 | 1248         | >100 (16%) b | >100 | >100 |
|                 | 1577         | 2.5    | 3     | 25    |
|                 | 1868         | >100 (34%) b | 12.5 | >100 |
|                 | 1949         | >100 (38%) b | >100 | >100 |
|                 | 3028         | >100 (32%) b | >100 | >100 |
|                 | 3492         | 50     | >100  | >100  |
|                 | 3499         | >100 (19%) b | >100 | >100 |
| 1248-diastereoisomers | (S,S)-1248 | 90     | 100   | >100  |
|                 | (S,R)-1248   | 32     | 100   | 100   |
|                 | (R,S)-1248   | >100 (0%) b | 100  | 100   |
|                 | (R,R)-1248   | >100 (10%) b | 100  | 100   |
| Commercial products | Jasmonic acid | 25     | >100  | >100  |
|                 | Estradiol    | 75     | >100  | >100  |
|                 | Estriol      | 50     | >100  | >100  |

*Values are in μg/ml.

**In brackets, inhibition (%) at 100 μg/ml.

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Figure 4. In vitro Ti plasmid transfer frequency in Agrobacterium. The Ti plasmid transfer frequencies were measured in the presence of QSI (A) and the four 1248-diastereoisomers (B) at 0.1 mg/ml. Histograms represent the cell density of transconjugants (CFU/ml), while black diamonds and squares, those of the donor and recipient strains, respectively. Measurements were performed in quadruplicate and the experiment was repeated twice. The cell densities of transconjugants in the presence of QSI were compared to that of the control in the presence of DMSO with a Mann and Whitney test (α = 0.05). Statistically different values are noted by asterisks.

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Compounds: General Procedures

Procedure A-Preparation of carboxamides IIIa-d: To a solution of the indolinylcarboxylic acid I (1 eq) in dry methylene chloride (0.3M) at 0°C, was added the amine II (1.05 eq), 1-hydroxybenzotriazole (HOBt, 1.05 eq), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 1.05 eq) and triethylamine (1.05 eq). The mixture was stirred at 0°C for 1 h and at room temperature for...
5 h. The reaction mixture was quenched with water and extracted with methylene chloride. The organic phase was washed successively with saturated aqueous Na₂SO₄ and saturated aqueous NaCl, dried over MgSO₄, filtered and concentrated under reduce pressure. The residue was purified by flash chromatography on silica gel (elution with heptane/EtOAc 0 to 20%).

Procedure B-Reduction of carboxamides IIIa-d: To a solution of the amide derivatives IIIa-d (1 eq) in dry THF (0.03 M) at 0 °C, was added BH₃·THF (1 M, 1 eq) and the mixture was stirred at reflux for 16 h. The reaction mixture was cooled to 0 °C, acidified with aqueous HCl (2 M) and refluxed for an additional 30 min. The mixture was then extracted with methylene chloride and the organic phase was washed successively with saturated aqueous Na₂CO₃, dried over MgSO₄, filtered and concentrated under reduce pressure. The residue was purified by flash chromatography on silica gel (elution with heptane/EtOAc 0 to 30%). The pure amine product was dissolved in diethyl ether, a solution of HCl in diethyl ether (2 M) was added and the precipitated hydrochloride salt was collected by filtration, washed with diethyl ether and dried under vacuum.

(R)-N-((S)-1-(naphthalen-1-yl)ethyl)indoline-2-carboxamide (IIIb). Following general procedure A using (R)-indoline-2-carboxylic acid (0.61 mmol, 100 mg), (S)-1-(1-naphthyl)ethyamine (0.64 mmol, 103 µL), HOBt (0.64 mmol, 86 mg), EDCI (0.64 mmol, 123 mg) and Et₃N (0.64 mmol, 46 µL) in methylene chloride (2 mL), IIIb was obtained as a white solid (105 mg, 73%). 1H NMR (CDCl₃): δ 8.12 (d, J = 8.3 Hz, 1H), 7.86 (d, J = 7.6 Hz, 1H), 7.80 (d, J = 8.3 Hz, 1H), 7.56–7.40 (m, 4H), 7.36–7.31 (m, 1H), 7.04 (t, J = 7.6 Hz, 2H), 6.79 (td, J = 7.3, 1.2 Hz, 1H), 6.69 (d, J = 7.6 Hz, 1H), 6.05–5.95 (m, 1H), 4.53–4.47 (m, 1H), 4.15 (bs, 1H), 3.60–3.49 (m, 1H), 2.99–2.91 (m, 1H), 1.72 (d, J = 6.8 Hz, 3H); 13C NMR (CDCl₃): δ 172.4, 149.5, 138.3, 133.9, 131.1, 128.8, 128.3, 127.9, 127.6, 126.4, 125.8, 125.2, 124.8, 123.3, 122.3, 120.6, 110.8, 62.0, 44.1, 35.5, 21.2; IR (neat) 3298, 2900, 1651, 1608, 1512, 1484, 1467, 1245; MS (ESI): [M+H]+ m/z 317.2.

(S)-N-((S)-1-(naphthalen-1-yl)ethyl)indoline-2-carboxamide (IIIc). Following general procedure A using (S)-indoline-2-carboxylic acid (0.61 mmol, 100 mg), (S)-1-(1-naphthyl)ethyamine (0.64 mmol, 103 µL), HOBt (0.64 mmol, 86 mg), EDCI (0.64 mmol, 123 mg) and Et₃N (0.64 mmol, 46 µL) in methylene chloride (2 mL), IIIc was obtained as a white solid (141 mg, 73%). 1H NMR (CDCl₃): δ 8.12 (d, J = 8.3 Hz, 1H), 7.86 (d, J = 7.6 Hz, 1H), 7.80 (d, J = 8.3 Hz, 1H), 7.56–7.40 (m, 4H), 7.36–7.31 (m, 1H), 7.04 (t, J = 7.6 Hz, 2H), 6.79 (td, J = 7.3, 1.2 Hz, 1H), 6.69 (d, J = 7.6 Hz, 1H), 6.05–5.95 (m, 1H), 4.50–4.48 (m, 1H), 4.15 (bs, 1H), 3.61–3.49 (m, 1H), 3.00–2.87 (m, 1H), 1.72 (d, J = 6.8 Hz, 3H); 13C NMR (CDCl₃): δ 172.4, 149.5, 138.3, 133.9, 131.1, 128.8, 128.3, 127.9, 127.6, 126.4, 125.8, 125.2, 124.8, 123.3, 122.3, 120.6, 110.8, 62.0, 44.1, 35.5, 21.2; IR (neat) 3352, 2900, 1651, 1608, 1511, 1484, 1466, 1244; MS (ESI): [M+H]+ m/z 317.2.

(S)-N-((R)-1-(naphthalen-1-yl)ethyl)indoline-2-carboxamide (IIId). Following general procedure A using (R)-indoline-2-carboxylic acid (0.61 mmol, 100 mg), (R)-1-(1-naphthyl)ethyamine (0.64 mmol, 103 µL), HOBt (0.64 mmol, 86 mg), EDCI (0.64 mmol, 123 mg) and Et₃N (0.64 mmol, 46 µL) in methylene chloride (2 mL), IIId was obtained as a white solid (116 mg, 60%). 1H NMR (CDCl₃): δ 8.12 (d, J = 8.3 Hz, 1H), 7.86 (d, J = 7.6 Hz, 1H), 7.80 (d, J = 8.3 Hz, 1H), 7.56–7.40 (m, 4H), 7.36–7.31 (m, 1H), 7.04 (t, J = 7.6 Hz, 2H), 6.79 (td, J = 7.3,
1.2 Hz, 1H), 6.69 (d, J = 7.6 Hz, 1H), 6.05–5.95 (m, 1H),
4.50–4.48 (m, 1H), 4.01 (bs, 1H), 3.61–3.49 (m, 1H),
3.00–2.87 (m, 1H), 1.68 (d, J = 6.8 Hz, 3H); 13C NMR (CDCl 3):
172.6, 149.5, 138.3, 133.9, 131.1, 128.8, 128.3, 127.9, 127.6,
126.4, 125.8, 125.2, 124.8, 123.3, 122.3, 120.6, 110.8, 61.4, 44.0,
35.6, 20.7; IR (neat) 3352, 2925, 2900, 1651, 1608, 1511, 1484,
1466, 1246; MS (ESI): [M + H] m/z 317.2.

(S)-N-((S)-indolin-2-ylmethyl)-1-(naphthalen-1-yl)ethanamine ((S,S)-1248).
Following the general procedure B using amide IIIa (0.25 mmol, 80 mg),
BH3.THF (1M, 1.21 mL) in dry THF (8 mL) afforded ((S,S)-1248 (42 mg, 55%) as a colorless oil.
1H NMR (CDCl3): 8.19 (dd, J = 7.1, 2.1 Hz, 1H), 7.87 (dd, J = 7.1,
2.1 Hz, 1H), 7.76 (d, J = 8.1 Hz, 1H), 7.64 (d, J = 8.1 Hz, 1H),
7.54–7.45 (m, 3H), 7.06–6.98 (m, 2H), 6.66 (t, J = 7.1 Hz, 1H),
6.62 (d, J = 8.1 Hz, 1H), 4.75–4.60 (m, 1H), 3.94–3.84 (m, 1H),
3.09 (dd, J = 15.9, 8.9 Hz, 1H), 2.72–2.65 (m, 3H), 1.51 (d,
J = 6.7 Hz, 3H); 13C NMR (CDCl3): 150.8, 141.3, 134.0, 131.3,
129.0, 128.7, 127.3, 127.2, 125.9, 125.7, 125.4, 124.8, 123.0,
122.7, 118.7, 109.7, 59.3, 54.0, 53.3, 34.1, 23.6; IR (neat) 3361,
2973, 2926, 2853, 1609, 1485, 1465, 1115; HRMS (ESI): calcd.
for C21H23N2 [M + H] m/z 303.1861, found m/z 303.1863.

Hydrochloride derivative: Anal. Calcd. C, 67.20; H, 6.45; N, 7.46.
Found C, 62.07; H, 6.22; N, 6.76.

(R)-N-((S)-indolin-2-ylmethyl)-1-(naphthalen-1-yl)ethanamine ((S,R)-1248).
Following general procedure B using amide IIIb (0.30 mmol, 95 mg),
BH3.THF (1M, 1.45 mL) in dry THF afforded ((R,S)-1248 (74 mg, 81%) as a colorless oil.
1H NMR (CDCl3): 8.31 (d, J = 8.5 Hz, 1H), 7.97 (d, J = 7.2 Hz, 1H),
7.83 (d, J = 7.2 Hz, 1H), 7.74–7.53 (m, 3H), 7.15–7.08 (m, 2H),
6.78 (t, J = 7.2 Hz, 1H), 6.68 (d, J = 7.8 Hz, 1H), 4.77–4.70 (m, 1H),
4.04–3.92 (m, 1H), 3.20–3.12 (m, 1H), 2.86–2.78 (m, 2H),
2.72–2.66 (m, 1H), 1.62 (d, J = 6.5 Hz, 3H); 13C NMR (CDCl3):
150.9, 140.9, 134.1, 131.4, 129.1, 128.7, 127.3, 127.0, 125.8, 125.8,
125.4, 124.9, 123.1, 122.9, 118.7, 109.7, 59.1, 53.9, 52.8, 33.9, 23.8;
IR (neat) 3365, 2973, 2927, 2864, 1608, 1484, 1464, 1247, 1114; HRMS (ESI): calcd.
for C21H23N2 [M + H] m/z 303.1861, found m/z 303.1873.
Hydrochloride derivative: Anal. Calcd. C, 67.20; H, 6.45; N, 7.46. Found C, 67.27; H, 6.78; N, 7.25.

(S)-N-(R)-indolin-2-ylmethyl-1-(naphthalen-1-yl)ethanamine ([(R),R]-1248). Following general procedure B using amide HIl (0.35 mmol, 110 mg), BH₃·THF (1M, 1.70 mL) in dry THF (10 mL) afforded (S,R)-1248 (76 mg, 72%) as a colorless oil. ¹H NMR (CDCl₃): 8.27 (d, J = 8.3 Hz, 1H), 7.96 (d, J = 7.1 Hz, 1H), 7.83 (d, J = 8.3 Hz, 1H), 7.74 (d, J = 7.2 Hz, 1H), 7.39 (d, J = 7.1 Hz, 1H), 7.39 (d, J = 7.1 Hz, 1H), 7.24 (d, J = 7.2 Hz, 1H), 7.14–7.07 (m, 2H), 6.78 (t, J = 7.2 Hz, 1H), 6.68 (d, J = 7.1 Hz, 1H), 4.04–3.92 (m, 1H), 3.20–3.12 (m, 1H), 2.86–2.78 (m, 2H), 2.72–2.66 (m, 1H), 1.62 (d, J = 6.5 Hz, 3H). ¹³C NMR (CDCl₃): 150.8, 141.4, 134.1, 131.4, 131.4, 129.1, 129.1, 128.7, 127.3, 127.0, 125.8, 125.4, 124.9, 123.1, 122.9, 118.7, 109.7, 59.1, 53.9, 53.0, 33.9, 23.8; IR (neat) 3364, 2973, 2927, 2863, 1609, 1484, 1464, 1247, 1114; HRMS (ESI): calcd. for C₂₁H₂₃N₂ [M⁺] m/z 263.1866, found m/z 263.1865.

(R)-N-(R)-indolin-2-ylmethyl-1-(naphthalen-1-yl)ethanamine ([(R),R]-1248). Following general procedure B using amide HIl (0.35 mmol, 110 mg), BH₃·THF (1M, 1.70 mL) in dry THF (10 mL) afforded (R,R)-1248 (76 mg, 72%) as a colorless oil. ¹H NMR (CDCl₃): 8.27 (d, J = 8.3 Hz, 1H), 7.96 (d, J = 7.1 Hz, 1H), 7.83 (d, J = 8.3 Hz, 1H), 7.74 (d, J = 7.2 Hz, 1H), 7.39 (d, J = 7.1 Hz, 1H), 7.39 (d, J = 7.1 Hz, 1H), 7.24 (d, J = 7.2 Hz, 1H), 7.14–7.07 (m, 2H), 6.78 (t, J = 7.2 Hz, 1H), 6.68 (d, J = 7.1 Hz, 1H), 4.04–3.92 (m, 1H), 3.20–3.12 (m, 1H), 2.86–2.78 (m, 2H), 2.72–2.66 (m, 1H), 1.62 (d, J = 6.5 Hz, 3H). ¹³C NMR (CDCl₃): 150.8, 141.4, 134.1, 131.4, 129.1, 129.1, 128.7, 127.3, 127.0, 125.8, 125.4, 124.9, 123.1, 122.9, 118.7, 109.7, 59.1, 53.9, 53.0, 33.9, 23.8; IR (neat) 3364, 2973, 2927, 2863, 1609, 1484, 1464, 1247, 1114; HRMS (ESI): calcd. for C₂₁H₂₃N₂ [M⁺] m/z 263.1866, found m/z 263.1865.

Hydrochloride derivative: Anal. Calcd. C, 67.20; H, 6.45; N, 7.46. Found C, 67.27; H, 6.78; N, 7.20.

Library screening for QSI

When the screening was performed, the chemical library of the Institut de Chimie des Substances Naturelles (ICSN, Gif-sur-Yvette, France) contained more than 3500 synthetic and natural compounds, which were individually dissolved in dimethylsulfoxide (DMSO) at 1mg/ml and stored in 96 microwell plates. A first screening was performed by mixing biosensor CV026 with the AHL hexanoylhomoserine lactone (C₆-HSL) at 0.5 μM and the tested compounds at 50 μg/ml. Final volume was adjusted to 100 μl with a synthetic medium (10% LBm and 0.4% sucrose).
After 24 hours of incubation at 30°C, the presence or absence of violacein pigment was quoted by visual reading. A second screening was performed using biosensor *A. tumefaciens* NT1[pZLR4], the AHL octanoylhomoserine lactone (C8-HSL) at 10 nM and the tested molecules at 5 µg/ml. Final volume was adjusted to 100 µl by the addition of AB minimal medium supplemented with mannitol (0.2%) and TY medium (10%). After 4 hours of incubation at 30°C, β-galactosidase activity was measured in *A. tumefaciens* cultures as previously described [44]. In the two screenings, 4-nitrophenyl-N-oxide (4-NPO) was used as a QSI reference [17].

**Measurement of IC50, MIC and MBC values of the QSI**

To determine half maximal inhibitory concentration (IC50) of QSI, β-galactosidase activity of the *Agrobacterium* biosensor was measured, as described in the screening protocol, in the presence of AHL and QSI, which were introduced at final concentrations ranging from 1.5 µg/ml to 100 µg/ml. Aside from the effect of QSI on QS regulation, the toxicity of these compounds was tested on bacterial cells by measuring two parameters [16]: the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). MIC, which is the lowest concentration of QSI (µg/ml) inhibiting any visible culture after 36 h of incubation at 30°C, was estimated by culturing 10^5 CFU/ml of the *Agrobacterium* biosensor cells in the presence of different concentrations of the QSI. MBC, which is the lowest concentration of QSI (µg/ml) that results in a 99.9% reduction of the initial bacterial population (10^5 CFU/ml) after 36 h of incubation at 30°C in the presence of different concentrations of QSI, was estimated by plating 100 µl of the *Agrobacterium* cultures on agar LBm plates. After an incubation of 48 h at 30°C, CFU were counted and the MBC values were calculated.

**QS-regulated plasmid-transfer in *A. tumefaciens***

In *A. tumefaciens*, the transfer of the Ti-plasmid from a donor cell to a recipient one is controlled by QS. QSI were evaluated for their capacity to reduce plasmid transfer frequency in *Agrobacterium*. In the plasmid transfer assay, two modified *Agrobacterium* strains were used: a Ti-plasmid donor, which requires exogenous AHL to transfer a kanamycin-resistant (Km') Ti-plasmid [45], and a rifampicin-resistant (Rif') recipient strain, which is free of Ti-plasmid. Overnight cultures of the donor and recipient strains were mixed with 500 µM of oxo-octanoylhomoserine lactone (OC8-HSL) and 0.1 mg/ml of QSI in LBm medium. Each combination was repeated 4 times in microwell plates. After 72 hours of incubation at 24°C, the recipient and donor cells, as well as recipient cell which had acquired the Ti-plasmid from the donors, were counted on LBm agar plates supplemented with the antibiotics Km and Rif.

**QS-regulated genes in Pseudomonas aeruginosa and AHLs quantification**

QS gene transcription in *P. aeruginosa* PA01 was monitored using PA01 strains carrying various gene promoters fused to a lacZ reporter gene, as described in Vandeputte et al., 2010 and Table 1. PA01 reporter strains were prepared according to Vandeputte et al. [29]. Briefly, 18-hours-old liquid cultures (50 µl) were diluted in order to obtain a starting OD_600 nm_ comprised between 0.02 and 0.03 in fresh LB medium supplemented with 50 mM MOPS pH 7.0 (1 ml), carbenicillin (300 µg/ml) and 10 µl of the QSIs to be tested (OD were measured using a SpectraMax M2 device from Molecular Devices). Test and control QSIs were diluted in 100% DMSO (resulting after addition to the growth medium in a 1% final concentration of DMSO). Cultures were incubated for 18 hours at 37°C with agitation. After incubation, cell densities were assessed spectrophotometrically (OD_600 nm_) and β-galactosidase assays were performed using the substrate o-nitrophenyl-β-D-galactopyranoside as described previously [19,29]. Promoterless-lacZ fusions were used as controls. AHLs were quantified as described in Vandeputte et al. [19]. All tests were performed in quintuplicates and three biological repetitions.

The statistical significance of each test was evaluated by conducting Student’s t tests and two-way ANOVA combined with the Tukey post-analysis test using the GraphPad Prism software and *p* values ≤ 0.01 were considered significant.

**Heterologous expression of the Pseudomonas QS-receptors in *E. coli***

*Escherichia coli* JLD271 biosensor strains harboring LasR- and RhlR-based plasmids pAL105 and pAL101 and control plasmids pAL106 (LasR) and pAL102 (RhlR) (Table 1) were prepared according to Vandeputte et al. [19]. Briefly, these strains were grown in LB medium supplemented with tetracycline (10 µg/ml) and chloramphenicol (25 µg/ml) for 24 h [21]. Then 50-µl portions of the cultures were subcultured in 1 ml of LB medium (the starting OD_600_ ranged between 0.02 and 0.025 corresponding to 3.10^6 CFU) supplemented with 10 µl of DMSO (1% [vol/vol] final), 10 µl of naringenin or naringin dissolved in DMSO (2 mM, final concentration), or 10 µl of the molecule to be tested. To induce the expression of the lux operon, 0, 1, 10 or 100 nM of C4-HSL was added to pAL101 and pAL102, while OC12-HSL was added to pAL105 and pAL106. After incubation for 2 h at 37°C with agitation (175 rpm), 200 µl of culture was transferred to 96-well OptiPlate-96 F plates from Perkin-Elmer, and the luminescence of each sample was measured by using a TopCount NXT device from Perkin-Elmer. The LasR(pAL106) and RhlR(pAL102) biosensors were used for background subtraction, and the OD_600_ values were measured to account for the differences in cell density. All experiments were performed in six replicates. The statistical
significance of each test was evaluated by conducting Student’s t tests using the GraphPad Prism software, and p values ≤0.05 were considered significant. Naringenin (4',5,7-trihydroxyflavone), naringin (4',5,7-trihydroxyflavonanone 7-rhamnoside), the AHLs OC12-HSL and C6-HSL were purchased from Sigma-Aldrich and dissolved freshly in 100% DMSO before use. Tested QSIs were dissolved in 100% DMSO before use.

Modeling of the interactions between QSIs and QS-receptors

PDB codes of the estradiol, estritol and estrone were respectively 2YJA (name EST), 1x8 V (name ESL) and 3HM1 (name J3Z) [46–47]. Docking experiments were performed on each hormone using Arguslab [48] with X-ray protein structures of LasR (pdb code 2UV0) [49] and TraR (pdb code 1L3L) [50]. The docking engine GA (genetic algorithm) was employed with default parameters using a 15 Å docking box centered on natural ligands (OC12-HSL and C6-HSL). Docking results for each protein were then superimposed with PyMol (http://www.pymol.org/) to generate figures presented in Figure 8 A and B (top). Simplified binding modes (Figure 8 C and D) were constructed with Arguslab using the docking results of estradiol (taken as example) within LasR and TraR by hiding some binding site residues. Hydrogen bonds were assigned within a distance of 3.0 Å.

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Author Contributions

Conceived and designed the experiments: ABC MT LS RHD YQ YD CG. Wrote the paper: ABC MT LS RHD YQ YD CG OMV DF.

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