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Synthesis of fluorescently tagged isoprenoid bisphosphonates that inhibit protein geranylgeranylation

Mona A. Maalouf,[a,†] Andrew J. Wiemer,[b,†] Craig H. Kuder,[c] Raymond J. Hohl[d,c,b] and David F. Wiemer[a,c,*]

[a]*Department of Chemistry, University of Iowa, Iowa City, IA 52242-1294, USA*
[b]*Program in Molecular & Cellular Biology, University of Iowa, Iowa City, IA 52242-1294, USA*
[c]*Department of Pharmacology, University of Iowa, Iowa City, IA 52242-1294, USA*
[d]*Department of Internal Medicine, University of Iowa, Iowa City, IA 52242-1294, USA*

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Abstract—Geminal bisphosphonates can be used for a variety of purposes in human disease including reduction of bone resorption in osteoporosis, treatment of fractures associated with malignancies of the prostate, breast, and lung, and direct anticancer activity against bone marrow derived malignancies. Previous research led to identification of some novel isoprenoid bisphosphonates that inhibit geranylgeranyl pyrophosphate (GGPP) synthesis and diminish protein geranylgeranylation. Described here is the synthesis of fluorescent anthranilate analogues of the most active isoprenoid bisphosphonates and examine their ability to impact post-translational processing of the small GTPases Ras, Rap1a, and Rab6. Similar to their non-fluorescent counterparts, some of these fluorescent isoprenoid bisphosphonates diminish protein geranylgeranylation. Their biological activity and fluorescent character suggest that they may be useful in studies of bisphosphonate localization both in cultured cells and in whole organisms.

1. Introduction

Several geminal bisphosphonates are used clinically for treatment of bone-related human diseases.[1] For example, risedronate (**1**, Fig. 1) is used for treatment of post-menopausal osteoporosis.[2] Zoledronate (**2**) is used to treat excess bone resorption caused by bone metastases from diseases such as multiple myeloma and prostate, breast, and lung cancers.[3] These bisphosphonates can be viewed as structural analogues of pyrophosphate (**3**) that contain a carbon in place of the central oxygen atom. The central carbon results in greater metabolic stability and also provides a scaffold that can be modified with additional substituents, such as the hydroxyl group and heteroaromatic rings found in risedronate and zoledronate. It has been postulated that through modification of their substituents, bisphosphonates can be targeted to specific enzymes involved in isoprenoid metabolism. Previous studies have focused on geminal bisphosphonates bearing one[4–6] or more isoprenoid[7] substituents, and recently some were prepared (e.g., **4**) that diminish protein geranylgeranylation.[8] The structures of these isoprenoid bisphosphonates differ significantly from current clinical compounds because they lack the –OH group on the geminal carbon that has been identified as a key part of the molecule that enhances localization to bone. These novel compounds also are much more hydrophobic than the clinical compounds due to the presence of the isoprenoid chains. The impact of these structural differences on intracellular and tissue localization is not yet known. Fluorescent analogues, as reported herein, will afford the opportunity to elucidate these characteristics.

Despite extensive studies,[9] the mechanisms of bisphosphonate action are not completely defined. On an organismal level, the clinically used bisphosphonates are known to localize to the bone based on studies with radiolabeled compounds. On a cellular level bisphosphonates have been shown to inhibit growth of a variety of cancer cell lines.[10] On a molecular level, it is known that the nitrogen-containing bisphosphonates target the isoprenoid biosynthetic pathway and impact

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* Corresponding author. Tel.: +1 319 335 1365; fax: +1 319 335 1270; e-mail: david-wiemer@uiowa.edu
† These authors contributed equally to this work, in chemistry and biology, respectively.

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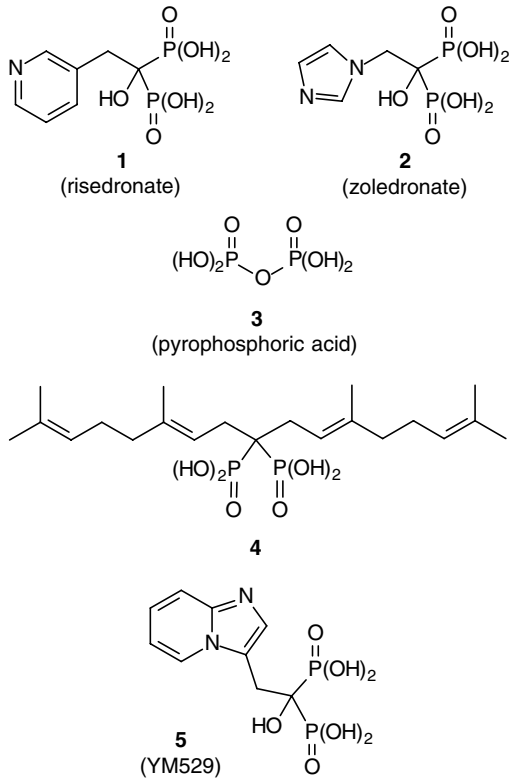
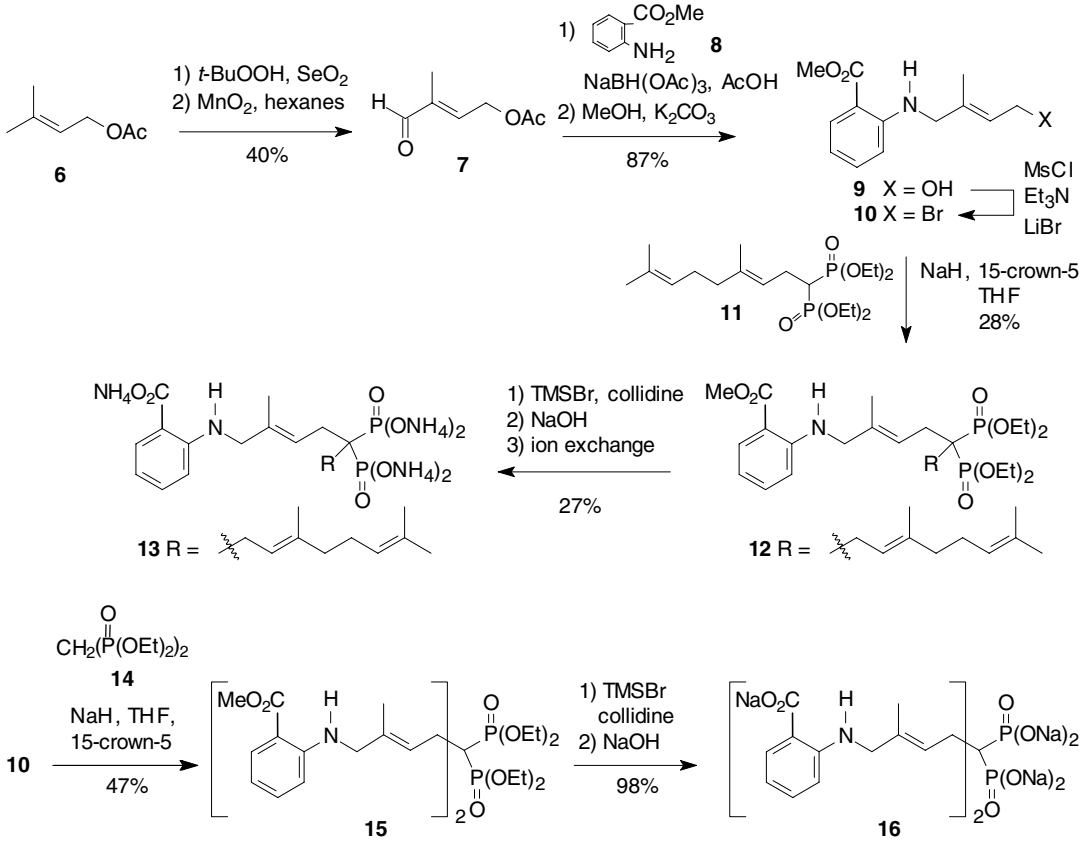


Figure 1. Representative bisphosphonates and pyrophosphoric acid.

isoprenylation of small GTPases.[11] Even some of the earliest studies suggested that there may be multiple target sites in living systems, including squalene synthase.[12] The clinically utilized nitrogenous bisphosphonates, such as risedronate, alendronate, and zoledronate, have been reported to inhibit farnesyl pyrophosphate (FPP) synthase,[13,14] and indeed the structure of FPP synthase complexed with zoledronate recently has been revealed.[15] Other bisphosphonates have been demonstrated to inhibit IPP isomerase.[16] Yet others,[17,18] including the digeranyl bisphosphonate **4**,[7] inhibit protein geranylgeranylation with little or no effect upon protein farnesylation, indicating that they target the isoprenoid pathways more selectively and further downstream.

One approach to further characterize the mechanism of bisphosphonate action is to study their organismal and/or cellular localization. Fluorescent bisphosphonates such as YM529 (**5**)[18,19] have been reported to be readily detectable at very low levels in plasma, urine, and bone,[19] and this can simplify the study of their pharmacokinetics. Additionally, a fluorescent pamidronate analogue, Pam78, has been shown to allow for visualization of microcalcifications in a mouse breast cancer model system.[20] As a tool for further study of localization of isoprenoid bisphosphonates, and to facilitate studies of the metabolism of isoprenoid bisphosphonates, fluorescent analogues of digeranyl bisphosphonate were designed (**4**). Despite incorporation of an anthranilate



Scheme 1. Preparation of bisphosphonates **13** and **16**.

fluorophore, some of these compounds still show a significant impact on protein geranylgeranylation but not upon protein farnesylation. In this manuscript, the synthesis and biological activity of the new isoprenoid bisphosphonates that incorporate a fluorescent label in a modified isoprenoid chain is reported.

2. Chemical synthesis

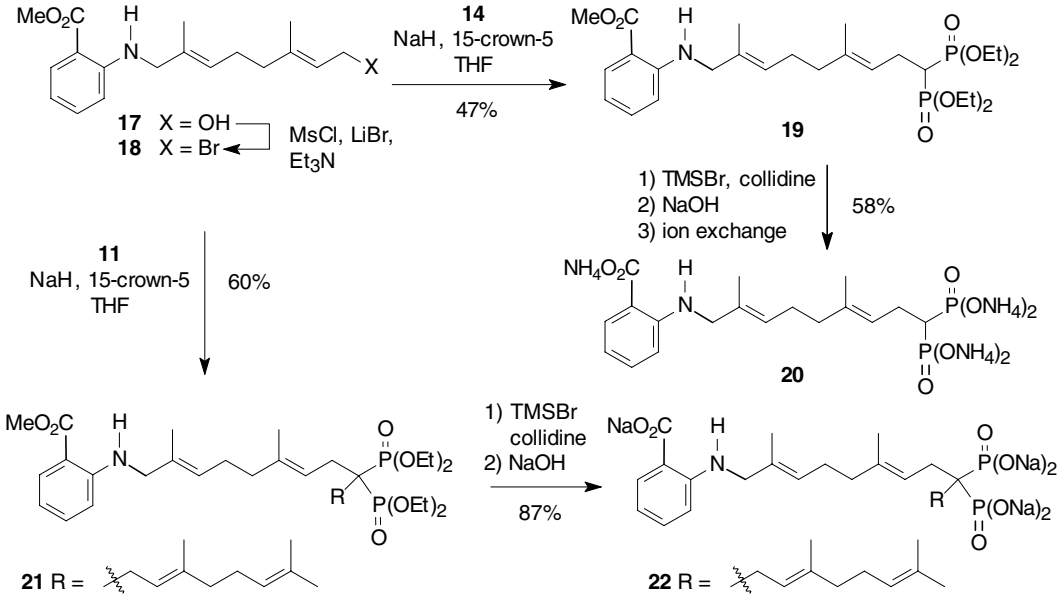
Synthesis of the allylic alcohol **9** begins with commercially available prenyl acetate **6** (Scheme 1). Reaction with selenium dioxide followed by oxidation with MnO_2 afforded the aldehyde **7**.[21] Treatment of the aldehyde with methyl anthranilate (**8**) under reductive amination conditions afforded allylic alcohol **9**.[22] The alcohol was then converted to the corresponding bromide (**10**) via the mesylate, providing entry to the fluorescent bisphosphonates with one isoprene unit between the fluorescent group and the bisphosphonate head. Treatment of bromide **10** with the anion of either geranyl bisphosphonate (**11**)[4] or tetraethyl methylenebisphosphonate (**14**) afforded bisphosphonate esters **12** and **15**, respectively. Finally, the corresponding salts **13** and **16** were obtained employing standard hydrolysis conditions.[23]

The second set of compounds (Scheme 2) was prepared from the known farnesol analogue **17**.[24] The alcohol **17** was first converted to the bromide **18** through reaction with MsCl followed by LiBr.[25] The allylic bromide **18** was treated with the anion of tetraethyl methylenebisphosphonate (**14**) to afford the bisphosphonate ester **19** in modest yield. Standard hydrolysis conditions resulted in the sodium salt which was passed through an ion-exchange column (ammonium form) to yield the fluorescently tagged bisphosphonate **20** as the ammonium salt in 58% yield. The dialkyl bisphosphonate ester **21** was prepared through treatment of the allylic bromide **18** with the anion of geranyl bisphosphonate **11**. Standard hydrolysis afforded the sodium salt **22** in 87% yield.

3. Biological assays

In order to gauge their impact on cell growth, compounds **13**, **16**, **20**, and **22** were tested for their ability to inhibit DNA synthesis in K562 leukemia cells. Similar to the digeranyl bisphosphonate **4**,[7] 24 h IC_{50} values for all of the compounds toward inhibition of [^{3}H]thymidine incorporation were greater than 100 μM (data not shown). To determine the internalization of the fluorescent compounds, treated cells were examined by fluorescent microscopy. As shown in Figure 2, the bisphosphonates **13**, **22**, **20**, and **16** are internalized, even though all are highly charged compounds. The compounds do not co-localize with the nuclear stain DRAQ5, and instead appear to be present in the cytoplasm.

The compounds were subsequently tested for their ability to impair protein prenylation in this cell line (Fig. 3). Ras protein was studied as a model of farnesylation, while Rap1a and Rab6 were studied as models for geranylgeranylation by GGPTase I and II, respectively. As positive controls, cells also were treated with lovastatin or digeranyl bisphosphonate (**4**).[7] Lovastatin has been shown to inhibit HMG-CoA reductase and to deplete cells of mevalonate and its derivatives FPP and GGPP,[26] which in turn diminishes both protein farnesylation and geranylgeranylation. This effect can be visualized by the appearance of slower migrating, double bands in the Ras and Rab6 Western blots. Because the Rap1a antibody only detects unmodified Rap1a and not modified Rap1a, lovastatin causes the appearance of a single band which is not seen in control cells. Digeranyl bisphosphonate (**4**) has been shown to inhibit geranylgeranylation[7] which occurs through depletion of



Scheme 2. Synthesis of bisphosphonates **20** and **22**.

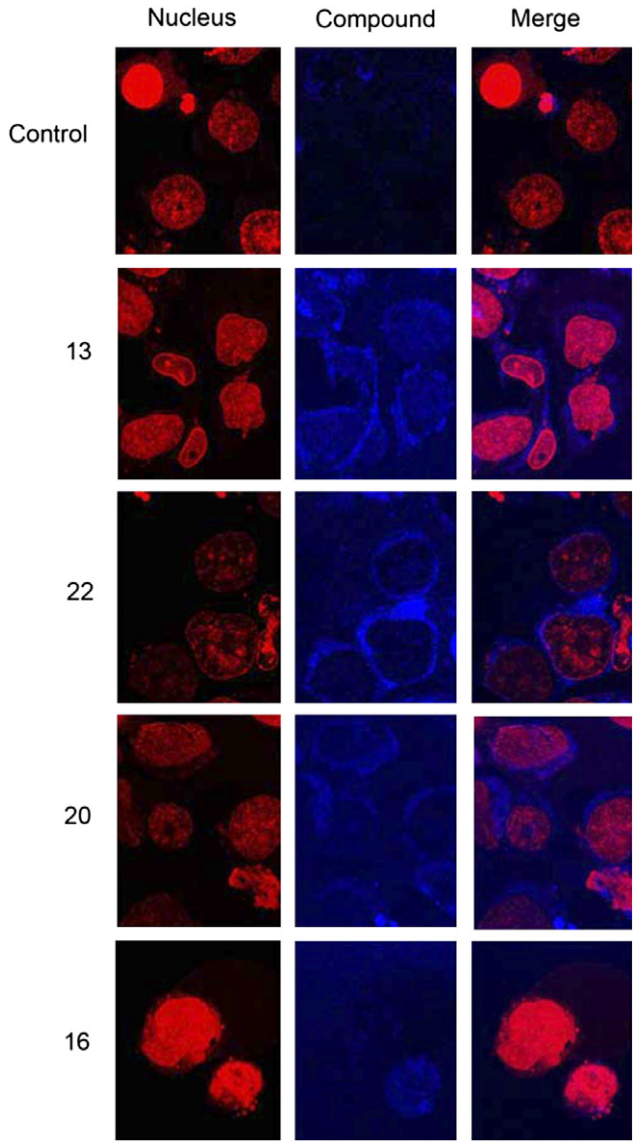


Figure 2. Compound cellular internalization. Fluorescent images of K562 cells treated for 24 h with 50 μM of active compounds **13** and **22** and inactive compounds **20** and **16**. Cells were stained with DRAQ5 nuclear stain as described in methods. Nucleus, compound, and merged images are displayed for each test compound.

GGPP. This can be visualized by both the appearance of the unmodified Rap1a band and the appearance of the upper band or widening of the band on the Rab6 blot. Digeranyl bisphosphonate (**4**) does not affect farnesylation, and as such no upper band appears on the Ras blot. Compounds **13** and **22** inhibited Rap1a and Rab6 geranylgeranylation at concentrations similar to digeranyl bisphosphonate and like digeranyl bisphosphonate had no effect on Ras farnesylation. Compounds **16** and **20** did not inhibit prenylation of Ras, Rap1a, or Rab6 at the concentrations tested.

4. Conclusions

In conclusion, the synthesis of four novel isoprenoid bisphosphonates incorporating an anthranilate moiety is reported. The key feature of the synthetic path is the use of the geraniol and farnesol analogues as building blocks for a diverse family of compounds. These data provide the first examples of visualization of isoprenoid bisphosphonates in cells. The ability of compounds **13** and **22**, but not compounds **16** or **20**, to inhibit geranylgeranylation further supports the hypothesis that at least one isoprenoid chain is required on the bisphosphonate template for inhibition of geranylgeranylation.[7] The ability of fluorescent bisphosphonates containing either a five- or ten-carbon linker to inhibit geranylgeranylation could indicate a GGPP synthase binding pocket[27] with sufficient size to accommodate further modifications, or a region of the molecule that is not directly involved in binding to the enzyme. Distinguishing between possibilities such as these, as well as investigations of the localization and activity in animal models, will form the basis for further studies of these intriguing compounds.

5. Experimental

5.1. General experimental conditions

Tetrahydrofuran (THF) was freshly distilled from sodium/benzophenone, and CH_2Cl_2 and Et_3N were freshly distilled from CaH_2; other solvents were purchased from commercial sources and used as provided. All reactions in non-aqueous solvents were conducted in oven-dried glassware under positive pressure of argon with magnetic stirring. Oil-free NaH was prepared by washing mineral oil dispersions with hexanes. NMR spectra were recorded at 300 MHz for ^1H, and 75 MHz for ^{13}C with $CDCl_3$ as solvent and $(CH_3)_4Si$ (^1H) or $CDCl_3$ (^{13}C, 77.2 ppm) as internal standards unless otherwise noted. The ^{31}P chemical shifts are reported in ppm relative to 85% H_3PO_4 (external standard). High resolution mass spectra were obtained at the University of Iowa Mass Spectrometry Facility or at the Washington University Resource for Biomedical and Bioorganic Mass Spectrometry. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA).

5.2. 4-Acetoxy-2-methyl-2-*E*-butenal (7)

To a stirred suspension of 4-hydroxybenzoic acid (0.64 g, 4.7 mmol) and selenium dioxide (2.1 g, 19 mmol) in CH_2Cl_2 was added 70% *tert*-butyl hydroperoxide (20 mL, 140 mmol). After 1 h, the mixture was cooled to 0 °C, stirred for 10 min, and prenyl acetate (5.7 g, 45 mmol) was added. The reaction mixture was allowed to warm to rt over a period of 24 h after which saturated $NaHCO_3$ was added. The mixture was extracted with CH_2Cl_2 and the organic extract was dried ($MgSO_4$) and filtered. The filtrate was concentrated to afford a yellow oil that was dissolved in hexanes and cooled to −10 °C. Solid MnO_2 (57 g, 560 mmol) was added to the vigorously stirred reaction, and after 24 h at −10 °C the mixture was filtered through a pad of Celite. The filtrate was concentrated in vacuo and purified by flash chromatography (hexanes/EtOAc; 80:20) to afford aldehyde **7** (5.0 g, 40%) with ^1H, ^{13}C spectra identical with literature data.[28]

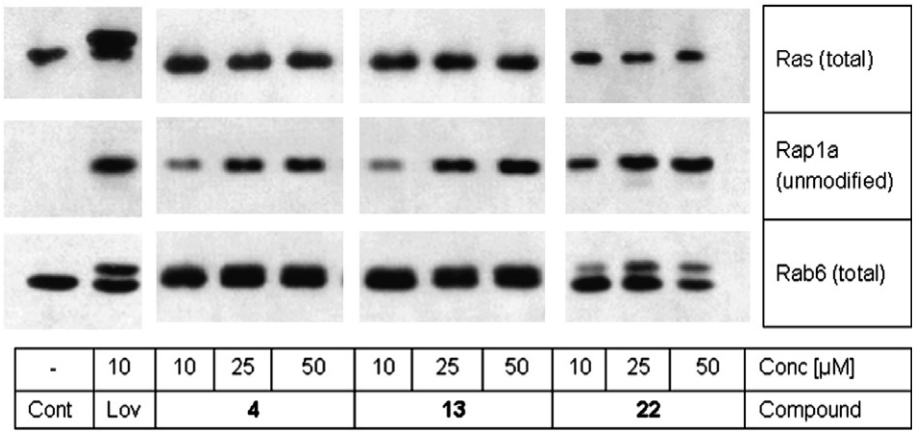


Figure 3. Western blot analysis of K652 cells treated for 24 h with indicated concentrations of compounds **4**, **13**, and **22**. Samples were compared to untreated K562 cells as a negative control (Cont), and to K562 cells treated with 10 μM lovastatin (Lov) as a positive control for inhibition of protein prenylation. Antibodies for total Ras and Rab6, and for unmodified Rap1a, were used for visualization of proteins.

5.3. Alcohol 9

To a solution of aldehyde **7** (1.9 g, 13 mmol) and methyl anthranilate (**8**, 1.9 mL, 15 mmol) in anhydrous dichloroethane were added molecular sieves (4 Å) and glacial acetic acid (0.94 mL, 16 mmol). The resulting mixture was allowed to stir at rt for 5 min, and then sodium triacetoxyborohydride (4.2 g, 19 mmol) was added at 0 °C. The reaction mixture was allowed to warm to rt over 2.5 h after which saturated $NaHCO_3$ was added dropwise at 0 °C. The aqueous layer was extracted with ether and the combined organic extract was dried ($MgSO_4$) and filtered. The filtrate was concentrated in vacuo and the residue was dissolved in methanol at rt. After potassium carbonate (4.6 g, 33 mmol) was added, the reaction mixture was left to stir for 2 h. Saturated NH_4Cl was added and the mixture was extracted with ether. The combined organic extract was dried ($MgSO_4$), filtered, and the filtrate was concentrated in vacuo and purified by flash chromatography (hexanes/EtOAc; 85:15) to give allylic alcohol **9** (2.8 g, 87%) as a yellow oil: ^{1}H NMR δ 7.92 (br s, 1H, exchanges with D_2O), 7.89 (dd, J = 8.0, 1.5 Hz, 1H), 7.30 (dd, J = 8.0, 1.5 Hz, 1H), 6.61–6.54 (m, 2H), 5.63 (t, J = 6.4 Hz, 1H), 4.16 (d, J = 6.7 Hz, 2H), 3.83 (s, 3H), 3.75 (d, J = 5.6 Hz, 2H; br s upon addition of D_2O), 1.99 (br s, 1H, exchanges with D_2O), 1.70 (s, 3H); ^{13}C NMR δ 169.2, 151.2, 135.2, 134.6, 131.7, 124.6, 114.8, 111.6, 110.0, 59.0, 51.5, 49.9, 14.7; HRMS (ESI, *m*/*z*): calcd $C_{13}H_{18}NO_8$ $[M+H]^+$, 236.1287; found 236.1283.

5.4. Bisphosphonate ester 12

To a solution of allylic alcohol **9** (0.51 g, 2.2 mmol) in CH_2Cl_2 at −50 °C were added triethylamine (0.39 mL, 2.8 mmol) and MsCl (0.20 mL, 2.6 mmol) consecutively. After 30 min, a solution of LiBr (0.47 g, 5.4 mmol) in THF was added to the cooled reaction mixture via cannula. The reaction flask was transferred to an ice bath for 1.5 h, and then water was added. The mixture was extracted with CH_2Cl_2, washed with ice-cold brine, dried ($MgSO_4$), and filtered. The solvent was removed in vacuo to obtain the allylic bromide as a yellow oil that was used without further purification. To a suspension of NaH (0.17 g, 60% dispersion in mineral oil, 4.3 mmol) in anhydrous THF at 0 °C was added 15-crown-5 (0.04 mL, 0.22 mmol) followed by geranyl bisphosphonate **11** (1.8 g, 4.3 mmol). After 1 h, the allylic bromide (0.81 g, 2.7 mmol) in THF was added via cannula. The reaction mixture was allowed to warm to rt over 2 h and then filtered. The filtrate was concentrated in vacuo and the resulting residue was purified by flash chromatography (4% MeOH in ether) to give bisphosphonate **12** (0.39 g, 28%) as a yellow oil: ^{1}H NMR δ 7.87 (dd, J = 8.0, 1.5 Hz, 1H), 7.29 (t, J = 7.7 Hz, 1H), 6.65 (d, J = 8.5 Hz, 1H), 6.55 (t, J = 7.6 Hz, 1H), 5.72 (t, J = 6.5 Hz, 1H), 5.40 (t, J = 6.8 Hz, 1H), 5.09 (t, J = 6.4 Hz, 1H), 4.15 (t, J = 7.0 Hz, 8H), 3.84 (s, 3H), 3.77 (d, J = 5.4 Hz, 2H), 2.64 (dd, J = 15.6, 6.7, 4H), 2.07–1.97 (m, 4H), 1.68 (s, 3H), 1.67 (s, 3H), 1.59 (s, 3H), 1.56 (s, 3H), 1.29 (dt, J = 7.1, 1.5 Hz, 12H); ^{13}C NMR δ 169.1, 151.5, 137.4, 134.6, 133.6, 131.6, 131.4, 124.4, 121.6 (t, J_{CP} = 7.3 Hz), 119.2 (t, J_{CP} = 7.2 Hz), 114.5, 111.9, 109.9, 62.6 (t, J_{CP} = 3.2 Hz, 4C), 51.5, 51.0, 45.8 (t, J_{CP} = 131.4 Hz), 40.2, 29.3 (t, J_{CP} = 4.6 Hz), 29.1 (t, J_{CP} = 4.4 Hz), 26.8, 25.8, 17.8, 16.6 (t, J_{CP} = 3.0 Hz, 4C), 16.3, 14.7; ^{31}P NMR δ 27.1 ppm; HRMS (ESI, *m*/*z*): calcd $C_{32}H_{53}NO_8P_2Na$ $[M+Na]^+$, 664.3144; found 664.3147.

5.5. Bisphosphonate salt 13

To a solution of bisphosphonate ester **12** (0.39 g, 0.61 mmol) in anhydrous CH_2Cl_2 at 0 °C were added 2,4,6-collidine (0.81 mL, 6.1 mmol) and TMSBr (0.80 mL, 6.1 mmol). The reaction mixture was allowed to warm to rt for 24 h, and toluene was added. The volatiles were removed in vacuo to afford a white solid that was dissolved in aqueous NaOH (10 mL, 1 N) at rt. After 24 h, the mixture was lyophilized to afford a gray solid. This solid was dissolved in a buffer solution (1:49 v/v isopropyl alcohol: 25 mM aqueous NH_4HCO_3), passed through an ion-exchange column using Dowex resin (50WX8-200) hydrogen form, washed with ammonium hydroxide solution (1 N), and then allowed to equilibrate with ion-exchange buffer solution. The

sample was eluted with the buffer solution and the eluant was lyophilized to give bisphosphonate salt **13** (0.36 g, 27%) as a white solid: ^{1}H NMR (25% ND_4OD in D_2O) δ 7.80 (d, J = 7.8 Hz, 1H), 7.55 (t, J = 7.1 Hz, 1H), 7.09–7.01 (br m, 1H), 6.93 (t, J = 7.8 Hz, 1H), 6.13 (br s, 1H), 5.82 (br s, 1H), 5.41 (br s, 1H), 3.97 (br s, 2H), 2.88–2.66 (m, 4H), 2.35–2.18 (m, 4H), 1.89 (s, 6H), 1.83 (s, 3H), 1.80 (s, 3H); ^{13}C NMR (100 MHz) δ 176.4, 150.5, 135.6, 133.6, 132.9, 132.1 (2C), 126.8, 125.5, 123.9, 120.2, 116.4, 113.7, 52.3, 44.4 (t, J_{CP} = 110.6 Hz), 40.2, 30.8, 26.8, 25.5, 17.6, 16.0, 14.5 (2C); ^{31}P (D_2O) δ 25.0 ppm; HRMS (ESI, *m/z*): calcd $C_{23}H_{34}NO_8P_2$ $[M-H]^-$, 514.1756; found 514.1781.

5.6. Ester 15

To a solution of allylic alcohol **9** (2.8 g, 12 mmol) in CH_2Cl_2 at −50 °C were added triethylamine (2.1 mL, 15 mmol) and MsCl (1.1 mL, 14 mmol) consecutively. After 30 min, a solution of LiBr (2.5 g, 29 mmol) in THF was added to the cooled reaction mixture via cannula. The reaction flask was transferred to an ice bath and after 1.5 h water was added. The mixture was extracted with CH_2Cl_2, washed with ice cold brine, dried ($MgSO_4$), and filtered. The solvent was removed in vacuo to obtain the allylic bromide **10** as a yellow oil that was used without further purification. To a suspension of NaH (0.94 g, 60% dispersion in mineral oil, 24 mmol) in anhydrous THF at 0 °C was added 15-crown-5 (0.23 mL, 1.2 mmol) followed by tetraethyl methylenebisphosphonate (1.7 g, 5.8 mmol). After 1 h, the allylic bromide was added via cannula as a solution in THF. The reaction mixture was allowed to warm to rt overnight and then quenched by addition of water. The mixture was extracted with ether and the combined organic extract was dried ($MgSO_4$) and filtered. The filtrate was concentrated in vacuo and the resulting residue was purified by flash chromatography (4% MeOH in ether) to give bisphosphonate **15** (2.0 g, 47%) as a yellow oil: ^{1}H NMR δ 7.87 (dd, J = 8.0, 1.7 Hz, 2H), 7.28 (dt, J = 8.7, 1.6 Hz, 2H), 6.62 (d, J = 8.5 Hz, 2H), 6.54 (dt, J = 7.5, 1.1 Hz, 2H), 5.69 (t, J = 6.7 Hz, 2H), 4.12 (t, J = 7.8 Hz, 8H), 3.83 (s, 6H), 3.74 (d, J = 5.6 Hz, 4H), 2.65 (dt, J = 15.9, 7.1 Hz, 4H), 1.62 (s, 6H), 1.26 (t, J = 7.1 Hz, 12H); ^{13}C NMR δ 169.1 (2C), 151.4 (2C), 134.5 (2C), 133.7 (2C), 131.5 (2C), 121.2 (t, J_{CP} = 7.3 Hz, 2C), 114.5 (2C), 111.8 (2C), 109.9 (2C), 62.6 (t, J_{CP} = 3.3 Hz, 4C), 51.4 (2C), 50.8 (2C), 45.6 (t, J_{CP} = 131.6 Hz), 29.1 (t, J_{CP} = 4.4 Hz, 2C), 16.5 (t, J_{CP} = 2.8 Hz, 4C), 14.7 (2C); ^{31}P NMR δ 27.5 ppm; HRMS (ESI, *m/z*): calcd $C_{35}H_{53}N_2O_{10}P_2$ $[M+H]^+$ 723.3175; found 723.3162.

5.7. Bisphosphonate 16

To a solution of bisphosphonate **15** (71 mg, 0.01 mmol) in anhydrous CH_2Cl_2 at 0 °C were added 2,4,6-collidine (0.19 mL, 15 mmol) and TMSBr (0.19 mL, 15 mmol). The reaction mixture was allowed to warm to rt over a period of 24 h, and toluene was then added. The volatiles were removed in vacuo to afford a white solid that was dissolved in aqueous NaOH (5 mL, 1 N). After 24 h, the mixture was poured into acetone and stored at 0 °C for 24 h. The mixture was filtered and the residue was washed with cold hexanes and dried in vacuo to afford bisphosphonate **16** (69 mg, 98%) as a white solid: ^{1}H NMR (400 MHz, D_2O-dioxane) δ 7.76 (dd, J = 7.8, 1.6 Hz, 2H), 7.32 (dt, J = 7.7, 1.6 Hz, 2H), 6.87 (d, J = 8.3, 2H), 6.72 (dt, J = 7.7, 1.1 Hz, 2H), 5.90 (t, J = 6.4 Hz, 2H), 3.73 (s, 4H), 2.55 (dt, J = 15, 6.4 Hz, 4H), 1.61 (s, 6H); ^{13}C NMR (100 MHz, 67.4 ppm with respect to dioxane) δ 177.0 (2C), 150.5 (2C), 133.3 (2C), 132.6 (2C), 132.2 (2C), 127.0 (t, J_{CP} = 6.4 Hz, 2C), 120.7 (2C), 117.0 (2C), 114.4 (2C), 52.6 (2C), 44.4 (t, J_{CP} = 111.3 Hz), 31.1 (2C), 14.6 (2C); ^{31}P (D_2O) 24.8 ppm; HRMS (ESI, *m/z*): calcd. $C_{25}H_{31}N_2O_{10}P_2$ $[M-H]^-$ 581.1454; found 581.1439.

5.8. Bisphosphonate ester 19

To a solution of allylic alcohol **17** (2.8 g, 12 mmol) in CH_2Cl_2 at −50 °C were added triethylamine (2.1 mL, 15 mmol) and MsCl (1.1 mL, 14 mmol) consecutively. After 30 min, a solution of LiBr (2.5 g, 29 mmol) in THF was added to the cooled reaction mixture via cannula. The reaction flask was transferred to an ice bath and allowed to stir for 1.5 h after which water was added. The mixture was extracted with CH_2Cl_2, washed with ice cold brine, dried ($MgSO_4$), and filtered. The solvent was removed in vacuo to obtain the allylic bromide **18** as a yellow oil that was used without further purification. To a suspension of NaH (0.94 g, 60% dispersion in mineral oil, 24 mmol) in anhydrous THF at 0 °C was added 15-crown-5 (0.23 mL, 1.2 mmol) followed by tetraethyl methylenebisphosphonate (1.7 g, 5.8 mmol). After 1 h, the allylic bromide **18** was added via cannula as a solution in THF. The reaction mixture was allowed to warm to rt overnight and then quenched by addition of water. The mixture was extracted with ether and the combined organic extract was dried ($MgSO_4$) and filtered. The filtrate was concentrated in vacuo and the resulting residue was purified by flash chromatography (4% MeOH in ether) to give bisphosphonate **19** (2.0 g, 47%) as a yellow oil: ^{1}H NMR δ 7.89 (dd, J = 8.1, 1.7 Hz, 1H), 7.35–7.28 (m, 1H), 6.63 (d, J = 8.4 Hz, 1H), 6.56 (t, J = 7.3 Hz, 1H), 5.41 (t, J = 6.8 Hz, 1H), 5.31 (t, J = 7.2 Hz, 1H), 4.18–4.05 (m, 8H), 3.86 (s, 3H), 3.73 (d, J = 4.6 Hz, 2H), 2.73–2.54 (m, 2H), 2.31 (tt, J = 23.8, 5.7 Hz, 1H), 2.19–2.08 (m, 2H), 2.05–1.97 (m, 2H), 1.67 (s, 3H), 1.64 (s, 3H), 1.33 (t, J = 7.1 Hz, 12H); ^{13}C NMR δ 169.3, 151.5, 136.6, 133.7, 134.6, 131.7, 126.1, 122.2 (d, J_{CP} = 7.3 Hz), 114.5, 111.8, 109.9, 62.6 (t, J_{CP} = 7.6 Hz, 4C), 51.6, 50.7, 39.5, 37.7 (t, J_{CP} = 132.7 Hz), 30.5, 26.5, 24.2 (t, J_{CP} = 5.1 Hz, 2C), 16.6 (t, J_{CP} = 5.3 Hz, 2C), 16.3, 14.7; ^{31}P NMR δ 24.9 ppm. Anal. Calcd for $C_{27}H_{45}NO_8P_2$: C, 56.54; H, 7.84. Found: C, 56.70; H, 8.03.

5.9. Bisphosphonate salt 20

To a solution of bisphosphonate **19** (0.26 g, 0.45 mmol) in anhydrous CH_2Cl_2 at 0 °C were added 2,4,6-collidine (0.59 mL, 4.5 mmol) and TMSBr (0.58 mL, 4.5 mmol), and the reaction mixture was allowed to warm to rt over a period of 22 h. Toluene was then added, and the volatiles were removed in vacuo to afford a white solid.

This material was dissolved in aqueous NaOH (5 mL, 1 N) at rt. After 24 h, the mixture was lyophilized to afford a gray solid. This solid was dissolved in a buffer solution (1:49 v/v isopropyl alcohol: 25 mM aqueous NH_4HCO_3), passed through an ion-exchange column using Dowex resin (50WX8-200) hydrogen form, washed with ammonium hydroxide solution (1 N), and then allowed to equilibrate with ion-exchange buffer solution. The sample was eluted with the buffer solution and the eluant was lyophilized to give bisphosphonate salt **20** as a white solid (0.14 g, 58%): ^{1}H NMR (D_2O) δ 7.80 (d, J = 7.8 Hz, 1H), 7.38 (t, J = 7.8 Hz, 1H), 6.86 (d, J = 7.9 Hz, 1H), 6.78 (t, J = 7.8 Hz, 1H), 5.60 (t, J = 5.9 Hz, 1H), 5.55–5.47 (m, 1H), 3.76 (s, 2H), 2.61–2.39 (m, 3H), 2.17 (t, J = 6.9 Hz, 2H), 2.09–2.01 (m, 2H), 1.68 (s, 6H); ^{13}C δ 176.9, 150.3, 135.2, 133.2, 132.2, 131.6, 128.5 (t, J_{CP} = 7.0 Hz), 127.7, 120.7, 117.1, 114.3, 51.5, 42.6 (t, J_{CP} = 119.4 Hz), 39.8, 27.3, 27.1, 16.4, 14.6; ^{31}P NMR δ 22.7 ppm; HRMS (ESI, m/z): calcd $C_{18}H_{23}NO_8P_2Na_5$ $[M+H]^+$, 558.0385; found 558.0387.

5.10. Bisphosphonate 21

To a solution of allylic alcohol **17** (2.4 g, 7.8 mmol) in CH_2Cl_2 at −50 °C were added triethylamine (1.4 mL, 10 mmol) and MsCl (0.72 mL, 9.3 mmol) consecutively. After 30 min, a solution of LiBr (1.7 g, 19 mmol) in THF was added to the reaction mixture via cannula. The reaction flask was transferred to an ice bath, allowed to stir for 1.5 h, and then water was added. The mixture was extracted with CH_2Cl_2, washed with ice-cold brine, dried ($MgSO_4$), and filtered. The solvent was removed in vacuo and the resulting residue was purified by flash chromatography (hexanes/EtOAc; 95:5) to afford the allylic bromide **18** (2.0 g, 70%) as a yellow oil. To a suspension of NaH (0.26 g, 60% dispersion in mineral oil, 6.5 mmol) in anhydrous THF at 0 °C was added 15-crown-5 (0.07 mL, 0.33 mmol) followed by the geranyl bisphosphonate **11** (1.4 g, 3.3 mmol). After 1 h, the allylic bromide **18** (1.2 g, 3.3 mmol) was added via cannula as a solution in THF. The reaction mixture was allowed to warm to rt overnight and then quenched by addition of water. The mixture was extracted with ether and the combined organic extract was dried ($MgSO_4$) and filtered. The filtrate was concentrated in vacuo and the resulting residue was purified by flash chromatography (4% MeOH in ether) to give bisphosphonate **21** (1.4 g, 60%) as a yellow oil: ^{1}H NMR δ 7.81 (dd, J = 8.1, 1.4 Hz, 1H), 7.24–7.21 (m, 1H), 6.56 (d, J = 8.5 Hz, 1H), 6.48 (dt, J = 7.6, 1.0 Hz, 1H), 5.40–5.31 (m, 3H), 5.03 (t, J = 6.4 Hz, 1H), 4.09 (t, J = 6.9 Hz, 8H), 3.78 (s, 3H), 3.65 (d, J = 5.3 Hz, 2H), 2.55 (dt, J = 16.0, 7.5 Hz, 4H), 2.10–1.89 (m, 8H), 1.59 (s, 6H), 1.54 (s, 6H), 1.52 (s, 3H), 1.24 (t, J = 7.1, 12H); ^{13}C NMR δ 169.2, 151.5, 137.1, 136.9, 134.6, 131.6, 131.5, 131.4, 126.4, 124.5, 119.3 (m, 2C), 114.5, 111.8, 109.9, 62.5 (t, J_{CP} = 3.0 Hz, 4C), 51.3, 50.8, 46.0 (t, J_{CP} = 131.3 Hz), 40.2, 39.9, 30.4, 29.2 (d, J_{CP} = 2.6 Hz), 26.8, 26.7, 25.8, 17.8, 16.6 (t, J_{CP} = 3.0 Hz, 4C), 16.4 (2C), 14.6; ^{31}P NMR 27.9 ppm; HRMS (ESI, *m/z*): calcd $C_{37}H_{62}NO_8P_2$ $[M+H]^+$, 710.3951; found 710.3937.

5.11. Bisphosphonate salt 22

To a solution of the bisphosphonate ester **21** (0.20 g, 0.28 mmol) in anhydrous CH_2Cl_2 at 0 °C were added 2,4,6-collidine (0.37 mL, 2.8 mmol) and TMSBr (0.36 mL, 2.8 mmol). The reaction mixture was allowed to warm to rt over a period of 19 h and then toluene was added. The volatiles were removed in vacuo to afford a white solid that was dissolved in aqueous NaOH (5 mL, 1 N) at rt. After 3.5 h, the mixture was poured into acetone and stored at 0 °C for 14 h. The mixture was filtered and the residue was washed with cold hexanes and dried in vacuo to afford the bisphosphonate salt **22** (0.17 g, 87%) as a white solid: ^{1}H NMR (D_2O) δ 7.84–7.79 (m, 1H), 7.32–7.24 (m, 1H), 6.65 (d, J = 8.2 Hz, 1H), 6.52 (t, J = 7.5 Hz, 1H), 5.90–5.79 (br m, 2H), 5.44–5.39 (br m, 1H), 5.11–5.02 (br m, 1H), 3.70 (bs, 2H), 2.62–2.43 (br m, 4H), 2.15–1.90 (m, 8H), 1.60 (s, 6H), 1.54 (s, 6H); ^{13}C NMR δ 176.7, 150.8, 135.2, 135.1, 132.8, 132.5, 132.4, 131.5, 126.5, 125.7, 124.3, 119.3, 116.0, 112.7, 110.2, 51.2, 44.3 (t, J_{CP} = 113.5 Hz), 40.6, 40.4, 29.6, 27.5, 27.3, 26.0 (2C), 18.0, 16.5, 16.3, 14.9; ^{31}P NMR δ 27.2 ppm; HRMS (ESI, *m/z*): calcd $C_{28}H_{39}NO_8P_2Na_5$ $[M+H]^+$, 694.1638; found 694.1632.

5.12. Cell culture

K562 cells[29] were cultured at 37 °C in the presence of 5% CO_2 in RPMI-1640 media containing 10% heat-inactivated fetal bovine serum. Bisphosphonates were diluted in water and added at the concentrations indicated.

5.13. [^{3}H]Thymidine assay

K562 cells (2×10^5 cells/200 μL) were incubated in a 96-well plate with the test compounds for 24 h. During the last 2 h, cells were labeled with [^{3}H]thymidine (0.04 μCi/well). Cells were collected and [^{3}H]thymidine incorporated into cellular DNA was determined by liquid scintillation counting as described previously.[30]

5.14. Western blot analysis

K562 cells (5×10^6 cells/5 mL) were incubated for 24 h in the presence or absence of test compounds and lovastatin. Western blotting was performed as previously described. Pan-Ras antibody was obtained from Inter-Biotech Corporation (Tokyo, Japan). Rab6, unmodified Rap1a, and HRP-conjugated anti-goat antibodies were purchased from Santa Cruz Biotech (CA). The antibody for Rap1a detects only the unmodified form of Rap1a, and not the isoprenylated form. The HRP-conjugated anti-mouse and anti-rabbit antibodies were purchased from Amersham/GE Healthcare.

5.15. Fluorescence microscopy

K562 cells were incubated with the test compounds at indicated concentrations for 24 h. Following three washes, nuclei were stained with 10 μM DRAQ5, which binds to cellular DNA, and imaged with a Bio-Rad Radiance 2100 MP multi-photon LSCM at the University of Iowa

Central Microscopy Research Facility. Test compounds containing the anthranilate fluorophore were excited with a Mai Tai multi-photon laser and their emission was detected between 400 and 450 nm. Nuclei were visualized via DRAQ5 excitation with a red diode laser and emission was detected between 650 and 700 nm.

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