Article

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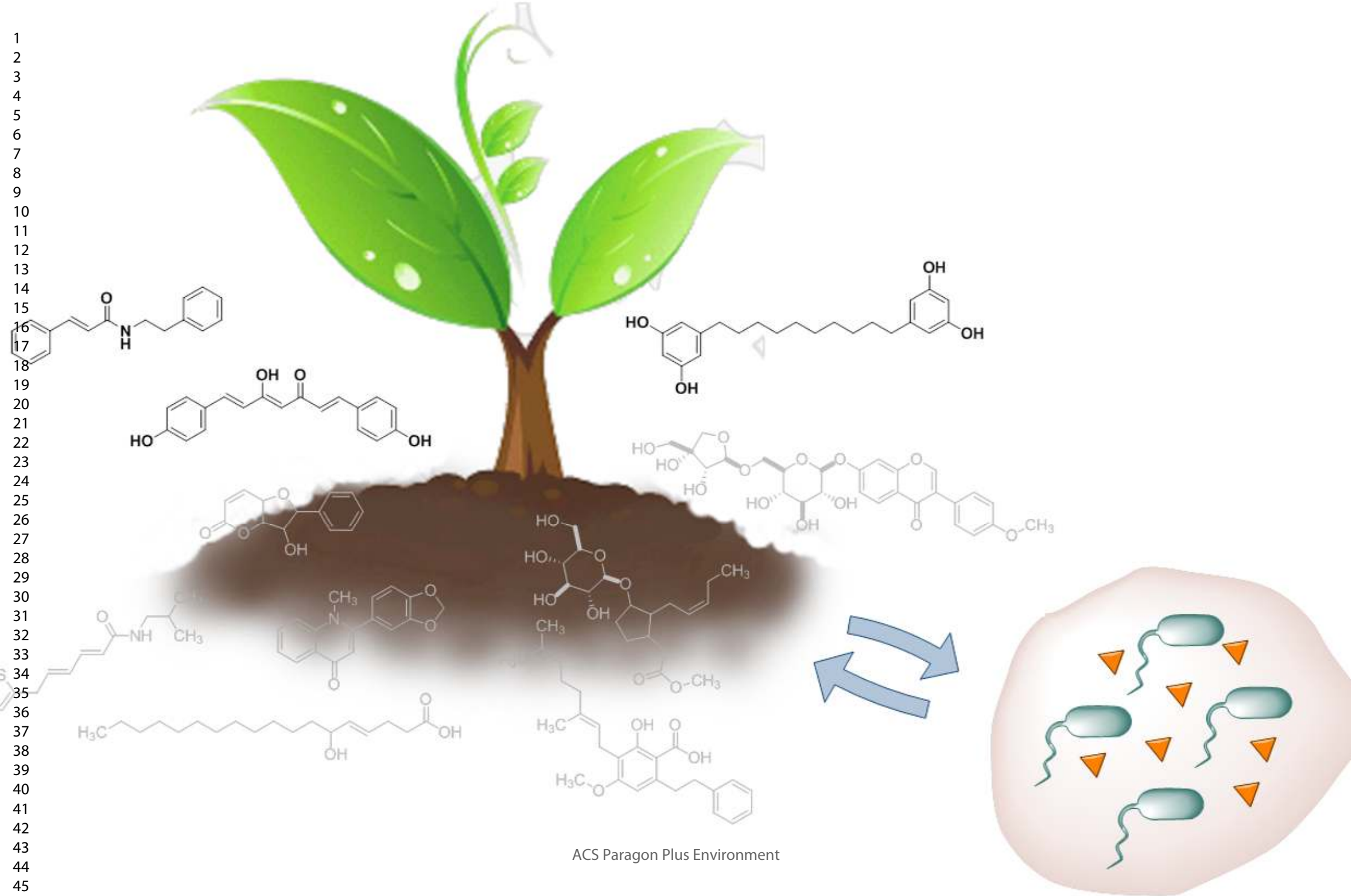

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Mining Plants for Bacterial Quorum Sensing Modulators

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ABSTRACT: The bacterial plant pathogen *Agrobacterium tumefaciens* uses quorum sensing (QS) in order to regulate the transfer of DNA into the host plant genome and this results in the induction of crown gall tumors. The deleterious results of these infections are widespread and affect many species of fruit and crops. In order to further our understanding of this process and to provide potential solutions we evaluated a library of 3800 natural products from plant sources and identified potent compounds that are able to strongly modulate plant-bacterial interactions.

Bacteria use chemical signaling to coordinate gene expression. This is a phenomenon called quorum sensing (QS) and it is based on the secretion of small signaling molecules and their recognition based on population density,[1-3] The plant pathogen *Agrobacterium tumefaciens* is one of the most extensively studied bacteria that use QS to regulate virulence and it can effectively induce crown gall disease and infect various plant species.[4-6] *A. tumefaciens* produces and secretes the QS molecule *N*-3-oxo-octanoyl-L-homoserine lactone (OC_8HSL), which at a threshold population density (and concentration) will bind the primary QS receptor, TraR, which results in the expression of virulence genes.[7] One of these genes is responsible for the conjugal transfer of the tumor inducing (Ti) plasmid between the bacteria and their plant host. Each bacterium holds a Ti plasmid that carries both virulence gens and a DNA fragment called transfer DNA (T-DNA). When the T-DNA is transferred and integrated into the host genome it will initiate over expression of phytohormones (i.e. auxin and cytokinin) and uncontrolled cell division that eventually will result in a formation of crown gall tumors.[8-9] The ability of the bacteria to exploit plants vulnerabilities by attacking wounded sites and spread infection has been thoroughly studied, but to date none of the current treatments against the disease have resulted in efficient methods to prevent bacterial infection the onset of crown gall disease. Since the QS mechanism of *A. tumefaciens* has a crucial effect on implementing and controlling infection in plants, a rational approach to deal with this question is to search for natural plant products that have evolved to attenuate bacterial QS activity through exogenous chemical interruption.

Due to their enormous structural and chemical versatility, natural products (secondary metabolites) in general and plant metabolites in particular can be used as a potential tool kit both to study and mimic plant-bacterial interactions.

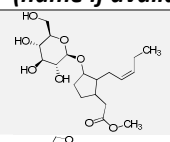
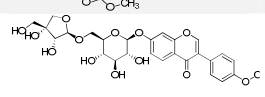
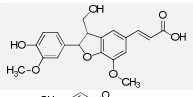
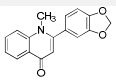
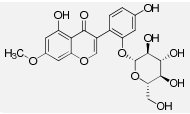
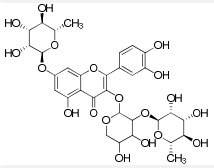
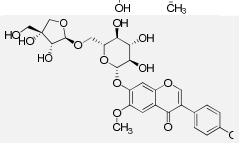
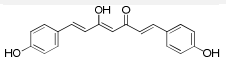
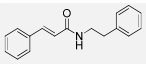
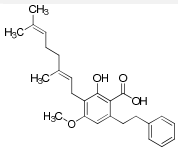
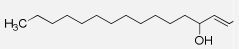
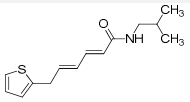
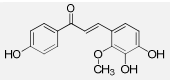
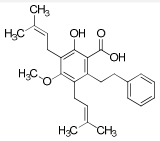
Here we report the screening of a library of 3800 purified and characterized natural products from plant sources on *A. tumefaciens* in order to find strong modulators that may affect the QS system. Based on the initial results of the screening, we prepared a synthetic

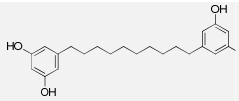
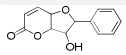
library of selected natural products and analogs that significantly agonize or antagonize the QS system of *A. tumefaciens.* We also validated the efficacy of the most potent modulators *in vivo* in a tumor formation model system in carrots.

RESULTS AND DISCUSSION

Library screening of natural products from plants source. Our initial library screening of the 3800 natural products (acquired from AnalytiCon Discovery library) was performed systematically using a highly sensitive luminescent *A. tumefaciens* reporter strain - A136 (pCF218)(pMV26) (see methods) - that responds to picomolar concentration of 3-oxo-C_8-AHL (the *A. tumefaciens* autoinducer). The compounds that revealed more than 50% inhibition or stimulation of the QS system with and without 400 pM 3-oxo-C_8-AHL (the *A. tumefaciens* autoinducer) were selected as potential hits (listed in **Table 1**). We observed interesting trends regarding structural features of active compounds. For instance, many of the agonists contain the flavonoid scaffold. This is in line with previous reports on flavonoid activity in bacteria; they have been shown to act as antimicrobial agents in plants [10-11], while they also were found to mediate symbiotic interactions between plants and *Rhizobium* bacteria[12-13]. Among the stronger antagonists, most of the hits contain long alkyl chains substituted with aromatic moieties at the terminal positions, or polyphenol containing compounds.

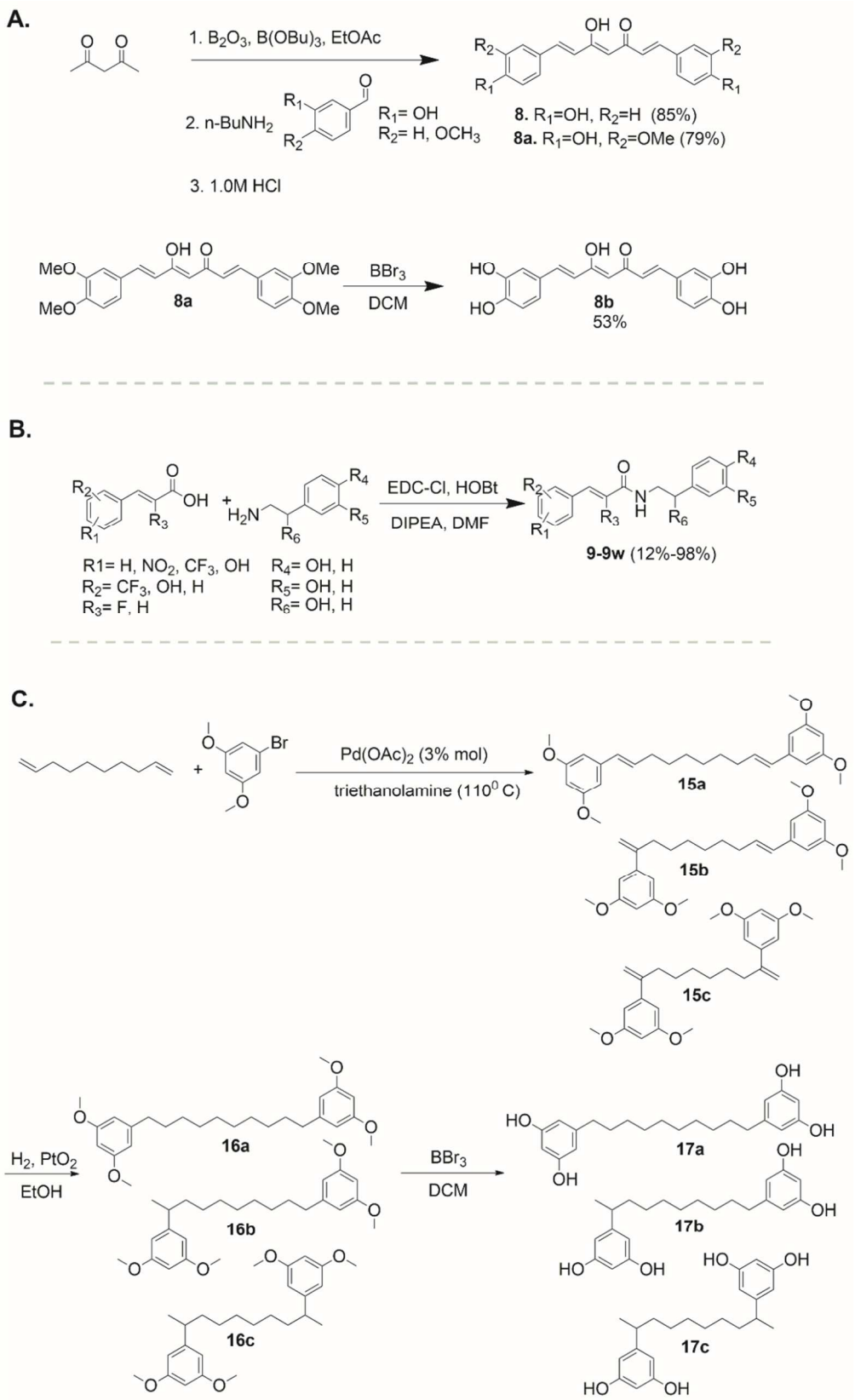
Table 1. Selected active compounds**.** Compounds that showed more than 50% inhibition or activation of QS at 30 µM

No.	*Structure (name if available)*	*Source (genus)*	*QS activity*
1		*Panax*	Agonist
2		*Acacia*	Agonism
3		*Momordica*	Agonism
4	 Graveoline	*Ruta*	Agonism
5		*Millettia*	Agonism
6		*Verbascum*	Agonism
7		*Millettia*	Agonism
8	 Bisdesmethoxycurcumin	*Chrysanthe-mum*	Antagonism
9	 2-N-Phenylethylcinnamamide	*Fissistigma*	Antagonism
10	 Amorfrutin B	*Amorpha*	Antagonism
11		*Polygala*	Antagonism
12		*Chrysanthe-mum*	Antagonism
13	 Licochalcone B	*Glycyrrhiza*	Antagonism
14		*Glycyrrhiza*	Antagonism

17a		*Grevillea*	Antagonism
16	 Altholactone	*Calophyllum*	Antagonism

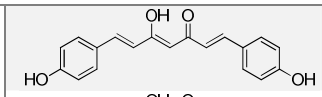
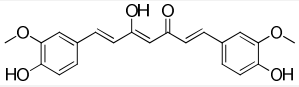
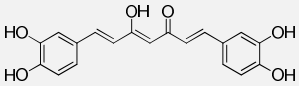
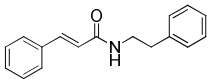
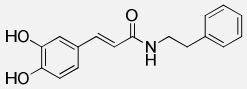
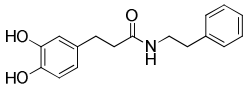
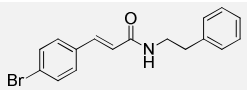
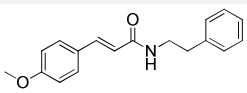
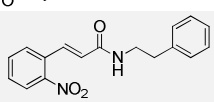
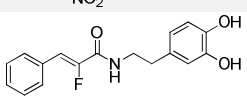
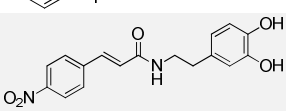
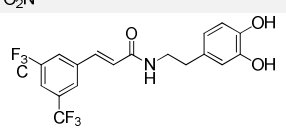
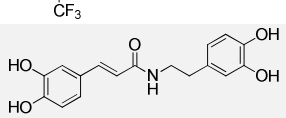
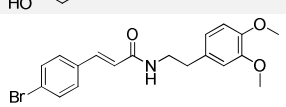
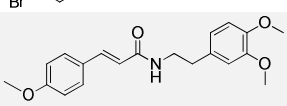
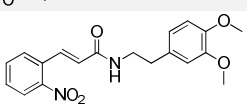
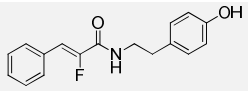
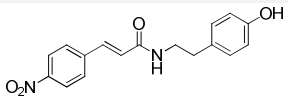
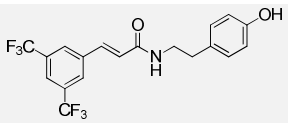
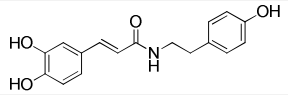
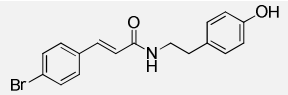
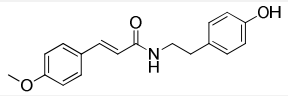
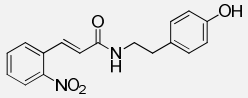
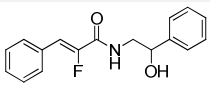
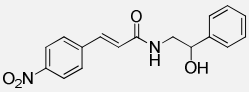
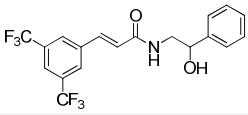
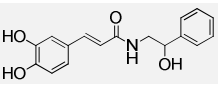
Hits to leads - focused antagonist libraries with structural refinements. To further investigate structure-activity relationships among the antagonists we selected compounds **8**, **9**, and **17a** (bisdesmethoxycurcumin, N-phenethylcinnamamide and 5,5'-(1,10-decanediyl)bis[1,3-benzenediol], respectively). The synthesis of compound **8** and its analog **8a** (curcumin, **Scheme 1A**) were performed following the Pabon method[14] with minor modifications described by Venkateswarlu *et al*[15]. For analog **8b** (**Scheme 1A**) we used an alternative method, due to the likelihood of side reactions on the unprotected catechol moiety. Instead, we reacted compound **8a** (curcumin) with boron tribromide to obtain the demethylated product. Then, compound **9** and several analogues were obtained by simple amide coupling reactions (**Scheme 1B**). The synthetic route to compound **17a** was designed based on three main steps (**Scheme 1C**); a Heck cross coupling reaction described by Li *et al*.[16] with slight modifications, hydrogenation of conjugated double bonds and demethylation. Interestingly, the Heck reaction resulted in two additional isomers, which proved hard to separate (their synthesis and biological evaluation is part of ongoing investigations).

Biological evaluation of synthetic library. To study the activities of the natural and synthetic derivatives on the QS system of *A. tumefaciens*, we used the luminescent reporter strain A136 (pCF218)(pMV26) in the presence (antagonist assay) or absence (agonist assay) of 400 pM 3-oxo-C_8-AHL (table 2). We observed mild inhibitory effects for compounds **8** and **8a**, which is not surprising given that bis-desmethoxycurcumin (**8**) and curcumin (**8a**), which are found in the flowering plants of the *Chrysanthemum* genus and in turmeric (*Curcuma longa*), respectively, have anti-microbial effects, among other therapeutic properties.[15, 17-18] Compound **9**, which originates from plants belonging to the *Fissistigma* genus showed moderate inhibitory effects , but we saw no significant inhibitory effect for the other analogs **9a-9w**. As far as we know, the effect of these compounds has never been tested on bacteria before, but in previous studies it has been shown that this family of compounds induces apoptosis in cancer cells[19]. Compound **17a**, which can be found in the *Grevillea* genus flowering plants, was examined along with its analogs **17b** and **17c**, and while we observed strong QS inhibition for compound **17a** in the initial library screening, we could not separate the mixture of the three isomers **17a, 17b** and **17c** (IC_{50} = 12.4 μM, preliminary data – full characterization of the purified isomers will be reported elsewhere). Compound **8b** and **9a**, which contain catechol moieties, show relatively strong inducing effects on the QS response cascade. It was previously reported that certain soil microbes are chemoattracted to phenolic compounds. In *A. tumefaciens*, phenolic compounds participate in chemotaxis based interactions, which could lead to activation of virulence genes, which may in turn prompt the Ti plasmid transfer and accelerate the infection process by *A. tumefaciens*[20-21]. We believe that this phenomenon affects the QS system in an indirect way. We came across another interesting phenomenon when we compared the activities of compounds **9a** and **9b**. It appears that conjugated aromatic compounds such as compound **9a** have a better inducing effect than the non-conjugated compound **9b**.



Scheme 1. Synthetic routes for selected natural products and their analogs. A) Synthesis of bisdesmethoxycurcumin (**8**) and analogs (**8-8b**). B) Synthesis of 2-N-phenethylcinnamamide (**9**) and analogs (**9a-9w**). C) Synthesis of 5,5'-(1,10-decanediyl)-bis-[1,3-benzenediol] (**17a**) and analogs (**17b-17c**).

Table 2. Activities of the natural products and their synthetic derivatives on the QS system of *A. tumefaciens*

No.	Compound	EC_{50} μM	IC_{50} μM
8		>100	33.3
8a		>100	15.0
8b		19.2	>100
9		>100	4.8
9a		13.6	>100
9b		65.1	>100
9c		>100	>100
9d		>100	>100
9e		>100	>100
9f		>100	>100
9g		>100	>100
9h		>100	>100
9i		>100	>100
9j		>100	>100
9k		>100	>100
9l		>100	>100
9m		>100	>100
9n		>100	>100
9o		>100	>100
9p		>100	>100
9q		>100	>100
9r		>100	>100
9s		>100	>100
9t		>100	>100
9u		>100	77.4
9v		>100	>100
9w		>100	>100

***In vitro* carrot disc infection assay.** To assess the virulence level of *A. tumefaciens* in the presence of the most potent anti-QS compounds, we performed tumor induction assays on carrot discs[22], which provide both visual and quantitative information on the selected compounds effect (see methods). Compounds **8a** and **9** were selected as potent inhibitors based on their QS activity in the previous tests. The assay was performed on carrot discs, eight replications for each treatment under sterile conditions (**Figure 1**). The tumor induction on carrots was tested at a concentration range between 0 μM - 50 μM. *A.*

tumefaciens induced tumor formation which could be inhibited strongly by both compounds at 50 µM (>90%, **Figure 2**).

SUMMARY AND CONCLUSION

Over the past half a billion years plants have co-evolved with microbes in general and plant pathogens in particular. In order to deal with threats and adjust to changing and often hostile environments, plants have developed multiple mechanisms to cope with adverse effects. We Humans have therefore used plants as a powerful and rich source of agents to promote health and combat microbial pathogens. Here we focus on the pathogen *A. tumefaciens* which induces crown gall tumors in plants. Since this disease is regulated and controlled by the cell dependent mechanism-QS, using natural products which are originated from plants could help finding potential strong anti-QS that will enable to attenuate infection. To achieve this goal, we performed a wide library screening of natural products on the QS system of *A. tumefaciens*, we selected potential hits with strong activity and we generated a synthetic library of analogs. by evaluating the QS activity of the synthetic library, we manage to acquire knowledge on activity based structural elements of analogs. the strongest QS inhibitors were selected and tested *in vitro* by monitoring over the tumor formation on surfaces of carrot discs. Based on these results and the mechanistic QS activity assays we can conclude that compounds **8a**, **9** and **17a** are indeed effective QS system inhibitors. To further understand their inhibitory role in the QS complex system in detail, additional studies are needed. Notably, both the QS reporter and wild-type virulence assays are somewhat limited in terms of quantitative assessment of virulence inhibition, and they do not take in account the ability of bacteria to swarm or engage in chemotaxis, which under natural conditions contribute greatly to their pathogenicity.

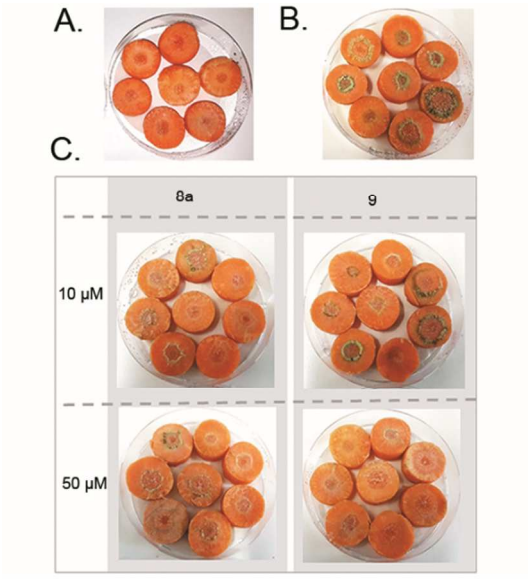
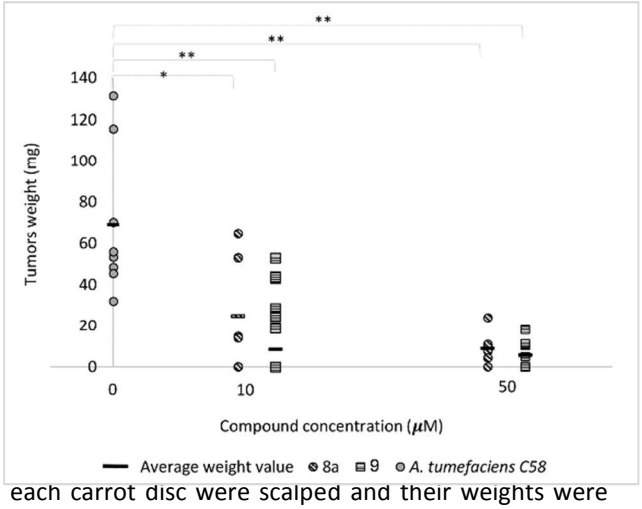


Figure 1. Carrot disc infection assay after 4 weeks: (A) carrot disc with LB medium, (B) carrot discs infected by *A. tumefaciens* C58. (C) *A. tumefaciens* C58 in the present of selected QS inhibitors at 10 µM (upper row) and 50 µM (bottom row)



each carrot disc were scalped and their weights were measured. Data are represented as the mean weight of eight experiments. P values of infected carrots in the presence of QS inhibitors vs. infected carrots without the tested compounds: (*) P < 0.02, (**) P<0.01

METHODS

Reagents and general procedures. All solvents and reagents were of reagent grade quality (Acros, Sigma-Aldrich or Fisher Scientific, Difco™) and used without further purification. Luria–Bertani (LB) medium was prepared as instructed. Compound Handling; Stock solutions of synthetic compounds (10 mM) were prepared in DMSO and stored at -20 °C in sealed vials. The amount of DMSO used in small molecule screens did not exceed 1 % (v/v).

Compound synthesis. Synthetic procedures and characterization data can be found in the Supporting Information.

Library screening -Luminescence Reporter Assay Protocol. The effect of the library compounds (AnalytiCon Discovery library) on *A. tumefaciens* A136 (pCF218)(pMV26) (lacking Ti plasmid, TraR response regulator, *traI*⁻ and TraI promoter fused to *luxCDABE*) was measured as described by Brenier *et al* [23]. an overnight culture of *A. tumefaciens* A136 (pCF218) (pMV26) was grown in Luria-Bertani broth (LB) (Miller's broth) supplemented with 25 µg ml^{-1} of kanamycin and 4.5 µg ml^{-1} of tetracycline, at 28 – 30 °C for 24-48 h. The overnight culture was diluted to absorbance density (OD_{600}) of 0.05 by fresh LB medium. The initial library screening of 3800 natural products was performed in Grenier white 384-well microliter plate. The activity of each compound was tested at approximate concentration of 30 µM. Two types of experiments were performed; competition assay in the presence of 400 pM 3-oxo-C_8-HSL and the other was performed in the absence of 3-oxo-C_8-HSL to measure agonistic activity of the test compounds. The control contained all the substances besides the tested compounds. Luminescence was measured every 20 min for 18 h with continuous shaking at 28.5 °C, using a Microtiter Plate Reader (Varioskan Flash, Thermo). Based on the luminescence values of the tested compounds hits were selected (compound that showed more than 50% activation or inhibition effect).

***A. tumefaciens* A136 (pCF218) (pMV26) QS activation assays.** The effect of the synthetic compounds on *A. tumefaciens* A136 (pCF218) (pMV26) was measured as described by Brenier *et al*[23]. an overnight culture of *A. tumefaciens* A136 (pCF218) (pMV26) was grown in Luria-Bertani broth (LB) (Miller's broth) supplemented with 25 µg ml^{-1} of kanamycin and 4.5 µg ml^{-1} of tetracycline, at 28–30 °C for 24-48 h. This overnight culture was diluted to absorbance density (OD_{600}) of 0.05 by fresh LB medium. A white/clear bottom 96-well microliter plate was prepared with wells containing test compounds serially diluted into LB medium (triplicates). 100 µL of the diluted cells were added to each well. In case of the A136 (pCF218) (pMV26) strain we performed two different types of experiments: one in which the test compounds were incubated with the addition of 400 pM of 3-oxo-C_8-HSL to monitor antagonist properties, and the other was performed in the absence of exogenous 3-oxo-C_8-HSL to measure agonistic activity of the test compounds. The control contained all the substances besides the tested compound. Luminescence was measured every 20 min for 18 h with continuous shaking at 28.5 °C, using a Microtiter Plate Reader (Varioskan Flash, Thermo). Average luminescence values divided by OD_{600} values were plotted against the added compound concentration.

***A. tumefaciens* carrot disc infection assay.** The effect of the potent synthetic compounds on *A. tumefaciens* tumor formation was tested as described by Trigui *et al*[22] with slight modifications. *A. tumefaciens* C58 strain was cultured on Luria Bertani (LB) agar medium. A single colony was transferred into LB broth medium and incubated at 25.7 °C for 24 h. Carrots (*Daucas carota*) were disinfected by scrubbing under running water

and soap with a brush, then immersed in 95% ethanol for 20 min. and 0.3 % NaClO for another 20 min. The carrots were then rinsed twice with sterile water. Carrot were sliced into discs (25 mm x 5 mm) by a sterile apple slicer and immersed in autoclaved distilled water for 30 min. After rinsing, the discs were removed from the distilled water, and blotted on sterile paper towels. 8 discs were placed on Petri plates containing autoclaved wet filter paper. Suspensions of *A. tumefaciens* in LB broth medium (OD_{600}: 0.99 ± 0.01) and either 10 or 50 µM of test compounds (from a 5 mM stock solution in DMSO) or without test compound (only 1% DMSO) were incubated for 30 min, then each disc was overlaid with 250 µL of bacterial suspension (8 replicates). Petri plates were sealed by parafilm to maintain and incubated at 27.5 °C. Carrot discs were examined after 14 days for young galls developing from meristematic tissue around the vascular system. The tumors were scratched carefully off the carrot surface and their weight was measured.

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Supporting Information Available:

Chemical compound synthesis, biological evaluation of synthetic library (figures 1S-10S), ^{1}HNMR spectra (figures 11S-32S), ^{13}CNMR spectra (figures 33S-55S) (PDF file). Natural product library screening data (agonist assays and competition assays) (Excel files). This material is available free of charge via the internet at http://pubs.acs.org.

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