Electronic Supplementary Information

Self-assembling bisphosphonates into nanofibers to enhance their inhibitory capacity on bone resorption

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1. General methods

Synthesis and characterization are in the online Electronic Supplementary Information. All the starting materials were obtained from GL Biochem or Accela ChemBio. Commercially available reagents were used without further purification, unless noted otherwise. All other chemicals were reagent grade or better. The Alpha modification (α-MEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco-BRL (Gaithersburg, MD, USA). The cell counting kit-8 (CCK-8) was obtained from Dojindo Molecular Technology (Tokyo,Japan). Soluble mouse M-CSF and RANKL were obtained from peprotech. The tartrate-resistant acid phosphatase (TRAP) staining kit and all other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). Total RNA from osteoclasts or bone tissue was extracted using the QiagenRNeasy® Mini kit (Qiagen, Valencia, CA, USA), and subjected to cDNA synthesis. HPLC analyses were performed on a Shimadzu UFLC system equipped with two LC-20AP pumps and an SPD-20A UV/vis detector using a Shimadzu PRC-ODS column, or on an Agilent 1200 HPLC system equipped with a G1322A pump and an in-line diode array UV detector using an Agilent Zorbax 300SB-C_{18} RP column, with CH_3CN (0.1% of TFA) and ultrapure water (0.1% of TFA) as the eluent. The spectra of electrospray ionization-mass spectrometry (ESI-MS) were recorded on a LCQ Advantage MAX ion trap mass spectrometer (Thermo Fisher). ^{1}H NMR and ^{13}C NMR spectra were obtained on a 300 MHz Bruker AV 300. Rheology test was done on a Haake Rheo Stress 6000 (Thermo Scientific), with cone-and-plate geometry (1°/20 mm) at the gap of 370 μm. Transmission electron micrographs (TEM) were obtained on a JEOL 2100 high resolution transmission electron microscope, operating at 200 kV. The samples were prepared as following: a copper grid coated with carbon was dipped into the suspension and placed into a culture dish. Real-time PCR was performed using the SYBR Premix Ex Tag kit (TaKaRa Biotechnology, Otsu, Japan) and an ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufactures' protocols.

Cell culture

Bone marrow derived monocytes/macrophages (BMMs) were isolated from the bone marrow of C57BL/6 mice and cultured in a T-75 flask, supplemented with α-MEM containing 10% FBS, 1% penicillin/streptomycin and 30 ng/mL of macrophage colony-stimulating factor (M-CSF) in a humidified atmosphere of 5% CO_2 at 37 °C for 4 days.

Cell viability assay

The effect of compounds on cell viability was assessed by Cell Counting Kit-8 (CCK-8) assay. Generally, CCK-8 reagent was added to each well for 1 hour before the endpoint of incubation. The optical density (OD) at 450 nm in each well was determined by a microplate reader. Experiments were repeated at least three times with each time in triplicate.

In vitro osteoclastogenesis assay

The BMMs were seeded in 96-well plate at a density of 8000 BMMs/well in the presence of 30 ng/mL of M-CSF, 100 ng/mL of RANKL and different concentrations of compounds as indicated. In addition, the

cells were also treated with different doses of compounds at day 1 (early stage) or day 3 (late stage) during osteoclastogenesis. Fresh cell culture medium was replaced every 2 days until mature osteoclasts formed. For TRAP staining, the cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min and stained using the TRAP kit. TRAP-positive cells with more than three nuclei were determined as osteoclasts.

RNA extraction and quantitative PCR assay

The sequences for the relevant primers are as followings: cathepsin K forward, 5′-CTTCCAATACGTGCAGCAGA-3′; cathepsin K reverse, 5′-TCTTCAGGGCTTTCTCGTTC-3′; TRAP forward, 5′-CTGGAGTGCACGATGCCAGCGACA-3′; TRAP reverse, 5′-TCCGTGCTCGGCGATGGACCAGA-3′; V-ATPase-d2 forward, 5′-AAGCCTTTGTTTGACGCTGT-3′; V-ATPase-d2 reverse, 5′-TTCGATGCCTCTGTGAGATG-3′; V-ATPase-a3 forward, 5′-AATCATGGACGACTCCTTGG-3′; V-ATPase-a3 reverse, 5′-GGCCACCTCTTCACTCCGGAA-3′; NFATc1 forward, 5′-CCGTTGCTTCCAGAAAATAACA-3′; NFATc1 reverse, 5′-TGTGGGATGTGAACTCGGAA-3′; GAPDH forward, 5′-ACCCAGAAGACTGTGGATGG-3′; GAPDH reverse, 5′-CACATTGGGGGTAGGAACAC-3′.All reactions were run in triplicate. Bax: Forward, 5’- TCATGAAGACAGGGGCCTTT-3’; Reverse: 5’-GTCCACGTCAGCAATCATCC-3’; Bcl2: Forward, 5’- CTTCAGGGATGGGGTGAACT-3’; Reverse: 5’- CAGCCTCCGTTATCCTGGAT-3’.

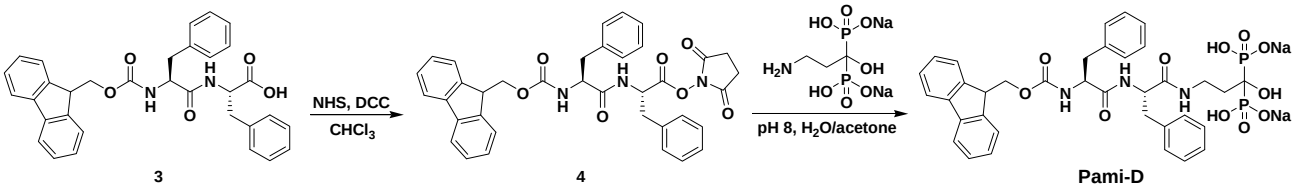
***In vitro* bone absorption assay**

BMMs were seeded at a density of 8×10^3 cells/well on bovine bone slices in 96-well plate, supplemented with 30 ng/mL of M-CSF, 100 ng/mL of RANKL and different doses of compounds as indicated. When mature osteoclast formed, adherent cells were removed from the bone slices. Bone resorption pits were imaged using a scanning electron microscope (SEM, FEI Quanta 250; FEI, Hillsboro, OR, USA) and the percentage of bone resorption area was quantified using Image J software.

2. Synthesis and Characterizations

The preparations of Fmoc-Phe-Phe-OH were synthesized following the literature method (Bernadett Bacsa & C Oliver Kappe. Rapid solid-phase synthesis of a calmodulin-binding peptide using controlled microwave irradiation. *Nat. Protoc.*, **2007**, 2(9), 2222-2227). The preparations of Fmoc-Phe-Phe-Pamidronate (**Pami-D**) and Fmoc-Phe-Phe-Alendronate (**Alen-D**) were synthesized following the literature method (Y. Gao, Y. Kuang, et al. Enzyme-instructed molecular self-assembly confers nanofibers and a supramolecular hydrogel of taxol derivative. *J. Am. Chem. Soc.*, **2009**, 131(38), 13576-13577).

Scheme S1. Synthetic route for **Pami-D**.



Characterizations of Fmoc-Phe-Phe-Pamidronate: ^{1}H NMR of **Pami-D** (300 MHz, DMSO-d_6) δ (ppm): 8.50-8.26 (d, 1 H), 8.12-8.02 (d, 1 H), 7.93-7.84 (d, 2 H), 7.67-7.58 (t, 2 H), 7.45-7.38 (t, 2 H), 7.26-7.17 (m, 10 H), 4.66-4.38 (m,1 H), 4.29-4.16 (m, 2 H), 4.16-4.05(m, 2 H), 3.08-2.89 (t, 2 H), 2.89-2.73 (m, 2 H), 2.73-2.56 (m, 2 H), 2.10-1.94 (m, 2 H). ^{13}C NMR of **Pami-D** (75 MHz, DMSO-d_6) δ (ppm): 171.64, 170.97, 156.12, 144.23, 144.16, 141.11 (2 C), 138.58, 138.06, 129.70 (4 C), 128.49 (2 C), 128.09 (2 C), 127.56 (2 C), 126.74 (2 C), 125.83 (2 C), 125.71 (2 C), 120.52 (2 C), 71.97, 66.18, 56.57, 54.46, 47.10, 38.58, 37.90, 34.96, 33.47. MS: calculated for $C_{36}H_{38}N_3O_{11}P_2$ [$(M-H)^-$]: 750.20, obsvd. ESI-MS: *m*/*z* 750.20.

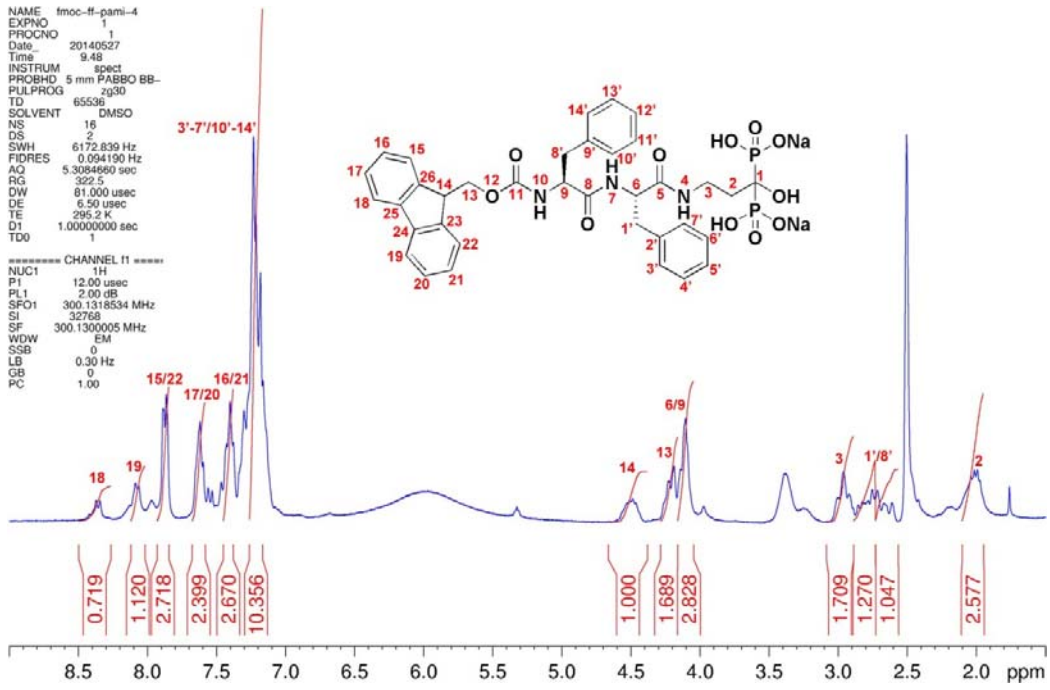


Figure S1. ^{1}H NMR spectrum of **Pami-D**.

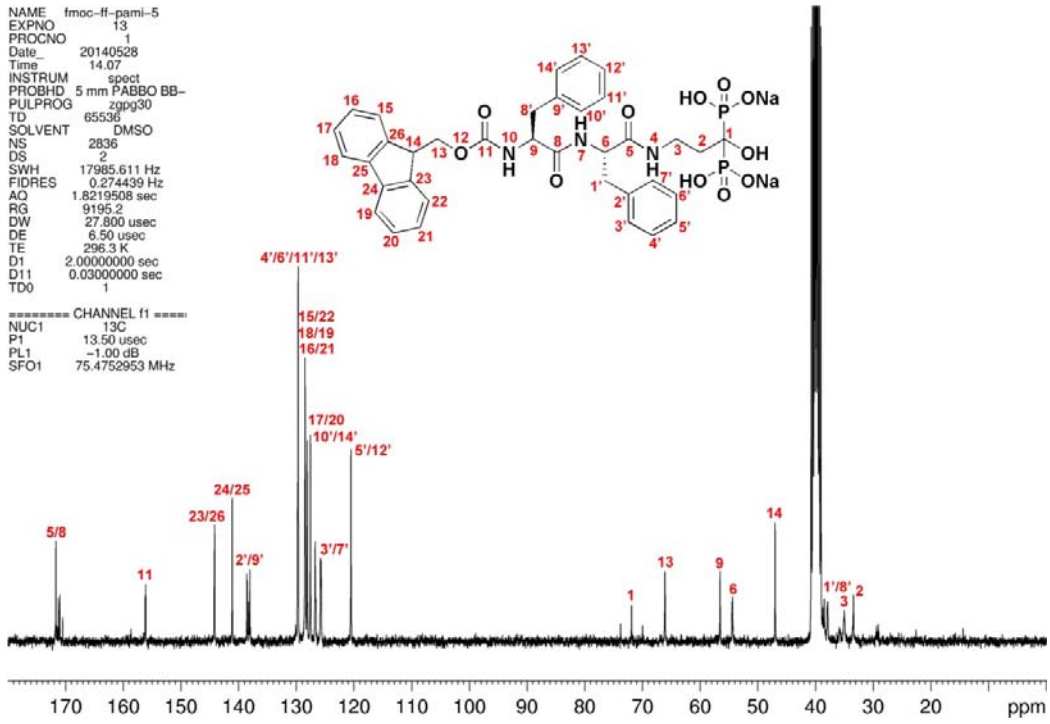
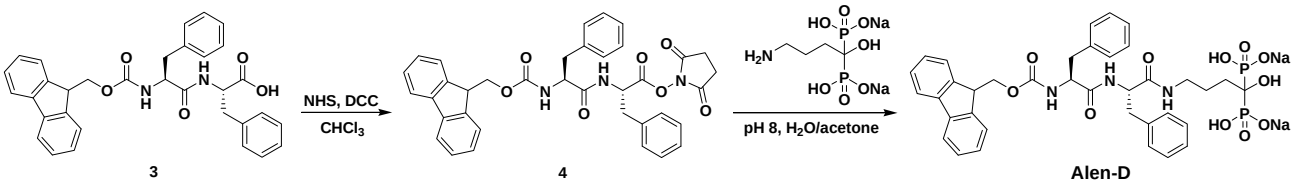


Figure S2. ^{13}C NMR spectrum of **Pami-D**.

Scheme S2. Synthetic route for **Alen-D**.



Characterizations of Fmoc-Phe-Phe-Alendronate: ^{1}H NMR of **Alen-D** (300 MHz, DMSO-d_6) δ (ppm): 8.49-8.33 (d, 1 H), 8.12-8.04 (d, 1 H), 7.98-7.84 (d, 2 H), 7.73-7.60 (t, 2 H), 7.53-7.39 (t, 2 H), 7.37-7.13 (m, 10 H), 4.74-4.44 (m, 1 H), 4.31-4.16 (m, 2 H), 4.15-4.07 (m, 2 H), 3.13-2.96 (m, 2 H), 2.96-2.78 (m, 2 H), 2.78-2.57 (m, 2 H), 2.04-1.83 (m, 2 H), 1.83-1.76 (m, 2 H). ^{13}C NMR of **Alen-D** (75 MHz, DMSO-d_6) δ (ppm): 171.13, 170.75, 155.72, 143.70, 143.64, 140.60 (2 C), 138.08, 137.77, 129.19 (4 C), 127.98 (2 C), 127.60 (2 C), 127.04 (2 C), 126.22 (2 C), 125.29 (2 C), 125.22 (2 C), 120.02 (2 C), 72.17, 65.60, 56.04, 53.80, 46.48, 38.07, 37.40, 31.22, 28.98, 23.55. MS: calculated for $C_{37}H_{42}N_3O_{11}P_2$ [$(M+H)^+$]: 766.23, obsvd. ESI-MS: *m*/*z*766.12.

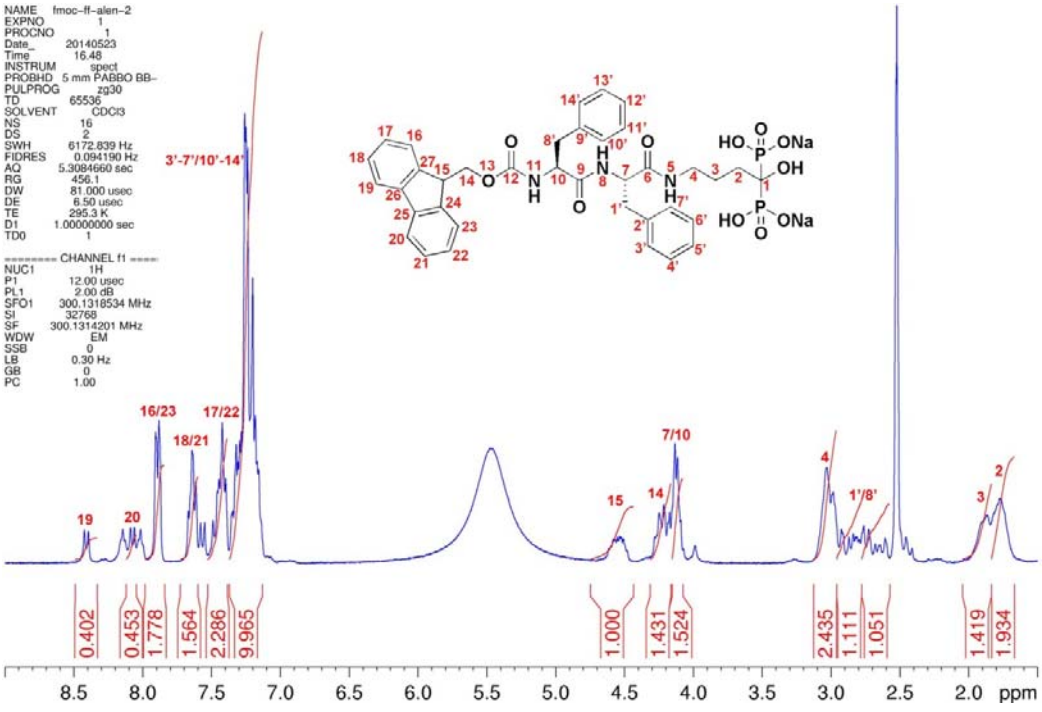


Figure S3. ^{1}H NMR spectrum of **Alen-D**.

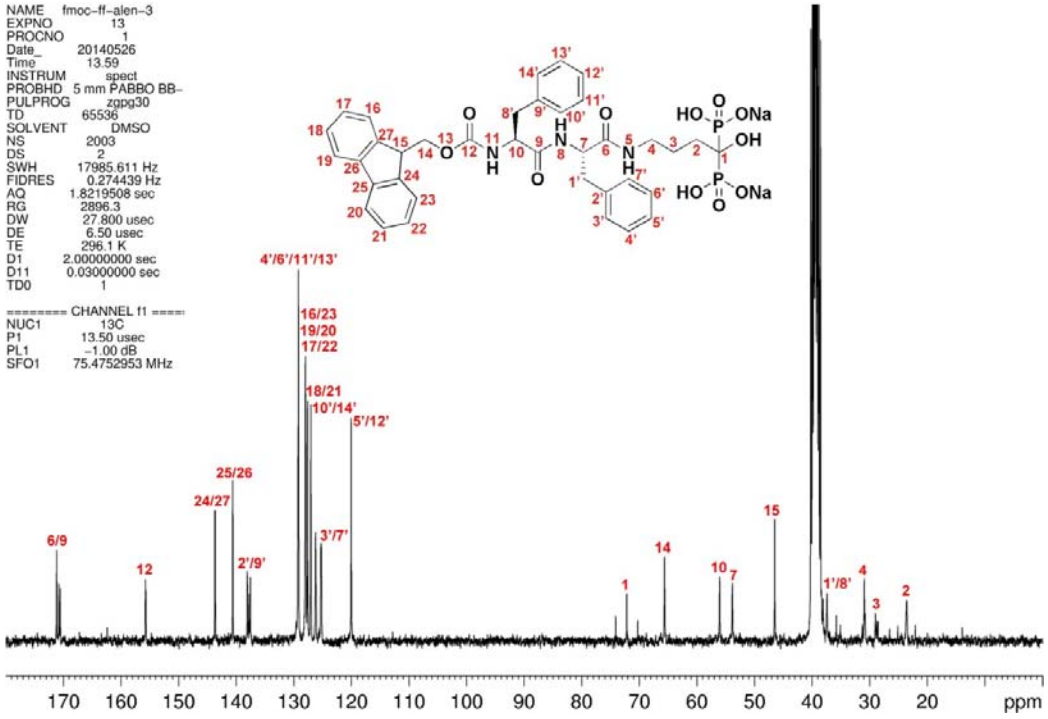


Figure S4. ^{13}C NMR spectrum of **Alen-D**.

3. Supporting Figures and Table

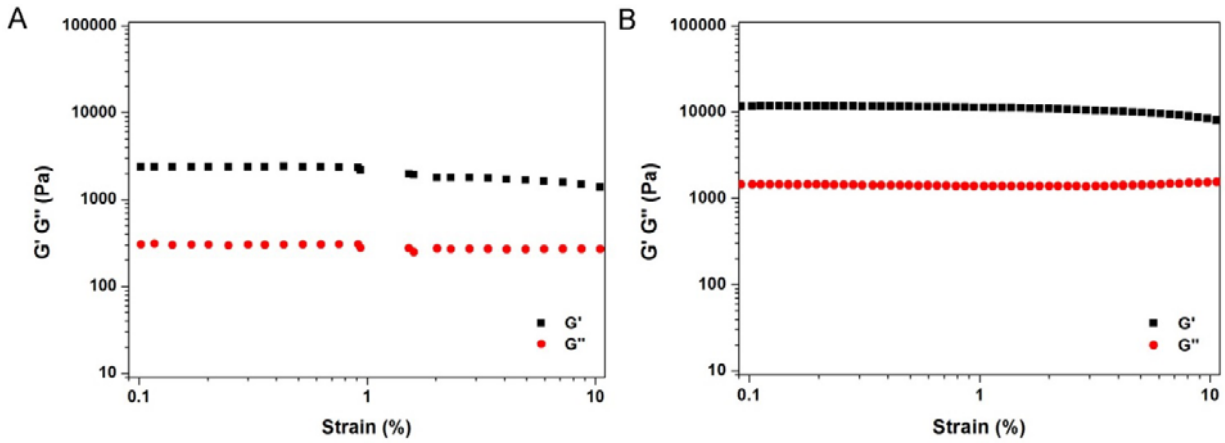


Figure S5. Strain dependence of the dynamic storage moduli (G') and the loss moduli (G") of hydrogels of **Pami-D** (A) and **Alen-D** (B).

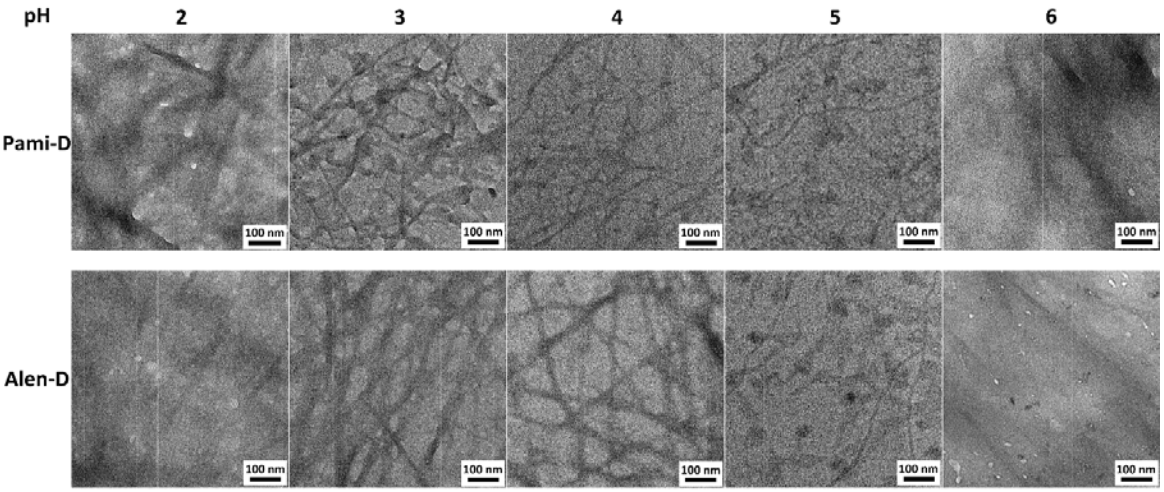


Figure S6. TEM images of 1.0 wt% hydrogelators in water at differernt pH values.

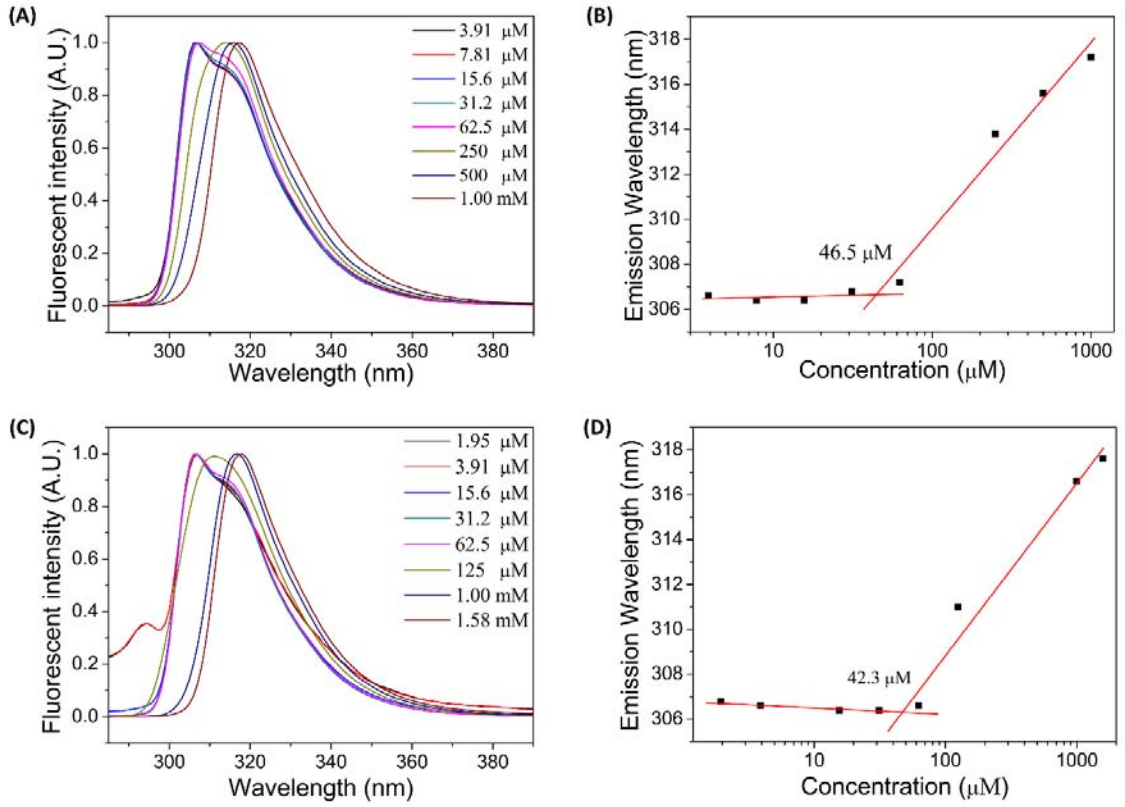


Figure S7. (A) Normalized fluorescence spectra of dilutions of **Pami-D** at different concentrations. Excitation: 265 nm. (B) Gelator concentration-dependent fluorescence emission maximum of dilutions of **Pami-D**. Excitation: 265 nm. (C) Normalized fluorescence spectra of dilutions of **Alen-D** at different concentrations. Excitation: 265 nm. (D) Gelator concentration-dependent fluorescence emission maximum of dilutions of **Alen-D**. Excitation: 265 nm.

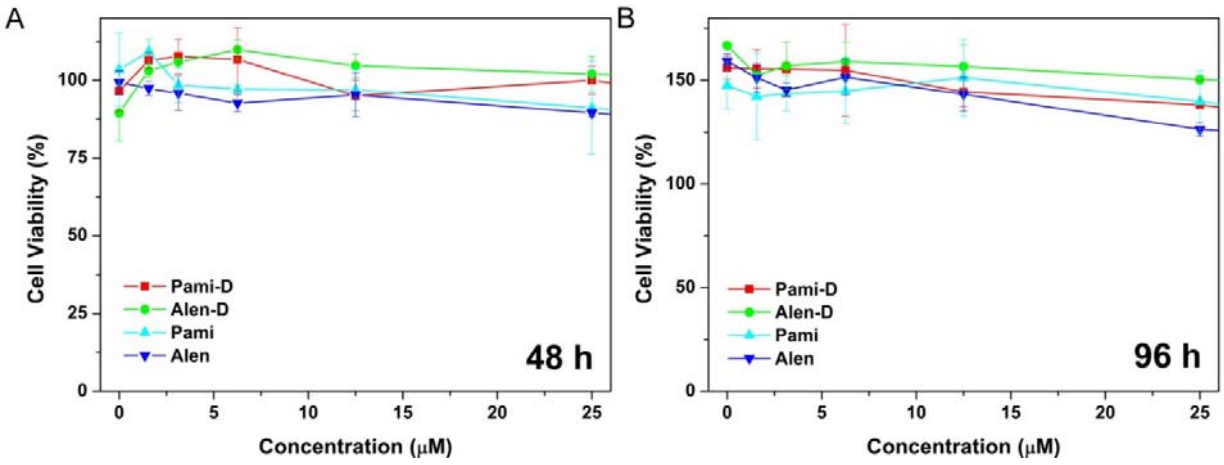


Figure S8. The effect of **Pami-D**, **Alen-D**, Pami, and Alen on osteoblasts (OBs) viability for 48 hours' treatment (A) and 96 hours' treatment (B).

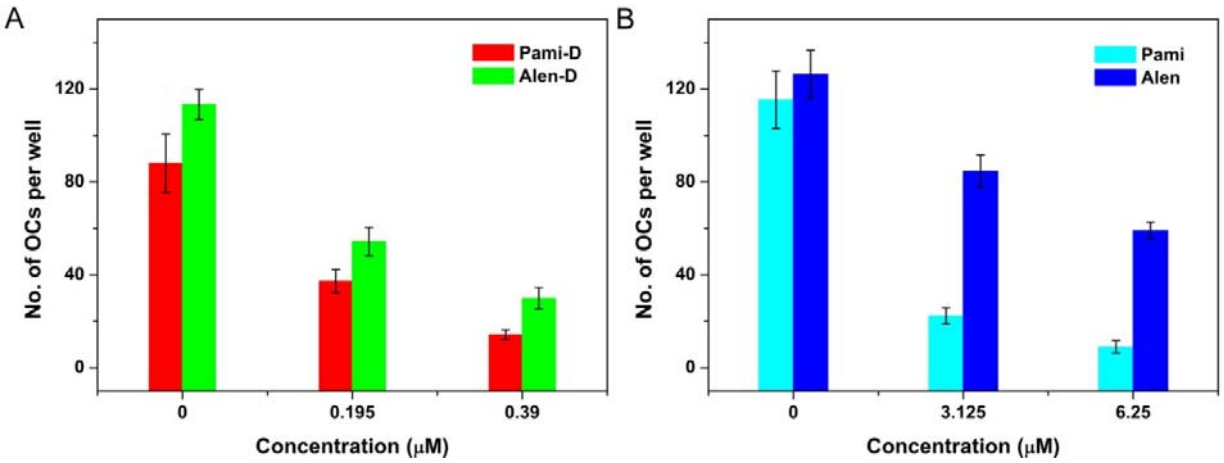


Figure S9. (A) The inhibitory effect of **Pami-D** and **Alen-D** on BMMs-derived osteoclast formation at the indicated concentrations. (B) The number of TRAP-positive osteoclasts after being treated with Pami or Alen at the indicated concentrations.

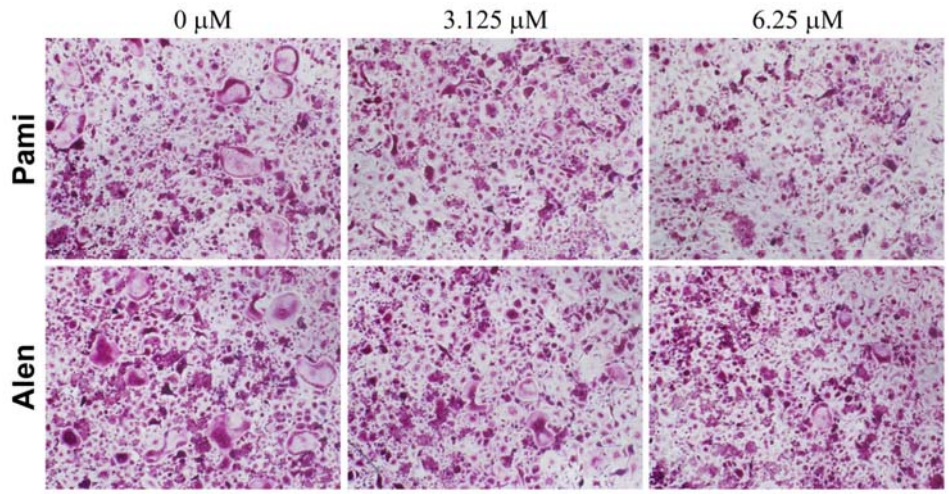


Figure S10. The inhibitory effect of Pami or Alen on osteoclastogenesis at the indicated concentrations.

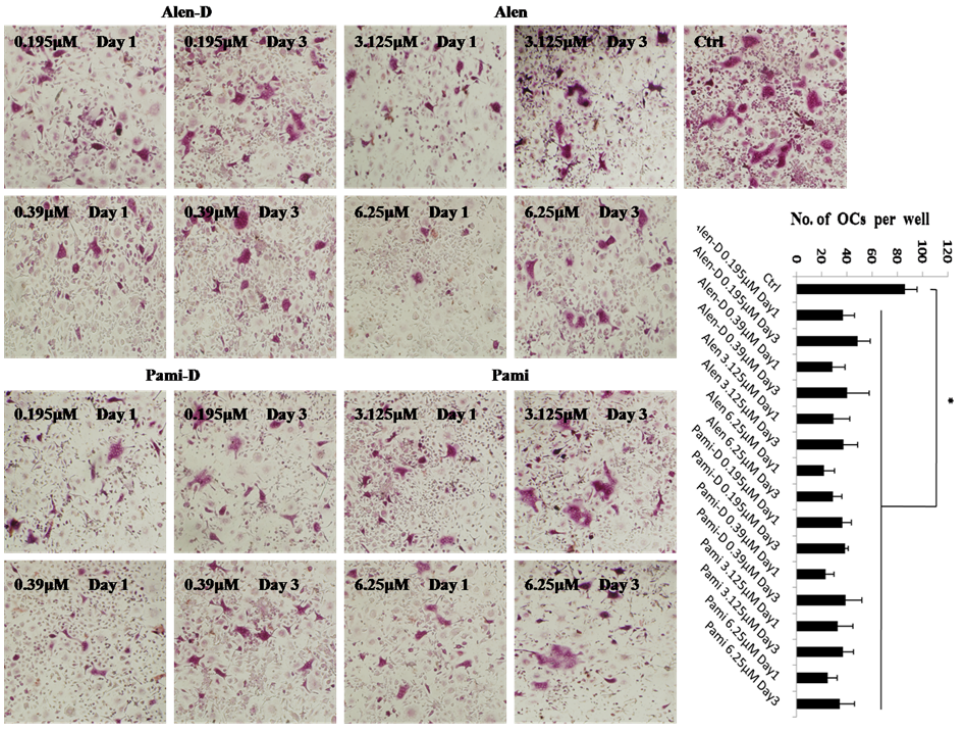


Figure S11. The effect of **Pami-D**, **Alen-D**, Pami, and Alen on osteoclast formation when the compounds were added at day 1 or day 3 during osteoclastogenesis.

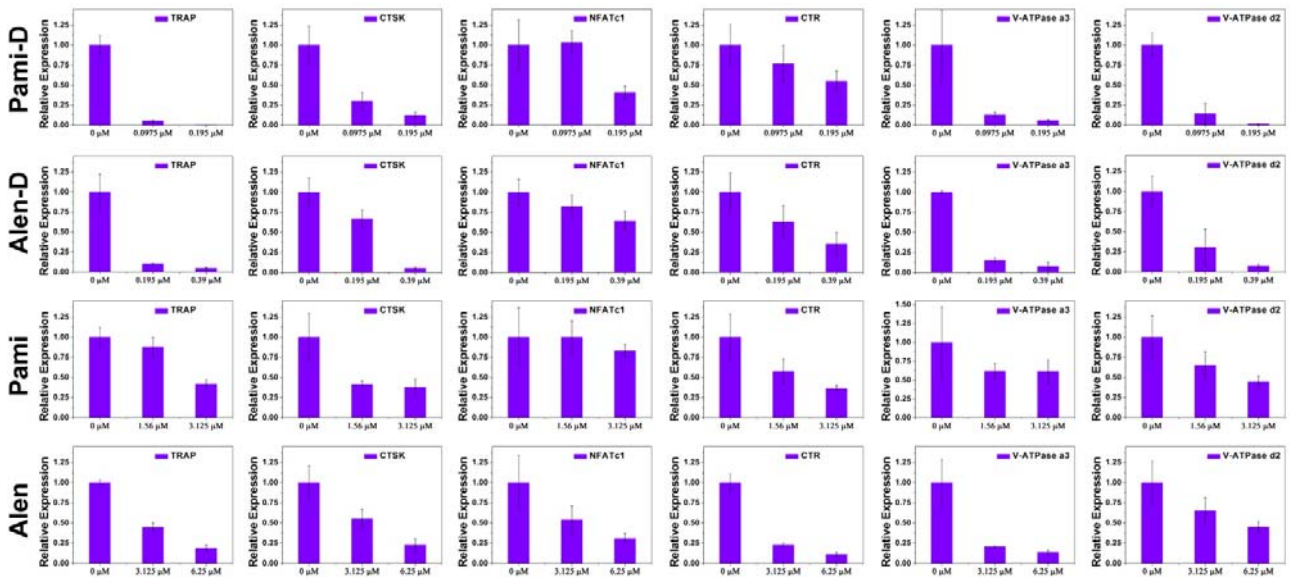


Figure S12. The effect of **Pami-D** (first row), **Alen-D** (second row), Pami (third row), and Alen (fourth row) on osteoclastic specific gene expression at the indicated concentrations.

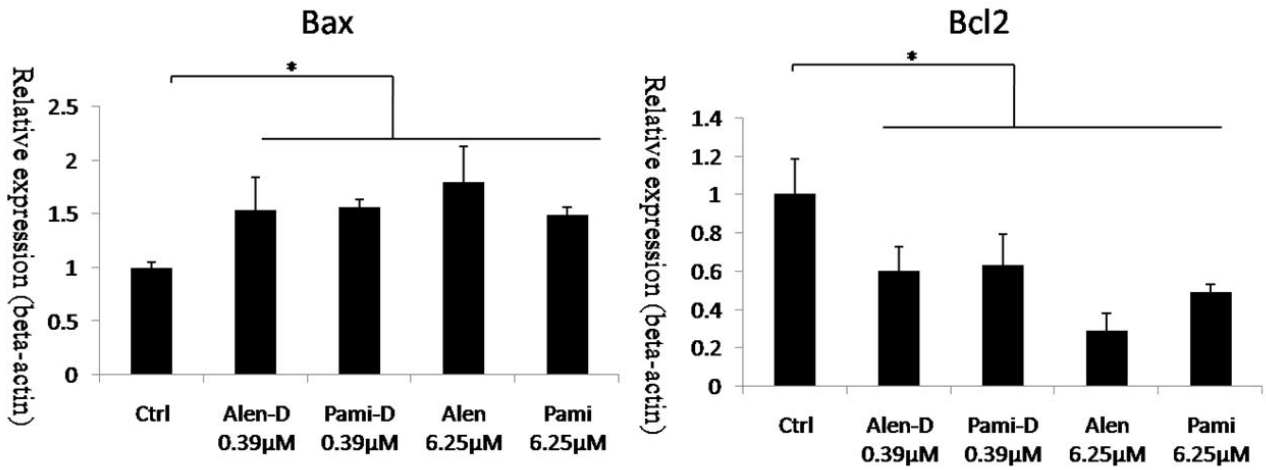


Figure S13. The expression of bcl2-associated X protein and B-cell chronic lymphocytic leukemia/lymphoma 2 in osteoclasts treated with the compounds at the indicated concentrations.

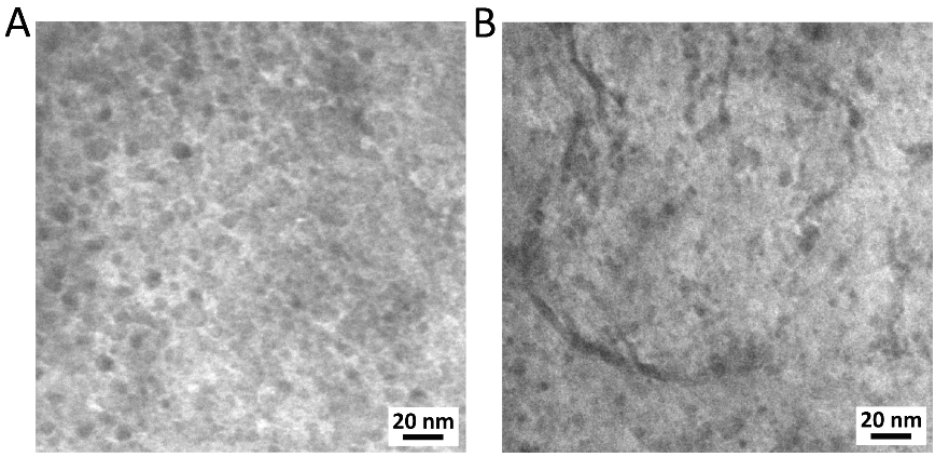


Figure S14. (A) TEM image of osteoclasts (OCs). (B) TEM image of the nanofibers formed on the surface of (or in) OCs after incubated with 200 μM **Pami-D** for 2 h at 37 ºC.

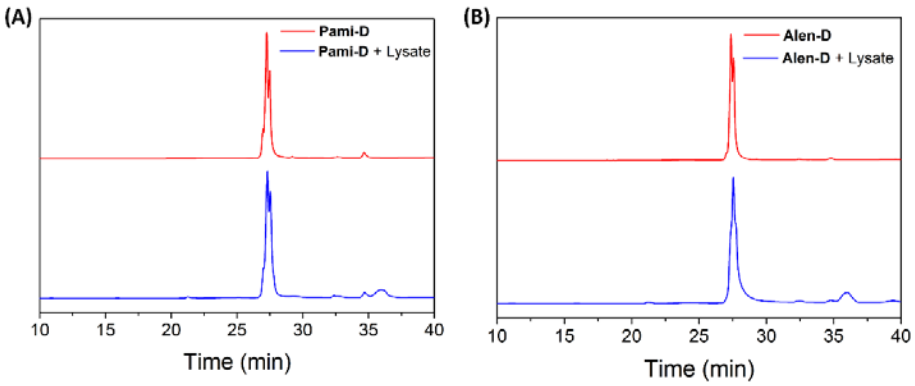


Figure S15. (A) The HPLC traces of **Pami-D**, 1.0 mM **Pami-D** after incubation with 100 μL HeLa cell lysate (2.34×10^6 cells) for 4 h at 37 ºC. (B) The HPLC traces of **Alan-D**, 1.0 mM **Alan-D** after incubation with 100 μL HeLa cell lysate (2.34×10^6 cells) for 4 h at 37 ºC. Absorbance wavelength: 254 nm.

Table S1. HPLC condition for the purification of **Pami-D** and **Alen-D**.

Time (minute)	Flow (mL/min.)	H_2O %	CH_3CN %
0	12.0	60	40
3	12.0	60	40
35	12.0	10	90
37	12.0	10	90
38	12.0	60	40
40	12.0	60	40

Table S2. HPLC condition for Figure S15.

Time (minute)	Flow (mL/min.)	H_2O %	CH_3CN %
0	3.0	90	10
3	3.0	90	10
35	3.0	30	70
37	3.0	30	70
38	3.0	90	10
40	3.0	90	10