General Methods. Amino Acids Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Phe-OH, and Fmoc-D-Phe-OH were obtained from Sigma-Aldrich. L-Threonine tert-butyl ester hydrochloride, Fmoc-D-allo-Thr(tBu)-OH and Fmoc-Gly-OH were from Chem-impex International, Inc and Fmoc-D-Leu-OH was from Advanced ChemTech.

Solution-phase reactions were done in a round-bottom flask. Organic solvent removal was performed using Eyela rotary evaporator with temperature not exceeding 40°C. Freeze-drying was done using a Labconco Lyophilizer or Labconco CentriVac. Solid-phase reactions were all performed at 25 °C in a glass column (15 mL) with a polyethylene porous disc and a stopcock using a protocol described by Ambiard et al. (2005) with some modifications. Solvents, excess soluble reagents and washings were removed by suction filtration. Deprotection of Fmoc group was done using 20% Piperidine in DMF (1 x 2mL x 2min; 1 x 2mL x 20min). Washings after each step (coupling and deprotection) were done with DCN (3 x 2mL x 2min), MeOH (3 x 2mL x 1min), and DMF (3 x 2mL x 1min).

Analytical HPLC was carried out on Phenomenex Luna C18 reversed-phase column (5 μm one-probe). Chemical shifts are reported in parts per million (ppm) and J (coupling constant) values are expressed in hertz, Hz. Multiplicity is indicated using the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), brs (broad singlet), and tt (triple triplet). QSTAR Elite Q-TOF (Applied Biosystems/AB Scieix) Electron Spray Ionization Mass spectrometer (ESI-MS) was used to generate the high-resolution mass spectra.

Purification of compounds by gravity column chromatography was performed using silica gel 60, 0.063-0.200 mm (70-230 mesh, Merck). Monitoring progress of solution-phase reactions was done by thin layer chromatography (TLC) using Silica gel 60 (Merck). The TLC plates were visualized first under a UV lamp set at long wavelength and stained using peroxymanganate staining solution. All solvents used in reactions and column chromatography were A.R. grade.

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Fmoc-Ala-(Z)-Dhb-OH (5): Fmoc-Ala-(Z)-Dhb-OBu (303 mg, 0.675 mmol) was dissolved in 2 mL TFA-DCM (95:5) and was stirred for 3 hrs at 25 °C and was concentrated in vacuo. The TFA was removed by co-evaporations with DCM and diethyl ether to give Fmoc-Ala-(Z)-Dhb-OH (242 mg, 0.615 mmol, 91.1 % yield). Appearance: White powder; TLC (SiO2, DCM-MeOH 9:1) Rf = 0.19; Analytical HPLC (tR = 20min), DMF (4 x 2mL x 1min). All deprotection solutions were kept for Fmoc monitoring. The Kaiser test was positive after deprotection. The loading, as calculated by Fmoc assay was 1.67 mmol/g, 98.2%.11,27

Nobilamidine B, Propanoyl-D-Phe-D-Leu-Phe-D-a-Thr–Val–Ala–(Z)-Dhb-OH (1): Barlos (chlorotriyl chloride) resin (86.9 mg, 0.148 mmol, 1 eq.; 1.7 mmol/g loading) was placed in a small column (described in 3.2). The resin was swelled by washing with DCM (3 x 2 mL x 1 min). A solution of Fmoc-Ala-(Z)-Dhb-OH (65.5 mg, 0.167 mmol, 1 eq.) and DIEA (77 µL, 0.442 mmol, 2.99 eq.) with the same cycle of washing, deprotection and coupling. In each treatment: DMF (3 x 1mL x 1min), 20% piperidine in DMF (1 x 2mL x 2min), 20% Piperidine in DMF (1 x 2mL x 2min) and DMF (3 x 2 mL x 2min). Fmoc-Ala-(Z)-Dhb-OH (5): Fmoc-Ala-(Z)-Dhb-OH (242 mg, 0.615 mmol, 91.1 % yield). Appearance: White powder; TLC (SiO2, DCM-MeOH 9:1) Rf = 0.19; Analytical HPLC (tR = 20min), DMF (4 x 2mL x 1min). All deprotection solutions were kept for Fmoc monitoring. The Kaiser test was positive after deprotection. The loading, as calculated by Fmoc assay was 1.67 mmol/g, 98.2%.11,27

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Dorsal Root Ganglion Primary Cell Culture Assay:

The neuroactivity of Nobilamide B was assessed using the dorsal root ganglion primary cell culture assay. DRG cells were harvested from the spine of a 21 day-old mouse using a dissecting microscope and the cells were plated and cultured in a 24-well plate. Individual cells were selected and monitored using the Olympus IX50 fluorescence microscope and Nikon camera attached to a computer running the NIS Elements Basic Research software. Intracellular calcium ions were viewed using Fluo-4 fluorescent probe.

Analytical HPLC profile
Nobilamide B

Conditions: C18 reversed-phase column; 5(A):95(B) to 1(A):0(B) over 40 min (A: 0.1% TFA in ACN, B: 0.1% TFA in H2O at 1.0 mL/min flow rate; wavelength detector set at 220 nm)

Co-elution of synthetic and isolated nobilamide B
Conditions: C18 reversed-phase column; 5(A):95(B) to 1(A):0(B) over 120 min (A: ACN, B: 0.5% TFA in H2O at 1.0 mL/min flow rate; wavelength detector set at 220 nm)
Mass Spectrum of nobilamide B

NMR data of Nobilamide B:

$^1$H NMR
$^{13}$C NMR

$^1$H-$^1$H COSY
TOCSY

NOESY