Supporting Information

Synthesis of Phakellistatin 13 and Oxidation to Phakellistatin 3 and Isophakellistatin 3

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Experimental Section

Reactions were run under an atmosphere of argon unless otherwise noted. Dichloromethane (DCM) and *N,N*-dimethyl formamide (DMF) were dried according to the method of Grubbs.¹ All other commercially obtained reagents and solvents were used without further purification. Reverse-phase HPLC was performed on C₁₈ microsorb columns with gradient elution using acetonitrile (MeCN) or methanol (MeOH) and aqueous buffer containing 0.1% (v/v) trifluoroacetic acid (TFA) or 100 mM acetic acid adjusted to pH 4.0 with sodium hydroxide.

ProThr(*Ot*-Bu)LeuTrpProPheGly•TFA (4). FmocGly (357 mg, 1.2 mmol)

was added to a suspension of 2-chlorotritylchloride resin in DCM. *N,N*-diisopropylethylamine (1.0 mL, 5.7 mmol) was added, and the mixture was stirred under argon for 2 h. The resin was filtered and washed with MeOH (3

× 20 mL) and DCM (3 × 20 mL). After deprotection with 20% piperidine in DMF (2 × 10 mL × 5 min) and washing of the resin with DMF (3 × 10 mL), the next amino acid (3 equiv.) was coupled using HBTU (3 equiv.) in the presence of HOBt (3 equiv.) and *N,N*-diisopropylethylamine (4 equiv.) for 1 h. The deprotection/coupling procedure was repeated for each remaining amino acid in the sequence. Following deprotection of the final residue, cleavage from the resin was effected with 1:1:98 TFA/1,2-ethanedithiol/DCM. The solution was concentrated in vacuo and the crude heptapeptide was purified by reverse-phase HPLC (25-75% CH₃CN in 0.1% aq. TFA) to give **4** as a white solid (827 mg, 69%): *R*_f = 0.25 (10:15:75 H₂O/HCO₂H/*n*-BuOH); ¹H-NMR shows two conformations at 298 K (80:20) and one

¹ Pangborn, A. B.; Giardello, M. A.; Grubbs, R. H.; Rosen, R. K.; Timmers, F. J. *Organometallics* **1996**, 15, 1518

conformation at 360 K; ^1H -NMR (500 MHz, d_6 -DMSO, 298 K, major conformation) δ 10.88 (s, 1H), 9.49 (br s, 1H), 8.50-8.60 (m, 2H), 8.23-8.28 (m, 1H), 8.16 (d, $J = 7.5$ Hz, 1H), 7.87 (d, $J = 8.0$ Hz, 1H), 7.77 (d, $J = 8.0$ Hz, 1H), 7.59 (d, $J = 7.7$ Hz, 1H), 7.34 (d, $J = 8.0$ Hz, 1H), 7.16-7.22 (m, 7H), 7.07 (t, $J = 7.6$ Hz, 1H), 7.00 (t, $J = 7.5$ Hz, 1H), 4.76-4.82 (m, 1H), 4.52-4.59 (m, 1H), 4.25-4.52 (m, 4H), 3.80-3.90 (m, 3H), 3.46-3.50 (m, 1H), 2.91-3.27 (m, 7H), 2.81-2.85 (m, 1H), 2.29-2.34 (m, 1H), 1.80-1.87 (m, 4H), 1.65-1.72 (m, 2H), 1.50-1.57 (m, 1H), 1.36-1.40 (m, 1H), 1.12 (s, 9H), 1.01 (d, $J = 5.7$ Hz, 3H), 0.87 (d, $J = 6.2$ Hz, 3H), 0.83 (d, $J = 6.2$ Hz, 3H); ^{13}C -NMR (125 MHz, d_6 -DMSO, 298 K) δ 171.6, 171.2, 171.1, 171.0, 170.3, 168.4, 168.3, 137.8, 136.2, 129.2, 128.0, 127.3, 126.2, 124.8, 120.9, 118.4, 118.0, 111.4, 109.8, 74.1, 67.1, 59.9, 58.9, 57.7, 53.6, 51.2, 50.9, 46.8, 45.8, 41.2, 40.8, 37.3, 29.9, 28.7, 28.0, 27.9, 27.1, 24.2, 23.5, 23.0, 21.7, 18.8; IR (thin film) 3282, 1657, 1543, 1200, 1133, 1023, 1000 cm^{-1} ; LRMS (ESI) m/z 873.50; HRMS (ESI) calcd for $\text{C}_{46}\text{H}_{64}\text{N}_8\text{O}_9\text{Na}$ $[\text{M} + \text{Na}]^+$ 895.4694, found 895.4678.

cyclo-ProThr(Ot-Bu)LeuTrpProPheGly (6). Peptide **4** (155 mg, 0.16 mmol) was dissolved in CH_3CN (160 mL) and treated with HOBt (22 mg, 0.16 mmol), HBTU (64 mg, 0.16 mmol) and N,N -diisopropylethylamine

(0.17 mL, 0.98 mmol). The solution was maintained at 23 $^\circ\text{C}$ for 4 days and concentrated in vacuo. The crude peptide was purified by reverse phase HPLC (45-60% CH_3CN in 0.1% aq. TFA) to give **6** as a white amorphous solid (82 mg, 60%): $R_f = 0.39$ (1:15:84 AcOH/MeOH/ CHCl_3); ^1H -NMR (500 MHz, d_6 -DMSO, 298 K) δ 10.79 (s, 1H), 9.34 (d, $J = 4.7$ Hz, 1H), 8.26 (d, $J = 7.6$ Hz, 1H), 7.58-7.60 (m, 1H), 7.46 (d, $J = 8.8$ Hz, 1H), 7.35-7.38 (m, 3H), 7.19-7.28 (m, 4H), 7.13-7.15 (m, 2H), 7.04 (t, $J = 7.6$ Hz, 1H), 6.91 (t, $J = 7.7$ Hz, 1H), 4.43-4.48 (m, 2H), 4.26 (dd, $J = 7.0, 7.9$ Hz, 1H), 4.16-4.19 (m, 2H), 4.13 (dd, $J = 3.4, 18.0$ Hz, 1H), 4.01 (dd, $J = 4.0, 18.0$ Hz, 1H), 3.67-3.72 (m, 1H), 3.58-3.63 (m, 1H), 3.44 (d, $J = 7.2$ Hz, 1H), 3.18 (dd, $J = 3.7, 13.3$ Hz, 1H), 3.08-3.10 (m, 2H), 2.95 (t, $J = 13.1$ Hz, 1H), 2.91-2.97 (m, 1H), 2.40 (t, $J = 9.9$ Hz, 1H), 2.26-2.33 (m, 1H), 1.94 (quint, $J = 6.9$ Hz, 2H), 1.77-1.86 (m, 1H), 1.65-1.74 (m, 2H), 1.56-

1.60 (m, 1H), 1.50-1.53 (m, 1H), 1.27-1.31 (m, 1H), 1.12 (s, 9H), 1.06 (d, $J = 6.0$ Hz, 3H), 1.02-1.00 (d, $J = 6.3$ Hz, 3H and m, 1H), 0.89 (d, $J = 6.3$ Hz, 3H), 0.20-0.40 (m, 2H); ^{13}C -NMR (125 MHz, d_6 -DMSO, 298 K) δ 171.6, 171.3, 170.9, 170.5, 169.7, 169.6, 168.1, 137.6, 136.1, 128.6, 128.3, 127.0, 126.7, 124.1, 121.2, 118.4, 118.1, 111.4, 108.7, 73.5, 66.6, 61.6, 59.8, 59.1, 56.4, 53.5, 51.5, 46.0, 45.5, 42.9, 40.4, 37.3, 29.7, 28.9, 28.8, 28.6, 26.6, 24.5, 24.2, 23.7, 21.9, 20.7; IR (thin film) 3368, 1633, 1519, 1433, 1024, 995 cm^{-1} ; LRMS (ESI) m/z 855.47; HRMS (ESI) calcd for $\text{C}_{46}\text{H}_{62}\text{N}_8\text{O}_8\text{Na}$ $[\text{M} + \text{Na}]^+$ 877.4588, found 877.4577.

Phakellistatin 13 (1). Peptide **6** (23 mg, 0.027 mmol) was dissolved in 50:50:1 TFA/ CHCl_3 /1,2-ethanedithiol (0.3 mL) and allowed to stir at 23 °C for 30 min. The reaction mixture was concentrated in vacuo and purified by silica gel flash chromatography (0%, 5%, 10% MeOH/ CHCl_3) to give

phakellistatin 13 as a white amorphous solid (20 mg, 94%): $R_f = 0.81$ (10:15:75 $\text{H}_2\text{O}/\text{HCO}_2\text{H}/n\text{-BuOH}$); ^1H -NMR shows two conformations at 298 K ($>95:<5$) and one conformation at 370 K; ^1H -NMR (500 MHz, d_6 -DMSO, 298 K, major conformation) δ 10.80 (s, 1H), 9.36 (d, $J = 4.6$ Hz, 1H), 8.29 (d, $J = 7.7$ Hz, 1H), 7.85 (d, $J = 9.1$ Hz, 1H), 7.55 (t, $J = 3.7$ Hz, 1H), 7.31-7.35 (m, 2H), 7.32 (s, 1H), 7.24 (t, $J = 7.2$ Hz, 2H), 7.19 (d, $J = 7.0$ Hz, 1H), 7.13 (d, $J = 6.2$ Hz, 1H), 7.10 (d, $J = 7.2$ Hz, 2H), 7.04 (t, $J = 7.6$ Hz, 1H), 6.91 (t, $J = 7.6$ Hz, 1H), 4.86 (br s, 1H), 4.42-4.49 (m, 1H), 4.37-4.42 (m, 1H), 4.20-4.23 (m, 2H), 4.14-4.17 (m, 1H), 4.09-4.13 (m, 2H), 3.94 (dd, $J = 17.9, 3.7$ Hz, 1H), 3.63-3.69 (m, 1H), 3.46-3.52 (m, 1H), 3.44 (d, $J = 7.4$ Hz, 1H), 3.19 (dd, $J = 13.2, 3.3$ Hz, 1H), 3.05-3.09 (m, 1H), 2.95 (t, $J = 13.2$ Hz, 1H), 2.81-2.89 (m, 1H), 2.38 (t, $J = 10.1$ Hz, 1H), 2.20-2.29 (m, 1H), 1.87-1.94 (m, 2H), 1.78-1.82 (m, 1H), 1.63-1.71 (m, 1H), 1.56-1.64 (m, 1H), 1.48-1.51 (m, 1H), 1.44-1.49 (m, 1H), 1.03 (d, $J = 6.1$ Hz, 3H), 0.97-1.01 (m, 1H), 1.00 (d, $J = 6.6$ Hz, 3H), 0.89 (d, $J = 6.5$ Hz, 3H), 0.20-0.30 (m, 1H), 0.07-0.17 (m, 1H); ^{13}C -NMR (125 MHz, d_6 -DMSO, 298 K) δ 171.9, 171.6, 170.9, 170.5, 169.9, 169.7, 168.2, 137.6, 136.1, 128.6, 128.3, 127.0, 126.6, 124.1, 121.2, 118.4, 118.1, 111.4, 108.7, 65.5, 61.7, 59.8, 58.8, 56.4, 53.5, 51.5, 46.1, 45.4, 42.8, 40.3, 37.1,

30.0, 28.8, 26.6, 24.4, 23.6, 21.9, 20.6; IR (thin film) 3301, 2960, 2930, 2878, 1636, 1534 cm^{-1} ; HRMS (ESI) calcd for $\text{C}_{42}\text{H}_{54}\text{N}_8\text{O}_8\text{Na}$ $[\text{M} + \text{Na}]^+$ 821.3962, found 821.3950.

Phakellistatin 3 (2) and isophakellistatin 3

(3). Phakellistatin 13 (20 mg, 0.025 mmol) was dissolved in MeOH (25 mL). Rose bengal (5 mg, 0.005 mmol) was added and the solution

was cooled to $-40\text{ }^{\circ}\text{C}$ by means of recirculating cooling bath. Oxygen gas was then bubbled through the solution with a diffuser while the solution was irradiated with a 500 W halogen lamp. After 4 h, dimethyl sulfide (0.1 mL) was added and the reaction warmed to $4\text{ }^{\circ}\text{C}$ over 6 h. The solution was concentrated in vacuo, and the sensitizer quickly removed by chromatography over silica gel (10% MeOH/ CHCl_3). Purification by reverse-phase HPLC (30-70% MeOH in acetate buffer) afforded pure phakellistatin 13 (5 mg, 25% recovery) and a mixture of phakellistatin 3 and isophakellistatin 3 (4 mg, 20% combined yield). After repeated reverse-phase HPLC purification, phakellistatin 3 was obtained in $> 95\%$ purity as a white amorphous solid: $R_f = 0.18$ (4:2:1 hexanes/EtOAc/MeOH); $^1\text{H-NMR}$ (500 MHz, d_4 -MeOH, 298 K) δ 7.29-7.33 (m, 4H), 7.23-7.26 (m, 1H), 7.18-7.20 (m, 1H), 7.13 (td, $J = 1.0, 7.4\text{ Hz}$, 1H), 6.77 (td, $J = 1.0, 7.4\text{ Hz}$, 1H), 6.72 (d, $J = 7.4\text{ Hz}$, 1H), 5.54 (s, 1H), 4.87-4.90 (m, 1H), 4.82 (dd, $J = 4.3, 9.6\text{ Hz}$, 1H), 4.47 (d, $J = 7.7\text{ Hz}$, 1H), 4.39 (dd, $J = 3.9, 12.6\text{ Hz}$, 1H), 4.35-4.38 (m, 1H), 4.27 (d, $J = 3.6\text{ Hz}$, 1H), 4.21-3.25 (m, 2H), 4.06 (d, $J = 18.0\text{ Hz}$, 1H), 3.69-3.74 (m, 1H), 3.59-3.64 (m, 1H), 3.34-3.37 (m, 1H), 3.09-3.24 (m, 2H), 2.85-2.89 (m, 1H), 2.55-2.65 (m, 1H), 2.31-2.38 (m, 1H), 2.22 (dd, $J = 6.3, 12.6\text{ Hz}$, 1H), 2.04-2.11 (m, 2H), 1.88-2.02 (m, 4H), 1.77-1.79 (m, 1H), 1.52-1.58 (m, 2H), 1.21 (d, $J = 6.4\text{ Hz}$, 3H), 1.08 (d, $J = 6.6\text{ Hz}$, 3H), 0.99 (d, $J = 6.6\text{ Hz}$, 3H), 0.64-0.74 (m, 1H); $^{13}\text{C-NMR}$ (125 MHz, d_4 -MeOH, 298 K) δ 176.6, 173.8, 173.0, 172.9, 171.9, 171.5, 170.8, 149.6, 138.8, 132.7, 130.7, 130.1, 129.9, 128.2, 123.3, 120.5, 112.4, 90.7, 87.2, 67.4, 63.9, 62.8, 62.4, 61.5, 58.3, 51.6, 47.7, 47.6, 43.5, 41.8, 41.6, 37.9, 32.1, 30.9, 26.1, 26.1, 24.0, 22.7, 22.2, 21.0; IR (thin film) 3331, 2914, 2853, 1636, 1524, 1439 cm^{-1} ; HRMS (ESI) calcd for

$C_{42}H_{54}N_8O_9Na$ $[M + Na]^+$ 837.3912, found 837.3942. Isophakellistatin 3 was obtained in 85% purity along with 15% phakellistatin 3: 1H -NMR (500 MHz, d_4 -MeOH, 298 K) δ 7.28-7.32 (m, 2H), 7.26-7.24 (m, 3H), 7.19 (d, $J = 7.3$ Hz, 1H), 7.09-7.12 (m, 1H), 6.87 (d, $J = 7.9$ Hz, 1H), 6.69-6.77 (m, 1H), 5.75 (s, 1H), 4.90-4.87 (m, 1H), 4.78 (dd, $J = 4.3, 9.3$ Hz, 1H), 4.56 (d, $J = 7.4$ Hz, 1H), 4.45 (dd, $J = 3.2, 9.0$ Hz, 1H), 4.39-4.42 (m, 2H), 4.18-4.28 (m, 2H), 3.99 (d, $J = 18.2$ Hz, 1H), 3.67-3.73 (m, 1H), 3.45-3.60 (m, 1H), 3.34-3.43 (m, 1H), 3.20-3.23 (m, 1H), 3.00-3.05 (m, 1H), 2.81-2.85 (m, 1H), 2.59-2.65 (m, 1H), 2.35-2.40 (m, 1H), 2.20-2.26 (m, 2H), 2.04-2.09 (m, 2H), 1.93-2.02 (m, 2H), 1.76-1.90 (m, 2H), 1.61-1.68 (m, 1H), 1.52-1.58 (m, 1H), 1.22 (d, $J = 6.1$ Hz, 3H), 1.07 (d, $J = 6.4$ Hz, 3H), 0.99 (d, $J = 6.6$ Hz, 3H), 0.64-0.74 (m, 1H); LRMS (ESI) m/z 815.52, 837.52.

cyclo-ProThr(Ot-Bu)LeuTrp(Boc)ProPheGly (7). Phakellistatin 13 (20 mg, 0.025 mmol) was dissolved in DCM (2 mL). To this solution was added triethylamine (10 mg, 0.1 mmol), *N,N*-dimethylaminopyridine (1 mg, 0.008 mmol) and di-*tert*-butyldicarbonate (6 mg, 0.027 mmol). The solution was

maintained under argon for 2 h and then concentrated in vacuo. Purification by reverse phase HPLC (50-80% CH_3CN in 0.1% aq. TFA) afforded **7** as an amorphous white solid (18 mg, 75%): $R_f = 0.40$ (93:7 $CHCl_3/MeOH$); 1H -NMR (500 MHz, d_6 -DMSO, 298 K) δ 8.45 (d, $J = 7.4$ Hz, 1H), 8.09 (d, $J = 8.0$ Hz, 1H), 7.70-7.71 (m, 1H), 7.57 (s, 1H), 7.42 (d, $J = 7.8$ Hz, 1H), 7.12-7.31 (m, 10H), 4.58-4.62 (m, 1H), 4.48-4.50 (m, 1H), 4.42 (dd, $J = 2.3, 6.3$ Hz, 1H), 4.31-4.26 (m, 2H), 4.16-4.20 (m, 1H), 4.13-4.14 (m, 1H), 3.96-4.02 (m, 1H), 3.79 (d, $J = 7.2$ Hz, 1H), 3.67-3.73 (m, 1H), 3.57-3.61 (m, 1H), 3.24-3.25 (m, 1H), 3.18-3.20 (m, 1H), 2.96-3.07 (m, 2H), 2.62-2.68 (m, 1H), 2.37-2.45 (m, 1H), 2.06-2.12 (m, 3H), 1.90-1.99 (m, 2H), 1.83-1.92 (m, 1H), 1.71-1.73 (m, 3H), 1.66 (s, 9H), 1.18 (s, 9H), 1.16 (d, $J = 6.3$ Hz, 3H), 1.10 (d, $J = 5.7$, 3H), 0.96 (d, $J = 5.9$ Hz, 3H), 0.45-0.53 (m, 1H), 0.35-0.45 (m, 1H); LRMS (ESI) m/z 955.5, 977.5;

Conformational Search

Monte Carlo conformational searches were carried out using the Maestro interface for Macromodel 8.1 using Amber*/H₂O.

To test the validity of molecular modeling an unconstrained Monte Carlo search was used to generate plausible conformations of isophakellistatin 3 for which a crystal structure was available. Five hundred structures were generated and minimized. Structures within 1.89 kcal/mol of the global minimum were saved. The family of structures corresponding to the global minimum (found 37 times) did not have the same backbone conformation as the crystal structure of isophakellistatin 3•H₂O•acetone. Fortunately, the family of structures with the second-lowest energy (0.46 kcal/mol above the global minimum, found 21 times) exhibited the same backbone conformation as the crystal structure, with a slight pyramidalization of the indoline nitrogen. In the crystal structure, isophakellistatin 3 exhibits one hydrogen bond to the water molecule and four hydrogen bonds to neighboring molecules in the crystal lattice. The Monte Carlo search does a good job of reproducing the crystal structure even though it cannot account for the additional forces that were present in the crystal lattice.

An unconstrained Monte Carlo search was used to generate plausible conformations. Ten thousand structures were generated and minimized. Two families of conformations, with various sidechain rotamers, were within 1 kcal/mol of the global minimum. In both families of conformations the Pro2 amide has an *E* configuration and the Pro5 amide has a *Z* configuration, consistent with NOE data. Surprisingly, neither family exhibited cross-ring hydrogen bonds with good geometries. The lowest energy family of conformers is stabilized by a hydrogen between the Thr6 OH and the Pro5 carbonyl, however the distance between Trp H α and Gly4 NH is 5.4 Å, inconsistent with the observable strong NOE. The second family of conformers (0.7 kcal/mol above the global minimum) appears to be consistent with all of the spectroscopic data.

Parameters:

Ring closure on Gly CH₂-NH bond.

Both Pro amides allowed to be *E* or *Z* All other amides *E*.

Family A

Energy: Global minimum (-563.99408)

Found 405 times (399 + 6 closely related)

Pro 2: *E*

Pro 5: *Z*

GlyCH₂: Bisected

H-Bonds: Thr(OH) to Pro5(CO)

W $_{\alpha}$ -F $_{NH}$: 3.24 Å

W $_{\alpha}$ -G $_{NH}$: 5.40 Å

Family B

Consistent with NMR data

Energy: +2.95 kJ/mol (-561.042358)

Found 80 times (65 + 15 closely related)

Pro 2: *E*

Pro 5: *Z*

GlyCH₂: Bisected

W $_{\alpha}$ -F $_{NH}$: 2.71 Å

W $_{\alpha}$ -G $_{NH}$: 2.53 Å

A Monte Carlo search was carried out on phakellistatin 3. Ten thousand structures were generated and minimized. Only one family of conformations was identified within 2.6 kcal/mol of the global minimum (found 118 times). In this minimum energy conformation both the Pro5 and Hpi1 amides exist in the *cis* configuration. There was one cross-ring hydrogen bond between the Hpi hydroxyl proton and the Gly4-CO. Although the Leu7-CO is in the proximity of Gly4NH and Phe3NH, the orientation does not suggest hydrogen bonding. This conformer closely resembles the published crystal structure for isophakellistatin 3, differing primarily in the Hpi1-OH hydrogen bond to Hpi1-CO in isophakellistatin 3 and to Gly4-CO in phakellistatin 3. A second family of conformations, with a gamma turn hydrogen bond between the Gly4-NH and the Pro2-CO was identified 2.7 kcal/mol above the global minimum. These structures are consistent with the absence of long range NOEs.

Parameters:

Ring closure on Gly CH₂-NH bond.

Pro and HPI amides allowed to be E or Z All other amides E.

Family A

Global minimum

(-478.637604 kJ/mol)

Times found: 330 (87% of structures)

Family B

Energy +11.27 kJ/mol (+2.69 kcal/mol)

(-467.363373 kJ/mol)

Times found 4 (1% of structures)

0-100% MeCN in 0.1 % aqueous TFA / 15 min

0-100% MeCN in 0.1% aqueous TFA / 15 min

30-70% MeCN in 0.1% aqueous TFA / 30 min

0-100% MeCN in 100 mM aqueous acetate buffer / 15 min

30-70% MeCN in 0.1% aqueous TFA / 15 min

30-70% MeCN in 0.1% aqueous TFA / 15 min

4

4

^1H -NMR spectrum of isolated phakellistatin 3 (300 MHz, top: *J. Org. Chem.* **1994**, 59, 1593) and synthetic phakellistatin 3 (500 MHz, bottom)

^1H -NMR spectrum of isolated isophakellistatin 3 (300 MHz, top: *J. Org. Chem.* **1994**, 59, 1593) and synthetic isophakellistatin 3 (500 MHz, bottom)