Supplementary material

Butenolides from Plant Smoke Modulate Bacterial Quorum Sensing

Aviad Mandabi,[1] Hadas Ganin,[1] Josep Rayo[1] & Michael M. Meijler*,[1]

[1]Department of Chemistry and The National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel;

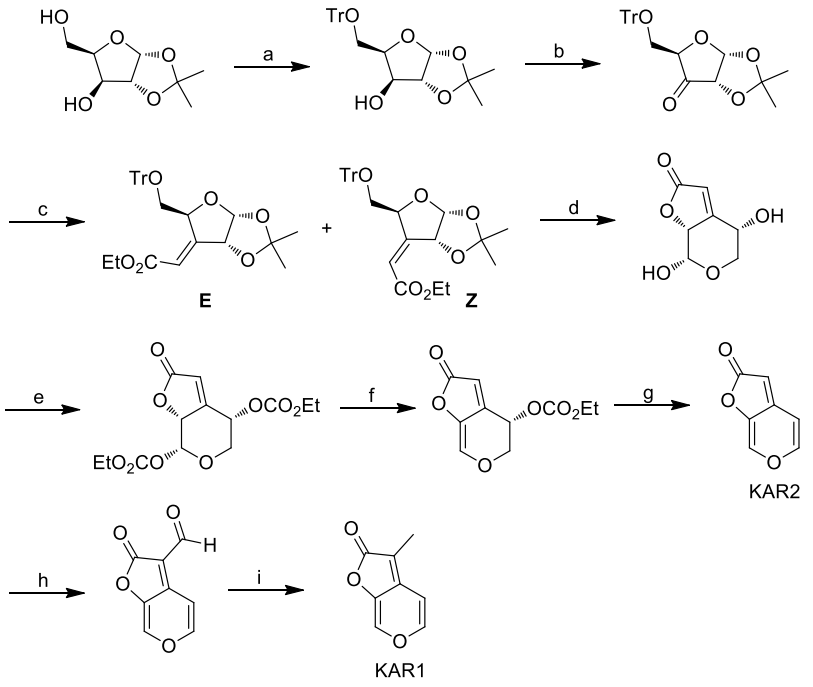
Table of contents:

i. Chemical synthesis

a. General

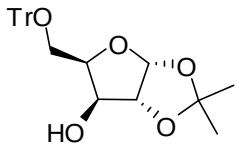
Unless stated otherwise, all reactions were performed under an inert atmosphere with dry reagents and solvents. All solvents and reagents were of reagent grade quality (Acros, Aldrich, Sigma or Fluka), and used without further purification. Thin-layer chromatography was performed using silica gel 60 with F254 indicator on glass plates (Merck). Flash chromatography was performed using Merck 40-63 μm silica gel. Solvent ratios for the purification of compounds by flash chromatography are reported as percent volume (v/v). NMR spectra were recorded using a Bruker Avance DPX400 (400 MHz) or Bruker Avance DMX500 (500 MHz) spectrometer. Spectra were calibrated on residual solvent signal.

b. Synthetic scheme

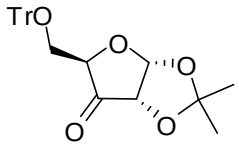


Scheme S1: Synthesis of KAR1 and KAR2. Reagents and conditions: (a) TrtCl, Et_3N, CH_2Cl_2 , 95%. (b) Dess-Martin periodinane, CH_2Cl_2, 85%. (c) NaH, $(EtO)_2POCH_2CO_2Et$, THF, 9%/88% (E/Z). (d) TFA/ CH_2Cl_2, 57%, crude. (e) ethyl chloroformate, pyridine, 0°C, 74%. (f) Et_3N, CH_2Cl_2, 73%. (g) $(Ph_3P)_4Pd$, THF, 54%. (h) $POCl_3$, DMF, 90%. (i) $AlCl_3$, $tBuNH_2{\cdot}BH_3$, CH_2Cl_2, 35%.

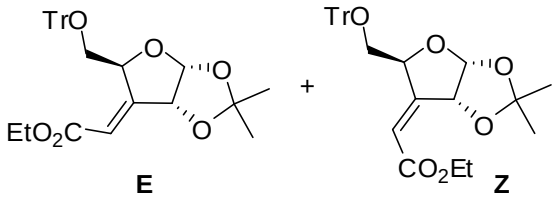
c. Synthetic procedures



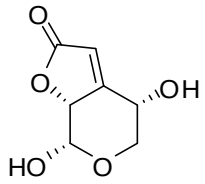
Synthesis of 1. To a solution of D-xylose (2 g , 10.51 mmol) in CH_2Cl_2 (20 mL) was added Et_3N (2.2 mL, 15.76 mmol) dropwise. The reaction mixture was stirred for 45-60 min. A solution of TrtCl (3.22 g, 11.56 mmol) in 6 mL CH_2Cl_2 was added to the reaction mixture dropwise. The reaction was stirred for 3 hours, followed by removal of the solvent *in vacuo*, dissolved in Et_2O and extracted from water (x3). The combined organic phases were washed with H_2O, dried over $MgSO_4$ and filtered. Concentration of the organic phase and purification of the product with flash chromatography (hexanes:ether 8:2 – hexanes:ether 5:5) afforded the desired product **1** as a white solid (4.318 g, 9.98 mmol, 95%). ^{1}H NMR (400 MHz, $CDCl_3$, δ 7.26 ppm) δ ppm 1.33 (s, 3H), 1.50 (s, 3H), 3.17 (d, J = 2.72 Hz, OH), 3.48 (dd, J = 3.17 Hz, J = 10.06 Hz, 1H), 3.57 (dd, J = 5.21 Hz, J = 10.03 Hz, 1H), 4.28 (m, 2H), 4.53 (d, J = 3.73 Hz, 1H), 6.02 (d, J = 3.72 Hz, 1H), 7.23-7.34 (m., 15H). ^{13}C NMR (400MHz, $CDCl_3$, 77.16 ppm) δ ppm 26.0, 26.7, 61.6, 76.2, 78.3, 85.0, 87.4, 104.9, 111.4, 127.2, 128.0, 128.3, 143.1.



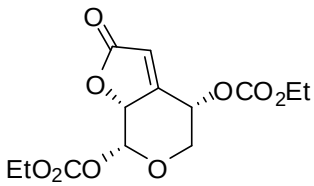
Synthesis of 2. Dess-Martin periodinane (3.86 g, 9.10 mmol) was dissolved in CH_2Cl_2 (15 mL). A solution of **1** (2.62 g, 6.07 mmol) in 5 mL CH_2Cl_2 was added dropwise to the reaction at RT. The reaction mixture was stirred at RT overnight. Afterwards, 20 mL CH_2Cl_2 was added, followed by addition of 15 mL $Na_2S_2O_3$ (0.1 g/mL) and 15 mL of saturated $NaHCO_3$, and the reaction was stirred for 30 min. The reaction mixture was then transferred to a separation funnel and the phases were separated. Extraction of the aqueous phase with CH_2Cl_2 (20 mL x 3) was followed by washing of the combined organic phases with brine and H_2O, dried with $MgSO_4$ and filtered. Concentration of the solvent and flash chromatography (CH_2Cl_2) gave the desired ketone **2** (2.23 g, 5.2 mmol, 85%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$, 7.26 ppm) δ ppm 1.48 (s, 6H), 3.31 (dd, J = 2.52 Hz, J = 10.11 Hz, 1H), 3.50(dd, J = 2.44 Hz, J = 10.11 Hz, 1H), 4.42 (m, 1H), 4.54 (d, J = 4.48 Hz, 1H), 6.34 (d, J = 4.49 Hz, 1H), 7.23-7.37 (m., 15H). ^{13}C NMR (400MHz, $CDCl_3$, 77.16 ppm) δ ppm 27.1, 27.3, 64.3, 77.3, 79.9, 87.3, 103.5, 114.1, 127.2, 127.9, 128.5, 143.1, 210.2.



Synthesis of 3. To NaH (173 mg, 7.20 mmol, 60% in mineral oil) under argon atmosphere, 7.20 mL of THF was added. The suspension was cooled to -10°C , Triethylphosphonate (1.43 mL, 7.20 mmol) was added dropwise at -10°C continues stirring for 30 min. Then ketone **2** dissolved in 7.2 mL THF was added dropwise at -10°C and stirring for 45-60 min at -10°C. After removal of the solvent, the residue was dissolved in EtOAc/water and extracted with EtOAc (20 mL x 3). The organic phase was dried over $MgSO_4$, filtered and concentrated. Flash chromatography (EtOAc:petroleum ether 5:95) yielded the E isomer (162 mg, 0.324 mmol, 9%), followed by the Z isomer (1.60 g, 3.196 mmol, 88%) as colorless oil. ^{1}H NMR and ^{13}C NMR spectra of both isomers were identical to literature spectra.[1]

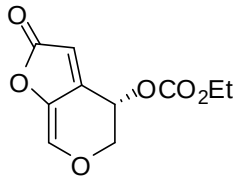


Synthesis of 4. Z isomer **3** (1.60 g, 3.196 mmol) was dissolved in 9.7 mL CH_2Cl_2. 15.5 mL of TFA: H_2O (4:1) was added dropwise to the solution at RT. The yellow solution was stirred for 30 min at RT, followed by concentration in vacuo, and addition of H_2O until the yellow color disappeared. Washing of the aqueous phase with EtOAc (x3), followed by concentration of the aqueous phase gave the crude diol **4** as a white solid (343 mg, 2 mmol, 62%) which was used without further purification.

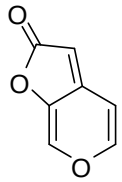


Synthesis of 5. Diol **4** (302 mg, 1.75 mmol) was dissolved in dry pyridine (4 mL) at 0°C . Ethyl chloroformate (770 µL, 8 mmol) was added dropwise to the reaction at 0°C and stirred for 10 min. Then the reaction mixture was warmed to RT and stirred for 3 hours. Concentration of the solvent yielded a residue that was dissolved and extraction with EtOAc (x3). The combined organic phases were dried over $MgSO_4$, followed by filtration and concentration of the solvent. Purification by flash chromatography (petroleum

ether:EtOAc, 3:1) gave the desired product **5** (412 mg, 1.30 mmol, 74%). ^{1}H NMR and ^{13}C NMR data corresponded to literature values.[1]

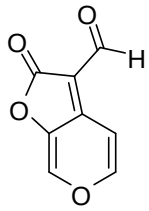


Synthesis of 6. 5 (412 mg, 1.30 mmol) was dissolved in CH_2Cl_2 (4.5 mL) under argon atmosphere. Et_3N (907 µL, 6.5 mmol) was added dropwise at RT. The reaction was stirred for 1 hour at RT. Concentration of the solvent followed by flash chromatography (petrol ethereum:EtOAc, 1:1) gave **6** (209 mg, 0.924 mmol, 71%). ^{1}H NMR and ^{13}C NMR data corresponded to literature values.[1]



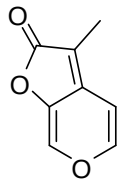
KAR2

Synthesis of KAR_2. 6 (209 mg, 0.924 mmol) and $Pd(PPh_3)_4$ (106 mg, 0.0924 mmol) were put under inert atmosphere and were dissolved in THF (2.5 mL). The reaction was heated at reflux overnight. Concentration of the solvent and flash chromatography (petroleum ether:EtOAc 1:1) gives **KAR_2** (67 mg, 0.496 mmol, 53%). ^{1}H NMR (400 MHz, Acetone-d6, 2.02 ppm) δ ppm 5.37 (dd, J = 0.27 Hz, J = 1.47 Hz, 1H), 6.88 (dd, J = 0.56 Hz, 5.43 Hz, 1H), 7.72 (d, J = 5.43 Hz, 1H), 7.90 (d, J = 1.47 Hz, 1H). ^{13}C NMR (400 MHz, Acetone-d6, 29.25, 205.27 ppm) δ ppm 89.7, 104.5, 128.4, 143.1, 145.2, 150.1, 169.5. MS (ESI): Measured = 137.00 [M+H], Calculated = 136.02 [M]. Spectral data were found to be identical to literature values.[1]



Synthesis of 7. KAR_2 (33 mg, 0.243 mmol) was dissolved in DMF (1.5 mL). $POCl_3$ (340 µL, 3.64 mmol) was added dropwise at RT. The reaction was warmed to 50˚C and stirred for 2 hours. The reaction was cooled down to RT and 2.5 mL CH_2Cl_2 was added, then 15 mL of $NaHCO_3$ was added slowly and the reac-

tion was stirred for 30 min. The reaction was transferred to a separation funnel and the phases were separated. The aqueous phase was extracted with CH_2Cl_2 (x3). The combined organic phases were dried over $MgSO_4$, filtered and concentrated. Flash chromatography (EtOAc: petroleum ether 8:2) gave aldehyde **7** (34 mg, 0.210 mmol, 86%). ^{1}H NMR and ^{13}C NMR data were found to be identical to literature values.[1]



KAR1

Synthesis of KAR$_1$. 7 (22 mg, 0.139 mmol) and $tBuNH_2*BH_3$ (76 mg, 0.834 mmol) were dissolved in CH_2Cl_2 under inert atmosphere and stirred for 20 min at RT. $AlCl_3$ (57 mg, 0.417 mmol) was added and the reaction was refluxed for 2 hours, and every 30 min 1 eq of $AlCl_3$ was added. The reaction was cooled down to 0^0 C. HCl 1M (5 mL) was added at 0^0 C. Extraction with CH_2Cl_2 (x4), were followed by drying over $MgSO_4$, filtration and concentration. Flash chromatography (EtOAc: petroleum ether 1:1) gives **KAR$_1$** (12 mg, 0.0845 mmol, 60%). ^{1}H NMR (500 MHz, Acetone-d6, 2.05 ppm) δ ppm 6.80 (dd, *J* = 0.55 Hz, 5.51 Hz, 1H), 7.62 (d, *J* = 5.51 Hz, 1H), 7.77 (s, 1H), 1.86 (s, 3H). ^{13}C NMR (500 MHz, Acetone-d6, 29.27, 205.28 ppm) δ ppm 6.72, 99.1, 103.2, 127.1, 139.7, 142.1, 148.9, 170.2. MS (ESI): Measured = 151.05 [M+H], Calculated = 150.03[M]. Spectral data were found to be identical to literature values.[1]

ii. Bioassays

a. Bioluminescence bioassays

MM32/BB170 synergistic-agonist assay

KAR1/KAR2 described above were tested for their agonist/antagonist activity in *V. harveyi* following the protocol reported by Schauder et al.[2] as follows: *Vibrio harveyi* strains MM32/BB170 were cultured for 18 h at 30˚C in AB medium and then diluted 1:1000 into fresh AB medium. A 96-well microliter plate was prepared with wells containing test compounds serially diluted into AB medium. 50 µL of the diluted cells were added to each well. In the case of MM32 strains we performed two different types of experiments, one in which the test compounds were incubated with the addition of 200 nM AI-2 to monitor antagonist activity, and the other was performed in the absence of exogenous AI-2 to measure agonist activity of the test compounds. The control contained all the substances besides the autoinducer tested. Boric acid was not added during these assays, as trace amounts are present in the medium. Luminescence was measured every 20 min at 30 ^{o}C, using a Microtiter Plate Reader (SpectraMax M2, Molecular Devices). Measurements were performed in triplicates and results are the average of three measurements. Luminescence values divided by OD_{600} values were plotted against the added compound concentration.

P. aeruginosa PAO1-*luxCDABE* and PAO-JP2-*luxCDABE* QS activation assays

PAO1-*luxCDABE* and PAO-JP2-*luxCDABE* (ant)agonist assays were performed as described previously by Ganin et al.[3] Briefly, strains which were used are: *P. aeruginosa* PAO1 wild type strain, harboring plasmid pKD201 containing a *lasI* promoter coupled to the *luxCDABE* luminescence system (pKD201, obtained from M. Surette, *J. Bacteriol*. 2007).[4] And PAO-JP2, a *lasI-rhlI* double mutant of PAO1, harboring a plasmid pKD201 with a *lasI* promoter coupled to the *luxCDABE* operon.[4] Each strain separately, was incubated overnight in LB medium containing 300 µg/mL of trimethoprim. A 96-well white/clear bottom microtiter plate (Greiner) was prepared with the desired concentrations of KAR1/2, a control well in each raw containing, and bacteria were added to reach a final absorbance density (OD600) of 0.015. For antagonist experiments, using the mutant strain PAO-JP2-*luxCDABE* a final concentration of 100 nM of the native 3-oxo-C12-HSL was added. The plate was then incubated for a period of 16 hours at 37^0C, and during this time luminescence measurements were performed at 20 minute intervals using a Microtiter Plate Reader (Varioskan Flash, Thermo). Luminescence values divided by OD_{600} values were plotted against the added compound concentration.

PAO-JP2 (*pKD-rhlA*) QS (ant)agonist assays

P. aeruginosa PAO-JP2 (pKD-*rhlA*) is a reporter strain for C4-HSL, which was developed by K. Duan et al.[4] in which the C4-HSL responsive *rhlA* promoter was fused upstream of the *luxCDABE* box, and the construct was introduced into PAO-JP2 (*lasI-rhlI* double-mutant strain). PAO-JP2 (pKD-*rhlA*) was incubated overnight in LB medium containing 300 µg/ml of trimethoprim. A 96-well white/clear bottom microtiter plate (Greiner) was prepared with the desired concentrations of KAR1/2, as well as control wells in each raw containing medium, and bacteria were added to reach a final absorbance density (OD600) of 0.015. For antagonist experiments, a final concentration of 10 µM of the native C4-HSL was added. The plate was then incubated for a period of 16 hours at 37^0C, and during this time luminescence measurements were performed at 20 minutes intervals using a Microtiter Plate Reader (Varioskan Flash, Thermo). Luminescence values divided by OD600 values were plotted against the added compound concentration.

A. tumefaciens A136 (pCF218) (pMV26) QS activation assays

The effect of KAR1/2 on KAR1/2 on *A. tumefaciens* A136 (pCF218) (pMV26) was measured as described by Brenier et al.[5] *A. tumefaciens* A136 (pCF218) (pMV26) was grown in Luria-Bertani broth (LB) (Miller's broth) at 28 – 30°C for 24-48 h with the addition of 25 µg/ml of kanamycin and 4.5 µg/ml of tetracycline. Overnight cultures of *A. tumefaciens* A136 (pCF218) (pMV26) were grown for 18-24 h in LB medium. This overnight culture was diluted 1:20 into fresh LB medium. A white/clear bottom 96-well microliter plate was prepared with wells containing test compounds serially diluted into LB medium. 50 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-C8-HSL to monitor antagonist properties, and the other was performed in the absence of exogenous 3-oxo-C8-HSL to measure agonistic activity of the test compounds. The control contained all the substances besides the autoinducer tested. Luminescence was measured every 20 min for 18 h with continuous shaking at 28.5 ^{o}C, using a Microtiter Plate Reader (Varioskan Flash, Thermo). Luminescence values divided by OD600 values were plotted against the added compound concentration.

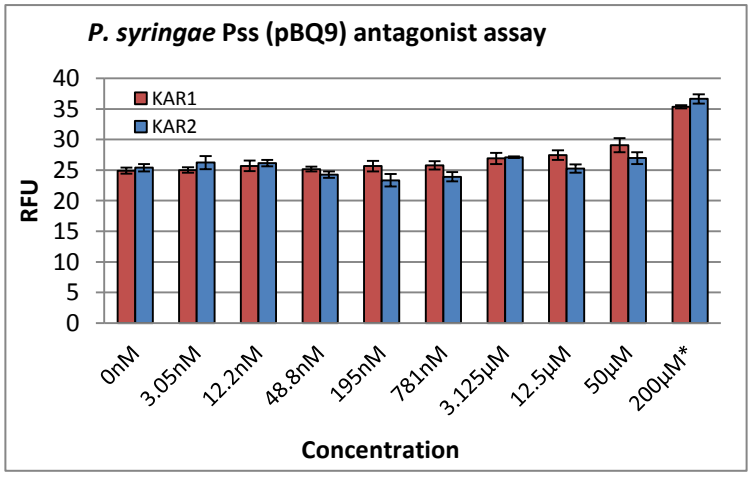
Pyocyanin assay

The effect of KAR1/2 on pyocyanin production in *P. aeruginosa* PAO1 wild-type (*Seattle*) strain was measured as described previously by Essar et al.[6] Briefly, after incubation for 24 h at 37 ^{0}C, 5 mL of cultures grown in the presence of 50 µM (final concentration) KAR1/2 in LB medium OD600 was recorded (bacteria in LB medium was used as control), then 1 mL of chloroform was added to the 5 mL culture supernatant. After extraction, 950 µL of the chloroform layer was transferred to a fresh tube and mixed with 300 µL of 0.2 N HCl. After centrifugation, the aqueous (top) layer was separated and its absorption was measured at 520 nm. We compared the average OD_{520} values of three measurements.

***P. syringae* QS activation / inhibition assays**

P. syringae Pss (pBQ9) is a reporter strain for 3-oxo-C6-HSL, which was developed by G. Dulla et al.[7] plasmid: p-ahlI, contains 500bp upstream of *ahlI* in pPROBE-Tagless (km). GFP is produced in response 3-oxo-C6-HSL, in all strains tested where *ahlR* is intact. *P. syringae* Pss (pBQ9) was incubated overnight in KB medium at 28^{0}C containing 100 µg/ml of kanamycin. A black 96-well microtiter plate (Greiner) was prepared with the desired concentrations of KAR1/2, as well as control wells in each raw containing medium, and bacteria were added to reach a final absorbance density (OD_{600}) of 0.05. For antagonist experiments, a final concentration of 1 µM of the native 3-oxo-C6-HSL was added. The plate was then incubated for a period of 15 hours at 28^{0}C, and during this time GFP fluorescence measurements (ex. 488 nm, em. 510 nm) were performed at 20 minutes intervals using a Microtiter Plate Reader (Varioskan Flash, Thermo). Luminescence values divided by OD_{600} values were plotted against compound concentrations.

(a)



(b)

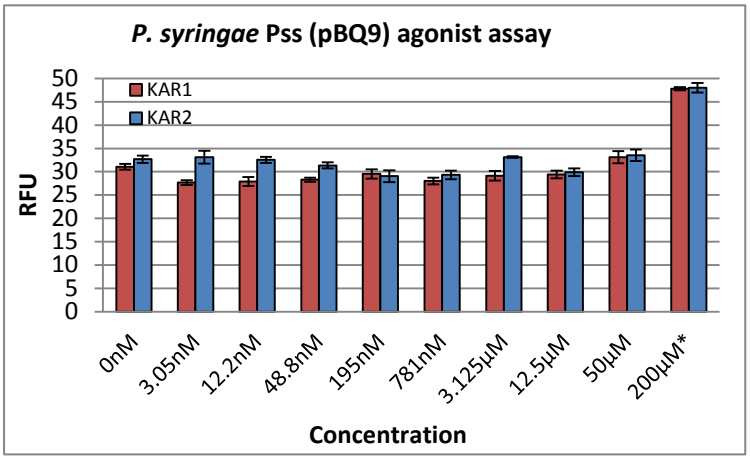


Figure S1: (a) Effect of QS by different concentration of KAR1/2, in the presence of 1 µM 3-oxo-C6-AHL in *P. syringae* Pss (pBQ9). (b) Effect of QS by different concentration of KAR1/2, in the absence of 3-oxo-C6-AHL in *P. syringae* Pss (pBQ9). *200 µM KAR1/2 exhibits growth inhibition, hence the increase in relative fluorescence units (FU/OD_{600}), RFU.

P. aeruginosa infection assays in lettuce midribs

This procedure is based on protocols described by Rahme and coworkers,[8] with minor modifications;

P. aeruginosa wild type strain PAO1 was grown overnight in LB medium at 37 °C, resuspended at an optical density (OD600) of 0.05 in LB medium, and divided into six eppendorfs (1 mL), containing different concentrations of KAR1 (from a stock solution of 10 mM in DMSO): 100, 33, 11, 3.7, 1.2 and 0 µM. Fresh crops of Romaine lettuce were washed with ddH_2O and and midribs of equal size were isolated and cut to fit into 10 cm petri dishes, containing sterile paper tissues moisturized with 1 mL H_2O. Each midrib was treated with three 5 µL injections of bacteria (using a pipette tip – the top of the midrib was punctured gently to a depth of approximately 1 mm) with and without KAR1 (vide supra), stored on the laboratory bench at room temperature, and the midribs were examined daily for rot formation.

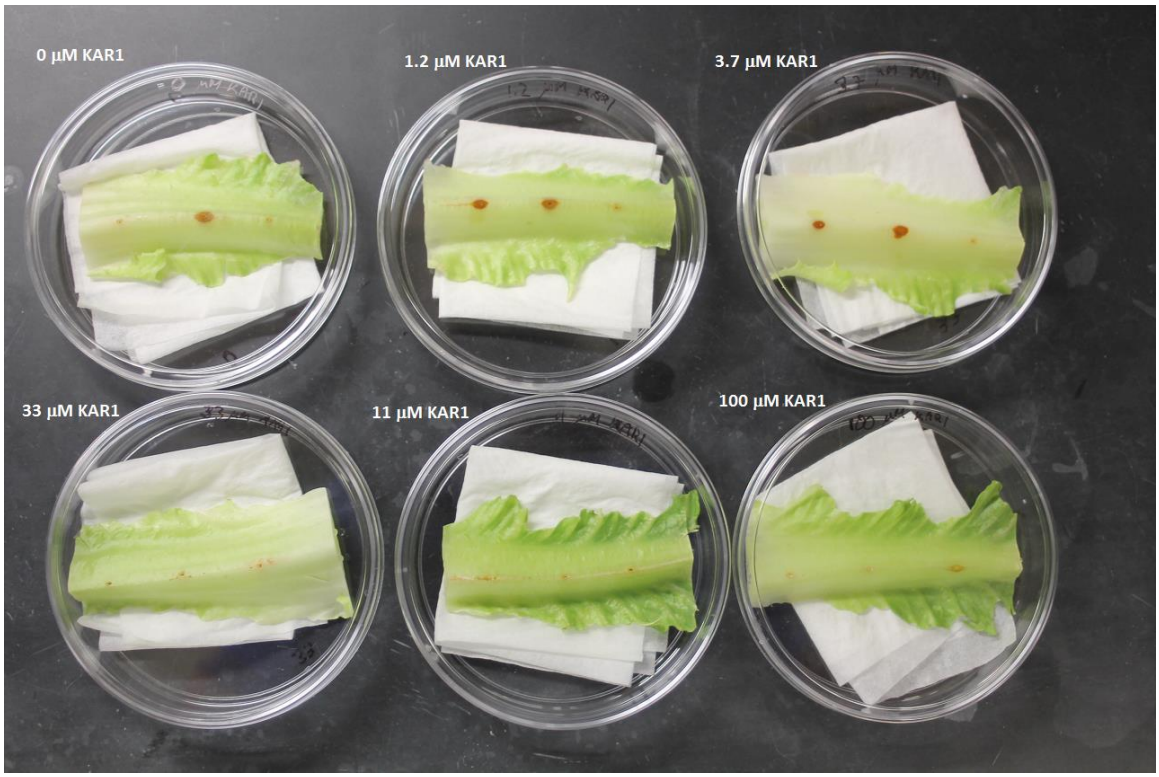


Figure S2: Romaine lettuce midribs infected with *P. aeruginosa* wild type strain PAO1 (OD_{600}=0.05), in the presence of varying concentrations of KAR1, 30 hours post injection.

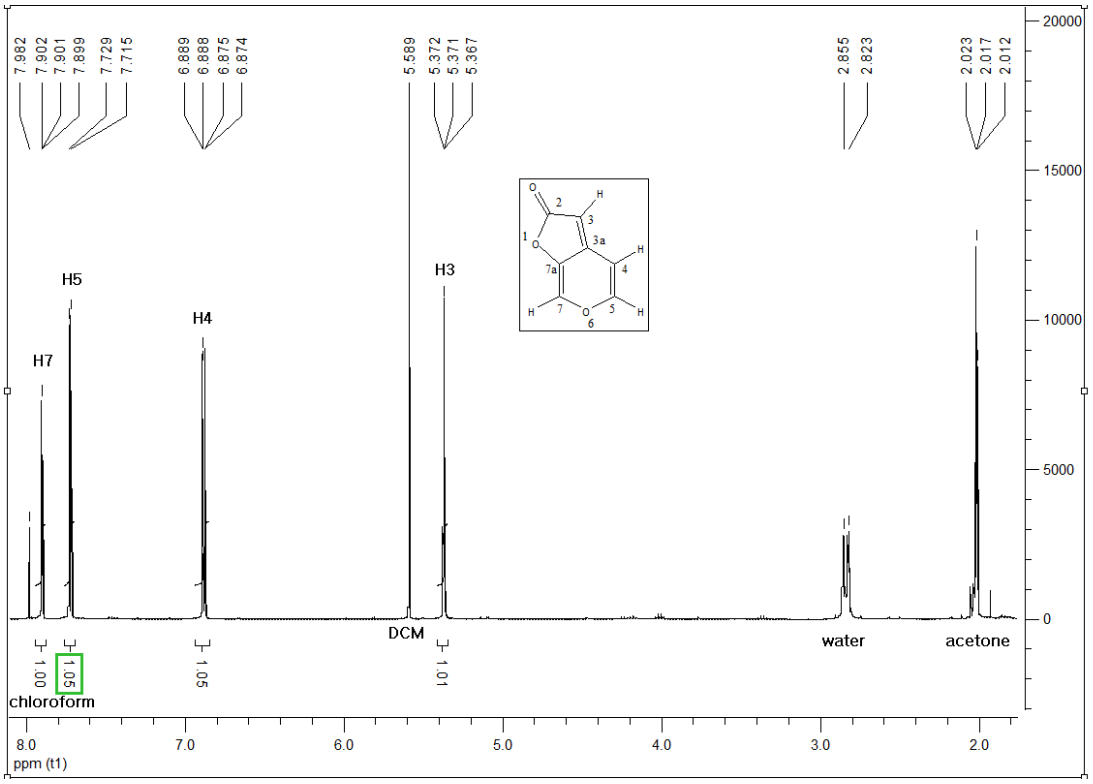
***P. aeruginosa* infection assays in *Arabidopsis thaliana* plants**

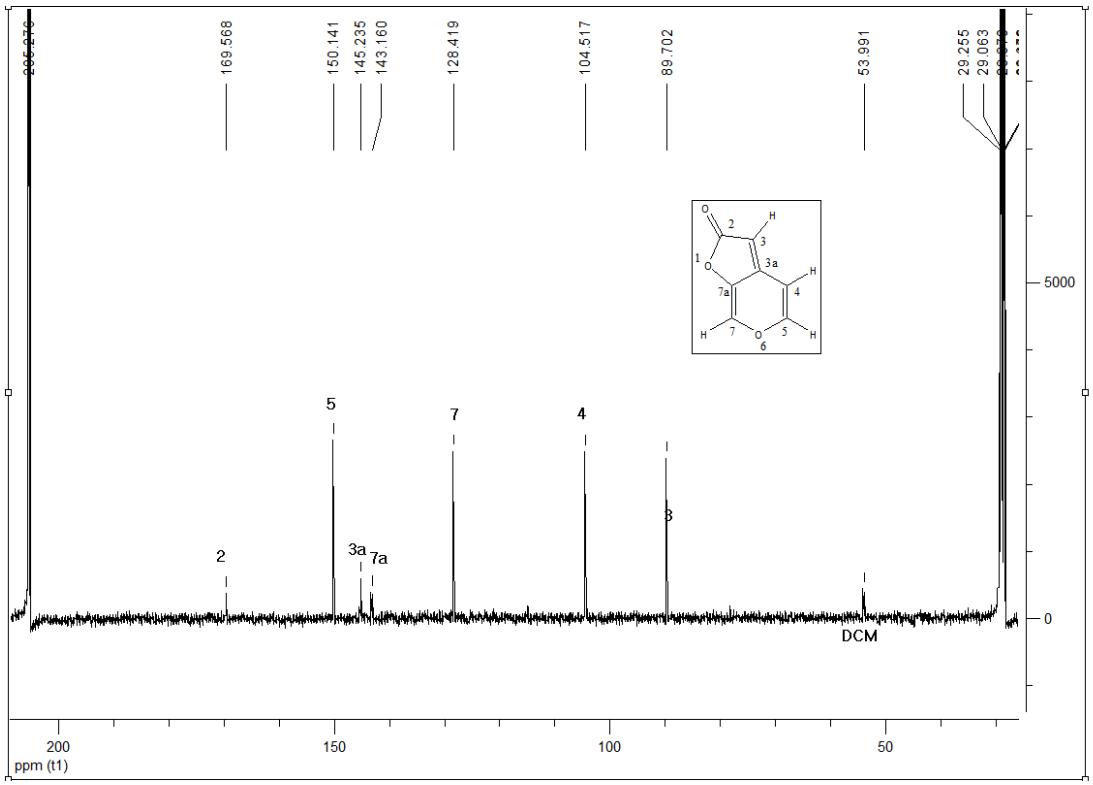
This procedure is based on protocols described by Rahme and coworkers,[8] with minor modifications;

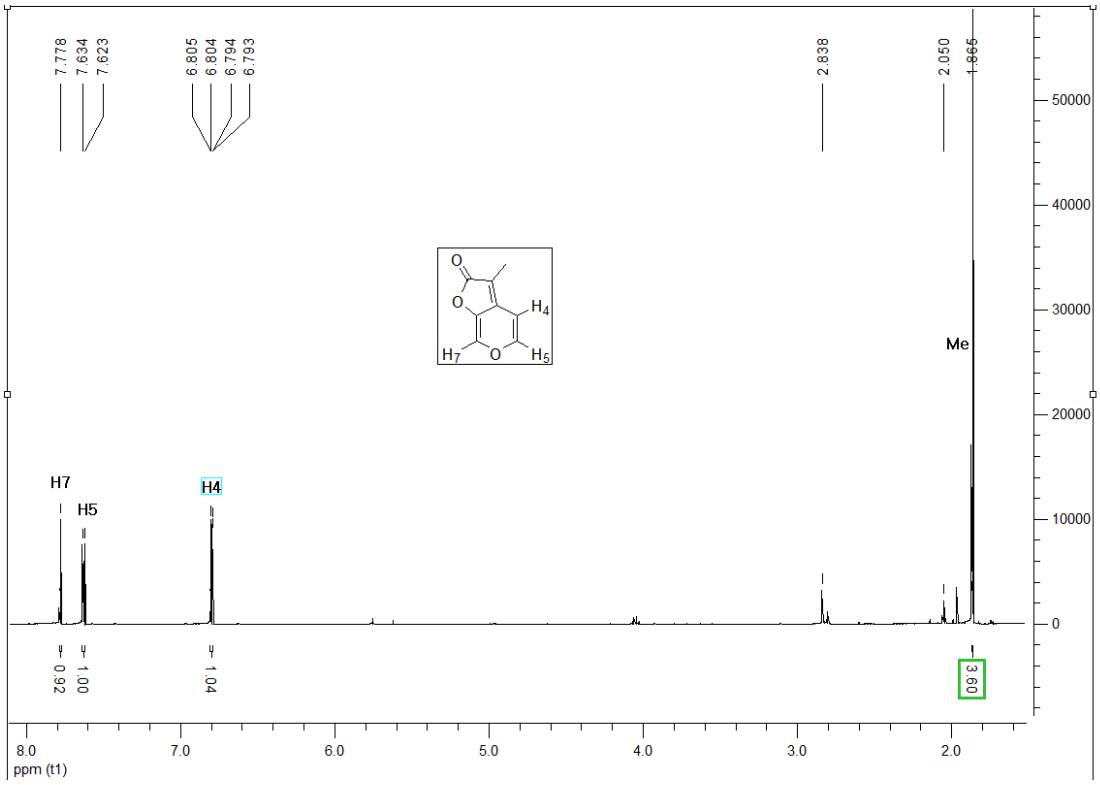
P. aeruginosa wild type strain PAO1 was grown overnight in LB medium at 37 °C, and resuspended at an optical density (OD_{600}) of 0.05 in LB medium into two falcon tubes containing 2 mL LB medium with either 100 µM KAR1 (from a 10 mM stock solution in DMSO) or only DMSO (20 µL, 1%). The suspensions were then dropwise added to trays of two-week old Arabidopsis thaliana plants (ecotype Columbia), each containing 8 or 9 plants, with half of the amount added to the center of the plants and the other half to the leaves. Pictures were taken after 24 h and after 48 h (Figure 5). As a control experiment plants were treated the same, only without addition of bacteria (Figure S3). It should be noted that while karrikins strongly induce germination of plant seedlings, their effect on leaf growth of grown plants is not significant.

Figure S3: Effect of KAR1 on *Arabidopsis thaliana*; upper panel, left: with 100 μM KAR1, after 24 h, and right: after 48 h; lower panel, left: without KAR1, after 24 h, and right: after 48 h. No significant differences in average leaf surface areas were observed between day 1 and day 2.

iii) **Spectral data**






7.778
7.634
7.623
6.805
6.804
6.794
6.793
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2.050
1.865
H7
H5
H4
Me
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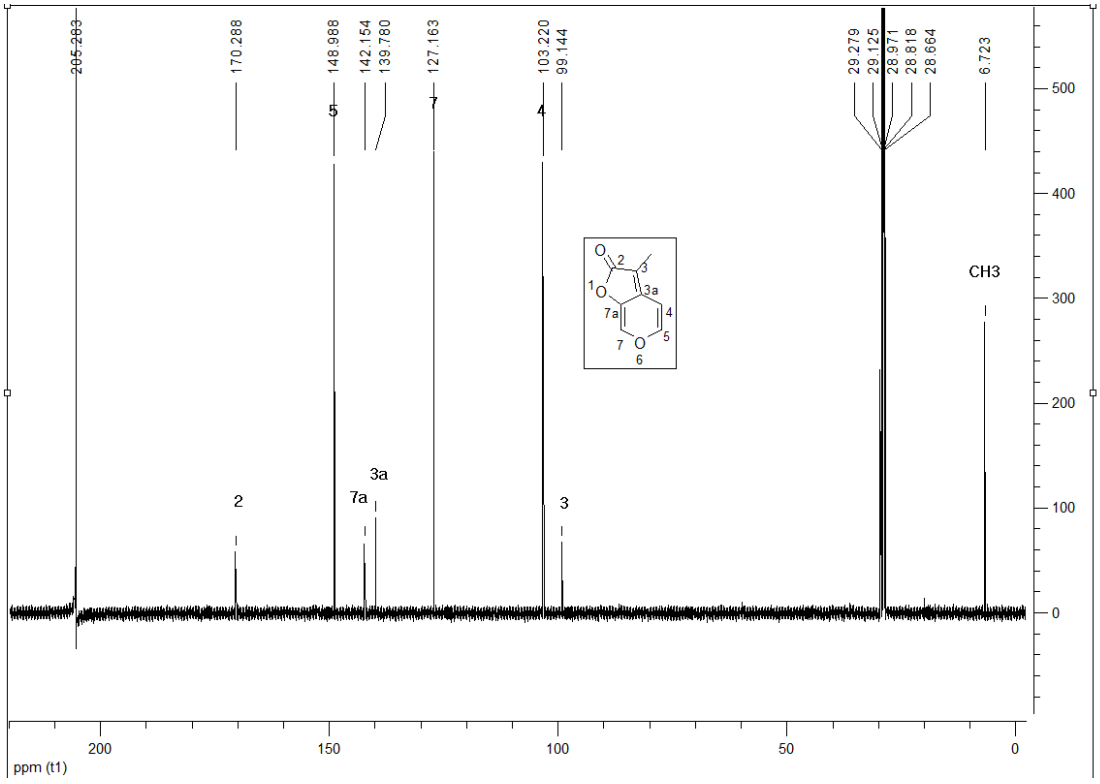
1.04
3.00
8.0
7.0
6.0
5.0
4.0
3.0
2.0
ppm (t1)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148.988
142.154
139.780
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29.125
28.971
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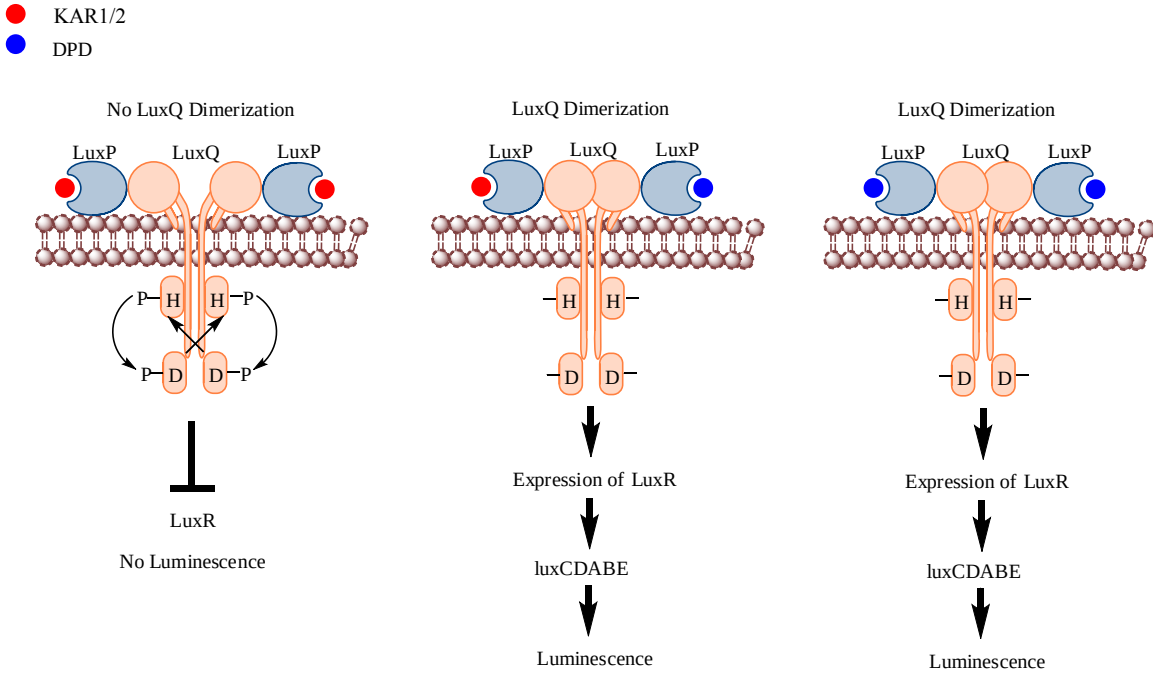
6.723
2
3a
7a
3
CH3
200
150
100
50
0
ppm (t1)

iv) Synergism in *V. harveyi*: the synergistic effect that we observed in *V. harveyi* may be explained as follows: due to the structure of *V. harveyi* AI-2/DPD receptor, LuxP, which yields two binding sites upon dimerization, as a dimer of LuxPQ is formed. Only when two AI-2 molecules bind both binding sites the complex triggers QS cascade. Thus it is reasonable to believe that KAR1/2 can enter one binding site (due to structural similarities and chemical properties) and in the other binding site have the native AI-2, causing stronger activation than with AI-2 alone.



v) References

1. E. D. Goddard-Borger, E. L. Ghisalberti and R. V. Stick, *Eur J Org Chem*, 2007, 3925-3934.
2. S. Schauder, K. Shokat, M. G. Surette and B. L. Bassler, *Mol Microbiology*, 2001, **41**, 463-476.
3. H. Ganin, X. Tang and M. M. Meijler, *Bioorg Med Chem Lett*, 2009, **19**, 3941-3944.
4. K. Duan and M. G. Surette, *J Bact*, 2007, **189**, 4827-4836.
5. S. P. Bernier, A. L. Beeston and P. A. Sokol, *Bmc Biotechnol*, 2008, **8**.
6. D. W. Essar, L. Eberly, A. Hadero and I. P. Crawford, *J Bact*, 1990, **172**, 884-900.
7. G. Dulla and S. E. Lindow, *Proc Natl Acad Sci U S A*, 2008, **105**, 3082-3087.
8. M. Starkey, L. G. Rahme, *Nat. Protoc*. 2009, **4**, 117.