Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

SUPPLEMENTARY INFORMATION

Sulforaphane and erucin, natural isothiocyanates from broccoli, inhibit bacterial quorum sensing

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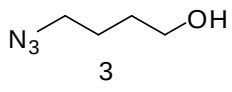
General. Unless otherwise stated, all reactions were performed under an inert atmosphere with dry reagents and solvents. All chemical reagents were purchased from Aldrich or Acros and used without further purification, with the exception of dichloromethane and tetrahydrofuran which were purified via an MBRAUN SPS solvent purification system before use. 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 DPX_{400} (400 MHz) or Bruker Avance DMX_{500} (500 MHz) spectrometer. Spectra were calibrated on residual solvent signal. Gas chromatography mass-spectrometry data were obtained using an Agilent 6850 GC instrument equipped with an Agilent 5973 mass spectrometer working under standard conditions and an Agilent HP5-MS column. Compounds 3-oxo-C_{12}-HSL and (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone were synthesized following modifications of procedures described by Chhabra et al.[1] and by Persson et al., respectively.[2]

Synthetic procedures: sulforaphane and erucin

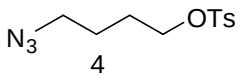


3

Compound 3. To a solution of 4-bromo-1-butanol (0.5 g, 3.3 mmol) in DMF (4 mL) was added sodium azide (0.85 g, 13.1 mmol). The reaction mixture was heated to 70 ^{o}C overnight. DMF was removed under reduced pressure, and the crude mixture was partitioned in water (10 mL) and diethyl

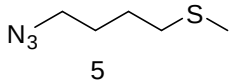
ether (20 mL). The organic layer was dried over anhydrous Na_2SO_4, and concentrated in *vacuo*. The desired product was obtained in 82% yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.66-1.68 (m, 4 H), 3.33 (t, J = 6.6 Hz, 2H), 3.68 (t, J = 6.0 Hz, 2H). ^{13}C NMR (400MHz, $CDCl_3$): δ 25.3, 29.7, 51.2, 62.1.



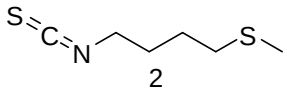
4

Compound 4. A solution of **3** (340 mg, 2.95 mmol) in dry CH_2Cl_2 (50 mL) together with triethylamine (0.5 mL, 3.5 mmol), and 4-(N,N-dimethylamino)pyridine (180 mg, 1.5 mmol) was cooled to 0 ^{o}C. p-toluenesulfonyl chloride (675 mg, 3.54 mmol) in CH_2Cl_2 (15 mL) was added dropwise to the mixture at 0^{o}C, and the reaction was stirred at RT over night. The mixture was washed with brine (20 mL), water (20 mL) and dried over $MgSO_4$. After filtration, and concentration in *vacuo*, the residue was purified by flash chromatography using a gradient mixture of 10-20 % ethyl acetate in hexanes to give product 4 in 52% yield. NMR Spectroscopic data are in agreement with previously reported data.[3] ^{1}H NMR (400MHz, $CDCl_3$): δ 1.58-1.77 (m, 4 H), 2.45 (s, 3H), 3.26 (t, J = 6.5 Hz, 2H), 4.05 (t, J = 6.07 Hz 2H), 7.35 (d, J = 8.1 Hz, 2H), 7.78 (d, J = 8.4 Hz, 2H). ^{13}C NMR (400MHz, $CDCl_3$): δ 21.6, 24.9, 26.0, 50.6, 69.6, 127.8, 129.8, 132.8, 144.8.



5

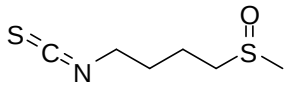
Compound 5. Sodium thiomethoxide (39.4 mg, 0.6 mmol) in THF (2 mL) was added to a two-necked flask fitted with a reflux condenser under nitrogen. The flask was cooled to 0 ^{o}C and compound **4** (100 mg, 0.4 mmol) dissolved in THF (15 mL), was added. The reaction was stirred for 4 h at 50 ^{o}C. The reaction mixture was dissolved in water (10 mL), extracted with diethyl ether (3*15 mL) and dried over Na_2SO_4. The product was filtered and carefully concentrated in vacuo. Further purification was done by flash chromatography using a gradient mixture of 0-20% EtOAc:hexanes to afford compound **5** at 39 % yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.68-1.71 (m, 4 H), 2.1 (s, 3H), 2.52 (t, J = 6.9 Hz, 2H), 3.30 (t, J = 6.3 Hz, 2H). ^{13}C NMR (400MHz, $CDCl_3$): δ 15.44, 26.11, 27.86, 33.64, 51.03.



2

Erucin

Compound 2 (erucin). To a solution of **5** (111.5 mg, 0.77 mmol) was added triphenylphosphine (393 mg, 1.5 mmol) in diethyl ether (6 mL), and the reaction was refluxed for 3 h. Then, the solvent was evaporated *in vacuo*, and carbon disulfide was added (1.15 mL, 19 mmol). After heating at reflux for 1 h, the product was concentrated *in vacuo* and purified by flash chromatography using a gradient mixture of 10-100% EtOAc in hexanes to give product **2** in 81% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.65-1.82 (m, 4 H), 2.06 (s, 3H), 2.49 (t, J = 6.9 Hz, 2H), 3.52 (t, J = 6.3 Hz, 2H). ^{13}C NMR (400MHz, $CDCl_3$): δ 15.25, 25.67, 28.69, 33.12, 44.59, 129.95.



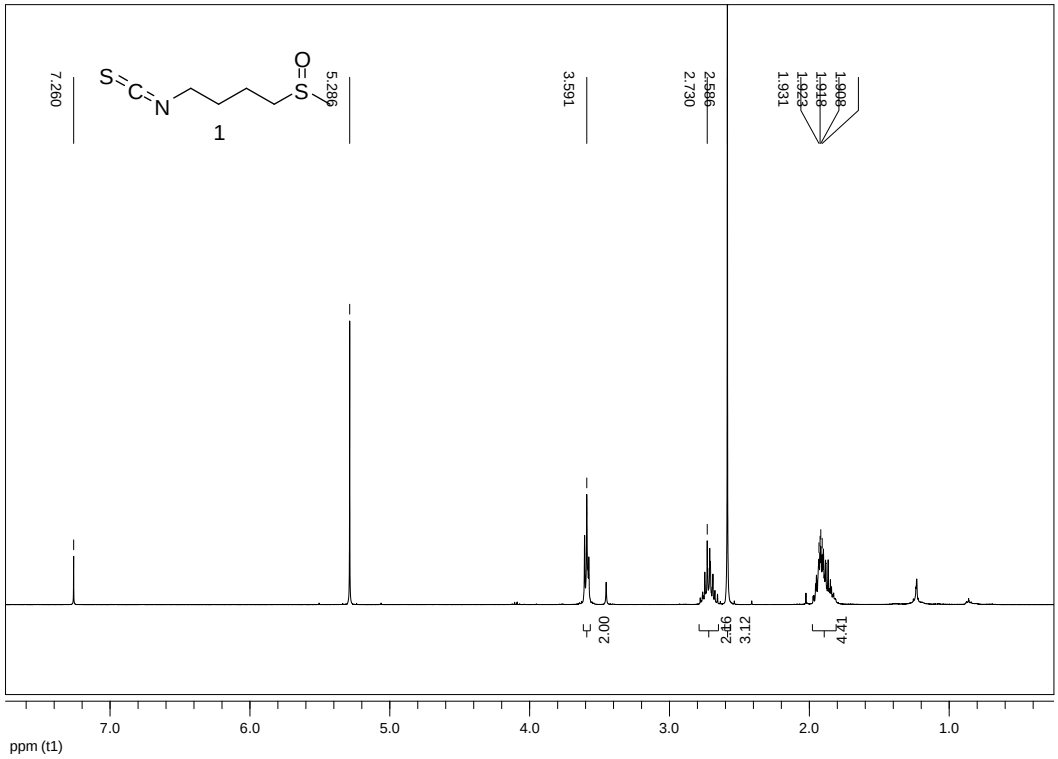
1

Sulforaphane

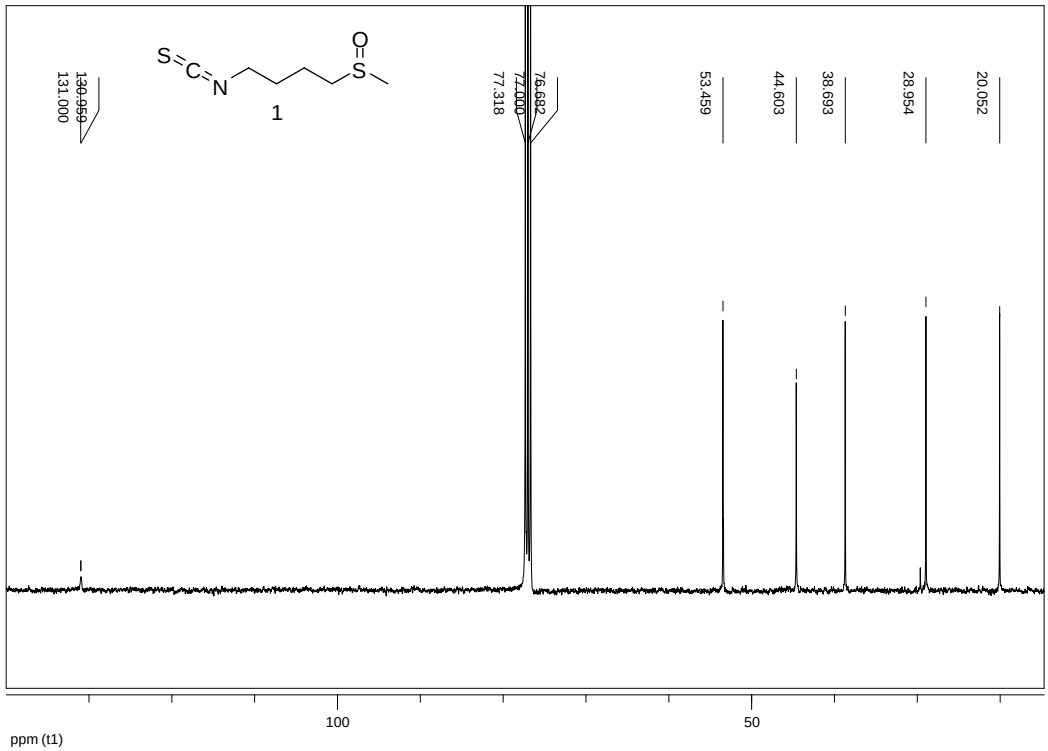
Compound 1 (sulforaphane). This compound was synthesized by addition of mCPBA to erucin, following a previously reported procedure,[4] affording the final product – sulforaphane - in 77% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.90-1.93 (m, 4 H), 2.59 (s, 3H), 2.73 (m, 2H), 3.59 (t, J = 6.3 Hz, 2H). ^{13}C NMR (400MHz, $CDCl_3$): δ 20.05, 29.63, 38.69, 44.60, 53.46, 131.0.

NMR spectra:

^{1}H-NMR for sulforaphane:

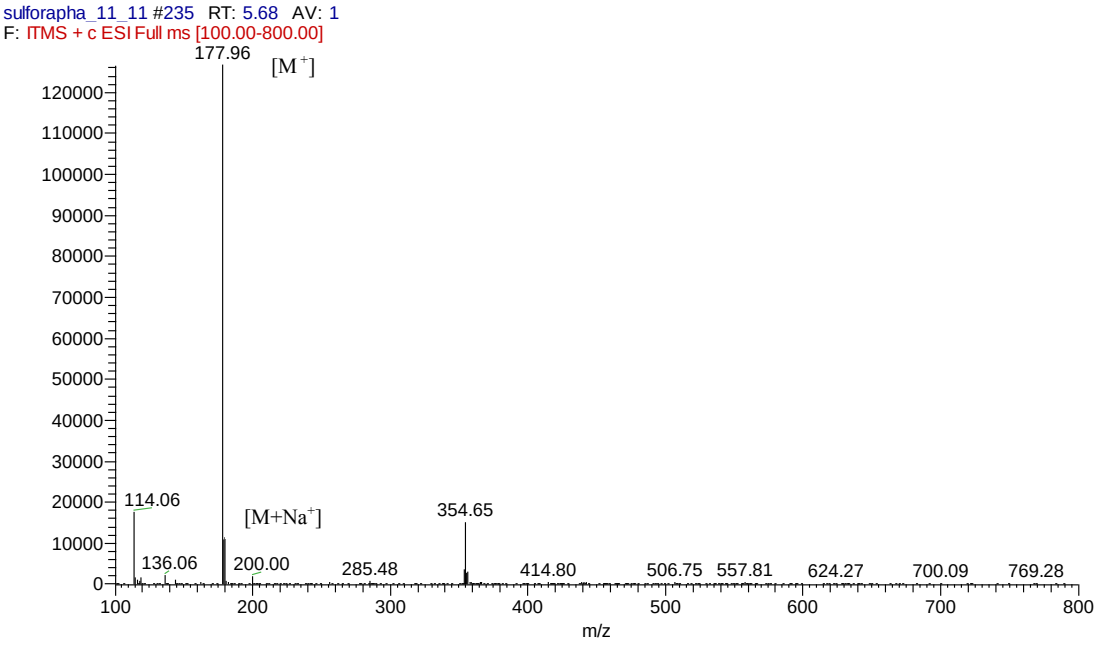


^{13}C-NMR for sulforaphane:



Mass Spectra of sulforaphane,1

Compound 1, m/z: calcd: [M+] 177.03, measured: [M+] 177.96.



GC/MS chromatogram and spectra of synthetic sulforaphane,1 and erucin,2

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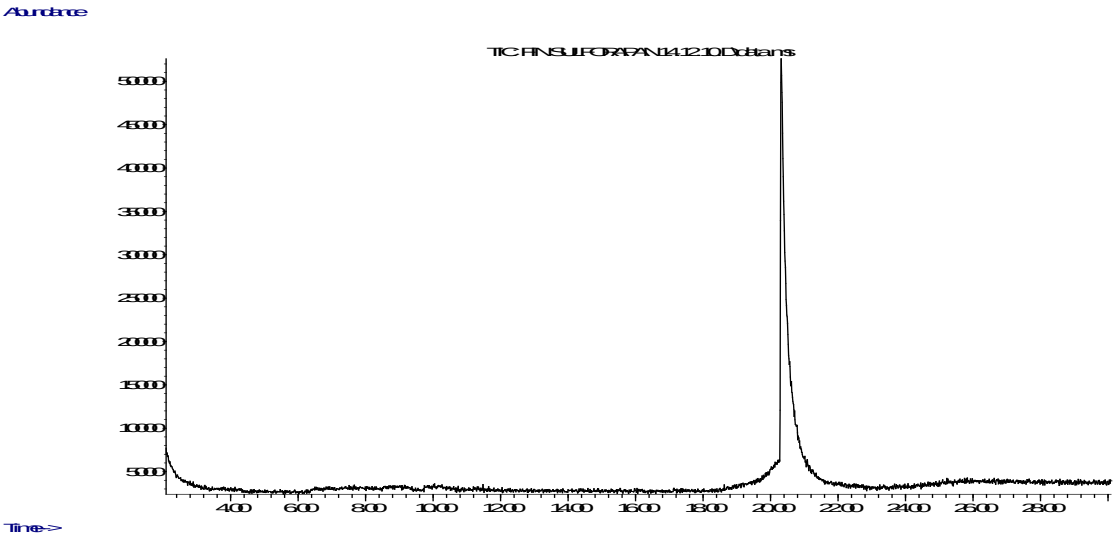

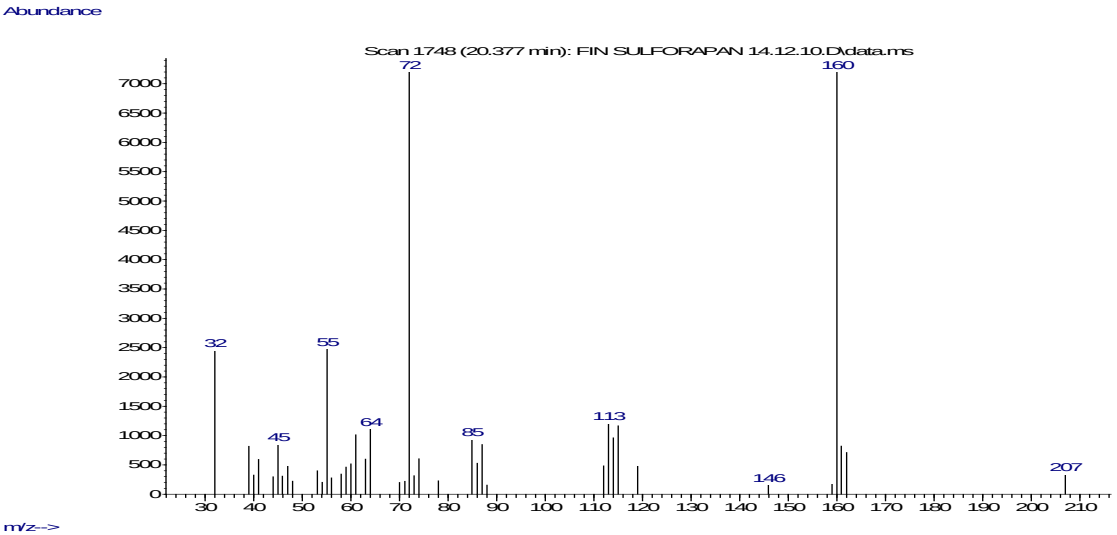

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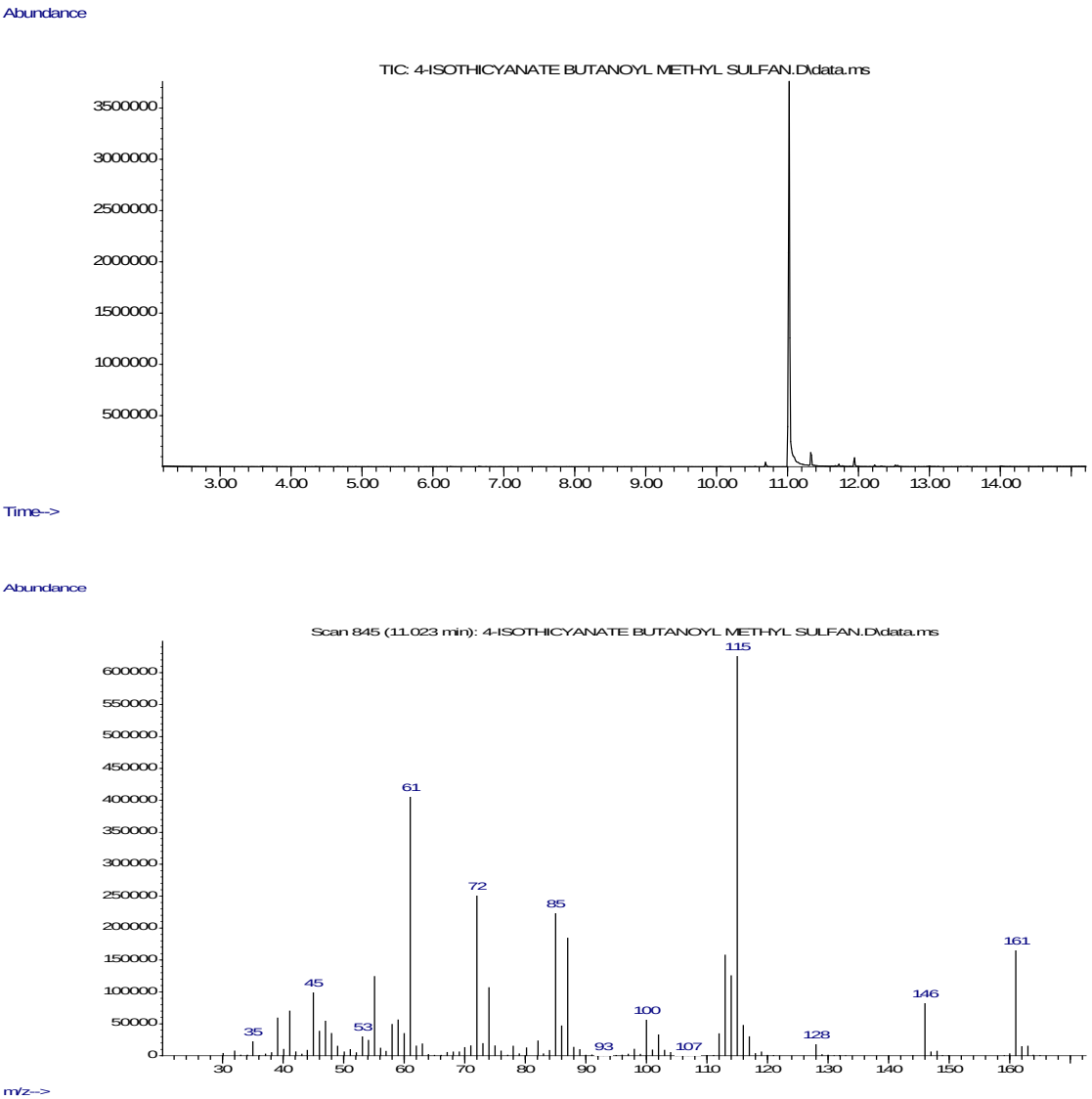

Figure S1**:** a) GC/MS analysis of synthetic (a) sulforaphane and (b) erucin; total ion count chromatogram and MS fragmentation of sulforaphane (20.3 min); m/z: 177 (M+) and m/z: 160, 72, 114; and erucin (11.0 min); m/z: 161 (M+) and m/z: 115, 61.

Bioassays

Bioluminescence Assays

PAO1-*luxCDABE* and PAO-JP2-*luxCDABE* (ant)agonist assays were performed as described previously by Ganin et al.[5] 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). [6] And PAO-JP2, a *lasI-rhlI* double mutant of PAO1, harboring a plasmid pKD201 with a *lasI* promoter coupled to the *luxCDABE* box. [6] Each strain separately, was incubated overnight in LB medium containing 300 μg/ml of trimethoprim. A 96-well black microtiter plate (Greiner) was prepared with the desired concentrations (0.003-200 μM) of SF and (R) ER, a control well in each raw containing medium and 0.2% DMSO, and bacteria were added to reach a final absorbance density (OD_{600}) of 0.015. For antagonist experiments, using the nutant strain PAO-JP2-*luxCDABE* a final concentration of 100 nM of the native 3-oxo-C_{12}-HSL was added. The plate was then incubated for a period of 16 hours at 37 ^{0}C, and during this time luminescence measurements were performed at 20 minute intervals using a Microtiter Plate Reader (SpectraMax M2, Molecular Devices). Luminescence values devided by OD_{600} vlaues were plotted against the added compound concentration.

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

P. aeruginosa PAO-JP2 (pKD-*rhlA*) is a reporter strain for C4-HSL, which was developed by K. Duan et al.[6] 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 black microtiter plate (Greiner) was prepared with the desired concentrations (1.6-200 μM) of sulforaphane and erucin, as well as control wells in each raw containing medium and 0.2 % DMSO, and bacteria were added to reach a final absorbance density (OD_{600}) 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 ^{0}C, and during this time luminescence measurements were performed at 20 minutes intervals using a Microtiter Plate Reader (SpectraMax M2, Molecular Devices). Luminescence values were plotted against the added compound concentration.

Pyocyanin production Assay

The effect of sulforaphane and erucin on pyocyanin production in *P. aeruginosa* PAO1 wild-type strain was measured as described previously by Essar et al.[7] Briefly, 2 mL of chloroform was added to 2 mL of culture supernatant, taken from 23 h cultures grown in the presence of each probe (sulforaphane and erucin – 100 μM from 100 mM stocks) or only LB medium with 0.1% DMSO as control. After extraction, 1 mL of the chloroform layer was transferred to a fresh tube and mixed with 300 μL of 0.2 N HCl. After centrifugation, the aquaeous (top) layer was separated and its absorption was measured at 520 nm. We compared the average OD_{520} values of three measurements.

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***E. coli* DH5α LasR Antagonist Assay:**

β-Galactosidase activity was measured following the addition of sulforaphane and erucin to *E. coli* DH5α comprising the LasR expression plasmid, pJN105L, and a reporter plasmid *lasI-lacZ* (pSC11),[8] as reported previously by Amara et al.[9] and according to the Miller assay method.[10]

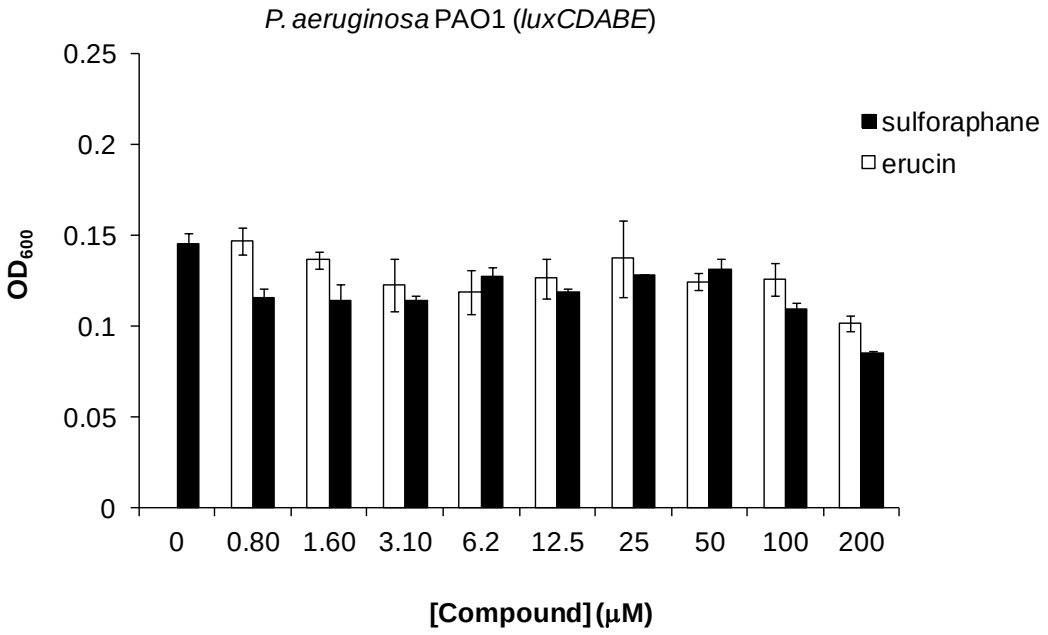
Bacterial growth inhibition measurements:

The effect of sulforaphane and erucin on *P. aeruginosa* PAO-JP2 (pKD-*rhlA*) growth, was evaluated as described previously by Ganin et al.[5]

Experimental biofilm:

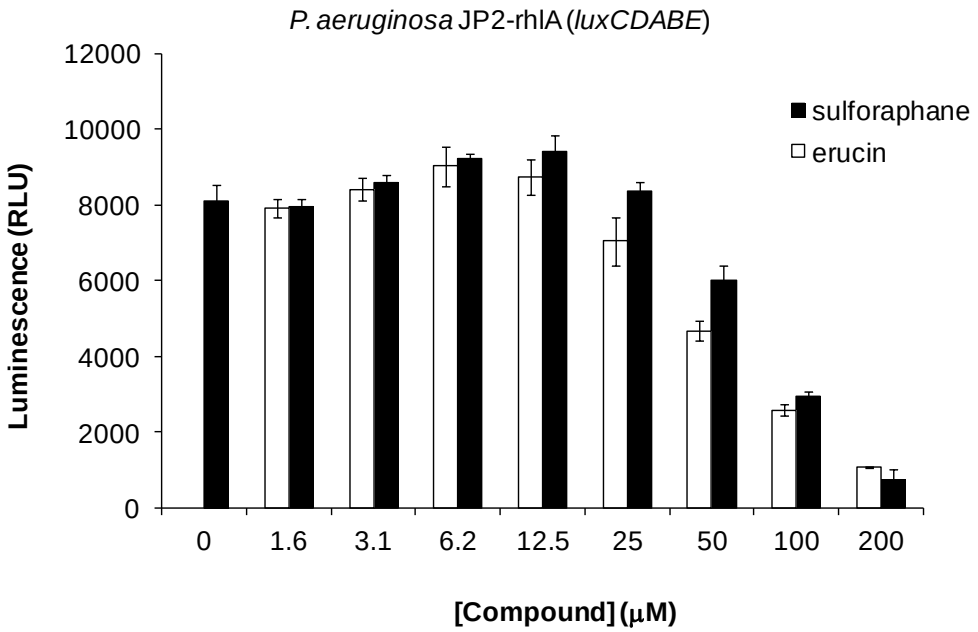
The effects of each compound on biofilm formation of the *P. aeruginosa* PAO1 wild-type strain were assessed following a modified procedure described by O'Toole et al.[11] Briefly, M9 minimal medium was inoculated with an overnight LB culture to a final OD_{600} of 0.05. After inoculation, plates (200 µL/well) were incubated statically in a humidified chamber at 37 °C for 18-24 h in the presence or absence (DMSO) of each compound. Then, OD_{600} was measured, planktonic cells were removed and the remaining biofilm was gently washed by immersion (4 times) in a beaker containing doubly deionized H_2O, fixed by thermally dehydrating at 37 °C, and stained with 210 µL of crystal violet (0.1%). After 10-20 min, the stain was removed and the plate was washed again by immersion (5 times). Finally the remaining biofilm was quantified by resolubilizing the crystal violet in 220 µL of 30% acetic acid, and the absorbance was measured at 590 nm.

1)



2)

(a)



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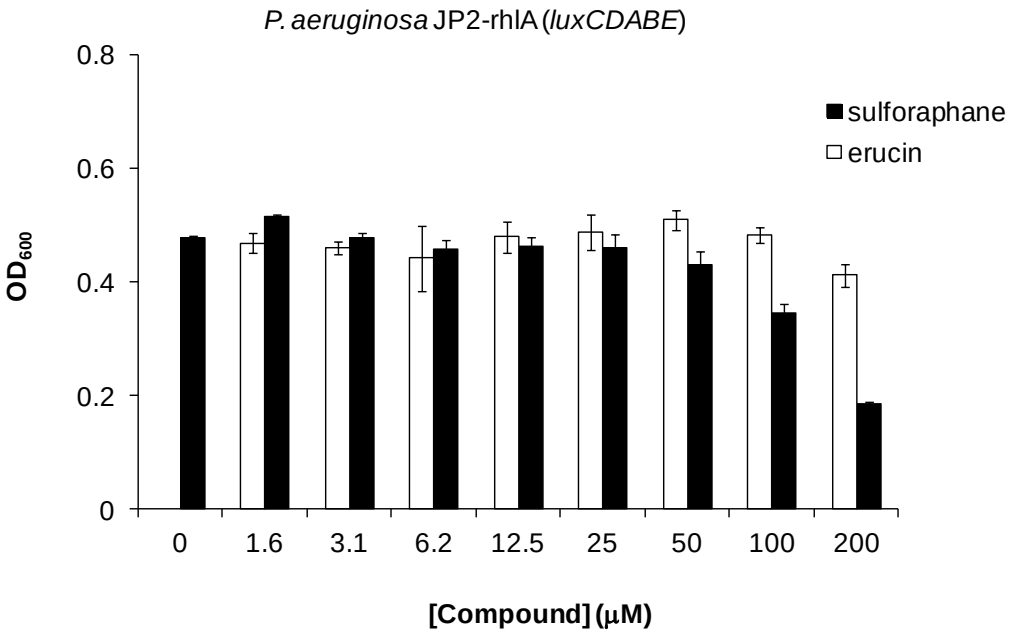

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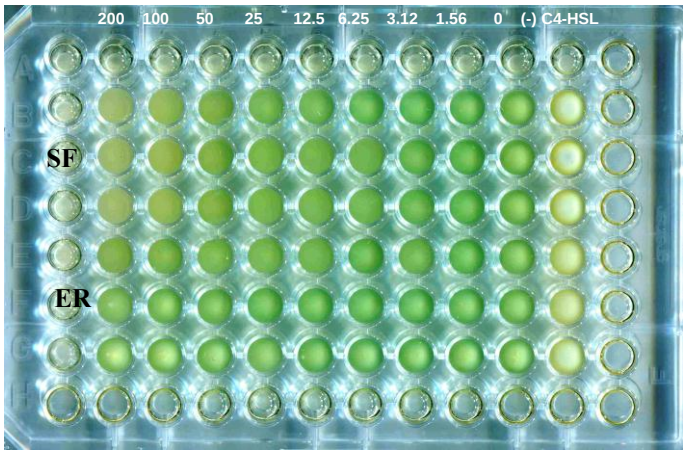
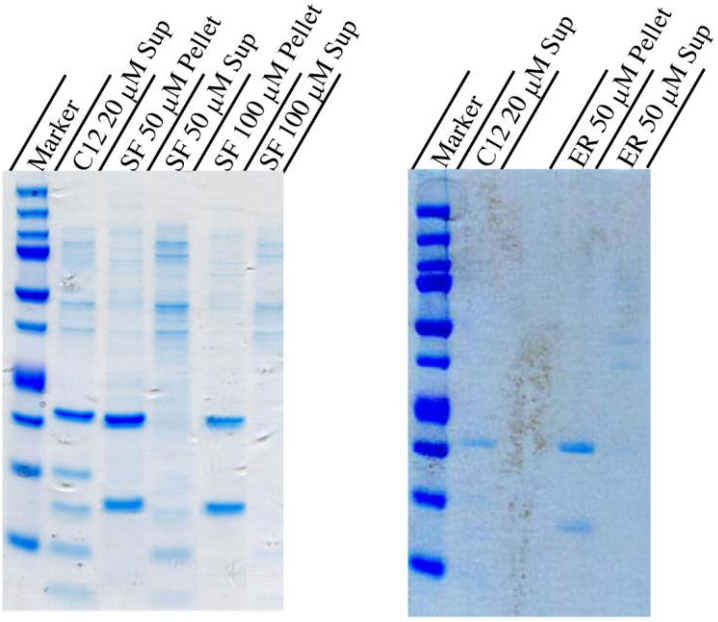
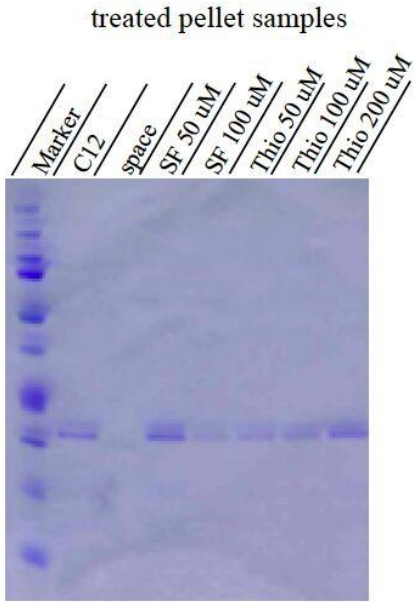


Figure S2. 1) OD_{600} vs. different concentrations of sulforaphane or erucin in *P. aeruginosa PAO1-luxCDABE*; 2) a) Inhibition of of QS activation vs. different concentrations of SF and ER in *P. aeruginosa* PAO-JP2 (pKD-rhlA), in the presence of C4-HSL (10 μM) (triplicates, after 5 h of incubation); b) OD_{600} vs. different concentrations of sulforaphane or erucin in *P. aeruginosa* PAO-JP2 (pKD-*rhlA*); c) Effects on pigment production by the probes in this assay.

Expression of LasR-LBD.

Expressions were performed using an *E. coli* BL21 LasR-ligand binding domain expression system, as previously described.[9, 12]





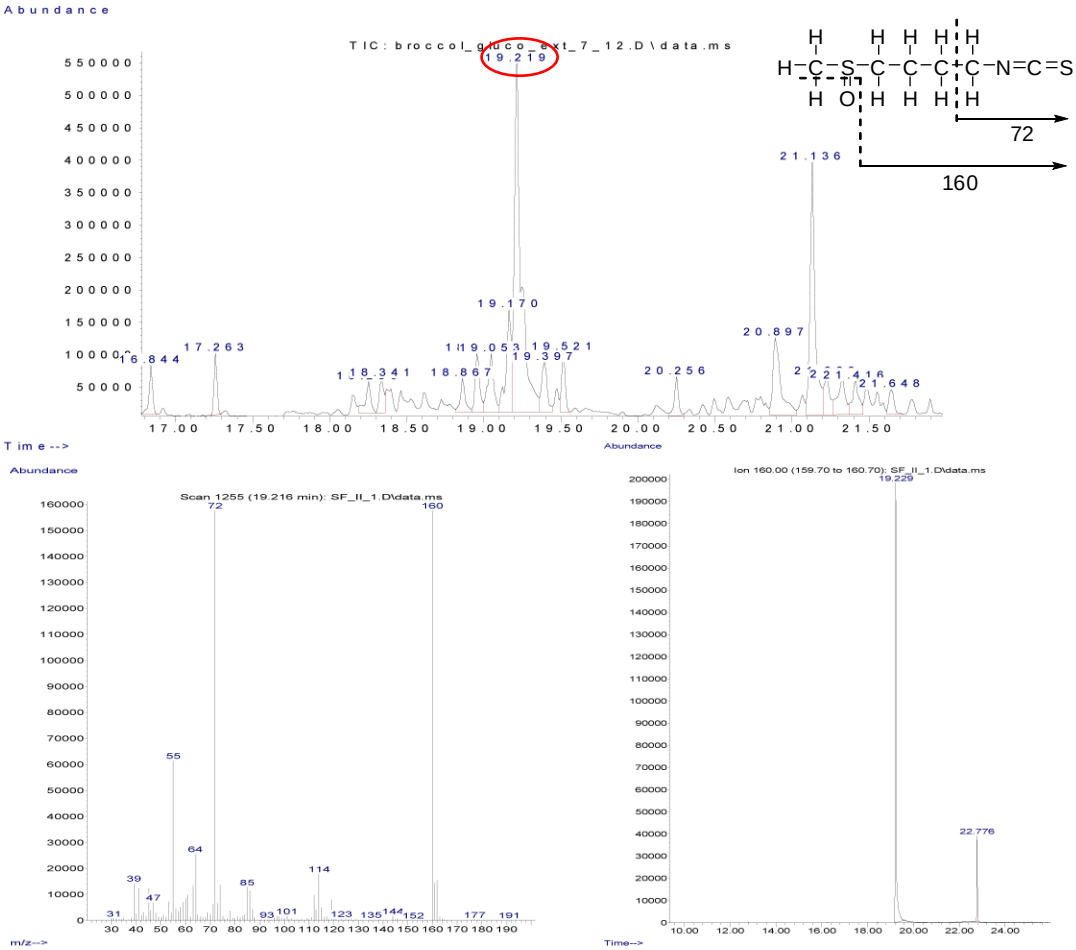
Isolation and Quantification of Sulforaphane rom Fresh Broccoli

Sulforaphane extraction

The extraction of biosynthetic sulforaphane was performed according to a previously published protocol[13] with a few modifications; 405 g of fresh organic broccoli was crushed in a blender with addition of 900 mL water. The resulting paste was pured into a beaker with a magnetic stirrer, and 25 units of thioglucosidase were added, followed by homogenization with stirring at 42 ^{o}C for 2 h. Then, the homogenate was centrifuged twice at 8000-10000 g. The supernatant was once more centrifuged at 4000 g, and filtered through a 0.22 mm filter. 5 mL fractions (three time) were extracted with dichloromethane (DCM,10 mL) by vigorously shaking the Falcon tube for 1 min. 5 mL of the DCM layer was then transferred to a 20 mL glass vial and evaporated. The remaining residue was dissolved in 200 μL DCM and analyzed for sulforaphane content by GC/MS.

GC/MS conditions: Injection volume: 5 μL, He carrier gas. Inlet; split, ratio: 25:1, flow: 25 mL/min. Heating sequence: 4 min at 50 ^{0}C, then 10 ^{0}C/min increase to 250 ^{0}C, held for 6 min. Total time: 30 min.

(a)



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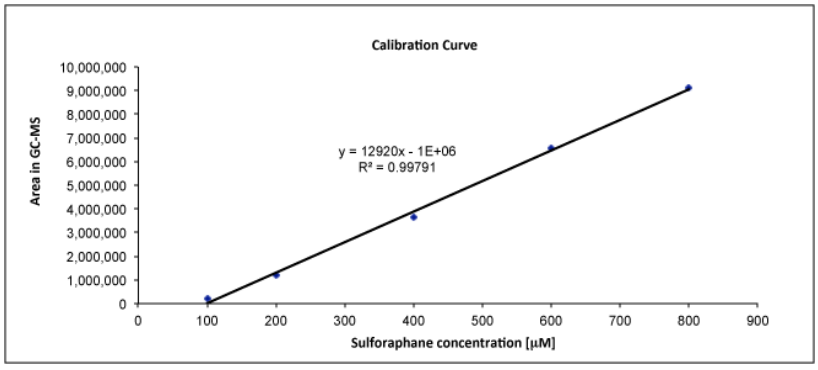

***Figure S3*:** a) GC/MS analysis of sulforaphane (SF) extracts from commercial broccoli extracts; total ion count chromatogram and extracted ion (160 Da) chromatogram; MS fragmentation of sulforaphane (19.2 min); m/z: 177 (M+) and m/z: 160, 72, 114; b) Calibration curve: peak area of the selected peak in the GC/MS chromatogram for five synthetic sulforaphane standard concentrations.

References

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