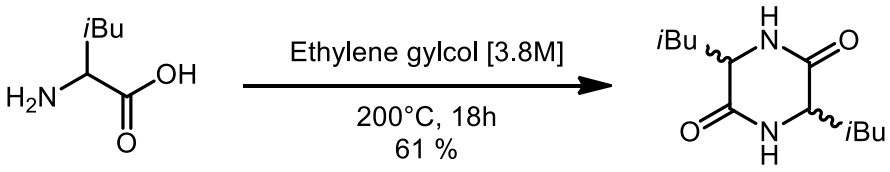
Supplementary data and Table S1-S3

Details on pulcherriminic acid (PA) synthesis

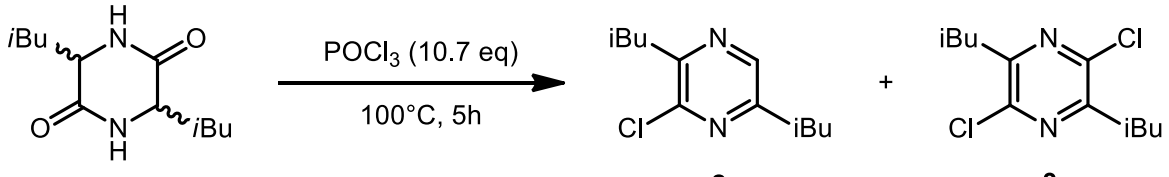
3,6-diisobutylpiperazine-2,5-dione (1)



1

Method based on a slightly modified procedure[1]. In a sealed tube, flame dried and under argon, (L)-leucine (3.28 g, 25 mmol) was put in suspension in Ethylene Glycol (6.58 ml, [3.8 M]). The white bread-like mixture was heated at 200°C for 18 h and became a brown/copper colored solution with agitation. After cooling to room temperature, the orange solid was washed with AcOEt then *i*-PrOH, then dried *in-vacuo* to obtain an inconsequential mixture of isomers of 3,6-diisobutylpiperazine-2,5-dione (1) (1.72 g, 61 %) as a white solid, which could be used directly for the next step. **^{1}H NMR** (400 MHz, Chloroform-*d*) δ (ppm) 6.42 (d, *J* = 26.5 Hz, 2H), 4.03 – 3.92 (m, 2H), 1.93 – 1.73 (m, 4H), 1.63 (dd, *J* = 9.3, 2.9 Hz, 2H), 1.07 – 0.89 (m, 12H). **^{13}C NMR** (100 MHz, Chloroform-*d*) δ (ppm) 168.98, 168.85, 53.47, 53.30, 43.46, 42.23, 24.45, 24.37, 23.40, 23.31, 21.40, 21.25. NMR analysis is consistent with the known compound[2]

3-chloro-2,5-diisobutylpyrazine (2) and 2,5-dichloro-3,6-diisobutylpyrazine (3)



1 **2** 60% **3** 14 %

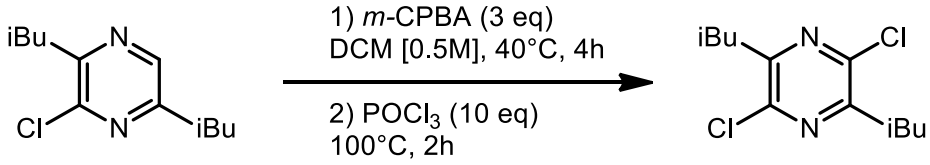
Procedure for chlorination, based on a slightly modified procedure[1]. In a sealed tube, flame dried and under argon, 3,6-diisobutylpiperazine-2,5-dione (1) (905 mg, 4 mmol) was solubilized in Phosphorus(V) Oxychloride (4 ml, 42.8 mmol), then heated at 100°C and stirred for 2h. After the indicated time, the red mixture was cooled to room temperature, then poured slowly in stirred ice-cooled water (50 ml). After addition and 15

min of stirring, CH_2Cl_2 (25 ml) was added, then the organic layer was extracted twice and washed with $NaHCO_3$ (sat.) (2 x 25 ml). The organic layer was dried with $MgSO_4$, filtered and concentrated. The products were separated by flash chromatography on silica, using 1% Et_2O in hexanes.

3-chloro-2,5-diisobutylpyrazine (2), pale yellow oil (542 mg, 60 %): **^{1}H NMR** ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm) 8.23 (s, 1H), 2.80 (d, *J* = 7.2 Hz, 2H), 2.61 (d, *J* = 7.2 Hz, 2H), 2.21 (dp, *J* = 13.8, 6.9 Hz, 1H), 2.10 (dp, *J* = 13.6, 6.8 Hz, 1H), 0.95 (dd, *J* = 10.9, 6.7 Hz, 12H); **^{13}C NMR** (100 MHz, Chloroform-*d*) δ (ppm) 154.22, 152.52, 148.26, 141.88, 43.60, 43.26, 28.95, 28.05, 22.51, 22.36; **Rf** (1% Et_2O / hexanes) : 0.05. NMR analysis is consistent with the known compound[3]

2,5-dichloro-3,6-diisobutylpyrazine (3), clear oil (150 mg, 14 %): **^{1}H NMR** (400 MHz, Chloroform-*d*) δ (ppm) 2.76 (d, *J* = 7.2 Hz, 4H), 2.21 (dp, *J* = 13.7, 6.8 Hz, 2H), 0.96 (d, *J* = 6.7 Hz, 12H); **^{13}C NMR** (100 MHz, Chloroform-*d*) δ (ppm) 152.69, 145.99, 42.82, 28.24, 22.51; **IR** (Neat) ν (cm^{-1}) 2950, 2925, 2875, 1475, 1400, 1325, 1100; **HRMS (ESI+)** (m/z) calcd for $C_{12}H_{18}Cl_2N_2$ $[M+Na]^+$: 283.0739, found : 283.0745; **Rf** (1% Et_2O / hexanes) : 0.30.

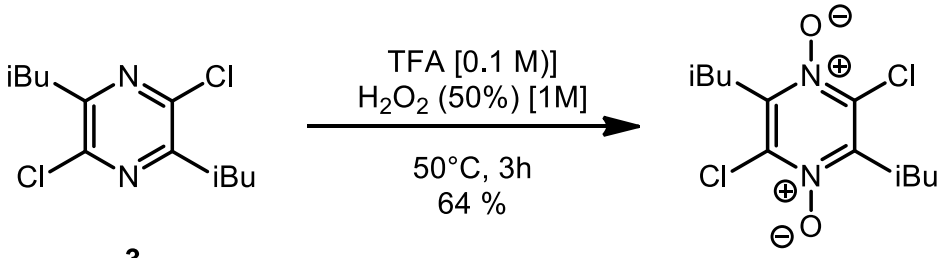
2,5-dichloro-3,6-diisobutylpyrazine (3)



2 **3**

Method based on a slightly modified procedure[1]. In a vial, 3-chloro-2,5-diisobutylpyrazine (2) (188 mg, 829 mmol) was solubilised in DCM (1.66 µl, [0.5M]), then *m*-CPBA (429 mg, 2.49 mmol) was added. The mixture was heated at 40°C and stirred for 4h. After the indicated time and cooling to room temperature, AcOEt (20 ml) was added, and the organic layer was washed with Na_2SO_3 (10 % in water, 3x 20ml), then $NaHCO_3$ (sat. 3x20ml). The organic layer was dried with $MgSO_4$, filtered and concentrated *in-vacuo*. Without purification, the procedure for chlorination was followed to obtain 2,5-dichloro-3,6-diisobutylpyrazine (3) (84.6 mg, 39 %)

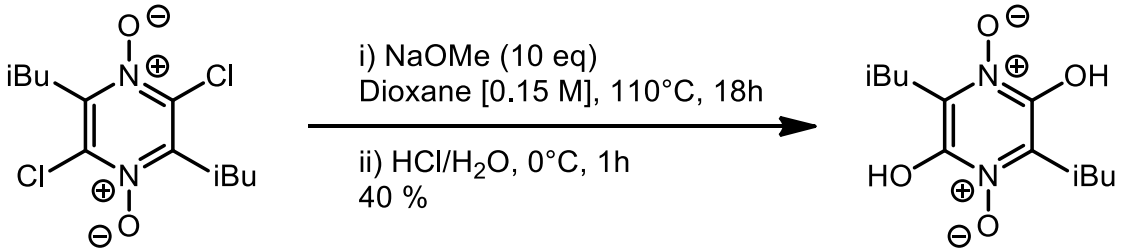
2,5-dichloro-3,6-diisobutylpyrazine 1,4-dioxide (4)



3 **4**

In a round bottom flask, 2,5-dichloro-3,6-diisobutylpyrazine (3) (360 mg, 1.38 mmol) was solubilized in THF (13.8 ml, [0.1 M]). The solution was cooled to 0°C, then H_2O_2 (50% in water, 1.38 ml, [1M]) was added dropwise. After addition, the mixture was heated at 50°C and stirred for 3h. After cooling to room temperature, DCM (10 ml) was added, then the mixture was poured slowly in saturated Na_2CO_3 with ice (c.a. 30 ml). Na_2SO_3 (10 % in water, 20 ml) was added, and the mixture was stirred 15 min at room temperature. The organic layer was extracted using DCM (2x25 ml), then dried over $MgSO_4$, filtered and concentrated. The product was purified using flash chromatography on silica, with 1 % MeOH in DCM as the eluant. 2,5-dichloro-3,6-diisobutylpyrazine 1,4-dioxide (4) was obtained as a yellow solid (260 mg, 64 %). **mp** 191°C; **^{1}H NMR** (400 MHz, Chloroform-*d*) δ (ppm) 3.03 (d, *J* = 7.3 Hz, 4H), 2.34 (dp, *J* = 13.8, 6.9 Hz, 2H), 1.02 (d, *J* = 6.7 Hz, 12H); **^{13}C NMR** (100 MHz, Chloroform-*d*) δ (ppm) 146.22, 139.81, 37.97, 26.35, 22.72; **IR** (Neat) ν (cm^{-1}) 2950, 2925, 2850, 1475, 1300, 1225, 1125, 1000; **HRMS (ESI+)** (m/z) calcd for $C_{12}H_{18}Cl_2N_2O_2$ $[M+Na]^+$: 315.0638, found : 315.0645; **Rf** (1% MeOH/DCM) 0.45

2,5-dihydroxy-3,6-diisobutylpyrazine 1,4-dioxide (Pulcherriminic acid) (5)



4 **5**

In a sealed tube, flame dried and under argon, sodium methoxide (326 µL, 4.88 mmol) was weighted (in a glove box), then, under argon flow, 2,5-dichloro-3,6-diisobutylpyrazine 1,4-dioxide (4) (143 mg, 488 µmol) and dry 1,4-dioxane (3.26 ml, [0.15M]) were added.

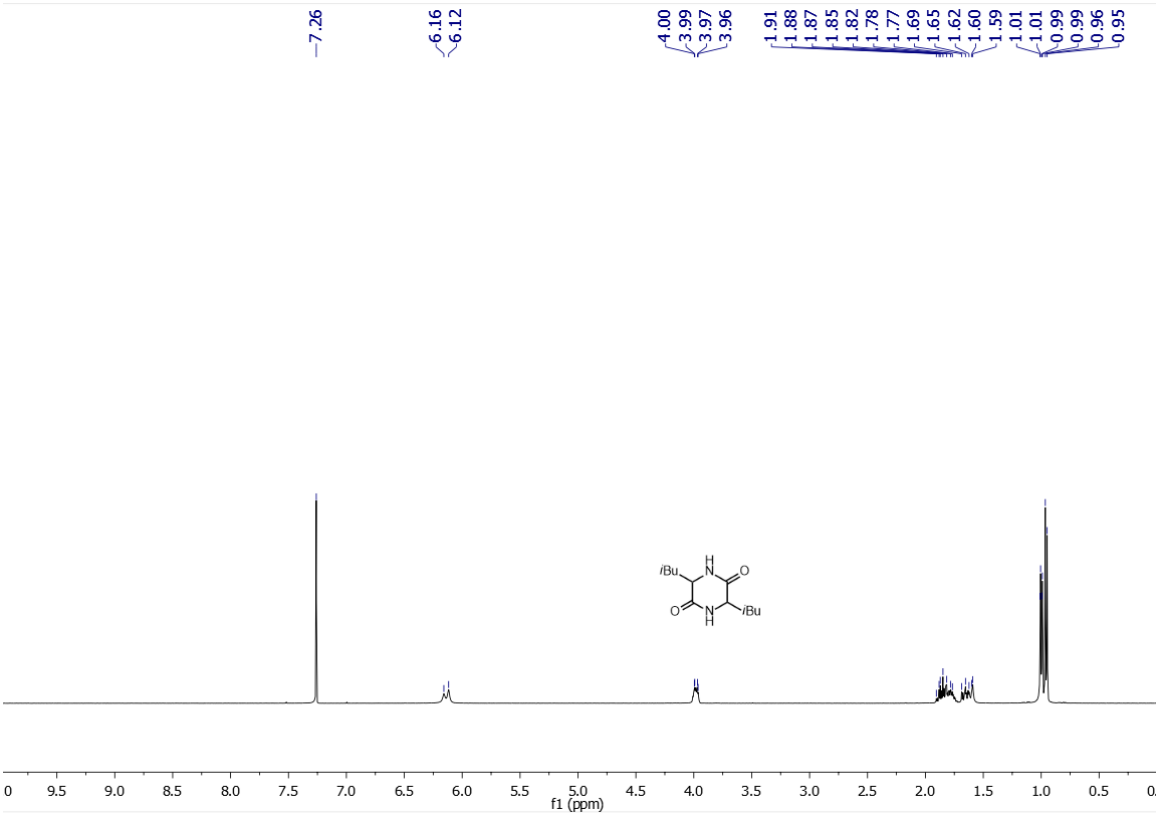
The mixture was heated at 110°C and stirred for 18h. After cooling, the solvent was evaporated, and the product was dissolved in water (20 ml). The aqueous layer was washed with ether (2x20ml), then the organic layer was removed. The aqueous layer was cooled to 0°C, then brought to pH 1 with concentrated HCl. The mixture was allowed to return to room temperature while stirring for 1h, then cooled back to 0°C before filtration. The product is purified by trituration with pentane and Et_2O to give pulcherrimine (49.9 mg, 40%) as a white solid. **^{1}H NMR** (400 MHz, DMSO-d_6) δ (ppm) 2.68 (d, *J* = 7.3 Hz, 4H), 2.12 (dp, *J* = 13.7, 6.8 Hz, 2H), 0.89 (d, *J* = 6.7 Hz, 12H).

(1) Usui, I.; Lin, D. W.; Masuda, T.; Baran, P. S. Convergent Synthesis and Structural Confirmation of Phellodonin and Sarcodonin ε. *Org. Lett.* **2013**, *15* (9), 2080–2083. https://doi.org/10.1021/ol400709f.

(2) Nonappa; Ahonen, K.; Lahtinen, M.; Kolehmainen, E. Cyclic Dipeptides: Catalyst/Promoter-Free, Rapid and Environmentally Benign Cyclization of Free Amino Acids. *Green Chem.* **2011**, *13* (5), 1203–1209. https://doi.org/10.1039/C1GC15043J.

(3) Dickschat, J. S.; Reichenbach, H.; Wagner-Döbler, I.; Schulz, S. Novel Pyrazines from the Myxobacterium Chondromyces crocatus and Marine Bacteria. *European Journal of Organic Chemistry* **2005**, *2005* (19), 4141–4153. https://doi.org/10.1002/ejoc.200500280.

95 **3,6-diisobutylpiperazine-2,5-dione (2)**



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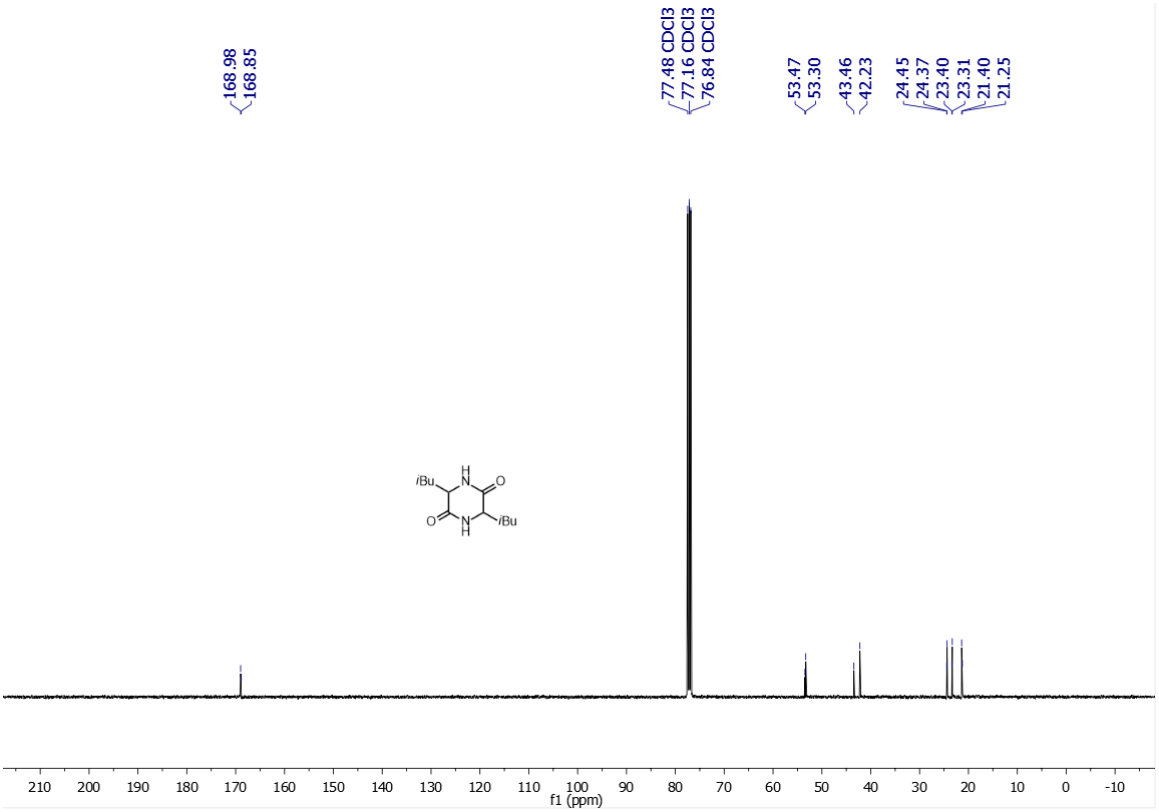

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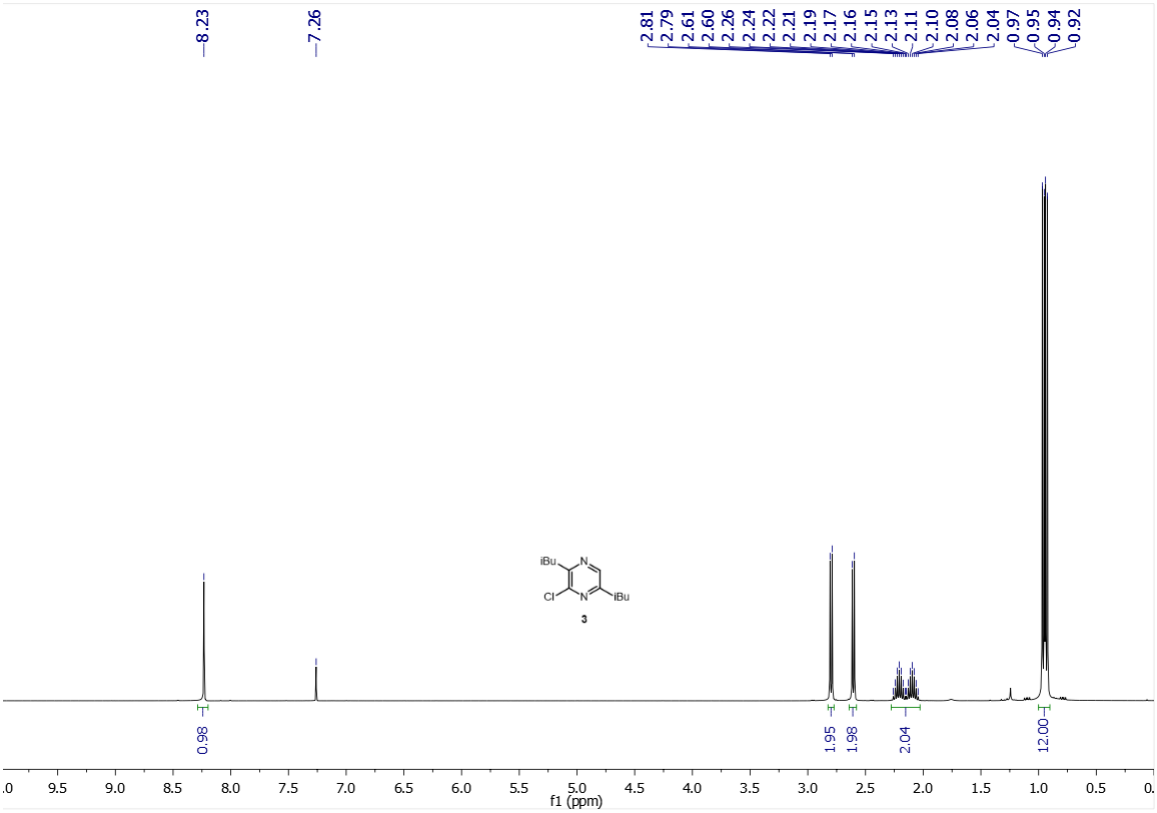
98 **3-chloro-2,5-diisobutylpyrazine** **(3)**



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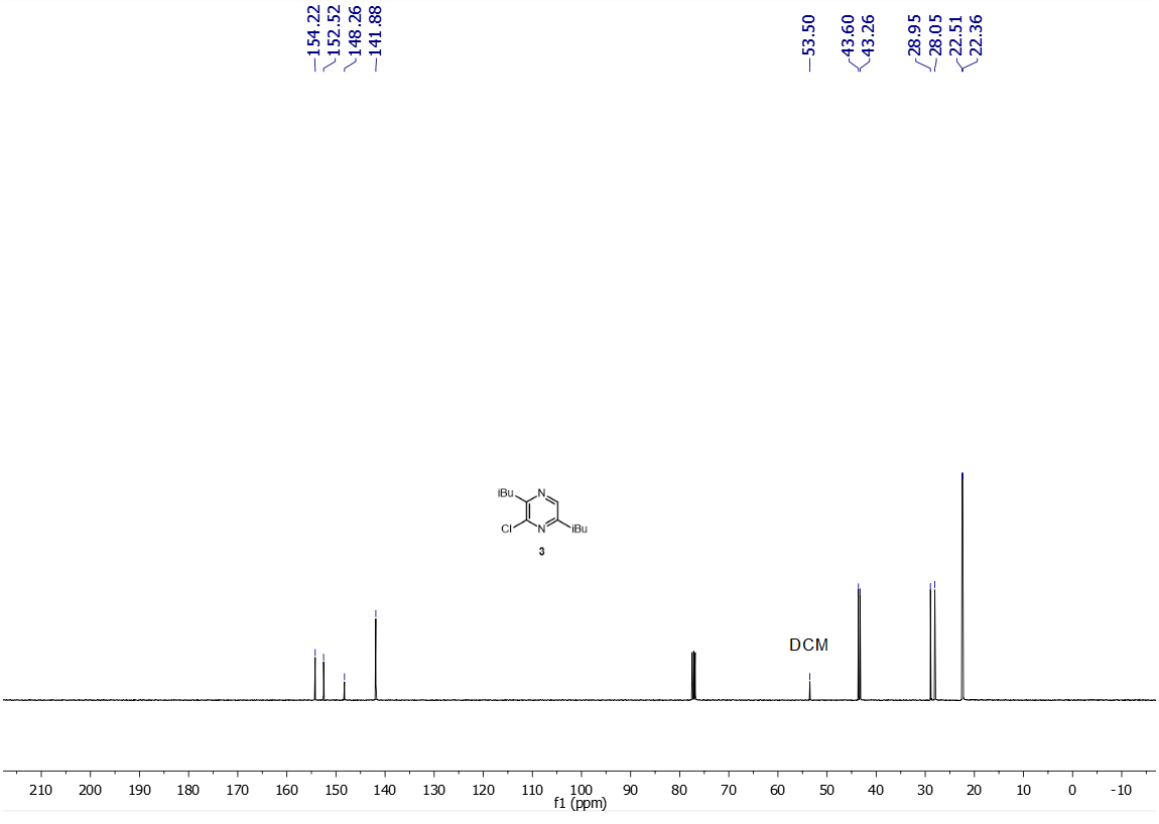

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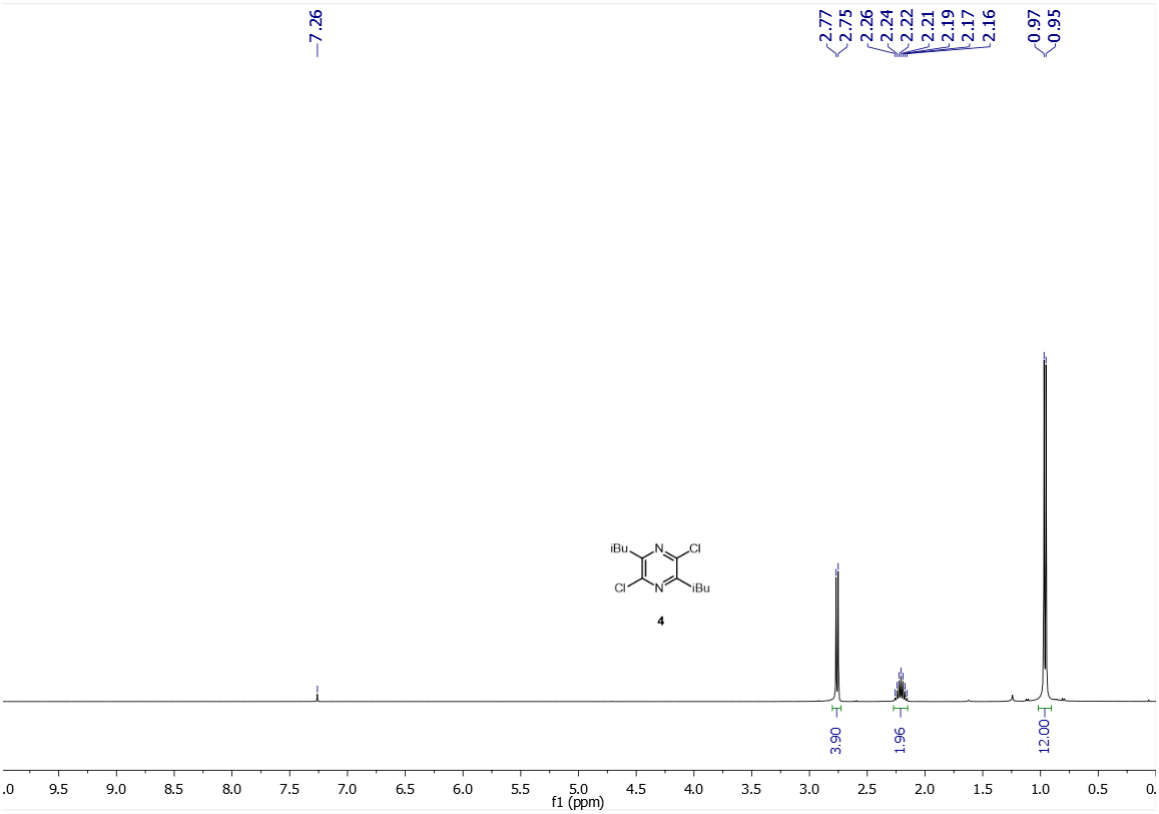

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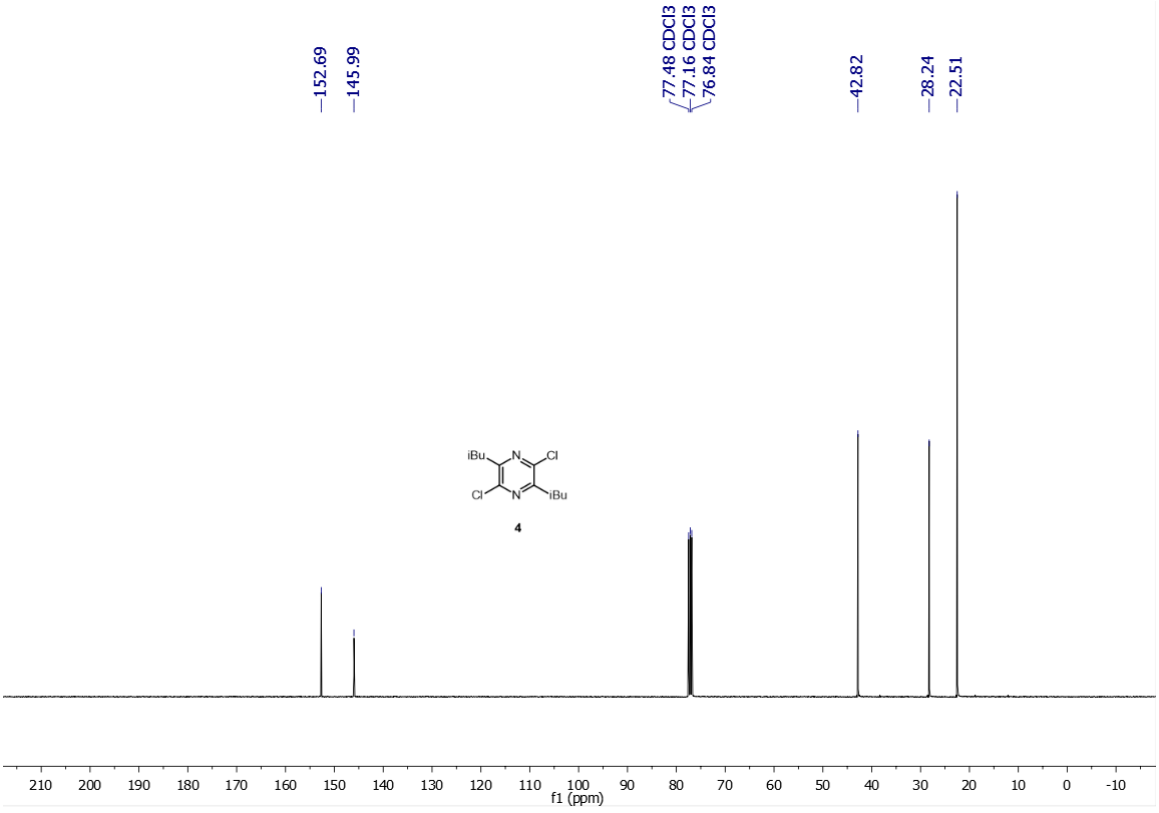
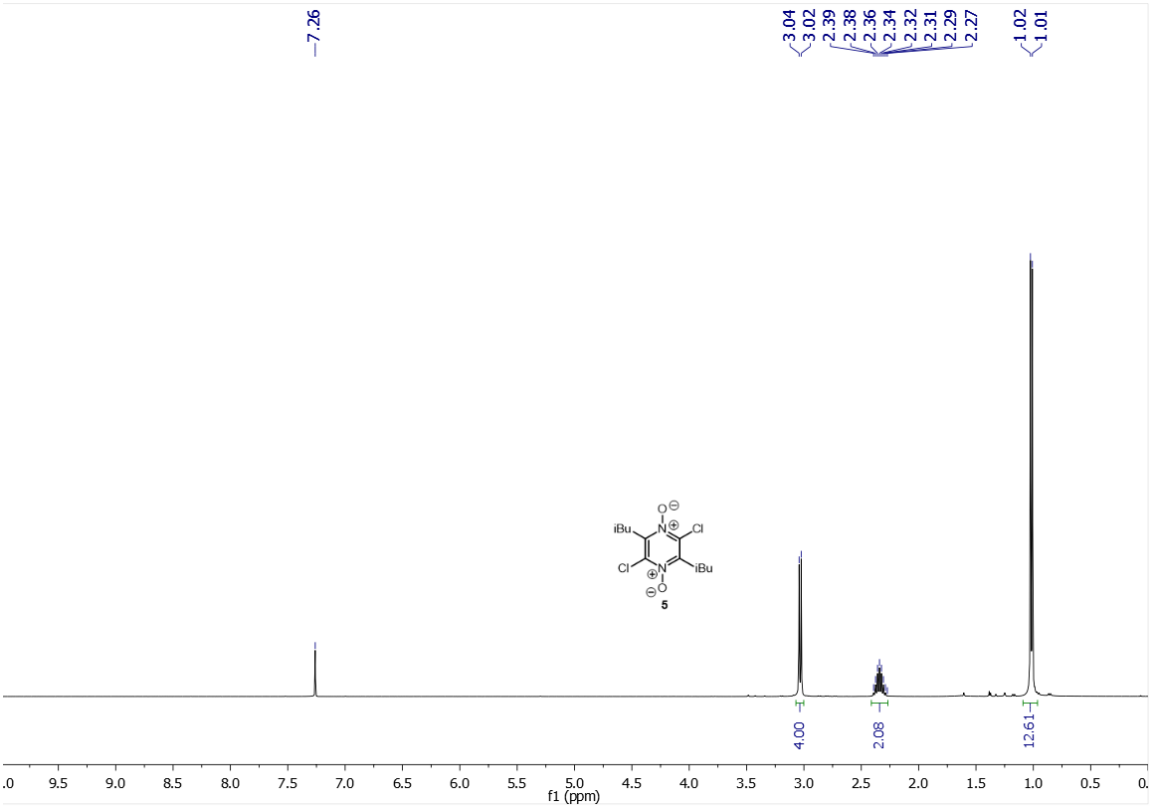


103

104 **2,5-dichloro-3,6-diisobutylpyrazine 1,4-dioxide (5)**



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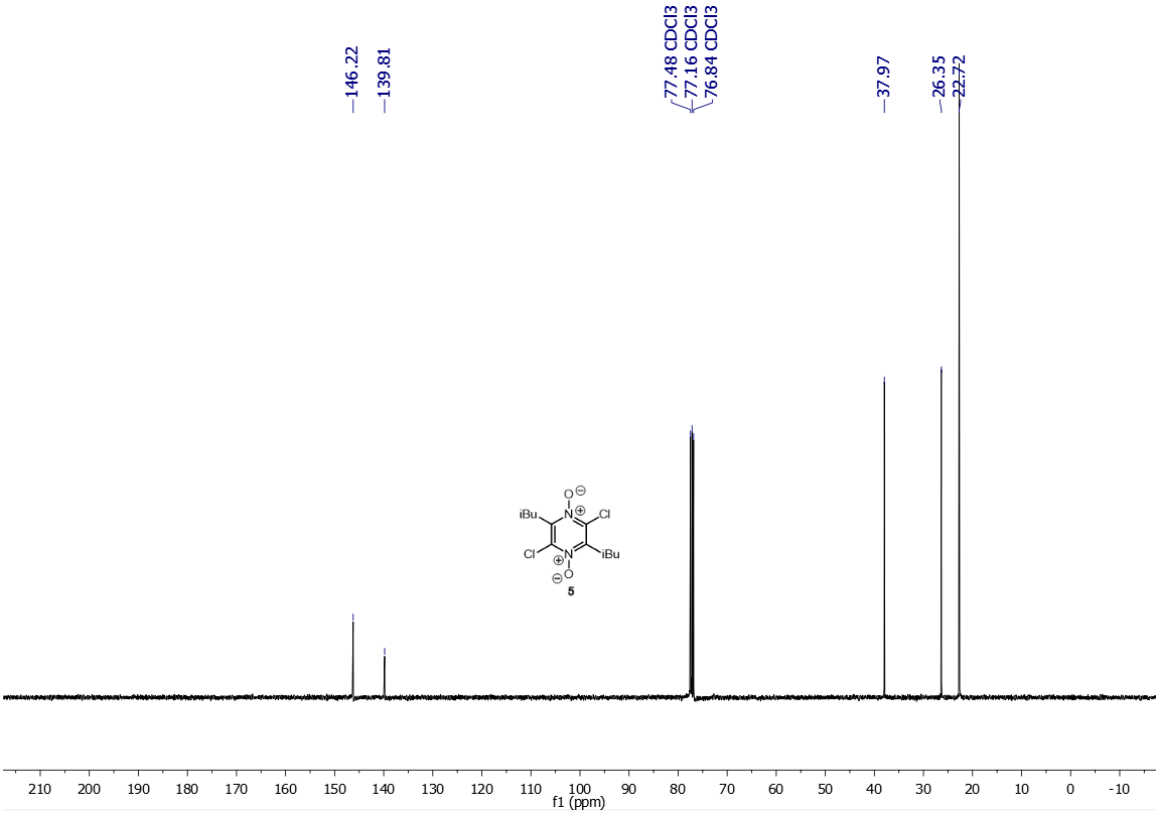

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2,5-dihydroxy-3,6-diisobutylpyrazine 1,4-dioxide (Pulcherrimin) (5)

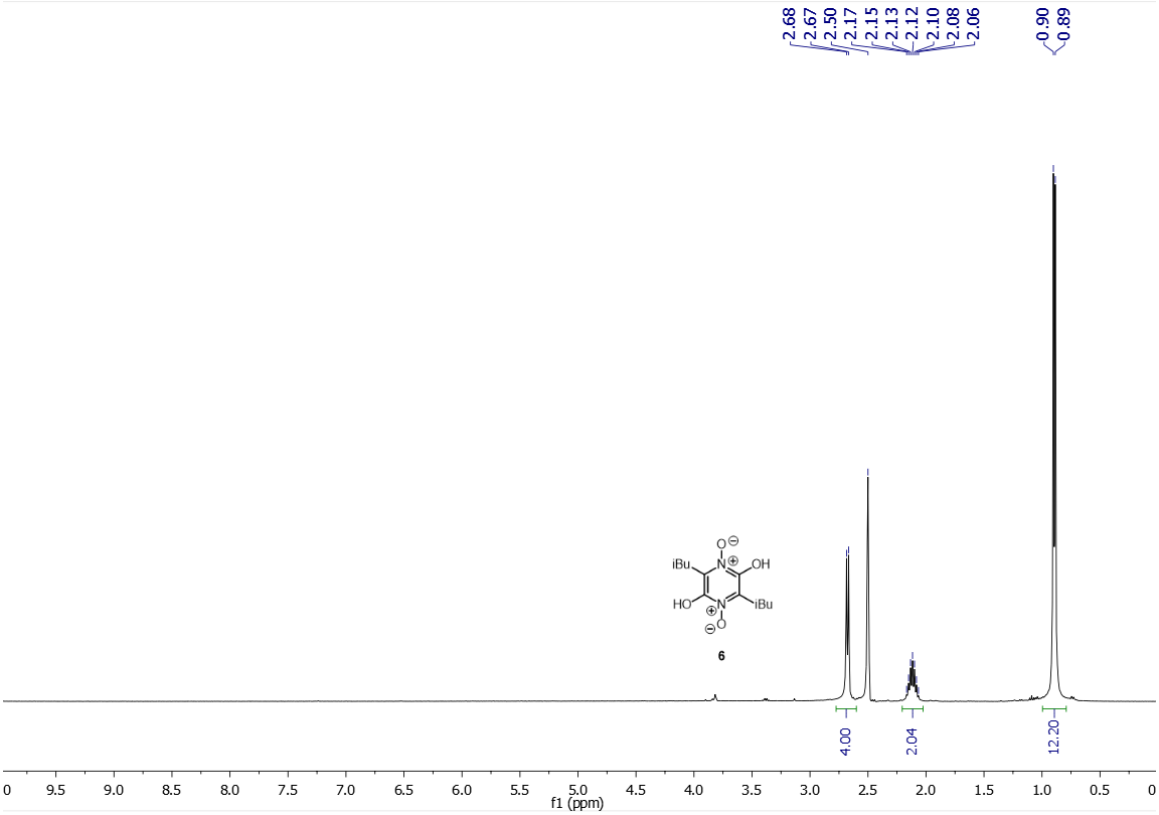

Table S1: Strains used in this study

Strain	Genotype	Reference
NCIB 3610	WT / undomesticated	Lab stock
168	Domesticated	Lab stock
Escherichia coli NEB5α	For molecular cloning	Lab stock
BKK35070	168 Δ*yvmC*::km	BGSC[1]
VCL97	3610 Δ*yvmC*::km	This study
BKK35080	168 Δ*pchR*::km	BGSC[1]
PB755	3610 Δ*pchR*::km	Lab stock
BKK31960	168 Δ*dhbF*::km	BGSC[1]
VCL109	3610 Δ*dhbF*::km	This study
VCL100	3610 *amyE*::P_{yvmC}-*yfp*::spc	This study
VCL111	3610 *amyE*::P_{dhbA}-*lacZ*::spc	This study
VCL112	3610 Δ*yvmC amyE*::P_{dhbA}-*lacZ*::spc	This study
VCL113	3610 Δ*pchR amyE*::P_{dhbA}-*lacZ*::spc	This study
VCL118	3610 Δ*yvmC amyE*::P_{yvmC}-*yvmC-cypX*::spc	This study
Pseudomonas fluorescens	WT	Lab stock
Pseudomonas capeferrum	WT	Lab stock
Pseudomonas protegens	WT	Lab stock
Pseudomonas protegens	Δ*phlD*::tet	Lab stock

Antibiotics abbreviations: Kanamycin (km), spectinomycin (spc), tetracycline (tet)

Reference

1. Koo, B. *et al.* Libraries for *Bacillus subtilis*. *Cell Syst* **4**, 291-305.e7. (2017).

Table S2: Regression parameters

Analytes	**Concentration** (ng/mL)	**Équation linéaire** Y=a X+b	R^2
Bacillibactin (BB)	0.01-2.5	Y= 10410 X – 1104	0.985
Pulcherriminic acid (PA)	0.1-10	Y= 595.77 X + 282.4	0.995

Table S3: Limit of quantification

Analytes	**Recovery (%)**	**RSD (%)**	**LOD (ng/mL)**	**LOQ (ng/mL)**
Bacillibactin (BB)	72.1	7.01	0.017	0.029
Pulcherriminic acid (PA)	83.9	8.59	0.145	0.211