Synthesis and Biological Evaluation of α-Halogenated Bisphosphonate and Phosphonocarboxylate Analogues of Risedronate

Mong S. Marma,[†] Zhidao Xia,[‡] Charlotte Stewart,[#] Fraser Coxon,[#] James E. Dunford,[‡] Rudi Baron,[⊥] Boris A. Kashemirov,[†] Frank H. Ebetino,[§] James T. Triffitt,[‡] R. Graham G. Russell,[‡] and Charles E. McKenna*[,†]

Department of Chemistry, University of Southern California, Los Angeles, California 90089-0744; Procter & Gamble Pharmaceuticals, Mason, Ohio 45040; Bone Research Group, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom; Nuffield Department of Orthopaedic Surgery, Oxford University, Institute of Musculoskeletal Sciences, Nuffield Orthopaedic Center, Headington, Oxford OX3 7LD, United Kingdom; and Cell and Molecular Biology, Division of Biomedical Sciences, Imperial College School of Medicine, London SW7 2AZ, United Kingdom

Received March 13, 2007

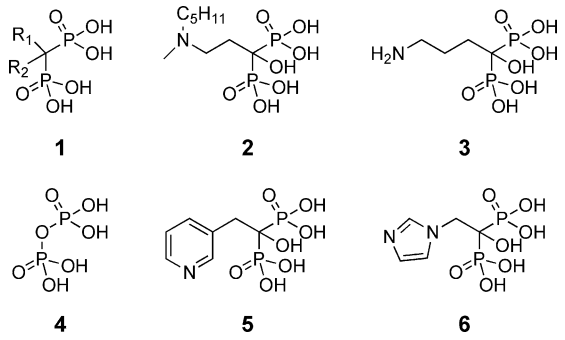
α-Halogenated analogues of the anti-resorptive bisphosphonate risedronate (**5**, Ris) and its phosphonocarboxylate cognate (**7**, 3-PEHPC) were synthesized and compared with **5**, **7**, and the corresponding desoxy analogues in bone mineral affinity and mevalonate pathway inhibition assays. The Ris (**5e**−**h**) and 3-PEHPC (**7e**−**h**) analogues had decreased bone mineral affinity, confirming that the α-OH group in **5** and **7** enhances bone affinity. The **5** α-halo-analogues potently inhibited farnesyl pyrophosphate synthase (FPPS) with IC_{50} values from 16 (α-F) to 340 (α-Br) nM (**5**, 6 nM). In contrast, **7** α-halo-analogues were ineffective versus FPPS ($IC_{50} > 600$ μM), but inhibited Rab geranylgeranyl transferase (RGGT) ($IC_{50} = 16-35$ μM) similarly to **7** itself ($IC_{50} = 24$ μM). The α-F analogue **7e** was 1−2 times as active as **7** in J774 cell viability and Rab11 prenylation inhibition assays.

Introduction

Bisphosphonates (BPs; general structure **1**), are widely used drugs for the treatment and prevention of excessive osteoclast-mediated bone resorption associated with osteoporosis, Paget's disease, tumor-induced osteolysis, and hypercalcemia.[1−5] In recent years, bisphosphonates have also been found to have antiparasitic,[6−9] antibacterial,[10] herbicidal,[11] and anticancer properties[12−19] and to stimulate γδT cells of the immune system, drawing interest in cancer immunotherapy.[20,21] These drugs are nonhydrolyzable analogues of the endogenous pyrophosphate (PP*i*, **4**) in which the oxygen bridge is replaced by a carbon atom. Attached to the bridging carbon atom are two substituents, forming a general "P−CR_1R_2−P" structure. The most potent bisphosphonates currently in use as anti-resorptive agents, such as ibandronate (**2**), alendronate (**3**), risedronate (**5**), and zoledronate (**6**), consist of a hydroxyl group in the R_1 position and a nitrogen-containing R_2 side chain (**1**, Scheme 1) and are therefore known as nitrogen-containing bisphosphonates (N-BPs).

Farnesyl pyrophosphosphate synthase (FPPS, also known as farnesyl diphosphate synthase, FDPS), one of the key enzymes involved in the mevalonate pathway, is the major molecular target of N-BPs.[22−29] This enzyme catalyzes the two-step synthesis of the C_{15} isoprenoid farnesyl pyrophosphate (FPP): first from isoprenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) to produce geranyl pyrophosphate (GPP), followed by condensation of an additional IPP with the resultant GPP to produce FPP (Scheme 2). Inhibition of the enzyme prevents the biosynthesis of FPP and other downstream products, such as geranylgeranyl pyrophosphate (GGPP), which are required for the post-translational prenylation of small GTPases, such as Ras, Rho, and Rab family proteins in osteoclasts.[30] Disruption of protein prenylation leads to loss of essential signaling processes that are necessary for osteoclast function and survival.[22,23,27,28] Although these pathways are crucial in all cell types, the extraordinary ability of N-BPs to selectively target bone mineral means that osteoclasts are exposed to high concentrations of the drug in vivo.[26,27,31−34]

Scheme 1. Generic N-BP Structure (**1**) and Structures of Common N-BP Drugs (All Represented in Acid Form) Compared to Pyrophosphoric Acid, **4**: **2**, Ibandronic Acid; **3**, Alendronic Acid; **5**, Risedronic Acid; **6**, Zolendronic Acid

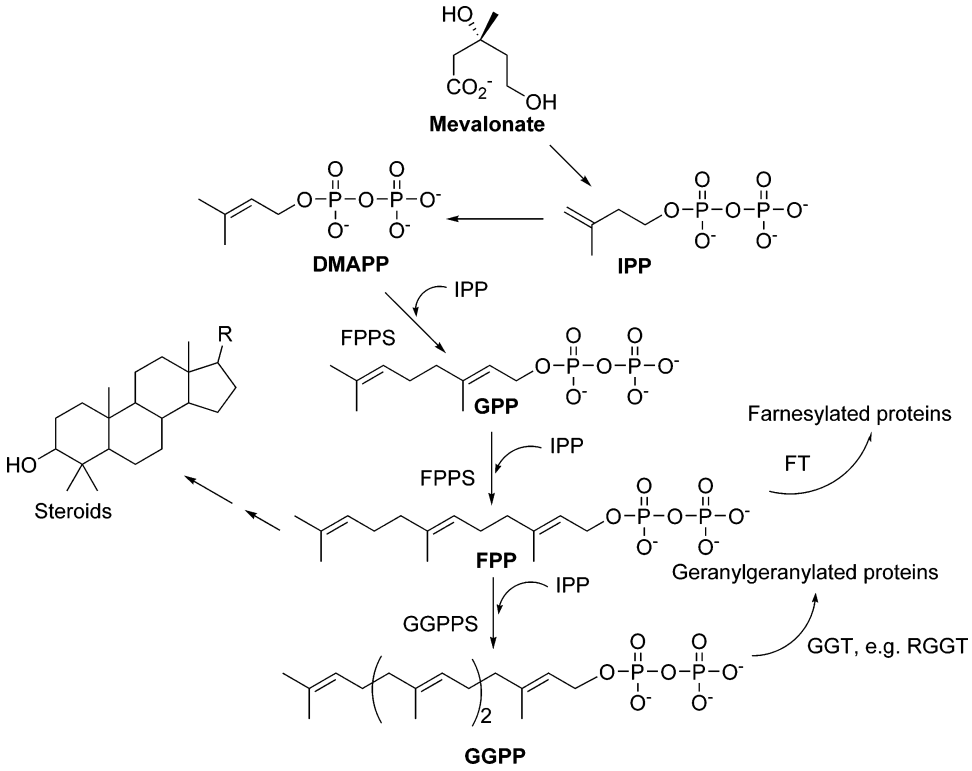


The exact mechanism by which N-BPs inhibit FPPS and their structure−activity relationships are still emerging. In general, the P−C−P substructure is required for selective targeting to bone mineral, and the R_n substituents influence potency. The presence of the α-OH group (i.e., R_1 = OH) is believed to enhance bone mineral binding[35−37] by allowing tridentate interaction with Ca^{2+}, but its role in potency at the cellular level is less clear. Analogues with an α-H in place of the α-OH have lowered bone affinities, presumably because their Ca^{2+} interaction is restricted to bidentate.[36−38] The enzyme inhibition potency of these analogues is also lower, despite the fact that in the crystal structure of the risedronate−FPPS complex, the α-OH group does not appear to participate directly in chelation to Mg^{2+}.[22,23,28] Therefore, it is possible that the α-OH group

* Author to whom correspondence should be addressed (e-mail mckenna@usc.edu; telephone +1-213-740-7007).
[†] University of Southern California.
[‡] Oxford University.
[⊥] Imperial College School of Medicine.
[#] University of Aberdeen.
[§] Procter & Gamble Pharmaceuticals.

10.1021/jm0702884 CCC: $37.00
Published on Web 11/02/2007

Scheme 2. Key Steps in Isoprenoid Biosynthesis

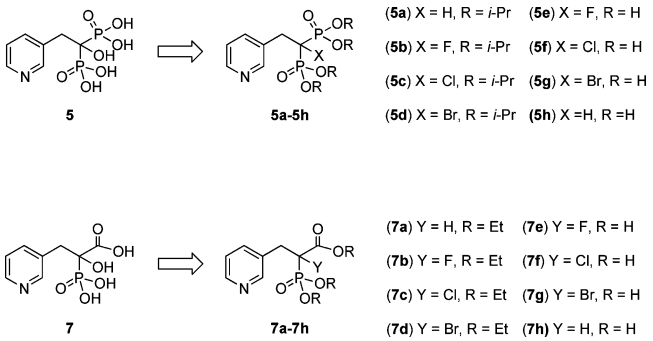


contributes indirectly to the phosphonate anion interaction with Mg^{2+} by the electron-withdrawing effect of its electronegative oxygen. In principle, this effect should be exerted by other electron-withdrawing groups, installed by replacing the α-OH and exploiting the sterically open space surrounding this group in the active site complex.

However, relatively little is known about the effect of replacing the α-OH group of N-BPs with other functional groups. Such substitutions could potentially alter the physicochemical properties of the bisphosphonate moiety significantly due to their proximity to the geminal phosphonate groups. A study on a small group of α-amino N-BPs has shown that antiresorptive potency can be affected dramatically by this substitution, depending on the parent structures—ranging from complete loss to full retention of activity.[36,39] However, in these compounds the amino replacement introduces an additional basic site of undefined protonation status, complicating interpretation. We hypothesized that replacement of the α-OH with a halogen atom might allow modulation of mineral affinity and/or enzyme inhibition potency, by varying only the substituent electronegativity and atomic size. Ideally, this could lead to a new class of anti-resorptive agents with properties not achievable with currently used α-hydroxyl N-BP analogues.

α-Fluorination is known to lower the acidity of methylenebisphosphonic acid such that there is a good match of pK_a values with PP*i*, creating a useful mimic of the latter compound.[40−42] Fluorine substitution for −H and −OH groups is frequently used to advantage in drug discovery and development.[43−45] The stability of the C−F bond, together with its high electronegativity and small atomic size, makes fluorine particularly valuable for modulating pharmacological properties of drug molecules without compromising their basic structural features. The fluorine atom occupies a van der Waals radius (1.47 Å) uniquely positioned between oxygen (1.52 Å) and hydrogen (1.20 Å), allowing bio-isosteric replacement of C−H, C═O, and C−OH groups.[45] It can also function as weak hydrogen bond acceptor

Scheme 3



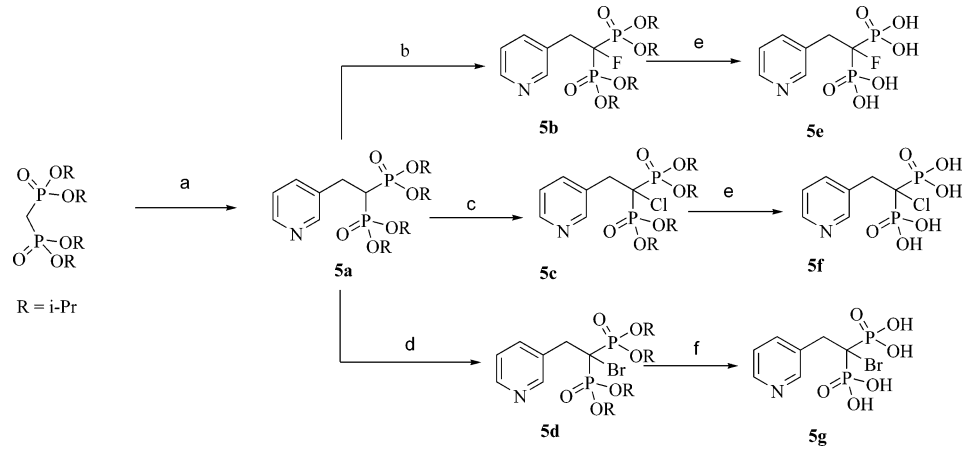
atom in enzyme active site or receptor environments.[43,45] In the present case, α-fluorination could allow separation of electron-withdrawing and H-donating contributions of the α-OH in N-BP drugs or their PC analogues.

In this paper, we report the synthesis of a series of α-halogenated analogues (Scheme 3) of the potent N-BP **5** (risedronate, a widely used anti-resorptive agent) and also of its phosphonocarboxylate analogue **7** (3-PEHPC, previously known as NE10790),[46−49] which is known to reduce bone mineral affinity while retaining some anti-resorptive properties in vitro and in vivo[46,47,49] and which can inhibit tumor cell invasion within bone.[48] It was recently shown that **7** acts upon a different enzyme target in the mevalonate pathway, Rab geranylgeranyl transferase (RGGT), thereby selectively preventing the prenylation of Rab family proteins.[46] The α-halo-analogues were evaluated for bone mineral affinity, inhibition of FPPS and RGGT in isolated enzyme assays, ability to block protein prenylation in J774 cells, and effects on J774 cell growth and viability.

Results and Discussion

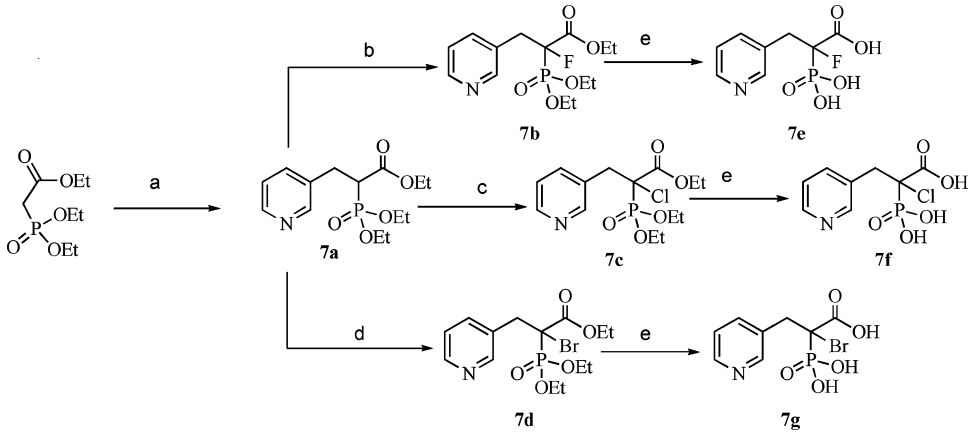
Synthesis. 1-Chloromethyl-4-fluoro-1,4-diazoniabicyclo-[2.2.2]octane bis(tetrafluoroborate) (**8**)[50−52] was employed for

Scheme 4[a]



[a] Reagents and conditions: (a) NaH in dry THF, 0 °C for 0.5 h then 1 h at room temperature, then 3-picolyl chloride−NaH mixture in dry DMF added, 8 h at 70 °C; (b) NaH in dry THF, at 0 °C for 0.5 h followed by 1 h at room temperature, **8** (1.5 equiv) in dry DMF, 5 h at room temperature; (c) NaH in dry THF, at 0 °C for 0.5 h followed by 1 h at room temperature, NCS (1.5 equiv) at 0 °C for 1 h, then overnight at room temperature; (d) same as in (c) except NBS was used instead of NCS; (e) 6 N HCl, overnight reflux; (f) 6 N HBr, overnight reflux.

Scheme 5[a]



[a] Reagents and conditions: (a) NaH in dry THF, 0 °C for 0.5 h followed by 1 h at room temperature, then 3-picolyl chloride−NaH mixture in dry DMF, 3 h at room temperature; (b) NaH in dry THF, at 0 °C for 0.5 h followed by 1 h at room temperature, **8** (1.5 equiv) in dry THF overnight at room temperature; (c) NaH in dry THF, at 0 °C for 0.5 h followed by 1 h at room temperature, NCS (1.5 equiv) at 0 °C for 1 h, then overnight at room temperature; (d) same as in (c) except NBS was used instead of NCS; (e) 6 N HCl overnight reflux.

convenient and safe α-fluorination of both bisphosphonate (**5a**) and phosphonoacetate (**7a**) esters (Schemes 4 and 5).[40,53] The intermediate **5a** was prepared by reaction of 3-picolyl chloride with tetraisopropyl methylenebisphosphonate sodiocarbanion. The alkylation is rather slow at room temperature, probably owing to steric hindrance, so to accelerate the reaction and improve the yield, the temperature was increased to 70 °C, giving a satisfactory yield within 8 h. In addition to the monosubstituted product **5a**, some disubstituted product was also formed (observed by ^{31}P NMR), which was removed by chromatography on a silica gel column. Ester **5a** was then halogenated by treating its carbanion with **8**, *N*-chlorosuccinimide (NCS), or *N*-bromosuccinimide (NBS) to produce **5b**, **5c**, or **5d**, respectively (Scheme 4). A previous report on fluorination of the tetraethyl ester of **5a** (where R = Et)[54] used **8** in THF/18-crown-6-ether; however, we found that for the tetraisopropyl ester, at least, a mixture of THF/DMF (1:1) sufficed as solvent, obviating the crown ether. Although in THF alone fluorination was undetectable by ^{31}P NMR, *N*-chlorosuccinimide and *N*-bromosuccinimide quantitatively halogenated the sodium tetraisopropyl methylenebisphosphonate in this solvent (by ^{31}P NMR) under similar conditions. After chromatographic separation, halogenated esters **5b**−**5d** were hydrolyzed to the corresponding acids **5e** (F-Ris), **5f** (Cl-Ris) and **5g** (Br-Ris) by overnight reflux in 6 N HCl or HBr. ^{31}P NMR spectra of halogenated products showed the expected upfield shifts relative to the resonance of the desoxy precursor (Table 1).

Table 1. ^{31}P NMR of Tetraisopropyl Esters and Acids Related to **5**

compound	δ ^{31}P	multiplicity (J)
5b, X = F[a]	12.12	d ($^2J_{PF}$ = 74.5 Hz)
5c, X = Cl[a]	14.02	s
5d, X = Br[a]	14.10	s
5a, X = H[a]	21.00	s
5e, X = F[b]	11.57	d ($^2J_{PF}$ = 66.6 Hz)
5f, X = Cl[b]	13.37	s
5g, X = Br[b]	13.50	s

[a] $CDCl_3$. [b] D_2O.

The syntheses of the corresponding α-halo-analogues of the phosphonocarboxlate **7**, **7e** (F-3-PEHPC), **7f** (Cl-3-PEHPC), and **7g** (Br-3-PEHPC), were carried out similarly. Desoxy ester **7a** was prepared by reacting triethyl phosphonoacetate sodiocarbanion with 3-picolyl chloride. The amount of double-substituted product was higher than in the bisphosphonate condensation described above, but could also be removed by silica gel column chromatography. As noted already, fluorination of **7a** was carried out in THF alone at room temperature (Scheme 5). The chlorination and bromination of **7a** by NCS and NBS, respectively, proceeded quantitatively.

Table 2. ^{31}P NMR of Triethyl Esters and Acids Related to **7**

compound	δ ^{31}P	multiplicity (J)
7b, X = F[a]	12.50	d ($^2J_{PF}$ = 82.9 Hz)
7c, X = Cl[a]	15.21	s
7d, X = Br[a]	15.48	s
7a, X = H[a]	21.78	s
7e, X = F[b]	7.85	d ($^2J_{PF}$ = 72.8 Hz)
7f, X = Cl[b]	10.30	s
7g, X = Br[b]	10.54	s
7h, X = H[b]	13.86	s

[a] $CDCl_3$. [b] D_2O.

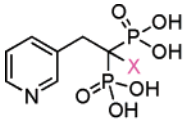
After chromatographic purification on silica gel, all esters were hydrolyzed by reflux in 6 N HCl. The phosphonocarboxylic triacids obtained (**7e**−**h**) were contaminated by ∼4% of an uncharacterized byproduct (by ^{1}H NMR), which was removed by washing with MeOH. As with the N-BP analogues, halogenation produced an upfield chemical shift in the ^{31}P NMR resonances relative to the desoxy analogue (Table 2).

In general, the esters **5a**−**d** and **7a**−**d** were viscous oils. The derived acids **5e**−**g** and **7e**−**h** were obtained as triacids (presumably in zwitterionic form) as white powders. All compounds were characterized by ^{1}H, ^{13}C, and ^{31}P NMR, HRMS, and combustion elemental analysis (target acids only).

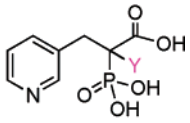
Bone Mineral Affinity of the Halo-analogues. Bone mineral affinity is an important property for selective targeting of BPs, enabling them to exert selective effects on bone-resorbing osteoclasts in vivo. Chromatography on columns of ceramic hydroxyapatite microspheres was used to determine the rank order of affinity of halo-analogues for bone mineral, which is indicated by their relative retention times. This new method produced reproducible results. Under the conditions utilized, high-affinity compounds can be distinguished one from another, exhibiting differing elution times, whereas compounds with low affinities are not retained. The halo-analogues (**5e**−**g**), as well as the H-analogue (**5h**), had similar retention times (5.7−6.0 min), showing decreased bone mineral affinity relative to **5**, the parent drug. This confirms that the α-OH of risedronate contributes to the high bone affinity of this drug and indicates that the effect involves direct interaction with the bone mineral, as an indirect effect arising from the electron-withdrawing properties of the α-substituent should have resulted in significant differences as the substituent was varied from H to F. Instead, absent an α-OH group, the bisphosphonate ligand appears to be the sole important factor in determining bone mineral affinity in the drug analogues. When one phosphonate group in the parent bisphosphonate drug was replaced by a carboxylate, as in **7**, the bone mineral affinity was significantly decreased (retention decreased to 4.6 min), as expected from previous studies,[47,49] showing that the carboxylate is a less effective ligand. Here the α-OH group is demonstrated to play a substantial, direct role in binding, because its replacement by either H or halogen resulted in a complete loss of retention on the HAP column (Table 3).

Biological Activity of the Desoxy- and Halo-Ris Analogues. The ability of the halogenated and desoxy derivatives of both risedronate and its PC analogue to inhibit FPPS in vitro was investigated. FPPS, acting on one of the branch points of the mevalonate pathway, has been identified as the key molecular target responsible for the overall anti-resorptive potency of risedronate. A number of cellular processes such as signaling are dependent on the FPPS function products (e.g., protein lipid modification by prenylation),[55,56] and inhibitors of this enzyme are potential anticancer, antiparasitic (e.g., antimalaria), and antibacterial agents, in addition to being bone agents.[57,58] The risedronate halo-analogues all exhibited inhibition of FPPS at nanomolar concentrations, with decreasing potency in the order F → Br (IC_{50} values of 16.4, 94.6, and 340.4 nM for **5e**, **5f**, and **5g**, respectively). The desoxy analogue **5h** was less potent (IC_{50} = 34.2 nM) than F-Ris, but more potent than the other two halo-analogues (Table 3). This SAR pattern is partly seen in terms of the ability of these compounds to both inhibit prenylation of Rap1A in J774 cells (Table 3 and Figure 1) and reduce the viable number of these cells (Table 3), although in these assays the bromo- and chloro-analogues **5f** and **5g** had comparable potencies. The relative abilities of these compounds to inhibit FPPS in vitro and cell viability differ, suggesting some difference in cellular uptake that compensates for the activity difference at the enzyme level. The latter results do not clearly distinguish electron-withdrawing and steric effects on the potency of these analogues. However, the substantial increase in FPPS IC_{50} for **5g** could be due to the combination of increased steric hindrance, which can make the molecule difficult to fit

Table 3. Relative Bone Mineral Affinity, FPPS and RGGT Inhibition Potencies, Effect on J774 Cell Viability and Inhibition of Rab1A/Rab11 Prenylation of Analogues Compared to **5** and **7**



5 Analogs, 5e-h



7 Analogs, 7e-h

5 analogues (−X)	HAP affinity (retention time), min	FPPS inhibition (IC_{50}), nM	reduction of J774 viability (IC_{50}), μM (mean ± SEM; n = 5)	inhibition of Rap1A prenylation (LED), μM
−OH (**5**)	9.97 ± 0.09	5.7 ± 0.54 [23]	31 ± 6	12
−F (**5e**)	5.93 ± 0.07	16.4 ± 0.5	140 ± 11	100
−Cl (**5f**)	6.03 ± 0.03	94.6 ± 16.1	679 ± 97	400
−Br (**5g**)	5.73 ± 0.15	340.4 ± 37	673 ± 111	400
−H (**5h**)	5.83 ± 0.17	34.2 ± 2	212 ± 29	50

7 analogues (−Y)	HAP affinity (retention time), min	FPPS inhibition (IC_{50}), nM	reduction of J774 viability (IC_{50}), mM (mean ± SEM; n = 4)	RGGT inhibition (IC_{50}), μM (mean ± SEM; n = 3)	inhibition of Rab11 prenylation (LED) μM
−OH (**7**)	4.6 ± 0.06	253500 ± 21000	2.3 ± 0.5	24.1 ± 5.7	62
−F (**7e**)	NA	>600000	1.8 ± 0.3	16.3 ± 0.3	31
−Cl (**7f**)	NA	>600000	5.0 ± 0.9	16.4 ± 0.3	125
−Br (**7g**)	NA	>600000	5.5 ± 0.8	17.7 ± 0.6	125
−H (**7h**)	NA	ND	2.6 ± 0.6	35.3 ± 8.7	62

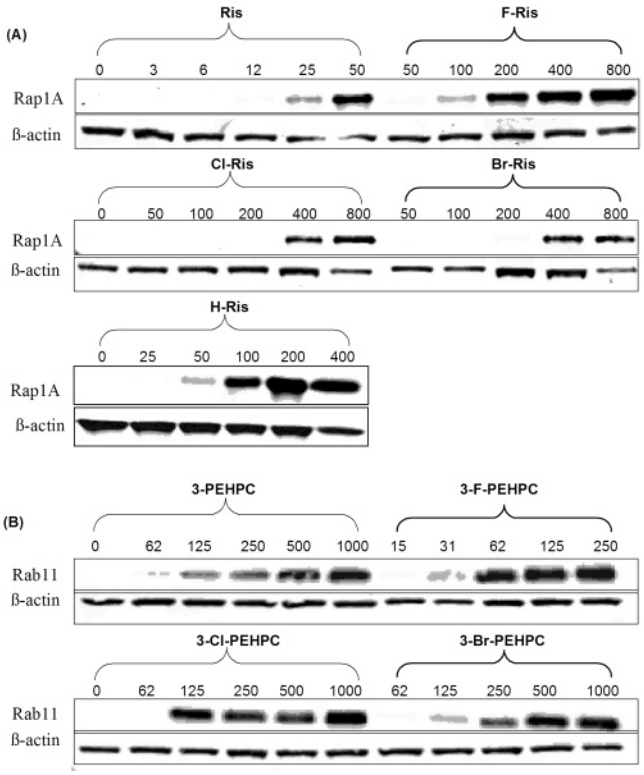


Figure 1. Effect of **5** and **7** analogues on protein prenylation, determined by Western Blot detection of unprenylated Rap1A or Rab11 with β-actin as the control protein. Data representative of three independent experiments: (A) Ris, **5**; F-Ris, **5e**; Cl-Ris, **5f**; Br-Ris, **5g**; H-Ris, **5h**; (B) 3-PEHPC, **7**; F-3-PEHPC, **7e**; Cl-3-PEHPC, **7f**; Br-3-PEHPC, **7g**. Data for **7h** are not shown.

in the active site, and of its decreased acidity, which could affect the phosphonic acid group chelation to the Mg^{2+} in the active site.

Replacement of a phosphonate group in **5** with a carboxylic acid group (i.e., **7**) drastically reduces potency for inhibition of FPP synthase (Table 3), as previously reported.[46] However, **7** selectively inhibits prenylation of Rab GTPases due to specific inhibition of a downstream mevalonate pathway enzyme, RGGT.[46,49] The halo-analogues of **7** were even poorer inhibitors of FPPS than the parent compound (Table 3). Because the substrate concentration in the assay is 10 μM, the weak inhibition detected may be due simply to competition with the substrate for Mg^{2+}, which is essential for enzyme activity. In contrast, all of the analogues **7e**−**g** were 1−1.5 times as potent as **7** (IC_{50} = 24.1 μM) in inhibiting RGGT. All of the analogues inhibited Rab prenylation in J774 cells and reduced J774 viability, showing up to a 4-fold difference in prenylation inhibitory potency, with the fluoro-analogue **7e** being most active (IC_{50} = 31 and 1.8 μM in the two assays, respectively; Table 3 and Figure 1). The α-OH group in this series has little or no influence on inhibitor activity at either the RGGT level or when determined by the prenylation assay, and the desoxy analogue **7h** was confirmed[49] to have an activity similar to that of **7** in all three assays. The lack of a requirement for an α-OH group in this group of inhibitors suggests the absence of strong **7** α-OH interactions with a divalent cation such as Mg^{2+} or with a local hydrogen-bond donor or acceptor in the active site of RGGT.

Conclusions

α-Halogenated analogues of the potent N-BP drug risedronate (**5**), and also of its PC analogue 3-PEHPC (**7**), were successfully synthesized. The reduced mineral affinity of all the desoxy- and halo-analogues confirms a direct role of the α-OH group in contributing to the bone affinity of analogues of both **5** and **7**. The halo-risedronate analogues all inhibited FPPS and inhibited protein prenylation in J774 cells, with the order of potency (−F > −Cl > −Br), demonstrating that a small, highly electron-withdrawing α-substituent such as fluorine in particular promotes inhibition of this enzyme. By contrast, the halo-analogues of **7** preferentially inhibited RGGT as the likely molecular basis for their interference with Rab prenylation, but did not provide evidence supporting a significant role for the α-OH group in **7**'s inhibition of RGGT.

Analogues such as **5e** and **7e**, which retain biological activity but have reduced bone affinity, may be useful therapeutically in situations when decreased drug retention is potentially advantageous. These may include pediatric use (such as treatment of osteogenesis imperfecta), when there are concerns regarding long-term skeletal retention of BPs,[60] minimization of the possibility of interfering with the anabolic response to PTH,[61] or treatment of cancers that cause osteolytic bone lesions (such as myeloma and metastatic breast cancer), in which there is some evidence that the beneficial effects of BPs may be due to direct effects on the cancer cells.[62]

Experimental Section

Abbreviations. N-BP, nitrogen-containing bisphosphonate; PC, phosphonocarboxylate; 3-PEHPC, 3-pyridinyl ethylidene hydroxyl phosphonocarboxylate; FPP, farnesyl pyrophosphate; IPP, isoprenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; RGGT, Rab geranylgeranyl transferase.

General Methods, Reagents, and Materials. All solvents and reagents were of reagent grade, purchased commercially and used without further purification, except as mentioned below. Dry tetrahydrofuran was obtained by distillation from sodium metal, and dry DMF was purchased from Van Waters & Rogers, Inc. Selectfluor (**8**), *N*-bromo- and *N*-chlorosuccinimide, 3-picolyl chloride−HCl, and NaH (60% in oil) were purchased from Aldrich. Triethyl phosphonoacetate and tetraisopropyl methylenebisphosphonate were gifts from Rhodia, Inc., and were freshly distilled under vacuum before use. Compound **5h** was provided by Procter & Gamble Pharmaceuticals. NMR spectra were measured on a Bruker AM-360 operated at frequencies of 360.14 MHz (^{1}H NMR), 90.56 MHz (^{13}C NMR), and 145.78 MHz (^{31}P NMR). Chemical shifts (δ) are reported in parts per million (ppm) relative to internal $CDCl_3$ (δ 7.24, ^{1}H; 77.0, ^{13}C) or external 85% H_3PO_4 (δ 0.00 ^{31}P). ^{13}C and ^{31}P NMR spectra were proton-decoupled. High-resolution mass spectra were recorded at the University of California at Riverside Mass Spectrometer Facility, and combustion elemental analysis was performed by Gallbraith Laboratories Inc., Knoxville, TN.

Synthesis of Tetraisopropyl 2-(3-pyridinyl)-ethylidene-1,1-bisphosphonate (5a). The method of Pohjala et al.[63] was modified to avoid using excess 3-picolyl chloride. To NaH (348.0 mg, 60% in oil, 8.71 mmol) in 10 mL of dry DMF was added 3-picolyl chloride−HCl (1.43 g, 8.71 mmol) in 15 mL of dry DMF at 0 °C with stirring under N_2. In another flask, to 348.0 mg of NaH (60% in oil, 8.71 mmol) dispersed in 15 mL of dry THF was added tetraisopropyl methylenebisphosphonate (3.0 g, 8.71 mmol) dropwise at 0 °C under N_2, and stirring was continued at 0 °C for 30 min then for 1 h at room temperature. The picolyl chloride solution was added to the tetraisopropyl methylenebisphosphonate carbanion solution and stirred for 8 h at 70 °C. The reaction was quenched by the addition of several drops of EtOH, dispersed in 100 mL of H_2O, and extracted with CH_2Cl_2 (100 × 3 mL). After drying over $MgSO_4$, the CH_2Cl_2 extracts were then concentrated and the residue was purified by silica gel column chromatography to obtain 1.68 g of **5a** (R_f = 0.40 EtOAc/acetone, 1:1) as a yellowish oil (44%): ^{1}H NMR ($CDCl_3$) δ 8.4 (1H, s), 8.3 (1H, d, J = 4.4 Hz), 7.5 (1H,

d, $J = 6.4$ Hz), 7.1 (1H, m), 4.6 (4H, m), 3.1 (2H, td, $J = 16.0$, 6.6 Hz), 2.3 (1H, m), 1.1−1.3 (24H, m); ^{13}C NMR ($CDCl_3$) δ 150.6, 147.8, 136.9, 135.7 (m), 123.3, 71.6 (d, $J = 6.5$ Hz), 71.4 (d, $J = 6.5$ Hz), 40.7 (t, $J = 134.3$ Hz), 29.3 (m), 24.3 (m); ^{31}P NMR ($CDCl_3$) δ 21.0 (s), lit.,[63] 20.13; HRMS, MH^+ *m/z* 436.201, calcd for $C_{19}H_{36}NO_6P_2$ 436.201.

Synthesis of Tetraisopropyl 1-fluoro-2-(3-pyridinyl)-ethylidene-1,1-bisphosphonate (5b). To 56.0 mg of NaH (60% in oil, 1.4 mmol) in 5 mL of dry THF was added 472.4 mg of **5a** (1.08 mmol) under N_2, and the mixture was stirred at 0 °C for 0.5 h and then for 1 h at room temperature. Selectfluor (576.5 mg, 1.5 equiv) was added at 0 °C and stirring continued for 2 h under N_2. The bath temperature was brought to room temperature, 5 mL of dry DMF was added, and stirring was continued for an additional 5 h. The reaction mixture was quenched by the addition of 50 mL of water and extracted with CH_2Cl_2 (3 × 100 mL), and the combined organic phases were dried over $MgSO_4$ and concentrated. The resulting oil was purified on a silica gel column to obtain 324.5 mg of **5b** (EtOAc/acetone, 4:1; $R_f = 0.5$, 66% yield): ^{1}H NMR ($CDCl_3$) δ 8.4 (1H, m), 8.3 (1H, m), 7.5 (1H, m), 7.1 (1H, m), 4.7 (4H, m), 3.3 (2H, m), 1.0−1.2 (24H, m); ^{13}C NMR ($CDCl_3$) δ 152.1, 148.0, 139.0, 130.4 (m), 122.8, 95.9 (C−F, m), 73.3 (t, $J = 3.5$ Hz), 72.9 (t, $J = 3.3$ Hz), 36.1 (d, $J = 18.4$ Hz), 24.38 (bt), 24.15 (bt), 23.83(t) 23.45 (t); ^{31}P NMR ($CDCl_3$) δ 12.28 (d, $^2J_{PF} = 74.5$ Hz); HRMS, MH^+ *m/z* 454.1915, calcd for $C_{19}H_{35}FNO_6P_2$ 454.1923.

General Method for the Preparation of 5c and 5d. To 32.0 mg of NaH (60% in oil, 0.8 mmol) in 5 mL of dry THF was added 266.0 mg of **5a** (0.6 mmol) in 2 mL of dry THF. The mixture was stirred for 0.5 h at 0 °C and for 1 h at room temperature under N_2. To the resultant carbanion solution was added 122.4 mg of *N*-chlorosuccinimide (NCS) (1.5 equiv) [for the bromo-analogue, equivalent *N*-bromosuccinimide (NBS) was added], and stirring was continued for 1 h at 0 °C and overnight at room temperature. In both cases quantitative conversions were observed by ^{31}P NMR. The reaction mixture was then dissolved in 100 mL of H_2O and extracted with CH_2Cl_2 (3 × 100 mL). The combined extracts were dried over $MgSO_4$, concentrated under vacuum, and purified on a silica gel column (EtOAc/acetone, 4:1) to obtain 245.0 mg of **5c** (87% yield). Similarly, **5d** was obtained in 93% yield.

Tetraisopropyl 1-chloro-2-(3-pyridinyl)-ethylidene-1,1-bisphosphonate (5c): ^{1}H NMR ($CDCl_3$) δ 8.5 (1H, d, $J = 1.2$ Hz), 8.3 (1H, dd, $J = 4.8$, 1.7 Hz), 7.6 (1H, d, $J = 7.8$ Hz), 7.1 (1H, dd, $J = 7.4$, 4.5 Hz), 4.7 (4H, m), 3.4 (2H, t, $J = 12.5$ Hz), 1.3−1.0 (24H, m); ^{13}C NMR ($CDCl_3$) δ 152.6, 148.3, 139.5, 130.9 (t, $J = 3.2$ Hz), 122.4, 73.7 (t, $J = 4.3$ Hz), 73.4 (t, $J = 4.0$ Hz), 62.5 (t, $J = 143.7$ Hz), 38.0, 24.3−23.3 (m); ^{31}P NMR ($CDCl_3$) δ 14.02 (s); HRMS, MH^+ *m/z* 470.161, calcd for $C_{19}H_{35}ClNO_6P_2$ 470.162.

Tetraisopropyl 1-bromo-2-(3-pyridinyl)-ethylidene-1,1-bisphosphonate (5d): ^{1}H NMR ($CDCl_3$) δ 8.5 (1H, br s), 8.4 (1H, dd, $J = 4.4$, 1.3 Hz, 7.6 (1H, d, $J = 8.0$ Hz), 7.1 (1H, dd, $J = 7.6$, 5.0 Hz), 4.8 (4H, m), 3.5 (2H, t, $J = 12.4$ Hz), 1.3−1.0 (24H, m); ^{13}C NMR ($CDCl_3$) δ 152.6, 148.2, 139.3, 131.3 (t, $J = 7.9$ Hz), 122.3, 73.8 (t, $J = 3.9$ Hz), 73.4 (t, $J = 4.0$ Hz), 55.2 (t, $J = 140.0$ Hz), 38.4, 24.5−23.3 (m); ^{31}P NMR ($CDCl_3$) δ 14.10 (s); HRMS, MH^+ *m/z* 514.113, calcd for $C_{19}H_{35}BrNO_6P_2$ 514.112.

General Method for the Hydrolysis of Intermediate Esters 5b−5d to 5e−5g. Both tetraisopropyl esters **5b** and **5c** were hydrolyzed by overnight reflux in excess concentrated HCl. Similarly, **5d** was hydrolyzed using concentrated HBr. A quantitative conversion was observed for each of the compounds by ^{31}P NMR. In both cases, solvent was removed under vacuum and the remaining residues were washed with acetone or EtOH, respectively, and then with acetone, leaving white powders that were dried under vacuum. Acid **5e** was previously reported to have been made by a different method (characterized by IR, ^{1}H, ^{13}C NMR and FAB$^+$ HRMS; no ^{31}P NMR or combustion analytical data were given).[54]

1-Fluoro-2-(3-pyridinyl)-ethylidene-1,1-bisphosphonic acid (5e): obtained in 94% yield; ^{1}H NMR (D_2O) δ 8.55 (1H, s), 8.47 (1H, d, $J = 5.6$ Hz), 8.39 (1H, d, $J = 8.0$ Hz), 7.80 (1H, dd, $J = 7.8$, 5.7 Hz), 3.50 (2H, m); ^{13}C NMR (D_2O) δ 151.85, 146.81, 141.09, 135.85 (m), 124.14, 100.00 (m), 37.69 (d, $^2J_{CF} = 18.4$ Hz); ^{31}P NMR (D_2O) δ 11.57 (d, $^2J_{PF} = 66.6$ Hz). Anal. [$C_7H_{10}FNO_6P_2 \cdot (H_2O)_{1.50}$] C, H, N. HRMS, $(M - H)^-$ *m/z* 283.987, calcd for $C_7H_9FNO_6P_2$ 283.988.

1-Chloro-2-(3-pyridinyl)-ethylidene-1,1-bisphosphonic acid (5f): obtained in 90.7% yield; ^{1}H NMR (D_2O) δ 8.65 (1H, s), 8.50−8.47 (2H, m), 7.81 (1H, dd, $J = 5.7$, 7.4 Hz), 3.55 (2H, t, $J = 9.7$ Hz); ^{13}C NMR (D_2O) δ 151.90, 147.22, 141.43, 135.12 (t, $J = 8.0$ Hz), 124.02, 67.61 (t, $^1J_{PC} = 116.0$ Hz), 37.91; ^{31}P NMR (D_2O) δ 13.37 (s). Anal. [$C_7H_{10}ClNO_6P_2 \cdot (H_2O)$] C, H, N. HRMS, $(M - H)^-$ *m/z* 299.958, calcd for $C_7H_9ClNO_6P_2$ 299.959.

1-Bromo-2-(3-pyridinyl)-ethylidene-1,1-bisphosphonic acid (5g): obtained in 75.4% yield; ^{1}H NMR (D_2O) δ 8.65 (1H, s), 8.50 (1H, d, $J = 8.0$ Hz), 8.47 (1H, d, $J = 5.7$ Hz), 7.79 (1H, t, $J = 7.8$ Hz), 3.7 (m); ^{13}C NMR (D_2O) δ 152.39, 147.44, 141.85, 136.56 (m), 124.12, 66.68 (t, $^1J_{PC} = 111.8$ Hz), 39.34; ^{31}P NMR (D_2O) δ 13.50 (s). Anal. [$C_7H_{10}BrNO_6P_2 \cdot (H_2O)_{1.5}$] C, H, N. HRMS, $(M - H)^-$ *m/z* 343.908, calcd for $C_7H_9BrNO_6P_2$ 343.908.

Synthesis of Triethyl 3-(3-pyridinyl)-2-phosphonopropionate (7a). To 658.0 mg of NaH (60% in oil, 16.4 mmol) dispersed in 10 mL of dry THF was slowly added 3.0 g of triethyl phosphonoacetate (13.5 mmol) at 0 °C under N_2, and stirring was continued at 0 °C for 0.5 h and for 1 h at room temperature. In another flask, 2.7 g of 3-picolyl chloride hydrogen chloride (16.4 mmol) was added to 658.0 mg of NaH (60% in oil, 16.4 mmol) in 10 mL of dry DMF. After about 0.5 h of stirring, it was added slowly to the triethyl phosphonoacetate enolate solution and stirred under N_2 for 3 h at room temperature. The reaction was quenched by the addition of 100 mL of H_2O and extracted with CH_2Cl_2 (4 × 100 mL). The organic extracts were combined, dried over $MgSO_4$, concentrated, and purified by silica gel chromatography (EtOAc/acetone, 4:1; $R_f = 0.45$) to obtain **7a** as a thick oil (1.44 g, 34%): ^{1}H NMR ($CDCl_3$) δ 8.4 (2H, m), 7.4 (1H, m), 7.1 (1H, m), 4.0 (6H, m), 3.1 (3H, m), 1.2 (6H, m), 1.0 (3H, m); ^{13}C NMR ($CDCl_3$) δ 168.1 (d, $J = 5.0$ Hz), 149.9, 148.1, 136.3, 134.0 (d, $J = 15.4$ Hz), 123.4, 63.0 (m), 61.6 (m), 47.2 (d, $J = 129.8$ Hz), 30.0 (d, $J = 4.2$ Hz), 16.4 (d, $J = 6.2$ Hz), 14.0; ^{31}P NMR ($CDCl_3$) δ 21.78 (s); HRMS, MH^+ *m/z* 316.130, calcd for $C_{14}H_{23}NO_5P$ 316.131.

Synthesis of Triethyl 2-fluoro-3-(3-pyridinyl)-2-phosphonopropionate (7b). To 120.0 mg of NaH (60% in oil, 3.0 mmol) dispersed in 2 mL of dry THF was added 785.1 mg of **7a** (2.49 mmol) in 2 mL of dry THF at 0 °C and stirred under N_2 at 0 °C for 0.5 h and for 1 h at room temperature. Selectfluor (1.3 g, 3.7 mmol) was added to the enolate solution and stirred at 0 °C for 1 h and overnight at room temperature. The reaction was quenched by adding several drops of EtOH. It was then dissolved in 50 mL of H_2O and extracted with CH_2Cl_2 (3 × 50 mL). The organic extracts were combined, dried over $MgSO_4$, and evaporated. The residue was chromatographed on a silica gel column using CH_2Cl_2/acetone, 2:3, as the mobile phase to produce **7b** as a thick oil (0.70 g, 84% yield): ^{1}H NMR ($CDCl_3$) δ 8.3 (1H, m), 8.2 (1H, s), 7.4 (1H, d, $J = 7.7$ Hz), 7.0 (1H, dd, $J = 7.7$, 4.2 Hz), 4.0 (6H, m), 3.3 (2H, m), 1.1 (6H, m), 0.96 (3H, t, $J = 6.6$ Hz); ^{13}C NMR ($CDCl_3$) δ 166.1 (dd, $J = 23.1$, 4.3 Hz), 151.1, 148.9, 137.9, 129.2 (d, $J = 12.7$ Hz), 123.3, 95.2 (dd, $J = 199.8$, 162.2 Hz), 64.4 (m), 62.6 (m), 36.5 (d, $J = 19.4$ Hz), 16.4 (d, $J = 6.0$ Hz), 14.0; ^{31}P NMR ($CDCl_3$) δ 12.50 (d, $J = 82.9$ Hz); HRMS, MH^+ *m/z* 334.121, calcd for $C_{14}H_{22}FNO_5P$ 334.121.

General Method for the Preparation of 7c and 7d. 7a (360.0 mg in 2 mL of dry THF) was added dropwise to 1.3 equiv of NaH (60% in oil) dispersed in 5 mL of dry THF at 0 °C under N_2 and stirred for 20 min at 0 °C and for 0.5 h at room temperature. To the enolate solution was added 1.5 equiv of NBS (NCS for **7c**) at 0 °C, and the reaction mixture was stirred at the same temperature for 1 h and overnight at room temperature. It was then quenched by the addition of 100 mL of H_2O and extracted with CH_2Cl_2 (5 × 100 mL). The organic extracts were combined, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified on a silica gel column using EtOAc/acetone, 4:1, as the mobile phase. Both **7d** (0.4 g, 88%, $R_f = 0.53$) and **7c** (0.37 g, 93%, $R_f = 0.50$) were obtained as thick oils on evaporation of the eluates.

Triethyl 2-chloro-3-(3-pyridinyl)-2-phosphonopropionate (7c): ^{1}H NMR ($CDCl_3$) δ 8.4 (2H, m), 7.5 (1H, d, J = 7.5 Hz), 7.1 (1H, dd, J = 7.6, 4.7 Hz), 4.2 (6H, m), 3.7 (1H, dd, J = 14.2, 6.5 Hz), 3.3 (1H, dd, J = 14.1, 8.5 Hz), 1.3 (6H, t, J = 7.5 Hz), 1.1 (3H, t, J = 7.5 Hz); ^{13}C NMR($CDCl_3$) δ 166.0, 151.9, 148.9, 138.6, 130.0 (d, J = 13.3 Hz), 123.1, 68.9 (d, J = 146.2 Hz), 65.5 (d, J = 8.0 Hz), 65.1 (d, J = 8.9 Hz), 63.6, 39.1, 16.6 (d, J = 6.7 Hz), 14.0; ^{31}P NMR ($CDCl_3$) δ 15.21 (s); HRMS, MH^+ m/z 350.092, calcd for $C_{14}H_{22}ClNO_5P$ 350.092.

Triethyl 2-bromo-3-(3-pyridinyl)-2-phosphonopropionate (7d): ^{1}H NMR ($CDCl_3$) δ 8.5 (1H, s), 8.4 (1H, m), 7.6 (1H, d, J = 7.9 Hz), 7.2 (1H, m), 4.2 (6H, m), 3.8 (1H, m), 3.4 (1H, m), 1.3 (6H, td, J = 7.4, 2.3 Hz), 1.2 (3H, td, J = 6.6, 2.4 Hz); ^{13}C NMR ($CDCl_3$) δ 166.0, 151.9, 148.8, 138.6, 130.9 (d, J = 10.5 Hz), 122.9, 65.5 (d, J = 7.5 Hz), 64.9 (d, J = 7.1 Hz), 63.5, 59.9 (d, J = 145.1 Hz), 39.4, 16.5 (d, J = 7.0 Hz), 13.9; ^{31}P NMR ($CDCl_3$) δ 15.48 (s); HRMS, MH^+ m/z 394.041, calcd for $C_{14}H_{22}BrNO_5P$ 394.041.

Hydrolysis of (7a,b−d) to (7h,e−g), Respectively. Each ester was hydrolyzed by overnight reflux in excess 6 M HCl. ^{31}P NMR spectra of the reaction mixtures showed quantitative conversion to the desired products. After solvent removal under vacuum, powdery substances ware obtained, which were washed with acetone and EtOH or MeOH, respectively, using a centrifuge, then pumped dry and left overnight in a vacuum desiccator giving **7e−g** as white powders.

3-(3-Pyridinyl)-2-phosphonopropionic acid (7h): ^{1}H NMR (D_2O) δ 8.55 (1H, s), 8.46 (1H, d, J = 5.2 Hz), 8.38 (1H, d, J = 7.1 Hz), 7.01 (1H, dd, J = 7.9, 5.5 Hz), 3.25−3.02 (3H, m); ^{13}C NMR (D_2O) δ 180.7 (d, J = 3.2 Hz), 148.9, 146.4, 138.6 (d, J = 16.3 Hz), 137.7, 124.3, 54.0 (d, J = 112.6 Hz), 32.7; ^{31}P NMR (D_2O) δ 13.86 (s). Anal. [$C_8H_{10}NO_5P(H_2O)_{0.25}$] C, H, N.

2-Fluoro-3-(3-pyridinyl)-2-phosphonopropionic acid (7e): obtained in 73% yield; ^{1}H NMR (D_2O) δ 8.57 (1H, s), 8.54 (1H, d, J = 7.4 Hz), 8.38 (1H, d, J = 7.8 Hz), 7.87 (1H, t, J = 7.2 Hz), 3.67−3.44 (2H, m); ^{13}C NMR (D_2O) δ 172.9 (d, $^2J_{CF}$ = 20.9 Hz), 149.75, 142.96, 141.12, 136.70 (d, $^3J_{PC}$ = 11.5 Hz), 128.28, 97.13 (dd, $^1J_{CF}$ = 194.0 Hz and $^1J_{PC}$ = 145.0 Hz), 37.36 (d, $^2J_{CF}$ = 19.1 Hz); ^{31}P NMR (D_2O) δ 7.85 (d, $^2J_{PF}$ = 72.8 Hz). Anal. [$C_8H_9FNO_5P\cdot(H_2O)_{0.2}$] C, H, N. HRMS, $(M - H)^-$ m/z 248.013, calcd for $C_8H_8FNO_5P$ 248.012.

2-Chloro-3-(3-pyridinyl)-2-phosphonopropionic acid (7f): obtained in 69.1% yield; ^{1}H NMR (D_2O) δ 8.63 (1H, s), 8.55 (1H, d, J = 5.0 Hz), 8.45 (1H, d, J = 8.6 Hz), 7.86 (1H, t, J = 5.7 Hz), 3.87 (1H, dd, J = 14.5, 5.4 Hz), 3.37 (1H, dd, J = 14.8, 5.4 Hz); ^{13}C NMR (D_2O) δ 172.29, 150.23, 143.44, 140.93, 137.60 (d, $^3J_{PC}$ = 12.4 Hz), 127.93, 71.9 (d, $^1J_{PC}$ = 128.4 Hz), 39.97; ^{31}P NMR (D_2O) δ 10.30 (s). Anal. [$C_8H_9ClNO_5P\cdot(H_2O)_{0.75}$] C, H, N. HRMS, $(M - H)^-$ m/z 263.983, calcd for $C_8H_8ClNO_5P$ 263.982.

2-Bromo-3-(3-pyridinyl)-2-phosphonopropionic acid (7g): obtained in 70.5% yield; ^{1}H NMR (D_2O) δ 8.66 (1H, s), 8.54 (1H, d, J = 5.7 Hz), 8.48 (1H, d, J = 8.0 Hz), 7.86 (1H, dd, J = 7.4, 5.9 Hz), 3.91 (1H, dd, J = 14.1, 6.3 Hz), 3.43 (1H, dd, J = 14.5, 7.3 Hz); ^{13}C NMR (D_2O) δ 172.06, 150.34, 143.61, 140.87, 138.20 (d, $^3J_{PC}$ = 11.6 Hz), 127.80, 64.94 (d, $^1J_{PC}$ = 123.5 Hz), 40.29; ^{31}P NMR (D_2O) δ 10.54 (s). Anal. [$C_8H_9BrNO_5P\cdot(H_2O)_{0.18}$] C, H, N. HRMS, $(M - H)^-$ m/z 307.932, calcd for $C_8H_8BrNO_5P$ 307.932.

Chromatographic Profiles of BP Mineral Affinity to Hydroxyapatite. Hydroxyapatite (HAP) ceramic spheres (20 μm diameter, Bio-Rad, Herts, U.K.) were packed in a 0.66 cm (diameter) × 6.5 cm (length) glass column (Omnifit, Cambridge, U.K.). The HAP columns were attached to a Waters 650E Advanced Protein Purification System (Millipore Corp., Waters Chromatography Division, Milford, MA) in a running buffer of 1 mM potassium phosphate at pH 6.8. Each compound was prepared in 1 mM potassium phosphate buffer at pH 6.8, and 400 μmol was injected into the FPLC system. BP compounds were eluted in a gradient of phosphate buffer, concentration increasing from 1 to 1000 mM, and detected by a Water 484 UV absorbance detector (Millipore Corp., Waters Chromatography Division) at their optimum wavelength. HAP retention profiles of each compound were determined in triplicate for statistical analyses.

FPP Synthase Assay. FPP synthase was purified and assayed as described.[23] Briefly, a clone encoding human FPPS (gi 61680822) as an N-terminally His_6-tagged fusion protein was expressed in *Escherichia coli* BL21(DE3). Cells were lysed using a high-pressure cell disruptor, and the protein was purified to near homogeneity using Ni-NTA resin (Qiagen). Gel filtration chromatography was then performed using a Superdex 200 column (GE/Amersham). For kinetic analysis 40 ng (1 pmol) of pure FPP synthase were assayed in a final volume of 100 μL of buffer containing 50 mM Tris, pH 7.7, 2 mM $MgCl_2$, 0.5 mM TCEP, and 20 μg/mL BSA. The concentrations of substrates, GPP and IPP (^{14}C-IPP, 400 kBq/μmol), were 10 μM each in the standard reaction. Preincubation of enzyme and N-BP was started by the addition of the appropriate amount of enzyme with inhibitor in a reaction buffer in a volume of 80 μL. After 10 min, 20 μL of substrate in water was added to start the reaction and also make the inhibitor and substrate at the final desired concentration. Reactions were timed such that a maximum of 10% of the available substrate was used. Assays were terminated by the addition of 0.2 mL of concentrated HCl/methanol, 1:4, followed by a further incubation of 10 min at 37 °C. The reactions were then extracted with 0.4 mL of ligroin, and after thorough mixing, the amount of radioactivity in the upper phase was determined by mixing 0.2 mL of the ligroin with 4 mL of general purpose scintillant. This was then counted using a Packard Tricarb 1900CA scintillation counter. Data were analyzed using Graphpad Prism.

RGGT Assay. RGGT activity was measured by determining the incorporation of [^{3}H]GGPP into His-tagged canine Rab1a protein as previously described.[59] The final concentrations in the prenylation reaction were 50 mM sodium HEPES, pH 7.2, 5 mM $MgCl_2$, 1 mM DTE, 1 mM NP-40, 4 μM Rab1a, 5 μM GGPP (8000 dpm/pmol), 2 μM REP-1, and 50 nM RGGT. All reactions were allowed for 30 min at 37 °C in a 25 μL volume in glass tubes (Fisher). The assay was conducted in duplicate and repeated in three independent reactions. The experimental data were fitted using the enzyme kinetic module from Systat Software, and data are expressed as mean ± SEM of three independent experiments.

Assessment of Effects on Protein Prenylation. The effect of the **5** and **7** analogues on protein prenylation was studied using Triton X-114 fractionation, in which prenylated proteins partition into the detergent-rich phase, whereas unprenylated proteins remain in the aqueous phase.[49] Briefly, cells were treated for 24 h and then lysed in 20 mM Tris, 150 mM NaCl, pH 7.5, and 1% Triton X-114; a sample was taken for determination of protein concentration, and then the remaining lysate was incubated at 37 °C for 10 min. Following centrifugation at 13000*g* for 2 min, the aqueous and detergent-rich phases were separated, and then Triton X-114 was added back to the aqueous phase to 1% v/v and the extraction process was repeated. Aqueous phases, equivalent to 20 μg of unfractionated lysate, were electrophoresed on 12% gels and western blotted for unprenylated Rab11 (an abundant, ubiquitous Rab), β-actin, or unprenylated Rap1A. Effects are expressed as the lowest effective dose (from three independent experiments), that is, the lowest dose at which unprenylated Rap1A or Rab11 was detected.

Measurement of Viable J774 Cell Number. The number of viable J774 cells following treatment with RIS and 3-PEHPC analogues for 48 h was assessed as previously described.[46] Experiments were carried out in replicates of six, and the data expressed are the mean ± SEM of at least four independent experiments.

Acknowledgment. This research was supported by Procter & Gamble Pharmaceuticals, Inc. C.E.M. and R.G.G.R. were consultants for Procter & Gamble Pharmaceuticals, Inc., at the time this paper was prepared and submitted. We thank Professor Michael Rogers, University of Aberdeen, for his interest in this research and for helpful discussions of preliminary data. C.E.M. dedicates this paper to the memory of Professor Yoshihiko Ito (1937−2006): vd. *Angew. Chem.* **2007**, 46, 3176.

Supporting Information Available: ^{1}H, ^{13}C, and ^{31}P NMR spectra and table of combustion elemental analysis data for **5e−5g**

and **7e**−**7h**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Green, J. R. Bisphosphonates: preclinical review. *Oncologist* **2004**, *9*, 3−13.

(2) Rogers, M. J.; Gordon, S.; Benford, H. L.; Coxon, F. P.; Luckman, S. P.; Monkkonen, J.; Frith, J. C. Cellular and molecular mechanisms of action of bisphosphonates. *Cancer* **2000**, *88*, 2961−2978.

(3) Russell, R. G. G.; Rogers, M. J. Bisphosphonates: from the laboratory to the clinic and back again. *Bone* **1999**, *25*, 97−106.

(4) Russell, R. G. G.; Croucher, P. I.; Rogers, M. J. Bisphosphonates: pharmacology, mechanisms of action and clinical uses. *Osteoporosis Int*. **1999**, *9*, 66−80.

(5) Fleisch, H. Bisphosphonates: mechanisms of action. *Endocrine Rev*. **1998**, *19*, 80−100.

(6) Ghosh, S.; Chan, J. M. W.; Lea, C. R.; Meints, G. A.; Lewis, J. C.; Tovian, Z. S.; Flessner, R. M.; Loftus, T. C.; Bruchhaus, I.; Kendrick, H.; Croft, S. L.; Kemp, R. G.; Kobayashi, S.; Nozaki, T.; Oldfield, E. Effects of bisphosphonates on the growth of entamoeba histolytica and plasmodium species in vitro and in vivo. *J. Med. Chem*. **2004**, *47*, 175−187.

(7) Martin, M. B.; Grimley, J. S.; Lewis, J. C.; Heath, H. T., III; Bailey, B. N.; Kendrick, H.; Yardley, V.; Caldera, A.; Lira, R.; Urbina, J. A.; Moreno, S. N. J.; Docampo, R.; Croft, S. L.; Oldfield, E. Bisphosphonates inhibit the growth of *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Roxoplasma gondii*, and *Plasmodium falciparum*: a potential route to chemotherapy. *J. Med. Chem*. **2001**, *44*, 909−916.

(8) Rodriguez, N.; Bailey, B. N.; Martin, M. B.; Oldfield, E.; Urbina, J. A.; Docampo, R. Radical cure of experimental cutaneous leishmaniasis by the bisphosphonate pamidronate. *J. Infect. Dis*. **2002**, *186*, 138−140.

(9) Yardley, V.; Khan, A. A.; Martin, M. B.; Slifer, T. R.; Araujo Fausto, G.; Moreno Silvia, N. J.; Docampo, R.; Croft, S. L.; Oldfield, E. In vivo activities of farnesyl pyrophosphate synthase inhibitors against *Leishmania donovani* and *Toxoplasma gondii*. *Antimicrob. Agents Chemother*. **2002**, *46*, 929−931.

(10) Wang, L.; Kamath, A.; Das, H.; Li, L.; Bukowski, J. F. Antibacterial effect of human Vγ2Vδ2 T cells in vivo. *J. Clin. Invest*. **2001**, *108*, 1349−1357.

(11) Cromartie, T. H.; Fisher, K. J.; Grossman, J. N. The discovery of a novel site of action for herbicidal bisphosphonates. *Pest. Biochem. Physol*. **1999**, *63*, 114−126.

(12) Das, H.; Wang, L.; Kamath, A.; Bukowski, J. F. Vγ2Vδ2 T-cell receptor-mediated recognition of aminobisphosphonates. *Blood* **2001**, *98*, 1616−1618.

(13) Coleman, R. E. Bisphosphonates in breast cancer. *Ann. Oncol*. **2005**, *16*, 687−695.

(14) Fromigue, O.; Lagneaux, L.; Body, J. J. Bisphosphonates induce breast cancer cell death in vitro. *J. Bone Miner. Res*. **2000**, *15*, 2211−2221.

(15) Chuah, C.; Barnes, D. J.; Kwok, M.; Corbin, A.; Deininger, M. W. N.; Druker, B. J.; Melo, J. V. Zoledronate inhibits proliferation and induces apoptosis of imatinib-resistant chronic myeloid leukaemia cells. *Leukemia* **2005**, *19*, 1896−1904.

(16) Ochiai, N.; Yamada, N.; Uchida, R.; Fuchida, S.-I.; Okano, A.; Okamoto, M.; Ashihara, E.; Inaba, T.; Shimazaki, C. Nitrogen-containing bisphosphonate incadronate augments the inhibitory effect of farnesyl transferase inhibitor tipifarnib on the growth of fresh and cloned myeloma cells in vitro. *Leukemia Lymphoma* **2005**, *46*, 1619−1625.

(17) Yuasa, T.; Nogawa, M.; Kimura, S.; Yokota, A.; Sato, K.; Segawa, H.; Kuroda, J.; Maekaw, T. A third-generation bisphosphonate, mindronic acid (YM529), augments the interferon α/β-mediated inhibition of renal cell cancer cell growth both in vitro and in vivo. *Clin. Cancer Res*. **2005**, *11*, 858−859.

(18) Segawa, H.; Kimura, S.; Sato, K.; Nogawa, M.; Yuasa, T.; Yokota, A.; Hodohara, K.; Fujiyama, Y.; Maekawa, T. The anti-leukemic efficacy of the third generation bisphosphonate ONO5920/YM529. *Leukemia Res*. **2005**, *29*, 451−457.

(19) Roelofs, A. J.; Hulley, P. A.; Meijer, A.; Ebetino, F. H.; Russell, R. G. G.; Shipman, C. M. Selective inhibition of Rab prenylation by a bhosphonocarboxylate analogue of risedronate induces apoptosis, but not s-phase arrest, in human myeloma cells. *Int. J. Cancer* **2006**, *119*, 1254−1261.

(20) Sanders, J. M.; Ghosh, S.; Chan, J. M. W.; Meints, G.; Wang, H.; Raker, A. M.; Song, Y.; Colantino, A.; Burzynska, A.; Kafarski, P.; Morita, C. T.; Oldfield, E. Quantitative structure−activity relationships for $\gamma\delta$ T cell activation by bisphosphonates. *J. Med. Chem*. **2004**, *47*, 375−384.

(21) Wilhelm, M.; Kunzmann, V.; Eckstein, S.; Reimer, P.; Weissinger, F.; Ruediger, T.; Tony, H.-P. $\gamma\delta$ T Cells for immune therapy of patients with lymphoid malignancies. *Blood* **2003**, *102*, 200−206.

(22) Rondeau, J.-M.; Bitsch, F.; Bourgier, E.; Geiser, M.; Hemmig, R.; Kroemer, M.; Lehmann, S.; Ramage, P.; Rieffel, S.; Strauss, A.; Green, J. R.; Jahnke, W. Structural basis for the exceptional in vivo efficacy of bisphosphonate drugs. *ChemMedChem* **2006**, *1*, 267−273.

(23) Kavanagh, K. L.; Guo, K.; Dunford, J. E.; Wu, X.; Knapp, S.; Ebetino, F. H.; Rogers, M. J.; Russell, R. G. G.; Oppermann, U. The molecular mechanism of nitrogen-containing bisphosphonates as antiosteoporosis drugs. *Proc. Natl. Acad. Sci*. **2006**, *103*, 7829−7834.

(24) van Beek, E.; Pieterman, E.; Cohen, L.; Lowik, C.; Papapoulos, S. Nitrogen-containing bisphosphonates inhibit isopentenyl pyrophosphate isomerase/farnesyl pyrophosphate synthase activity with relative potencies corresponding to their antiresorptive potencies in vitro and in vivo. *Biochem. Biophys. Res. Commun*. **1999**, *255*, 491−494.

(25) van Beek, E.; Pieterman, E.; Cohen, L.; Lowik, C.; Papapoulos, S. Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. *Biochem. Biophys. Res. Commun*. **1999**, *264*, 108−111.

(26) Keller, R. K.; Fliesler, S. J. Mechanism of aminobisphosphonate action: characterization of alendronate inhibition of the isoprenoid pathway. *Biochem. Biophys. Res. Commun*. **1999**, *266*, 560−563.

(27) Dunford, J. E.; Thompson, K.; Coxon, F. P.; Luckman, S. P.; Hahn, F. M.; Poulter, C. D.; Ebetino, F. H.; Rogers, M. J. Structure−activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates. *J. Pharmacol. Exp. Ther*. **2001**, *296*, 235−242.

(28) Hosfield, D. J.; Zhang, Y.; Dougan, D. R.; Broun, A.; Tari, L. W.; Swanson, R. V.; Finn, J. Structural basis for bisphosphonate-mediated inhibition of isoprenoid biosynthesis. *J. Biol. Chem*. **2004**, *279*, 8526−8529.

(29) Cheng, F.; Oldfield, E. Inhibition of isoprene biosynthesis pathway enzymes by phosphonates, bisphosphonates, and diphosphates. *J. Med. Chem*. **2004**, *47*, 5149−5158.

(30) Coxon, F. P.; Helfrich, M. H.; Van't Hof, R.; Sebti, S.; Ralston, S. H.; Hamilton, A.; Rogers, M. J. Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. *J. Bone Miner. Res*. **2000**, *15*, 1467−1476.

(31) Lin, J. H. Bisphosphonates: a review of their pharmacokinetic properties. *Bone* **1996**, *18*, 75−85.

(32) Sato, M.; Grasser, W.; Endo, N.; Akins, R.; Simmons, H.; Thompson, D. D.; Golub, E.; Rodan, G. A. Bisphosphonate action. alendronate localization in rat bone and effects on osteoclast ultrastructure. *J. Clin. Invest*. **1991**, *88*, 2095−2105.

(33) Azuma, Y.; Sato, H.; Oue, Y.; Okabe, K.; Ohta, T.; Tsuchimoto, M.; Kiyoki, M. Alendronate distributed on bone surfaces inhibits osteoclastic bone resorption in vitro and in experimental hypercalcemia models. *Bone* **1995**, *16*, 235−245.

(34) Masarachia, P.; Weinreb, M.; Balena, R.; Rodan, G. A. Comparison of the distribution of 3H-alendronate and 3H-etidronate in rat and mouse bones. *Bone* **1996**, *19*, 281−290.

(35) Jung, A.; Bisaz, S.; Bartholdi, P.; Fleisch, H. Influence of pyrophosphate on the exchange of calcium and phosphate ions on hydroxyapatite. *Calcif. Tissue Res*. **1973**, *13*, 27−40.

(36) Van Beek, E.; Lowik, C.; Que, I.; Papapoulos, S. Dissociation of binding and antiresorptive properties of hydroxybisphosphonates by substitution of the hydroxyl with an amino group. *J. Bone Miner. Res*. **1996**, *11*, 1492−1497.

(37) Ebetino, F. H.; Francis, M. D.; Rogers, M. J.; Russell, R. G. G. Mechanisms of action of etidronate and other bisphosphonates. *Rev. Contemp. Pharmacother*. **1998**, *9*, 233−243.

(38) Jung, A.; Bisaz, S.; Fleisch, H. Binding of pyrophosphate and two diphosphonates by hydroxylapatite crystals. *Calcif. Tissue Res*. **1973**, *11*, 269−280.

(39) Brown, R. J.; Van Beek, E.; Watts, D. J.; Lowik, C. W. G. M.; Papapoulos, S. E. Differential effects of aminosubstituted analogs of hydroxy bisphosphonates on the growth of *Dictyostelium discoideum*. *J. Bone Miner. Res*. **1998**, *13*, 253−258.

(40) McKenna, C. E.; Shen, P.-D. Fluorination of methanediphosphonate esters by perchloryl fluoride. synthesis of fluoromethanediphosphonic acid and difluoromethanediphosphonic acid. *J. Org. Chem*. **1981**, *46*, 4573−4576.

(41) Blackburn, G. M.; England, D. A.; Kolkmann, F. Monofluoro- and difluoromethylenebisphosphonic acids: isopolar analogs of pyrophosphoric acid. *Chem. Commun*. **1981**, 930−932.

(42) Berkowitz, D. B.; Bose, M. (α-Monofluoroalkyl)phosphonates: a class of isoacidic and "tunable" mimics of biological phosphates. *J. Fluorine Chem*. **2001**, *112*, 13−33.

(43) Boehm, H.-J.; Banner, D.; Bendels, S.; Kansy, M.; Kuhn, B.; Mueller, K.; Obst-Sander, U.; Stahl, M. Fluorine in medicinal chemistry. *ChemBioChem* **2004**, *5*, 637−643.
(44) Biffinger, J. C.; Kim, H. W.; DiMagno, S. G. The polar hydrophobicity of fluorinated compounds. *ChemBioChem* **2004**, *5*, 622−627.
(45) Ismail, F. M. D. Important fluorinated drugs in experimental and clinical use. *J. Fluorine Chem.* **2002**, *118*, 27−33.
(46) Coxon, F. P.; Helfrich, M. H.; Larijani, B.; Muzylak, M.; Dunford, J. E.; Marshall, D.; McKinnon, A. D.; Nesbitt, S. A.; Horton, M. A.; Seabra, M. C.; Ebetino, F. H.; Rogers, M. J. Identification of a novel phosphonocarboxylate inhibitor of Rab geranylgeranyl transferase that specifically prevents Rab prenylation in osteoclasts and macrophages. *J. Biol. Chem.* **2001**, *276*, 48213−48222.
(47) Van Beek, E. R.; Lowik, C. W. G. M.; Ebetino, F. H.; Papapoulos, S. E. Binding and antiresorptive properties of heterocycle-containing bisphosphonate analogs: structure−activity relationships. *Bone* **1998**, *23*, 437−442.
(48) Boissier, S.; Ferreras, M.; Peyruchaud, O.; Magnetto, S.; Ebetino, F. H.; Colombel, M.; Delmas, P.; Delaisse, J.-M.; Clezardin, P. Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. *Cancer Res.* **2000**, *60*, 2949−2954.
(49) Coxon, F. P.; Ebetino, F. H.; Mules, E. H.; Seabra, M. C.; McKenna, C. E.; Rogers, M. J. Phosphonocarboxylate inhibitors of Rab geranylgeranyl transferase disrupt the prenylation and membrane localization of Rab proteins in osteoclasts in vitro and in vivo. *Bone* **2005**, *37*, 349−358.
(50) Banks, R. E.; Mohialdin-Khaffaf, S. N.; Lal, G. S.; Sharif, I.; Syvret, R. G. 1-Alkyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane salts: a novel family of electrophilic fluorinating agents *Chem. Commun.* **1992**, 595−596.
(51) Singh, R. P.; Shreeve, J. N. M. Recent highlights in electrophilic fluorination with 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo-[2.2.2]octane bis(tetrafluoroborate). *Acc. Chem. Res.* **2004**, *37*, 31−44.
(52) Romanenko, V. D.; Kukhar, V. P. Fluorinated phosphonates: synthesis and biomedical application. *Chem. Rev.* **2006**, *106*, 3868−3935.
(53) (a) Marma, M. S.; Khawli, L. E.; Harutunian, V.; Kashemirov, B. A.; McKenna, C. E. Synthesis of α-fluorinated phosphonoacetate derivatives using electrophilic fluorine reagents: perchloryl fluoride versus 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate) (Selectfluor) *J. Fluor. Chem.* **2005**, *126*, 1467−1475. (b) McKenna, C. E.; Kashemirov, B. A.; Upton, T. G.; Batra, V. K.; Goodman, M. F.; Pederson, L. C.; Beard, W. A.; Wilson, S. H. (*R*)-β,γ-Fluoromethylene-dGTP−DNA ternary complex with DNA polymerase β. *J. Am. Chem. Soc.* **2007**, in press.
(54) Inoue, S.; Okauchi, T.; Minami, T. New synthesis of *gem*-bis-(phosphono)ethylenes and their applications. *Synthesis* **2003**, 1971−1976.
(55) Zhang, F. L.; Casey, P. J. Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.* **1996**, *65*, 241−269.
(56) Casey, P. J.; Seabra, M. C. Protein prenyltransferases. *J. Biol. Chem.* **1996**, *271*, 5289−5292.
(57) Eastman, R. T.; Buckner, F. S.; Yokoyama, K.; Gelb, M. H.; Van Voorhis, W. C. Fighting parasitic disease by blocking protein farnesylation. *J. Lipid Res.* **2006**, *47*, 233−240.
(58) Chakrabarti, D.; Da Silva, T.; Barger, J.; Paquette, S.; Patel, H.; Patterson, S.; Allen, C. M. Protein farnesyltransferase and protein prenylation in *Plasmodium falciparum*. *J. Biol. Chem.* **2002**, *277*, 42066−42073.
(59) Seabra, M. C.; James, G., L. Prenylation assays for small GTPases. *Methods Mol. Biol.* **1998**, *84*, 251−260.
(60) Russell, R. G. G. Determinants of structure−function relationships among bisphosphonates. *Bone* **2007**, *40*, S21−S25.
(61) Gasser, J. A.; Kneissel, M.; Thomsen, J. S.; Mosekilde, L. PTH and interactions with bisphosphonates. *J. Musculoskelet. Neuronal Interact.* **2000**, *1*, 53−56.
(62) Roelofs, A. J.; Thompson, K.; Gordon, S.; Rogers, M. J. Molecular mechanisms of action of bisphosphonates: current status. *Clin. Cancer Res.* **2006**, *12*, 6222s−6230s.
(63) Pohjala, E.; Heikkila-Hoikka, M.; Nikander, H.; Hanhijarvi, H. Methelenebisphosphonate acid derivatives, U.S. Patent 5,393,848, 1995.

JM0702884