1.Tissue Dissection/Preparation

Adult Strongylocentrotus purpuratus (purple sea urchin) were collected from the wild and obtained from Charles Hollahan, Santa Barbara Marine Biologicals. Animals were anesthetized by the injection of isotonic CaCl2 into the body cavity. Once spine movement was minimized, indicating anesthesia action, the test was equatorially bisected using surgical scissors. The upper and lower portions of the test were then separated, the gonads and internal organs were removed, and the body cavity was rinsed with ice cold artificial sea water consisting of 460 mM NaCl, 10 mM KCl, 10 mM CaCl2, 22 mM MgCl2, 6 mM MgSO4, and 10 mM HEPES adjusted to pH 7.7 with 1 M NaOH. All dissections were performed on ice and as rapidly as possible to limit protein degradation. The radial eater canals covering each radial nerve were removed with forceps and the radial nerve was separated from the surrounding tissues by cutting along the edge of the radial nerve with a scalpel. The radial nerve could then be lifted from the surrounding tissue with a pair of forceps and placed in a centrifuge tube for peptide extraction. Typically 2-3 animals were dissected for each extraction with five radial nerves being collected from each animal.
Collected tissues were homogenized and extracted in acidified acetonitrile (60:40:1 ACN:H2O:TFA) overnight at 4 ºC. Insoluble material was pelleted via centrifugation (5804R, Eppendorf, Hamburg, Germany) for 10 min at 15,000 x g. The supernatant was then removed, dried (Savant SpeedVac, Thermo, Milford, MA) and resuspended in minimal 5% ACN prior to use of a 10 kDa molecular weight cut-off filter to remove large proteins from the sample (Millipore, Billerica, MA). The supernatant was then dried and resuspended in minimal 5% ACN prior to LC separation.

2. 2D LC Q-tof MS/MS
Two-dimensional capillary LC/MS/MS experiments were conducted using an Ultimate 3000 (dionex) coupled to a Q-TOF hybrid quadrupole time-of-flight mass spectrometer (Micro-tofQ, Bruker Daltonics). The sample was dissolved in 25 µl of 5% acetonitrile in water and filtered through a spin-down filter (Millipore, USA). A 20 µl volume of this sample was injected on to a 500 µm x 15 mm Bio-SCX column (Dionex). The strong cation-exchange column was in-line with a reversed-phase C18 precolumn (µ-guard column MGU-30 C18, LC Packings). Sample loading was done at a flow-rate of 30 µl min-1 with water containing 2% acetonitrile and 0.1% formic acid. Loading took 10 min. Next, the SCX column was switched off-line. The reversed-phase precolumn was rinsed for another 5 min, then the reversed-phase trapping column was switched in-line with the capillary column, a 75 µm x 150 m C18 column (Dionex). The first fraction comprised those peptides that did not bind to the SCX column and that were immediately trapped by the reversed-phase column using a gradient from 2 to 50% acetonitrile containing 0.1% formic acid and a flow-rate at 200 nl min-1 in 50 min. The second fraction of peptides was eluted from the SCX column by injection of 20 µl of 20 mM ammonium acetate solution. The eluted peptides were again concentrated and desalted on the C18 precolumn prior to the MS analysis. This cycle was repeated 10 times including a breakthrough cycle (no salt). The successive concentrations of ammonium acetate used were 20, 50, 100, 200, 400, 600, 800, 1000 and 2000 mM.
The LC system was connected in series with the electrospray interface of the Q-TOF device. The column eluate was directed through a stainless-steel emitter (Proteon, Denmark). The needle voltage was set at 1650 V and the cone voltage at 35 V. Nitrogen was used as nebullizing gas. Parent ions with 2 or 3 charges were

automatically selected for fragmentation as they eluted from the column. Argon was used as the collision gas; the collision energy was set at 25-40 eV depending on the mass and charge state of the selected ion. The detection window in the survey scan was set from m/z 400 to 1500. Fragmentation spectra were acquired from m/z 50 to 2500.

3. LC-Maldi

The sample was dissolved in 15 µl of 5% acetonitrile in water and filtered through a spin-down filter (Millipore, USA). Ten microliters of the sample were loaded on the guard column with an isocratic flow of 2% acetonitrile in HPLC grade water, 0.1% formic acid, at a flow rate of 10 xl/min. After 2 min, the column-switching valve was switched, placing the guard column online with the analytical capillary column, a Pepmap C18, 3 µm 75 µm×150 mm nanocolumn (Dionex). Separation was conducted using a linear gradient from 95% solvent A, 5% solvent B to 5% solvent A, 65% solvent B in 35 min (solvent A: water/acetonitrile/formic acid (94.9:5:0.1, v/v/v); solvent B: acetonitrile/formic acid (99.9:0.1, v/v/v)). The flow rate was set at 200 nl/min.

Fractions were collected manually with intervals of 15 sec on a ground steel Maldi target plate (MTP 384 target plate ground steel, Bruker daltonics). Prior to fraction collection the sample plate was coated with a thin layer of x-cyano-4-hydroxycinnamic acid (HCCA). The thin layer was prepared using a 5X dilution of a saturated solution of HCCA in 100% acetone. 0,5 µl of this solution was deposited on each well of the target plate and left to dry. Fractions were collected in the centre of a well and left to dry. Fractions were collected from minute 10 to 35 of the LC separation.

The fractions were analysed in a Bruker Daltonics Ultraflex II. MS spectra for each were acquired in reflectron mode. Spectra were acquired between m/z 750 and 4000. Ions of sufficient intensity were selected manually for Lift MS/MS analysis.