Supporting Information

Kawatani *et al.* 10.1073/pnas.0712239105

SI Methods

Reagents and Proteins. The following were used: naphthol AS-MX phosphate, fast red violet LB salt, toluidine blue O, GGOH, mevastatin, Alizarin red-S, butylated hydroxytoluene, FPP, and *Saccharomyces cerevisiae* GLO1 (Sigma-Aldrich); Isogen (Nippon Gene); 4-[^{14}C]-IPP and Tri-Carb 2900TR (Perkin–Elmer); alendronate (Calbiochem); rhodamine-conjugated phalloidin (Molecular Probes); calcitonin (Elcitonin, Asahi Kasei); SuperScript II (Invitrogen); Ex TaqDNA polymerase (Takara); and anti-Rap1A, anti-Rap1, and anti-NFATc1 Abs (Santa Cruz Biotechnology).

Chemical Characterization of M-GFN. GFN was purified as described (1, 2). TMS-diazomethane (2 M, 0.345 ml) was added to a solution of GFN (100 mg, 0.34 mmol) in methylene chloride (3 ml) and methanol (1 ml), and the mixture was sequentially stirred for 30 min at 0°C. The reaction mixture was extracted with ethyl acetate and washed with brine. The resultant extracts were further purified with reverse-phase HPLC (Capcell Pak C_{18}, Shiseido Co., Ltd.; acetonitrile-0.5% phosphoric acid (70:30), 10 ml/min) to yield M-GFN as a brown powder (t_R 11.4 min). mp: 140–141°C; TLC (hexane:ethyl acetate, 2:1 vol/vol): R_f = 0.29; ^{1}H-NMR (300 MHz, DMSO-d_6):δ10.23 (s, 1H), 9.28 (s, 1H), 8.41 (s, 1H), 6.48 (d, J = 1.4 Hz, 1H), 6.26 (d, J = 2.2 Hz, 1H), 6.26 (d, J = 1.4 Hz, 1H), 6.10 (d, J = 2.2 Hz, 1H), 3.77 (s, 3H), 2.20 (s, 3H), 2.12(s, 3H); ^{13}C-NMR (75 MHz, DMSO-d_6):δ169.0, 160.4, 158.3, 146.9, 142.1, 139.1, 135.3, 127.9, 113.2, 113.0, 112.7, 109.4, 100.6, 51.8, 20.7, 20.4; IR (KBr): 3430, 2944, 1654, 1604, 1581, 1513, 1454, 1421, 1355, 1322, 1297, 1263, 1207, 1151, 1108, 1064, 1039, 997, 954 cm^{-1}; UV/Vis:λ_{max} 214.0, 262.0, 302 nm; HREIMS (m/z): $[M]^+$ calcd. for C16H16O2, 304.0947; found, 304.0964.

TRAP Staining. Cells were fixed in 3.7% formalin at room temperature for more than 30 min and treated with acetone/ethanol (1:1 vol/vol) at room temperature for 1 min, after which the cell surface was air dried. The fixed cells were incubated with 0.01% naphthol AS-MX phosphate and 0.05% fast red violet LB salt in the presence of 50 mM sodium tartrate and 90 mM sodium acetate (pH 5.0) for TRAP staining, and were then rinsed with water.

Drug Screening. In the screening with our chemical library, compounds were added to BMMs for 72 h in the presence of RANKL and M-CSF, and then TRAP$^+$ MNCs were counted, as inhibition of osteoclastogenesis. Also, compounds were added to osteoclasts for 48 h in the presence of RANKL and M-CSF as inhibition of osteoclast survival.

Phagocytosis Assay. Cells were treated with FL-zymosan A BioParticles (50 μg/ml) for 1.5 h. The cells were then rinsed with PBS twice to remove the particles that were not incorporated into the cells, and fixed in 3.7% formalin. A fluorescence microscope was used to observe the FL-zymosan A BioParticles in the cells.

Pit Formation Assay. BMMs were cultured on dentine slices (4 mm in diameter, 0.2 mm in thickness) in 96-well culture plates with RANKL (50 ng/ml) and M-CSF (50 ng/ml) for 72 h. After wiping the cells off dentine slices with cotton, the slices were immersed in 1% toluidine blue O to stain resorption pits formed by mature osteoclasts.

Preparation of M-GFN Beads. M-GFN beads were prepared as follows: photoaffinity linker-coated agarose beads (100 μl) (3) were washed with distilled water (3 × 400 μl) on a spin column and transferred to a glass sample vial. The transferred beads were suspended in 2-propanol (200 μl). Residual water absorbed on the beads was removed azeotropically using a rotary evaporator, and the beads were dried *in vacuo*. A solution of M-GFN (1 mg/150 μl in methanol) or methanol (as control beads) was added to the dried beads, and the mixture was concentrated and dried *in vacuo*. The beads were irradiated at 365 nm (4 J cm^{-2}) by a UV crosslinker, and the resulting beads were washed successively with 50% aq. methanol, methanol, DMSO, and methanol (3 × 400 μl each), suspended in PBS (200 μl).

RNAi and RT-PCR. *GLO1* and *SCP2* siRNA duplexes were designed from the mouse *GLO1* and *SCP2* cDNA sequences (Nippon EGT) with the following sequences: GLO1 #1, 5′-AGAAGUCCCUGGAUUUUUAdTdT-3′; GLO1 #2, 5′-GCAUGGACGUUUUCCAGAAdTdT-3′; SCP2 #1, 5′-ACAUGGGACUGGCCAUGAAdTdT-3′; SCP2 #2, 5′-GAAAAUCGGUGGCAUUUUUdTdT-3′. An siRNA duplex designed to target GFP (5′-GAACGGCATCAAGGTGAACdTdT-3′) was used as a control. The *SCP2* gene has two initiation sites coding for proteins that share a common 13-kDa SCP2 C terminus. One site codes for 58-kDa SCP-x, which is partially posttranslationally cleaved to 13-kDa SCP2 and a 45-kDa protein (peroxisomal 3-ketoacyl-CoA thiolase). A second site codes for 15-kDa pro-SCP2, which is completely posttranslationally cleaved to 13-kDa SCP2 (4). Therefore, SCP2 siRNAs will induce the knockdown of not only SCP2 but also of SCP-x and peroxisomal 3-ketoacyl-CoA thiolase. BMMs were transfected with siRNAs using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen). *GLO1* and *SCP2* knockdowns after 48-h siRNA treatment were measured by RT-PCR. Total RNA from the cells was prepared using Isogen. First-strand cDNA was synthesized for PCR using SuperScript II and subjected to amplification with Ex TaqDNA polymerase using the following specific primers: GLO1, 5′-CAGTGGCCTCACTGATGAGA-3′ (forward) and 5′-CGTCATCAGGCTTCTTCACA-3′ (reverse); *SCP2*, 5′-TCAAGGCAAACCTCGTCTTT-3′ (forward) and 5′-CAGTCCCATGTTACCAGCAA-3′ (reverse); *GAPDH*, 5′-AACTTTGGCATTGTGGAAGG-3′ (forward) and 5′-CCCTGTTGCTGTAGCCGTAT-3′ (reverse). The bands were quantitated by scanning the agarose gels with Typhoon 9400 Variable Mode Imager in conjunction with ImageQuant TL Image Analysis Software (GE Healthcare).

Actin Staining. Osteoclasts cultured on dentine slices were fixed in 3.7% formalin diluted with PBS and rinsed for 1 min with PBS containing 0.1% Triton X-100. F-actin was labeled with rhodamine-conjugated phalloidin by incubating for 30 min in the dark. The cells on dentine slices were washed with PBS and actin rings were visualized using fluorescence microscope.

***In Vitro* GGPP Synthase Assay.** Kinetic analysis of M-GFN against GGPP synthase was performed as previously described (1) with a slight modification. The reaction mixture was in a final volume of 0.4 ml containing 0.1 M Tris-HCl (pH 7.5), 0.3 mM $MnCl_2$, 0.2 mM $MgCl_2$, 10 μg/ml butylated hydroxytoluene, 1.02–4.06 nCi/ml 4-[^{14}C]-IPP, 55.3–221.0 nM FPP, and 0.013 μg recombinant human GST-GGPP synthase. The reaction was initiated by the addition of IPP and FPP, and was terminated by the addition

of 0.6 ml of 1-BuOH after 45 min at 37°C. Enzyme activity was evaluated for the radioactivity of 1-BuOH extract using Tri-Carb 2900TR.

Measurement of Intracellular MG. BMMs were treated with M-GFN or MG for 24 h. Cells were harvested, washed twice with PBS, equalized for cell number for each sample (2×10^7 cells), and then resuspended in distilled water. Cells were boiled for 5 min and centrifuged at 15,000 $\times$ *g* for 15 min at 4°C, and the supernatants were used as the source of MG. MG concentration was measured enzymatically using *Saccharomyces cerevisiae* GLO1 (5). The reaction was initiated by adding 5 nM GLO1 to the reaction buffer (100 mM sodium phosphate pH 7.0, 1 mM GSH, and 14.6 mM $MgSO_4$) contained in each supernatant.

***In Vitro* Mineralization of Osteoblastic Cells.** Mouse stromal/osteoblastic cell line UAMS-32 (kindly provided C.A. O'Brien, University of Arkansas) was cultured in differentiation medium (α-MEM containing 10% FCS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate) for 12 days in the absence or presence of various concentrations of M-GFN. The medium was changed every 3 days. Then, cells were washed with PBS, fixed in 70% ethanol for 30 min, stained with 40 mM Alizarin red-S (AR-S) (pH 4.2) for 10 min, and extensively rinsed with water. After photography, the bound staining was eluted with 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0), and the AR-S concentration was determined by absorbance measurement at 600 nm using an AR-S standard curve in the same solution.

1. Zenitani S, *et al*. (2003) Gerfelin, a novel inhibitor of geranylgeranyl diphosphate synthase from *Beauveria felina* QN22047 I Taxonomy, fermentation, isolation, and biological activities. *J Antibiot* 56:617–621.
2. Zenitani S, *et al*. (2003) Gerfelin, a novel inhibitor of geranylgeranyl diphosphate synthase from *Beauveria felina* QN22047 II Structural elucidation. *J Antibiot* 56:658–660.
3. Kanoh N, Honda K, Simizu S, Muroi M, Osada H (2005) Photo-cross-linked small-molecule affinity matrix for facilitating forward and reverse chemical genetics. *Angew Chem Int Ed* 44:3559–3562.
4. Gallegos AM, *et al*. (2001) Gene structure, intracellular localization, and functional roles of sterol carrier protein-2. *Prog Lipid Res* 40:498–563.
5. Maeta K, Izawa S, Okazaki S, Kuge S, Inoue Y (2004) Activity of the Yap1 transcription factor in. *Saccharomyces cerevisiae* is modulated by methylglyoxal, a metabolite derived from glycolysis. *Mol Cell Biol* 24:8753–8764.

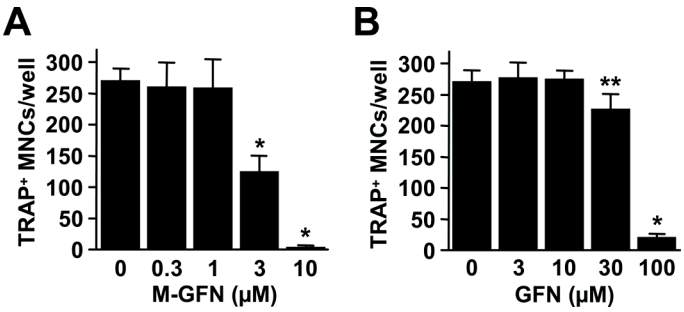

Fig. S1. Effects of M-GFN and GFN on osteoclastogenesis. (*A* and *B*) BMMs were treated with the indicated concentrations of M-GFN (*A*) or GFN (*B*) in the presence of RANKL and M-CSF for 72 h. Cells were fixed and stained for TRAP, and $TRAP^+$ MNCs were counted. Data are shown as the mean ± SD ($n = 4$). **, $P < 0.05$; *, $P < 0.01$ vs. Vehicle.

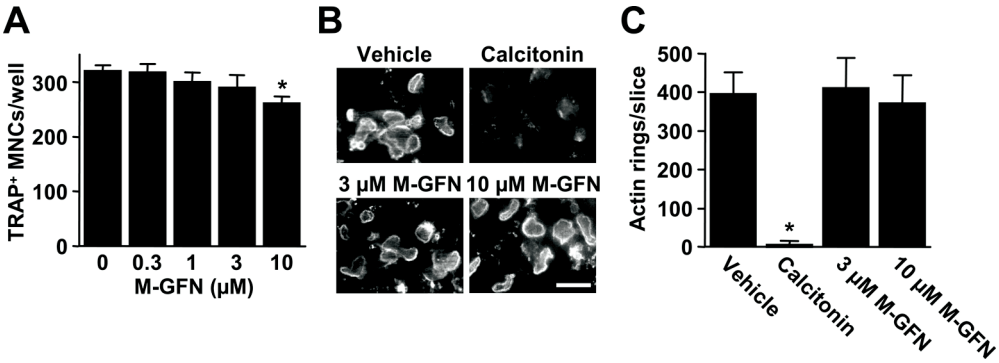

Fig. S2. Effect of M-GFN on the survival and the actin ring configuration of osteoclasts. (*A*) Osteoclasts were treated with the indicated concentrations of M-GFN for 48 h in the presence of RANKL and M-CSF. Cells were fixed and stained for TRAP, and $TRAP^+$ MNCs were counted. (*B* and *C*) Osteoclasts cultured on dentine slices were treated with M-GFN for 48 h or 0.2 units/ml calcitonin, which is known to disrupt actin rings, for 8 h in the presence of RANKL and M-CSF. Cells on dentine slices were fixed and labeled with rhodamine-conjugated phalloidin for visualization of actin rings (*B*). The number of actin rings on the slices was counted (*C*). Data are shown as the mean ± SD ($n = 4$). *, $P < 0.01$ vs. Vehicle. Scale bar, 100 μm. When osteoclasts were treated with M-GFN for 48 h, the number of osteoclasts decreased slightly, to about 90% and 85% at 3 and 10 μM, respectively. Actin rings, which are an essential morphological characteristic of osteoclasts carrying out their resorptive functions, were not disrupted after 48 h of treatment with concentrations of M-GFN up to 10 μM.

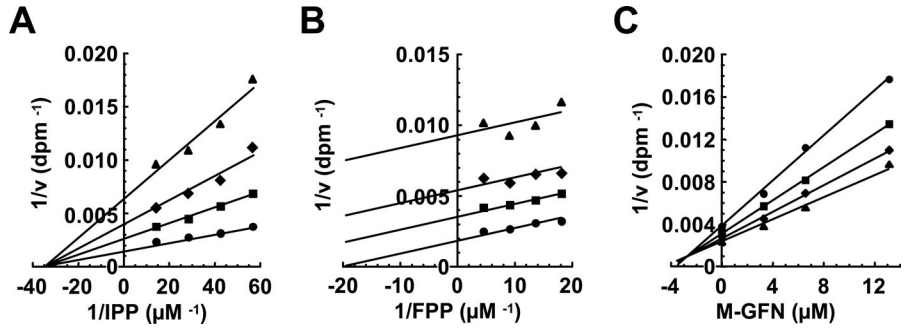

Fig. S3. Kinetic analysis of M-GFN against GGPP synthase. (*A* and *B*) Lineweaver-Burk plot of initial velocity vs. varying IPP concentrations (*A*), and vs. varying FPP concentrations (*B*). M-GFN concentrations are 0 (●), 3.3 (■), 6.6 (◆), and 13.2 (▲) μM, respectively. (*C*) Dixon plot of initial velocity vs. M-GFN concentration. IPP concentrations are 17.7 (●), 23.5 (■), 35.3 (◆), and 70.6 (▲) nM, respectively. M-GFN inhibited GGPP synthase in a noncompetitive manner against IPP, and the *K*i value was 2.46 $\pm$ 0.22 μM (mean $\pm$ SD of four independent experiments).

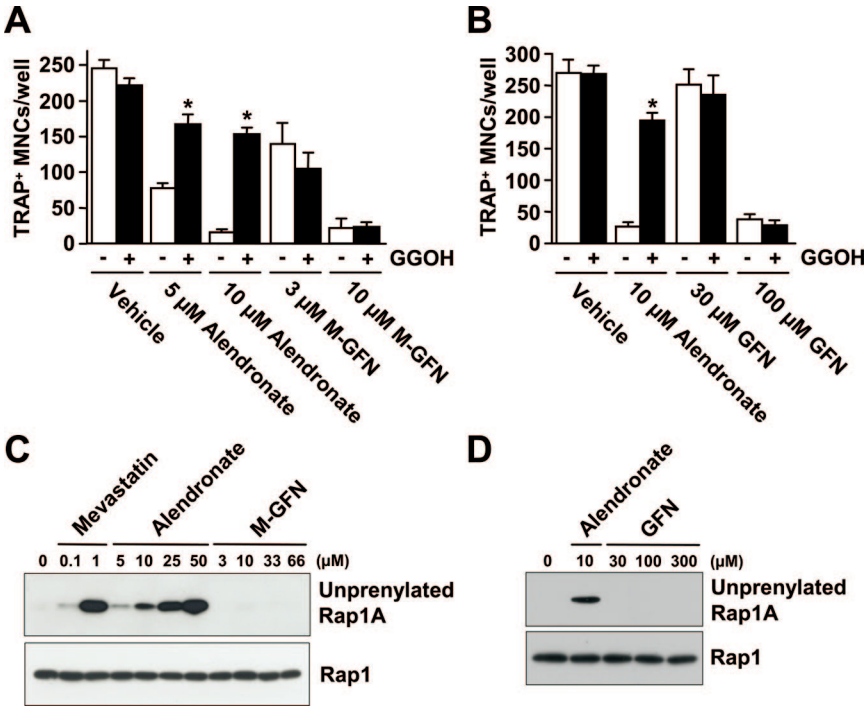

Fig. S4. Suppression of osteoclastogenesis by M-GFN and GFN is not involved in GGPP synthase inhibition. (*A* and *B*) Effect of GGOH on the inhibition of osteoclastogenesis by M-GFN and GFN. BMMs were treated in the presence of RANKL and M-CSF, and M-GFN, GFN, or alendronate was added to the cultures with or without 5 μM GGOH after 36 h. After 72 h of culture, cells were fixed and stained for TRAP, and TRAP$^+$ MNCs were counted. Data are shown as the mean $\pm$ SD ($n = 4$). *, $P < 0.01$ vs. alendronate without GGOH, as determined by Student's *t* test. (*C* and *D*) Effect of M-GFN and GFN on the prenylation of Rap1A. BMMs were treated with mevastatin, alendronate, M-GFN, or GFN for 24 h. Cell lysates were immunoblotted with anti-Rap1A Ab (*Top*), which preferentially hybridizes to the unprenylated form of Rap1A, and anti-Rap1 Ab *(Bottom)*.

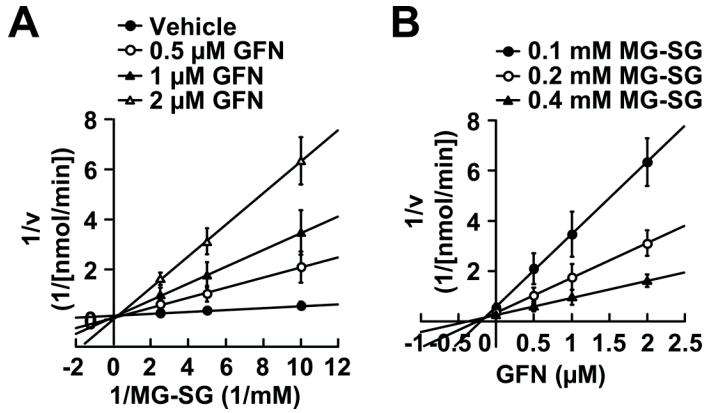

Fig. S5. GFN inhibits GLO1 activity in a competitive manner. (*A* and *B*) Kinetic analysis of GFN against mouse His-GLO1. (*A*) Lineweaver-Burk plot of initial velocity vs. varying MG-SG concentrations. (*B*) Dixon plot of initial velocity vs. varying GFN concentrations. Data are shown as the mean ± SD ($n = 3$).

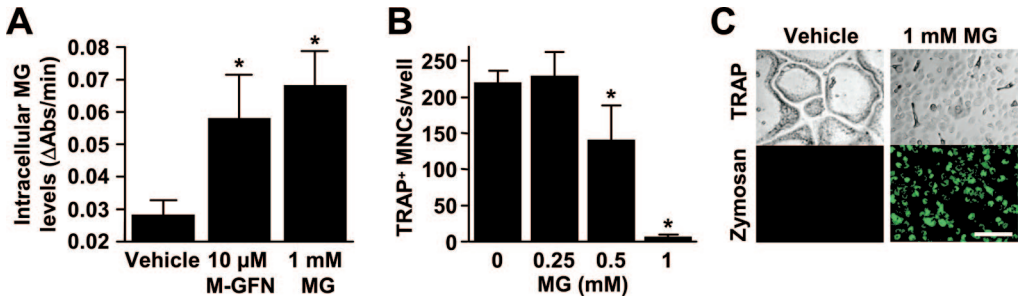

Fig. S6. Effect of MG on osteoclastogenesis. (*A*) Intracellular levels of MG in M-GFN-treated BMMs. BMMs were treated with M-GFN (10 μM) or MG (1 mM) for 24 h. Cells were washed, resuspended in distilled water, and boiled. The supernatants were used as the source of MG. *In vitro* GLO1 assay was performed with *Saccharomyces cerevisiae* GLO1. Data are shown as the mean ± SD ($n = 4$). *, $P < 0.01$ vs. Vehicle. (*B* and *C*) MG suppresses osteoclastogenesis. BMMs were treated with the indicated concentrations of MG in the presence of RANKL and M-CSF for 72 h. Then, cells were treated with FL-zymosan A and further stained for TRAP (*C*). TRAP$^+$ MNCs were counted (*B*). Data are shown as the mean ± SD ($n = 4$). *, $P < 0.01$ vs. Vehicle. Scale bar, 150 μm.

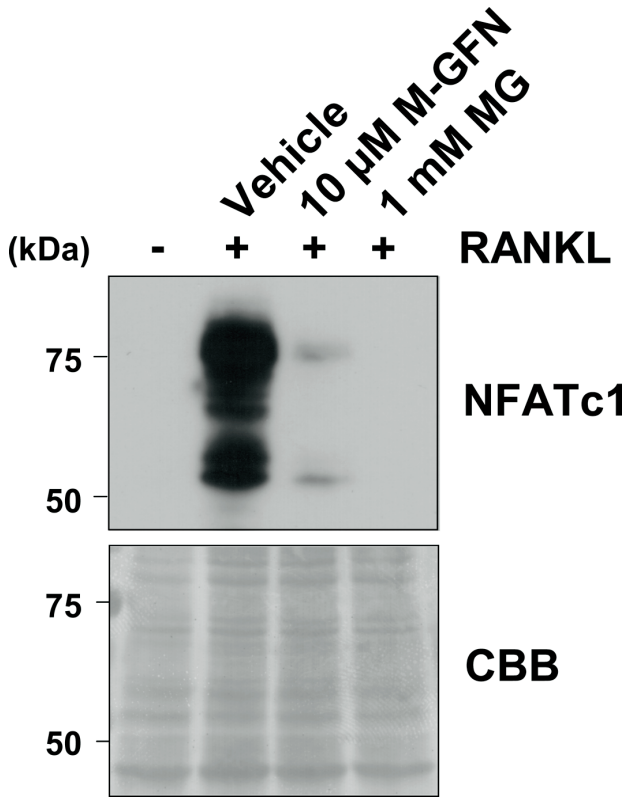

Fig. S7. M-GFN and MG inhibit RANKL-induced NFATc1 expression. BMMs were pretreated with M-GFN (10 μM) or MG (1 mM) for 2 h and then treated with RANKL (50 ng/ml) for 24 h. Cell lysates were immunoblotted with anti-NFATc1 Ab (*Top*). CBB staining of the filter is shown *(Bottom)*.

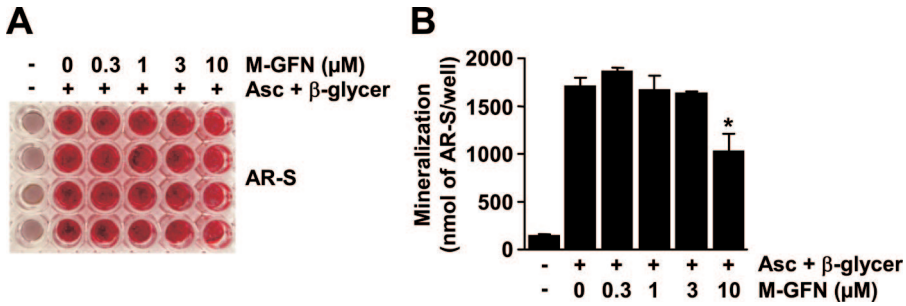

Fig. S8. Effect of M-GFN on the mineralization of osteoblastic cells *in vitro*. (*A* and *B*) UAMS-32 cells were treated with the indicated concentrations of M-GFN in the presence of ascorbic acid (Asc) and β-glycerophosphate (β-glycer) for 12 days. Cells were fixed and stained with AR-S (*A*), and AR-S concentration in samples was quantified (*B*). Data are shown as the mean $\pm$ SD ($n = 4$). *, $P < 0.01$ vs. Vehicle.

Table S1. Crystallographic data and refinement statistics

Data collection	
Space group	$P2_1$
Unit cell, Å	a = 42.0; b = 65.3; c = 66.2
°	a = 90; b = 101.2; g = 90
Resolution, Å*	50 - 1.70 (1.81 - 1.70)
Completeness, %	94.4 (100)
Mean I/σ	17.5 (3.2)
Redundancy	3.5 (3.7)
R_{merge}, %	6.8 (30.0)
Refinement	
Resolution, Å	23.4–1.70 (1.76–1.70)
Reflections	36,510
R_{work}/R_{free}, %	18.5 / 21.5
No. of atoms	
Protein	2,845
Ligand/ion	46
Water	392
rms deviaitons	
Bonds, Å	0.012
Angles, °	1.7
Average B value (protien), Å^{2†}	14.4, 14.8
Average B value (M-GFN), Å^{2†}	15.5, 18.1

*Values in parentheses are for the highest-resolution shells.
†Values are for molecules A and B, respectively.