Supporting Information

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SI Materials and Methods: Ghrelin hapten and substrate synthesis

Reagents and Materials. Fmoc amino acids and Boc-Gly were obtained from Novabiochem and the Rink Amide linker was purchased from CS Bio. Aminomethyl-derivatized PS-DVB resin was obtained from Polymer Laboratories at a loading of 1.11 mmol NH_2/g (75–150 μM). Side chain protections were as follows: Lys(Boc, Mtt or Alloc, see below), Arg(Pbf), Gln(Trt), Ser(tBu), Glu(tBu), His(Trt); Ser^3 residue was incorporated without side chain protection. Piperidine was obtained from Sigma–Aldrich, *N,N*-dimethylformamide (BioAnalyzed) was from J. T. Baker (St. Louis, MO), trifluoroacetic acid and dichloromethane (HPLC) were from EMD, 6-Cl-HOBt was from Luxembourg Industries, and *N,N′*-diisopropylcarbodiimide was from Advanced ChemTech. All other reagents, solvents, and chemicals were of the highest purity commercially available and used as received. RP-HPLC was performed using binary gradients of solvents A and B, where A is 0.1% TFA in water and B is 0.09% TFA in acetonitrile. Analytical RP-HPLC was performed using a Vydac 218TP5415 column at a flow rate of 1 ml/min, with detection at 214 nm during a linear gradient of 10–50% B over 30 min. Preparative RP-HPLC was performed using a custom-packed, 2-inch bore C18 column at a flow rate of 15 ml/min, with detection at 220 nm during a linear gradient of 10–50%B over 40 min. In all cases, fractions were analyzed off-line using an ABI/Sciex 150EX single quadrupole mass spectrometer and judged for purity after a consistent summing of 50 scans in multichannel analysis (MCA) mode. For preparative purification purposes, fractions that contained no consistent charged species, which accounted for >10% of the total ion intensity were designated "pure" and pooled; the homogeneity of this pool was verified by analytical RP-HPLC and was >95%. All purification and synthetic manipulation steps were performed at ambient temperature unless otherwise indicated.

Peptide Synthesis. The hapten and substrates were prepared on a 1.0 mmol scale as C-terminal amides using Rink amide AM resin and conventional Fmoc/tBu SPPS protocols (1) with standard DIC/6-Cl-HOBt coupling chemistry (2) on a CS Bio 136 automated peptide synthesizer (CS Bio, Menlo Park, CA). The Ser-3 side-chain acetylation and butyrylation were achieved by treating the partially protected peptide-resin with a DCM solution of 6 mmol of the corresponding acid anhydride/pyridine (1:1 vol/vol) for 16 hrs. The Ser-3 octanoyl- and palmitoyl-substrates were prepared by pre-activating 6 mmol of the requisite acid (octanoic or palmitic acid) with 6 mmol PyBrOP/12 mmol DIEA in 10 ml of DCM for 20 min (3), after which the resulting clear solution was added to the partially protected peptide-resin, followed by 0.5 mmol DMAP, and mixed by orbital shaking overnight. Test TFA cleavages were used to verify quantitative acylation, and the esterification step was repeated if necessary. For Lys(Mca) conjugations, the peptide-resin was shaken with 0.1 eq. $Pd(PPh_3)_4$ and 20 eq phenylsilane in DCM (2 × 30 min).(4) The newly unmasked free-amine was then neutralized by treatment with 5% DIEA/DMF, after which 7-methoxycoumarinyl-4-acetic acid was coupled according to standard DIC/HOBt protocols.

For *n*-butylphosphonic acid conjugations, any attempts to couple *n*-butylphosphonic dichloride onto the Ser-3 side chain, followed by on-resin alkaline hydrolysis of the remaining chlorophosphonate yielded an uncharacterizable mixture and therefore motivated us to explore an alternative synthetic approach for the installation of the key phosphonate moiety. Hypothesizing that these problems stemmed from either the extraordinarily high reactivity of the *n*-butylphosphonic dichloride acylating species, the need for on-resin alkaline hydrolysis of the chlorophosphonate intermediate, or a combination thereof, we prepared *n*-butylphosphonic acid in solution (via hydrolysis of the commercially available phosphonic dichloride) and coupled this derivative to the Ser-3 side chain via DIC/DMAP chemistry. This approach allowed for quantitative acylation to be achieved after two couplings. The optimized procedure involves preactivation of 5 mmol *n*-butylphosphonic acid with 5 mmol DIC in 10 ml of DCM for 20 min, after which 10 ml of DMF was added. The resulting slurry was added to the protected peptide-resin, followed by 0.5 mmol DMAP, and the entire procedure performed twice to achieve quantitative *n*-butylphosphonylation.

After washing with DMF, DCM, methanol, and diethyl ether (2 x each), the completed peptide-resins were dried in vacuo overnight. Global deprotection and cleavage were accomplished by treatment of the peptide-resin with 95:2.5:2.5 TFA:water:triisopropylsilane (TIPS) for 2 hrs, followed by filtration and removal of TFA by rotary evaporation. Peptides were then triturated from 10 volumes of Et_2O (pre-chilled at −20°C) and isolated by centrifugation. After likewise washing the pellet two times further with Et_2O, peptides were extracted into 50% AcOH and purified by RP-HPLC directly.

Ser-3(octanoyl) rat ghrelin amide. ESI-MS: Calculated, $M+H^+$ = 3314.9; $M+2H^+$ = 1657.9, $M+3H^+$ = 1105.6, $M+4H^+$ = 829.5. Observed, $M+2H^+$ = 1657.9, $M+3H^+$ = 1105.8, $M+4H^+$ = 829.3.

Ghrelin(1–5) Ser-3(butylphosphonyl) TS analog 1. ESI-MS: Calculated, FW = 628.3; $M+H^+$ = 629.3. Observed, $M+H^+$ = 629.5.

Ghrelin(1–5) Ser-3(butylphosphonyl) TSA hapten, 2. ESI-MS: Calculated, FW = 953.4; $M+H^+$ = 954.4. Observed, $M+H^+$ = 954.2.

Mca-labeled rat ghrelin(1–28) Ser-3(octanoyl) substrate, 3. ESI-MS: Calculated, $M+H^+$ = 3804.1; $M+2H^+$ = 1902.5, $M+3H^+$ = 1268.7, $M+4H^+$ = 951.8, $M+5H^+$ = 761.6. Observed, $M+2H^+$ = 1902.5, $M+3H^+$ = 1269.0, $M+4H^+$ = 952.0, $M+5H^+$ = 762.0.

Mca-labeled rat ghrelin(1–28) Ser-3-OH substrate, 4. ESI-MS: Calculated, $M+H^+$ = 3678.0; $M+2H^+$ = 1839.5, $M+3H^+$ = 1226.7. Observed, $M+2H^+$ = 1839.6, $M+3H^+$ = 1226.7.

Mca-labeled rat ghrelin(1–28) Ser-3(butyryl) substrate, 5. ESI-MS: Calculated, $M+H^+$ = 3748.0; $M+2H^+$ = 1874.5, $M+3H^+$ = 1250.0, $M+4H^+$ = 937.8, $M+5H^+$ = 750.4. Observed, $M+2H^+$ = 1875.0, $M+3H^+$ = 1250.0, $M+4H^+$ = 938.0, $M+5H^+$ = 751.0.

Mca-labeled rat ghrelin(1–28) Ser-3(acetyl) substrate, 6. ESI-MS: Calculated, $M+H^+$ = 3720.0; $M+2H^+$ = 1860.5, $M+3H^+$ = 1240.7, $M+4H^+$ = 930.8, $M+5H^+$ = 744.8. Observed, $M+2H^+$ = 1862.0, $M+3H^+$ = 1241.0, $M+4H^+$ = 931.0, $M+5H^+$ = 745.0.

Mca-labeled rat ghrelin(1–28) Ser-3(palmitoyl) substrate, 7. ESI-MS: Calculated, $M+H^+$ = 3916.2; $M+2H^+$ = 1958.6, $M+3H^+$ = 1306.1, $M+4H^+$ = 979.8, $M+5H^+$ = 784.0. Observed, $M+2H^+$ = 1959.0, $M+3H^+$ = 1306.0, $M+4H^+$ = 980.0, $M+5H^+$ = 785.0.

Mca-labeled Ala^4-rat ghrelin(1–28) Ser-3(octanoyl) substrate, 8. ESI-MS: Calculated, $M+H^+$ = 3728.1; $M+2H^+$ = 1864.5, $M+3H^+$ =

1243.4, $M+4H^+$ = 932.8, $M+5H^+$ = 746.4. Observed, $M+2H^+$ = 1865.0, $M+3H^+$ = 1244.0, $M+4H^+$ = 933.0, $M+5H^+$ = 747.0.

Mca-labeled Gly^2-rat ghrelin(1–28) Ser-3(octanoyl) substrate, 9. ESI-MS: Calculated, $M+H^+$ = 3774.1; $M+2H^+$ = 1887.5, $M+3H^+$ = 1258.7, $M+4H^+$ = 944.3, $M+5H^+$ = 755.6. Observed, $M+2H^+$ = 1888.0, $M+3H^+$ = 1259.0, $M+4H^+$ = 945.0, $M+5H^+$ = 756.0.

Mca-labeled Met^1,Gly^2-rat ghrelin(1–28) Ser-3(octanoyl) substrate, 10. ESI-MS: Calculated, $M+H^+$ = 3848.1; $M+2H^+$ = 1924.6, $M+3H^+$ = 1283.4, $M+4H^+$ = 962.8, $M+5H^+$ = 770.4. Observed, $M+2H^+$ = 1925.0, $M+3H^+$ = 1284.0, $M+4H^+$ = 963.0, $M+5H^+$ = 771.0.

Antibody Catalysis. Selection of monoclonal antibodies (mAbs) that catalyze ghrelin hydrolysis was performed by detection of *des*-octanoyl ghrelin formation upon incubation of native rat ghrelin with each member of a panel of 19 mAbs (10 μM) at 37 °C in phosphate buffered saline (0.5 ml), pH 7.4, for 10 h, using the analytical HPLC method. Reactions were quenched by dilution of 100 μL of sample with 100 μL of 50% AcOH in water, prior to HPLC analysis. Further characterization of selected catalytic antibodies was performed by incubation of the selected mAb (1 μM) with varying concentrations of methoxycoumarin-modified ghrelin analogs **3** and **5** (at 0.4, 0.8, 1.6, 3.1, 6.2, 12.5, 25, and 50 μM) for 5 h at 37 °C in phosphate buffered saline, pH 7.4. The extent of background hydrolysis of ghrelin substrates was evaluated by incubation of the of methoxycoumarin-modified ghrelin substrates in pH 7.4 PBS buffer for 5 h at 37 °C in the absence of mAb. Formation of product **4** was monitored by RP HPLC, with detection at 324 nm. Inhibition of catalysis by TS analog **2** was measured by incubation of **3** (400 μM) and mAb GHR-11E11 (1 μM) in the presence of varying concentrations of **2** (0 to 200 μM) for 5 h. The kinetic experiments were performed in triplicates, and the results were analyzed with Grafit 5.0 software. The chemical specificity of GHR-11E11 antibody was determined by incubation of the GHR-11E11 (1 μM) with 16 μM methoxycoumarin-modified ghrelin substrates **3**, **5–10** for 5 h at 37 °C in phosphate buffered saline, pH 7.4, followed by quenching of the hydrolysis by dilution of 100 μL of sample with 100 μL of 50% AcOH in water, and subsequent RP HPLC analysis of the resulting mixture. The extent of background hydrolysis of ghrelin substrates was evaluated by incubation of the of methoxycoumarin-modified ghrelin substrates **3**, **5–10** (16 μM) for 5 h at 37 °C in phosphate buffered saline, pH 7.4, in the absence of catalytic antibody, followed by HPLC analysis of the sample. The difference between the extent of catalyzed and uncatalyzed hydrolysis of the ghrelin substrates provided the basis for determining of the specificity of GHR-11E11. The ability of phenylmethylsulfonyl fluoride (PMSF) serine esterase inhibitor to arrest the catalytic properties of GHR-11E11 was determined by conducting the catalytic hydrolysis experiments using the methoxycoumarin-modified octanoyl-ghrelin substrate **3** in the presence of 5 μM PMSF.

Subjects. Mature male C57BL/6J mice (n = 17, The Jackson Laboratory) were group-housed in a 12 h:12 h lit (0600 hours lights on), humidity- (60%) and temperature-controlled (22°C) vivarium with continuous access to chow (LM-485 Diet 7012; Harlan Teklad) and water unless mentioned otherwise. After surgical implantation of jugular catheters under isoflurane anesthesia (1–3% in oxygen), mice were allowed to recover for at least 1 week and then acclimated to indirect calorimetry chambers (below). Procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication number 85–23, revised 1996) and the "Principles of laboratory animal care" (www.nap.edu/readingroom/bookslabrats) and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Metabolic and Food Intake Study. Indirect calorimetry was performed as described in ref. 5 in acclimated (>72 hr), singly-housed, mice using a computer-controlled, open-circuit system (Oxymax System) that was part of an integrated Comprehensive Lab Animal Monitoring System (CLAMS). Subjects were tested in two treatment-balanced cohorts in an apparatus containing 16 clear respiratory chambers (20 × 10 × 12.5 cm) with a stainless-steel elevated wire floor. Each of these chambers was equipped with a sipper tube delivering water, food tray connected to a balance, and 16 photobeams at 1.3 cm intervals situated in rows 3.3 and 7.3 cm above the floor of the cage to detect motor activity along the x and z axes, respectively. Room air was passed through chambers at a flow rate of ≈0.5 L/min. Exhaust air from each chamber was sampled at 16-min intervals for 1 min. Sample air was sequentially passed through O_2 and CO_2 sensors (Columbus Instruments) for determination of O_2 and CO_2 content, from which measures of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were estimated. Outdoor air reference values were sampled after every 8 measurements. Gas sensors were calibrated prior to the onset of experiments with primary gas standards containing known concentrations of O_2, CO_2, and N_2 (Airgas Puritan Medical). Respiratory exchange ratio (RER) was calculated as the ratio of carbon dioxide production (VCO_2) to oxygen consumption (VO_2). Energy expenditure measures (VO_2, VCO_2 and heat formation [$(3.815 + 1.232 \times \mathrm{RER}) \times VO_2$ (in liters)]) were corrected for estimated effective metabolic mass per Kleiber's power function (6). Chambers had photobeams to assess locomotor activity. Data were recorded under ambient room temperature (≈24–26 °C).

Statistics. ANOVA was used to identify group differences. Fisher's protected LSD tests were used for *posthoc* comparisons. The software package was Systat 11.0 (SPSS).

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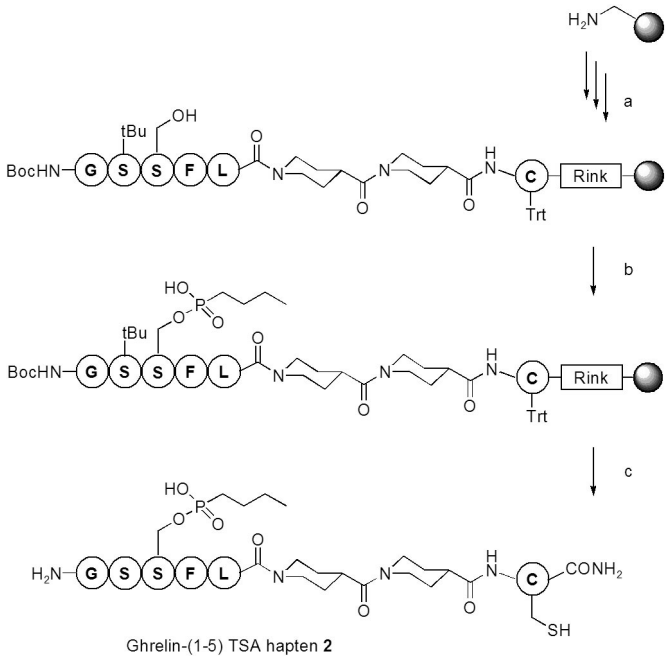

Fig. S1. Solid-phase synthesis of ghrelin TSA hapten 2. a) Fmoc/*t*Bu SPPS (DIC/HOBt, HOAt for Isn residues); b) *n*-butyl phosphonic acid, DIC/DMAP; c) TFA/water/triisopropylsilane (95/2.5/2.5).

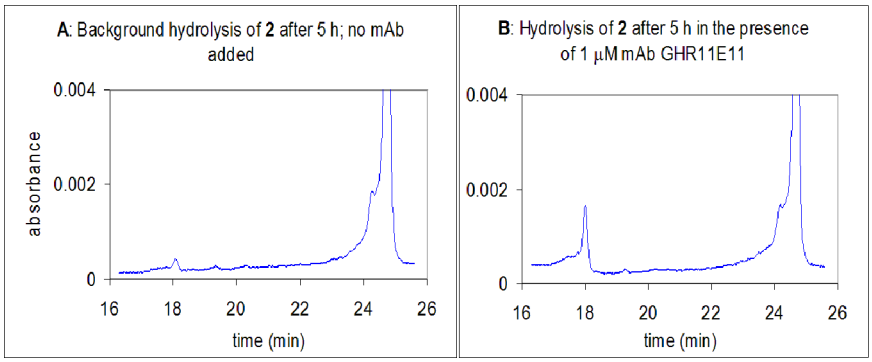

Fig. S2. HPLC analysis of ghrelin hydrolysis. (*A*) After 5 h incubation of 16 μM **3** in PBS, pH 7.4, at 37°C (0.8 nmol injected). (*B*) After 5 h incubation of 16 μM **3** in PBS, pH 7.4, at 37°C in the presence of 1 μM mAb GHR-11E11. Ghrelin analog **3** and its *des*-octanoyl form **4** elute at 24.5 and 18 min, respectively.