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Synthesis and biological activity of isoprenoid bisphosphonates

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Abstract—Bisphosphonates have been used in the clinic to treat osteoporosis and to reduce bone resorption and the accompanying pathological bone fractures that attend a number of malignancies including multiple myeloma and cancers of the prostate, breast, and lung. There is also evidence that some bisphosphonates have direct anticancer activity. Expansion of the current class of bisphosphonates may lead to compounds that more selectively and potently target these cancers through inhibition of the mevalonate pathway. To this end, a set of dialkyl bisphosphonates bearing isoprenoid chains of varying lengths has been synthesized. Some of these compounds were found to have biological activity on post-translational processing of the oncogenic small GTPases, Ras and Rap1a, in human-derived K562 leukemia cells. Most importantly, these compounds impair protein geranylgeranylation and not protein farnesylation.

1. Introduction

The geminal bisphosphonic acid **1** (Fig. 1), also referred to as methylene bisphosphonate, may be viewed as an analogue of pyrophosphoric acid (**2**) where the central oxygen has been replaced by a $-CH_2-$ group. This conceptually simple substitution imparts far greater metabolic stability and provides a scaffold that can be modified with varied substituents on the central carbon. Many examples are known where one of those substituents is a hydroxyl group, which appears to increase the affinity of the bisphosphonate for calcium and improve the likelihood that the compound can be used for treatment of diseases of the bone. For example, risedronate (**3**) is used clinically for treatment of osteoporosis[1] and zoledronate (**4**) is used to treat many malignant diseases that cause bone resorption, including multiple myeloma and prostate, breast, and lung cancers.[2]

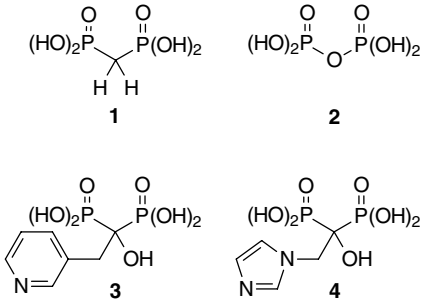

Figure 1. Methylene bisphosphonate, pyrophosphate, and some representative methylene bisphosphonate derivatives of clinical interest.

Despite extensive studies,[3] the molecular mechanism(s) responsible for the biological activity of bisphosphonates remains incompletely defined. Some bisphosphonates have been reported to inhibit farnesyl pyrophosphate (FPP) synthase,[4] an effect which may be of growing interest in cancer therapy due to the potential impact on post-translational isoprenylation of small GTPases. However, the effects of these compounds on other aspects of isoprenoid metabolism are much less clear. It is also unknown whether isoprenoid bisphosphonates can be targeted to specific enzymes involved in isoprenoid biosynthesis through modification of the isoprenoid chain length. We recently reported a

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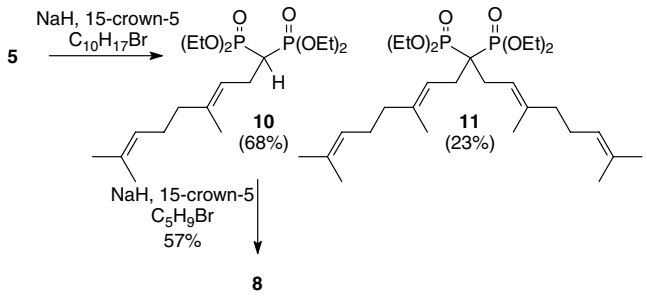
new strategy for modification of monoalkyl bisphosphonates through copper-mediated displacement of remote THP ethers,[5] and have now extended our studies to preparation of dialkyl bisphosphonates where the substituents on the bisphosphonate template are small isoprenoid chains. The syntheses of this set of compounds and our initial studies of the effects of isoprenoid chain length on biological activity are presented herein.[6]

2. Chemical synthesis

A number of strategies have been reported for synthesis of bisphosphonates.[7] Because isoprenoid halides are readily available and good electrophiles, the most attractive approach to synthesis of isoprenylated bisphosphonates involved alkylation of the anion of commercially available tetraethyl methylenebisphosphonate (**5**, Scheme 1).[8] For example, treatment of compound **5** with sodium hydride, 15-crown-5, and prenyl bromide provided a mixture of the mono- and disubstituted bisphosphonates **6** and **7**, respectively. When the ratio of base and prenyl bromide relative to compound **5** was kept low, the major product was the monoalkylated compound **6** while a high ratio favored the dialkylated product **7**. In principle, treatment of the monoalkyl compound **6** with base and geranyl bromide should give the dialkyl compound **8**, while treatment with base and farnesyl bromide would yield the larger terpenoid bisphosphonate **9**. In practice, separation of compounds **6** and **7** was difficult and we found it more convenient to add the larger isoprenoid chain to the bisphosphonate template first and then install the smaller chain as described below.

Bisphosphonates bearing other isoprenoid substituents were prepared through parallel reaction sequences. For example, reaction of the bisphosphonate **5** with base and geranyl bromide was used to prepare both the mono- and digeranyl bisphosphonates (**10** and **11**, respectively). As shown in Scheme 2, when the bisphosphonate **5** was treated with 1.1 equiv of base and geranyl bromide the monoalkylated compound predominated, but when compound **5** was treated with 2.5 equiv of base and geranyl bromide under the same reaction



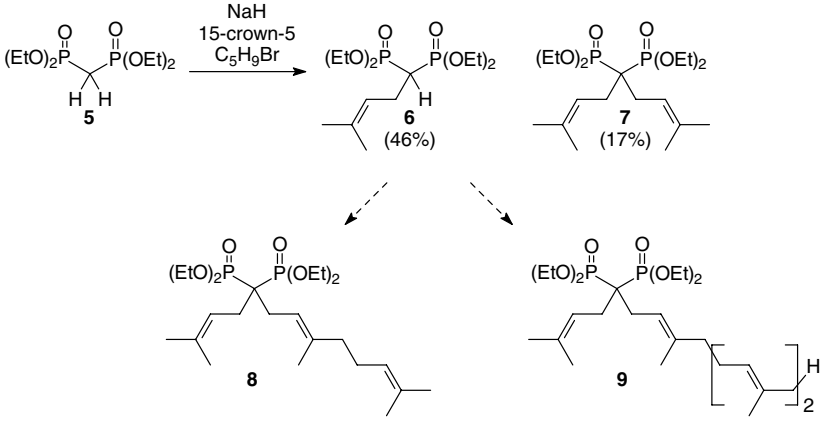
Scheme 2. Synthesis of bisphosphonates with a C_{10} (geranyl) substituent.

conditions the major product (85%) was the dialkylated compound **11**. The known monoalkyl compound **10**[9] was treated with base and prenyl bromide to afford compound **8** by a sequence complementary to that shown in Scheme 1. In the same manner, reaction of bisphosphonate **5** with base and farnesyl bromide gave the mono- and difarnesylated compounds (**12** and **13**, respectively; Scheme 3). The known monoalkyl compound **12**[10,11] then was treated with base followed by geranyl bromide to yield compound **14** or followed by prenyl bromide to yield compound **9**.

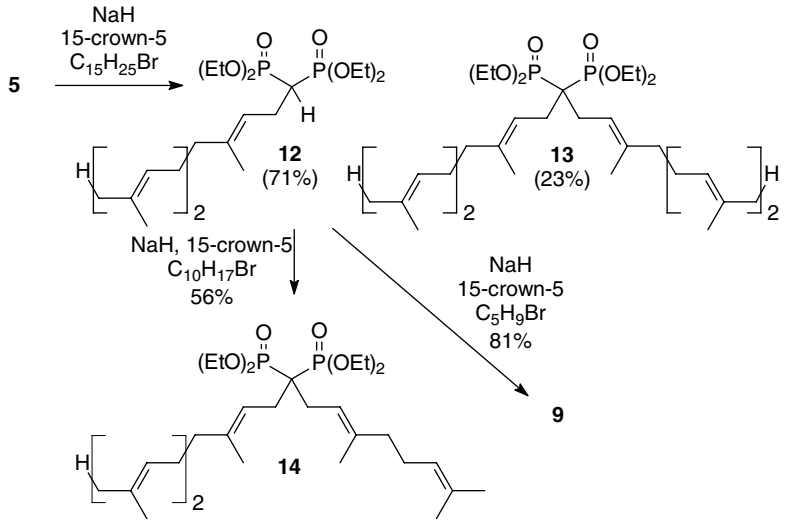
Once the desired set of dialkyl bisphosphonate esters was generated, ester hydrolysis was accomplished through standard reaction with trimethylsilyl bromide and collidine, followed by a basic workup to provide the corresponding bisphosphonic acid salt.[12] As shown in Figure 2, the yields for this final step were generally quite attractive.

3. Biological assays

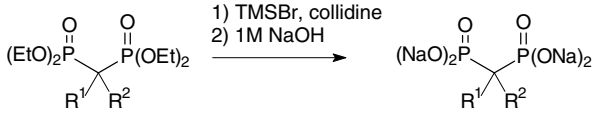
Before they were examined for their impact on isoprenoid biosynthesis, several of the isoprenoid bisphosphonates listed in Figure 2 were tested in [^{3}H]-thymidine incorporation assays[13] for their ability to inhibit growth of human-derived chronic myelogenous leukemia cells (K562). All of the tested compounds (**15**, **16**, **18**, and **19**) have IC_{50} values greater than 100 μM alone or in combination with 10 μM lovastatin (data not shown).



Scheme 1. Synthesis of bisphosphonates with a C_5 (prenyl) substituent.



Scheme 3. Synthesis of bisphosphonates with a C_{15} (farnesyl) substituent.



R^1	R^2	Reagent	Product	Yield
Prenyl	Prenyl	**7**	**15**	64%
Prenyl	Geranyl	**8**	**16**	100%
Prenyl	Farnesyl	**9**	**17**	81%
Geranyl	Geranyl	**11**	**18**	95%
Geranyl	Farnesyl	**14**	**19**	100%
Farnesyl	Farnesyl	**13**	**20**	82%

Figure 2. Hydrolysis of phosphonate esters.

The difarnesyl derivative **20** was not readily soluble under the test conditions and so this compound has not been studied in depth. The other five compounds (bisphosphonates **15**–**19**) were tested for their ability to impair protein farnesylation or geranylgeranylation in K562 cells. Ras and Rap1a were studied as indicators for alterations of farnesylation and geranylgeranylation, respectively.[14] Figure 3 is a compilation of Western blots for these two GTPases on lysates from control and lovastatin-treated cells also incubated with compounds **15**–**19**. Lovastatin, an inhibitor of HMG-CoA reductase, depletes cells of mevalonate and its subsequent derivatives including FPP and GGPP. Lovastatin treatment alone results in the accumulation of a more slowly migrating Ras band[15] and the appearance of an unmodified Rap1a band.[14] The latter occurs because the antibody for Rap1a only detects the non-geranylgeranylated Rap1a protein. All three of the dialkyl bisphosphonates that contained at least one ten-carbon chain, compounds **16**, **18**, and **19**, inhibited processing of Rap1a, as shown by the accumulation of unmodified Rap1a protein, but not Ras, as shown by the lack of accumulation of a more slowly migrating Ras protein (Fig. 3). Most evident is that the geranyl-containing compounds **16**, **18**, and **19** inhibit the geranylgeranylation of Rap1a in a concentration-dependent manner in the absence of lovastatin. Compounds **15** and **17** did

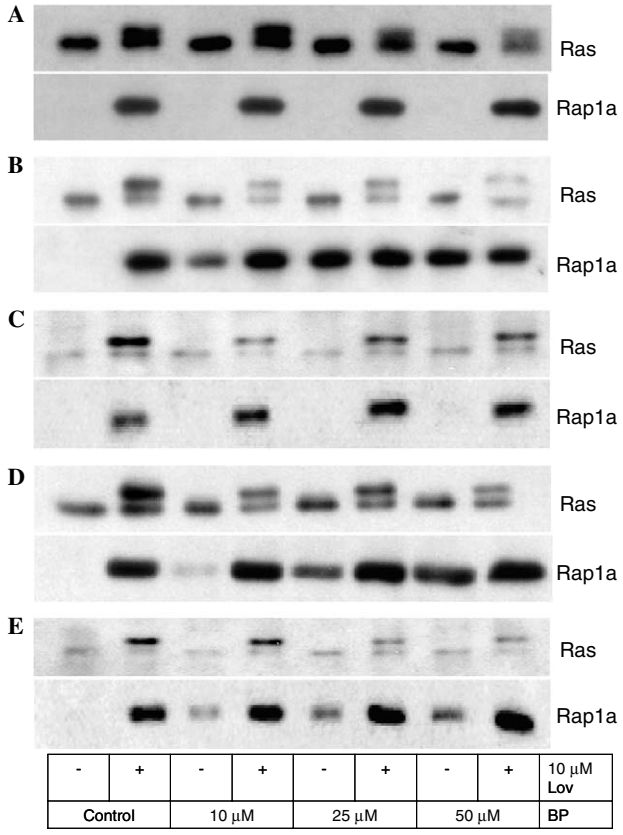


Figure 3. Effects of dialkylbisphosphonates on Ras and Rap1a processing. K562 cells were incubated with indicated concentrations of test compounds, in the absence (−) or presence (+) of 10 μM lovastatin. (A) Compound **15**; (B) compound **16**; (C) compound **17**; (D) compound **18**; and (E) compound **19**.

not inhibit Rap 1a processing up to a 50 μM concentration. Although none of the compounds **15**–**19** directly altered Ras expression, they do appear to alter the lovastatin-induced changes in Ras levels. For compounds **16**, **18**, and **19** there is primarily a reduction of unmod-

ified Ras levels, whereas for compounds **15** and **17** both unmodified and modified Ras levels are diminished.

4. Conclusions

Both symmetrical and unsymmetrical diisoprenoid bisphosphonates can be prepared in good yield by alkylation of tetraethyl methylene bisphosphonate, and the corresponding bisphosphonate salts display varying biological activity as a function of the isoprenoid substituents. The biological assays demonstrate differential effects on protein geranylgeranylation and farnesylation. Compounds **16**, **18**, and **19**, but not compounds **15** and **17**, impair geranylgeranylation which indicates a requirement for at least one ten-carbon isoprene chain as a substituent on the methylene bisphosphonate for this activity. Of the three geranyl-containing bisphosphonates, the geranyl/farnesyl compound **19** displayed the weakest inhibition of Rap1a processing, perhaps indicating that the bulky farnesyl group is disadvantageous for binding to the cellular target. That compounds **16**, **18**, and **19** mitigate the effects of lovastatin-induced accumulation of unmodified Ras may be a consequence of these compounds increasing cellular FPP levels. This might be expected if these compounds inhibit geranylgeranyl diphosphate synthase as compared to geranylgeranyl protein transferase.[16,17] While it may not be surprising that isoprenoid bisphosphonates impact an enzyme involved in isoprenoid metabolism, it would have been reasonable to expect compounds with farnesyl chains to have the greatest impact on geranylgeranyl diphosphate synthase given that its natural substrate is FPP. Further studies will be needed in this area to clarify this activity. Because aminobisphosphonates are used to treat patients with osteoporosis and select malignancies, it is conceivable that the novel dialkyl isoprene bisphosphonates reported here may be similarly, and perhaps more potently, active.

5. Experimental

5.1. General experimental conditions

THF was freshly distilled from sodium/benzophenone. All reactions in non-aqueous solvents were conducted in oven-dried glassware under a positive pressure of argon with magnetic stirring. NMR spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C with $CDCl_3$ as solvent and $(CH_3)_4Si$ (^{1}H) or $CDCl_3$ (^{13}C, 77.0 ppm) as internal standards unless otherwise noted. High-resolution mass spectra were obtained at the University of Iowa Mass Spectrometry Facility. Elemental analyses were preformed by an outside facility.

5.2. Tetraethyl 4-methyl-3-pentenyl-1,1-bisphosphonate (6) and tetraethyl 2,8-dimethyl-2,7-nonadienyl-5,5-bisphosphonate (7)

To a stirred solution of tetraethyl methylenebisphosphonate (4.90 mL, 19.3 mmol) in THF (20 mL) were added NaH (860 mg, 21.6 mmol) and 15-crown-5 (0.2 mL, 1 mmol) at 0 °C. Once hydrogen gas evolution had ceased, prenyl bromide (3.00 g, 20.2 mmol) was added as a neat liquid. The reaction mixture was allowed to stir for 1 h and quenched by addition of NH_4Cl (satd) and extracted with ether. The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. Purification by flash chromatography (2% methanol in ether) afforded two yellow oils, the mono- and dialkyl bisphosphonates **6** and **7** (46% and 17%, respectively):

For compound **6**: ^{1}H NMR δ 5.30 (t, $J = 7.2$ Hz, 1H), 4.23–4.12 (m, 8H), 2.71–2.56 (m, 2H), 2.32 (tt, $J_{HP} = 23.9$, $J = 6.1$ Hz, 1H), 1.70 (s, 3H), 1.65 (s, 3H), 1.34 (t, $J = 7.1$ Hz, 12H); ^{13}C NMR δ 133.1, 122.1 (t, $J_{CP} = 7.4$ Hz), 62.5–62.3 (m, 4C), 37.5 (t, $J_{CP} = 132.8$ Hz), 25.7, 24.2 (t, $J_{CP} = 5.0$ Hz,), 17.8, 16.5–16.4 (m, 4C); ^{31}P NMR +23.4 ppm. Anal. Calcd for $C_{14}H_{30}O_6P_2{\cdot}0.5H_2O$: C, 46.03; H, 8.55. Found: C, 46.21; H, 8.64.

For compound **7**: ^{1}H NMR δ 5.39 (t, $J = 7.0$ Hz, 2H), 4.21–4.11 (m, 8H), 2.61 (td, $J_{HP} = 15.9$, $J = 7.1$, 4H), 1.71 (s, 6H), 1.62 (s, 6H), 1.32 (t, $J = 7.1$ Hz, 12H); ^{13}C NMR δ 133.5 (2C), 119.6 (t, $J_{CP} = 7.4$ Hz, 2C), 62.6–62.3 (m, 4C), 46.1 (t, $J_{CP} = 131.4$ Hz), 29.4 (t, $J_{CP} = 4.5$ Hz, 2C), 26.2 (2C), 18.0 (2C), 16.7–16.5 (m, 4C); ^{31}P NMR +26.4 ppm. Anal. Calcd for $C_{19}H_{38}O_6P_2{\cdot}0.5H_2O$: C, 52.65; H, 9.07. Found: C, 52.62; H, 9.10.

5.3. Tetraethyl (*E*)-2,8,12-trimethyl-2,7,11-tridecatrienyl-5,5-bisphosphonate (8)

To a stirred suspension of NaH (80 mg, 1.88 mmol, washed with hexanes (3× 20 mL) and dried in vacuo) in THF (10 mL), 15-crown-5 (0.01 mL, 0.06 mmol) was added via syringe over 15 min at 0 °C. Geranyl bisphosphonate **10** (510 mg, 1.20 mmol) in THF (10 mL) was transferred via cannula to the NaH suspension over 10 min and the reaction mixture was allowed to stir for 30 min at 0 °C. Prenyl bromide (150 μL, 1.19 mmol) was added to the resulting mixture as a neat liquid. The reaction mixture was stirred for 3 h at 0 °C, filtered through Celite, and then concentrated in vacuo. The resulting yellow oil was purified by flash chromatography (0.5% methanol in Et_2O) to afford compound **8** as a clear oil (330 mg, 57%): ^{1}H NMR δ 5.44–5.30 (m, 2H), 5.10–5.05 (m, 1H), 4.16 (m, 8H), 2.62 (tt, $J_{HP} = 16.0$, $J = 7.0$ Hz, 4H), 2.10–1.90 (m, 4H), 1.71 (s, 3H), 1.67 (s, 3H), 1.62 (s, 6H), 1.60, (s, 3H), 1.32 (t, $J = 7.1$ Hz, 12H); ^{13}C NMR δ 137.2, 133.4, 131.5, 124.6, 119.9 (t, $J_{CP} = 7.2$ Hz), 119.5 (t, $J_{CP} = 7.5$ Hz), 62.6–62.5 (m, 4C), 46.1 (t, $J_{CP} = 131.3$ Hz), 40.3, 29.4–29.3 (m, 2C), 26.9, 26.3, 25.9, 18.1, 17.9, 16.8–16.6 (m, 4C), 16.5; ^{31}P NMR δ +26.5. Anal. Calcd for $C_{24}H_{46}O_6P_2{\cdot}0.5H_2O$: C, 57.47; H, 9.44. Found: C, 57.60; H, 9.38.

5.4. Tetraethyl (7*E*,11*E*)-2,8,12,16-tetramethyl-2,7,11,15-heptadecenyl-5,5-bisphosphonate (9)

To a stirred solution of farnesyl bisphosphonate **12** (507 mg, 0.95 mmol) in THF (20 mL) at 0 °C was added 15-crown-5 (0.02 mL, 0.1 mmol) as a neat liquid fol-

lowed by solid NaH (66.0 mg, 1.65 mmol). After 1 h, prenyl bromide was added as a neat liquid and the reaction mixture was allowed to stir for 20 min. The reaction mixture was quenched by addition of NH_4Cl (satd) and extracted with ether. The combined organic extracts were dried ($MgSO_4$) and concentrated in vacuo to produce a yellow oil. Final purification by flash chromatography (1% methanol in ether) afforded compound **9** as a yellow oil (428 mg, 81%): ^1H NMR δ 5.46–5.37 (m, 2H), 5.15–5.07 (m, 2H), 4.17 (quintet, $J = 7.4$ Hz, 8H), 2.70–2.55 (m, 4H), 2.08–1.94 (m, 8H), 1.71 (s, 3H), 1.68 (s, 3H), 1.62 (s, 6H), 1.60 (s, 6H), 1.32 (t, $J = 7.1$ Hz, 12H); ^{13}C NMR δ 137.1, 135.0, 133.2, 131.2, 124.4, 124.3, 119.7 (t, $J_{CP} = 7.2$ Hz), 119.3 (t, $J_{CP} = 7.3$ Hz), 62.5–62.2 (m, 4C), 46.0 (t, $J_{CP} = 131.3$ Hz), 40.2, 39.8, 29.3 (t, $J = 4.5$ Hz), 29.2 (t, $J = 4.5$ Hz), 26.8, 26.7, 26.1, 25.7, 17.9, 17.7, 16.5 (t, $J = 3.1$ Hz, 4C), 16.3, 16.0; ^{31}P NMR δ +26.4. Anal. Calcd for $C_{29}H_{54}O_6P_2$: C, 62.12; H, 9.71. Found: C, 62.33; H, 9.81.

5.5. Tetraethyl (6*E*,11*E*)-2,6,12,16-tetramethyl-2,6,11,15-heptadecenyl-9,9-bisphosphonate (11)

To a stirred solution of tetraethyl methylenebisphosphonate (12.6 mL, 49.7 mmol) in THF (40 mL) at 0 °C were added NaH (2.19 g, 54.8 mmol) and 15-crown-5 (0.47 mL, 2.32 mmol). Once hydrogen gas evolution had ceased, geranyl bromide (10.0 g, 46.1 mmol) was added as a neat liquid. The reaction mixture was allowed to stir for 1 h, then quenched by addition of NH_4Cl (satd) and extracted with ether. The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. Final purification by flash chromatography (1% methanol in ether) afforded two yellow oils, the mono- and dialkylated compounds **10** and **11** (68% and 23%, respectively):

For compound **10**: ^1H, ^{13}C, and ^{31}P NMR spectra are identical to the literature data.[5,9]

For compound **11**: ^1H NMR δ 5.43 (t, $J = 6.9$ Hz, 2H), 5.11 (tt, $J = 6.8$, 1.4 Hz, 2H), 4.22–4.12 (m, 8H), 2.63 (td, $J_{HP} = 16.0$, $J = 7.1$ Hz, 4H), 2.09–2.00 (m, 8H), 1.67 (s, 6H), 1.62 (s, 6H), 1.59 (s, 6H), 1.32 (t, $J = 7.1$ Hz, 12H); ^{13}C NMR δ 137.1 (2C), 131.4 (2C), 124.5 (2C), 119.5 (t, $J_{CP} = 7.3$ Hz, 2C), 62.6–62.4 (m, 4C), 46.1 (t, $J_{CP} = 131.1$ Hz), 40.3 (2C), 29.3 (t, $J_{CP} = 4.4$ Hz, 2C), 26.8 (2C), 25.8 (2C), 17.8 (2C), 16.7–16.6 (m, 4C), 16.4 (2C); ^{31}P NMR δ +26.7 ppm. Anal. Calcd for $C_{29}H_{54}O_6P_2 \cdot 0.5H_2O$: C, 61.14; H, 9.73. Found: C, 61.23; H, 9.70.

5.6. Tetraethyl (3*E*,7*E*)-4,8,12-trimethyl-3,7,11-tridecatrienyl-1,1-bisphosphonate (12) and tetraethyl (6*E*,10*E*,15*E*,19*E*)-2,6,10,16,20,24-hexamethyl-2,6,10,15,19,23-pentacosahexaenyl-13,13-bisphosphonate (13)

To a stirred suspension of NaH (140 mg, 3.52 mmol, washed with hexanes (3× 20 mL) and dried in vacuo) in THF (10 mL) at 0 °C, 15-crown-5 (0.03 mL, 0.15 mmol) was added via syringe over 15 min. Bisphosphonate **5** (800 μL, 3.16 mmol) was added as a neat liquid and the reaction mixture was allowed to stir for 30 min at 0 °C. Farnesyl bromide (1.00 g, 3.33 mmol) in THF (10 mL) was transferred via cannula over 10 min, and the reaction mixture was allowed to stir for 3 h. The resulting solution was then filtered through Celite and concentrated in vacuo. The resulting yellow oil was purified by flash chromatography (0.5% methanol in Et_2O) to afford the mono- and dialkylated products **12** and **13** (1.17 g, 71% and 260 mg, 23%, respectively):

For compound **12**: The ^1H, ^{13}C, and ^{31}P NMR spectra were identical to the literature data.[9–11]

For compound **13**: ^1H NMR δ 5.43 (t, $J = 6.8$ Hz, 2H), 5.15–5.05 (m, 4H), 4.22–4.11 (m, 8H), 2.63 (td, $J_{HP} = 19.0$, $J = 7.1$ Hz, 4H), 2.20–1.94 (m, 16 H), 1.68 (s, 6H), 1.63 (s, 6H), 1.60 (s, 12H), 1.32 (t, $J = 7.1$ Hz, 12H); ^{13}C NMR δ 137.2 (2C), 135.1 (2C), 131.3 (2C), 124.5 (2C), 124.3 (2C), 119.4 (t, $J_{CP} = 7.3$ Hz, 2C), 62.5–62.3 (m, 4C), 46.1 (t, $J_{CP} = 131.4$ Hz), 40.3 (2C), 39.8 (2C), 30.4 (2C), 29.2 (t, $J_{CP} = 4.4$ Hz, 2C), 26.9 (2C), 26.8 (2C), 25.8 (2C), 17.8 (2C), 16.6–16.4 (m, 4C), 16.1 (2C); ^{31}P NMR δ +26.6. Anal. Calcd for $C_{39}H_{70}O_6P_2 \cdot 0.5H_2O$: C, 66.36; H, 10.14. Found: C, 66.55; H, 10.16.

5.7. Tetraethyl (6*E*,11*E*,15*E*)-2,6,12,16,20-pentamethyl-2,6,11,15,19-henicosapentaenyl-9,9-bisphosphonate (14)

To a stirred solution of farnesyl bisphosphonate **12** (1.11 g, 2.25 mmol) in THF (40 mL) at 0 °C were added NaH (0.11 g, 2.68 mmol) and 15-crown-5 (0.05 mL, 0.25 mmol). After 20 min, the reaction mixture was allowed to warm to rt and geranyl bromide (790 μL, 3.39 mmol) was added as a neat liquid. The reaction mixture was stirred overnight and then quenched by addition of water (20 mL). The crude mixture was extracted with ether, dried ($MgSO_4$), and concentrated in vacuo. The resulting yellow oil was purified by flash chromatography (1% methanol in Et_2O) to provide compound **14** as a clear oil (1.22 g, 86%): ^1H NMR ($CDCl_3$) δ 5.43 (t, $J = 6.6$ Hz, 2H), 5.15–5.06 (m, 3H), 4.23–4.14 (m, 8H), 2.63 (td, $J_{HP} = 16.0$, $J = 7.0$ Hz, 4H), 2.17–1.94 (m, 12H), 1.67 (s, 6H), 1.62 (s, 6H), 1.59 (s, 9H), 1.32 (t, $J = 7.1$ Hz, 12H); ^{13}C NMR δ 137.0, 136.9, 134.9, 131.24, 131.18, 124.40, 124.38, 124.2, 119.40 (t, $J_{CP} = 7.2$ Hz), 119.37 (t, $J_{CP} = 7.3$ Hz), 62.4–62.2 (m, 4C), 46.0 (t, $J_{CP} = 131.2$ Hz), 40.15, 40.13, 39.8, 34.3, 29.2–29.1 (m, 2C), 26.8, 26.7, 25.69, 25.68, 17.7, 17.6, 16.5 (t, $J_{CP} = 3$ Hz, 4C), 16.34, 16.29, 16.0; ^{31}P NMR δ +27.3. Anal. Calcd for $C_{34}H_{62}O_6P_2 \cdot 0.5H_2O$: C, 64.03; H, 9.96. Found: C, 63.83; H, 9.94.

5.8. General procedure for phosphonate ester cleavage

The starting material (1.0 equiv) was dissolved in CH_2Cl_2 at 0 °C, and collidine (10 equiv) and TMSBr (10 equiv) were added as neat liquids. After 2 h, the reaction mixture was allowed to gradually warm to rt and stirred overnight. Toluene was added, the crude mixture was concentrated in vacuo, NaOH was added (5 mL, 1 M), and the mixture was stirred for 1 h. This

mixture then was poured into acetone (20 mL) and held at 4 °C for 18 h. The resulting solid, located between the two layers, was removed by filtration, dried in vacuo, and weighed.

5.9. 2,8-Dimethyl-2,7-nonadienyl-5,5-bisphosphonic acid, tetrasodium salt (15)

Yield 295 mg, 64%; ^{1}H NMR (D_2O) δ 5.61 (br t, J = 6.4 Hz, 2H), 2.47 (td, J_{HP} = 14.8, J = 6.8 Hz, 4H), 1.71 (s, 6H), 1.61 (s, 6H); ^{13}C NMR δ 134.6 (2C), 126.1 (t, J_{CP} = 6.9 Hz, 2C), 46.8 (t, J_{CP} = 112.3 Hz), 33.7 (t, J_{CP} = 3.2 Hz, 2C), 28.0 (2C), 20.0 (2C); ^{31}P NMR +24.2 ppm; HRMS (neg. ion ESI) m/z calcd for $(M-H)^-$ $C_{11}H_{21}O_6P_2$: 311.0813. Found: 311.0815.

5.10. (*E*)-2,8,12-Trimethyl-2,7,11-tridecatrienyl-5,5-bisphosphonic acid, tetrasodium salt (16)

Yield 310 mg, 100%; ^{1}H NMR (D_2O) δ 5.71–5.61 (m, 2H), 5.26–5.22 (m, 1H), 2.54–2.42 (m, 4H), 2.13–2.04 (m, 4H), 1.69 (s, 6H), 1.63 (s, 3H), 1.58 (s, 6H); ^{13}C NMR (with DSS) δ 137.6, 136.1, 133.9, 127.7, 126.9 (t, J_{CP} = 7.5 Hz), 126.8 (t, J_{CP} = 7.6 Hz), 47.2 (t, J_{CP} = 114.8 Hz), 42.3, 33.5–33.3 (m, 2C), 28.9, 28.0, 27.6, 20.1, 19.7, 18.0; ^{31}P NMR δ +24.6; HRMS (neg. ion ESI) m/z calcd for $(M-H)^-$ $C_{16}H_{29}O_6P_2$: 379.1439. Found: 379.1442.

5.11. (7*E*,11*E*)-2,8,12,16-Tetramethyl-2,7,11,15-heptadecenyl-5,5-bisphosphonic acid, tetrasodium salt (17)

Yield 156 mg, 81%; ^{1}H NMR (CD_3OD) δ 5.93–5.80 (m, 2H), 5.15–5.08 (m, 2H), 2.63–2.42 (m, 4H), 2.15–1.91 (m, 8H), 1.70 (s, 3H), 1.68 (s, 3H), 1.61 (s, 12H); ^{13}C NMR δ 135.3, 134.3, 132.1, 129.8, 126.9–126.6 (m, 2C), 126.5, 125.7, 41.9, 41.1, 32.0–31.7 (m, 2C), 28.6, 28.0, 26.5, 26.0, 18.5, 17.9, 16.4, 16.2; ^{31}P NMR δ +26.9; HRMS (neg. ion ESI) m/z calcd for $(M-H)^-$ $C_{21}H_{37}P_2O_6$: 447.2065. Found: 447.2052.

5.12. (6*E*,11*E*)-2,6,12,16-Tetramethyl-2,6,11,15-heptadecenyl-9,9-bisphosphonic acid, tetrasodium salt (18)

Yield 160 mg, 79%; ^{1}H NMR (CD_3OD) δ 5.80 (t, J = 6.3 Hz, 2H), 5.13 (t, J = 6.0 Hz, 2H), 2.69 (td, J_{HP} = 14.8, J = 6.9 Hz, 4H), 2.10–1.99 (m, 8H), 1.67 (s, 6H), 1.61 (br s, 12H). ^{13}C NMR δ 135.5 (2C), 131.7 (2C), 126.1 (2C), 124.2–124.1 (t, J_{CP} = 7.8 Hz, 2C), 45.4 (t, J_{CP} = 110.9 Hz), 41.6 (2C), 29.7 (t, J = 3.5 Hz, 2C), 28.3 (2C), 26.0 (2C), 17.9 (2C), 16.5 (2C); ^{31}P NMR δ +24.3 ppm; HRMS (neg. ion ESI) m/z calcd for $(M-H)^-$ $C_{21}H_{37}O_6P_2$: 447.2065. Found: 447.2072.

5.13. (6*E*,11*E*,15*E*)-2,6,12,16,20-Pentamethyl-2,6,11,15,19-henicosapentaenyl-9,9-bisphosphonic acid, tetrasodium salt (19)

Yield 1.09 g, 100%; ^{1}H NMR (D_2O) δ 5.67–5.63 (m, 2H), 5.25–5.21 (m, 2H), 5.18–5.15 (m, 1H), 2.57 (td, J_{HP} = 14.1, J = 5.7 Hz, 4H), 2.10–2.03 (m, 12H), 1.69 (s, 6H), 1.63 (s, 12H), 1.61 (s, 3H); ^{13}C NMR (with DSS) δ 138.5, 137.5, 137.4, 135.3, 135.1, 127.7, 127.65, 127.2, 126.3–125.9 (m, 2C), 46.2 (t, J_{CP} = 111.8 Hz), 42.5 (2C), 41.9, 32.2–31.9 (m, 2C), 29.3, 29.2, 28.9, 27.8, 27.79, 19.9, 19.85, 18.5, 18.4, 18.1; ^{31}P NMR δ +25.6; HRMS (neg. ion ESI) m/z calcd for $(M-H)^-$ $C_{26}H_{45}O_6P_2$: 515.2691. Found: 515.2706.

5.14. (6*E*,10*E*,15*E*,19*E*)-2,6,10,16,20,24-Hexamethyl-2,6,10,15,19,23-pentacosahexaenyl-13,13-bisphosphonic acid, tetrasodium salt (20)

Yield 107 mg, 82%; ^{1}H NMR (CD_3CN and D_2O, 2:1) δ 6.20–6.16 (m, 2H), 5.74–5.64 (m, 4H), 3.04 (td, J_{HP} = 14.0, J = 6.3 Hz, 4H), 2.65–2.47 (m, 16H), 2.21 (s, 6H), 2.15 (s, 12H), 2.11 (s, 6H); ^{13}C NMR (CD_3CN and D_2O) δ 134.9 (2C), 133.7 (2C), 131.7 (2C), 125.2 (2C), 124.4 (t, J_{CP} = 8.5 Hz, 2C), 124.2 (2C); 43.4 (t, J_{CP} = 116.6 Hz), 40.2 (2C), 39.5 (2C), 29.3–29.1 (m, 2C), 27.1 (2C), 26.6 (2C), 25.1 (2C), 17.0 (2C), 15.8 (2C), 15.3 (2C); ^{31}P NMR δ +25.4; HRMS (pos. ion ESI) m/z calcd for $(M-2H+3Na)^+$ $C_{31}H_{52}O_6P_2Na_3$: 651.2932. Found: 651.2934.

5.15. Cell culture

K562 cells[15] were cultured at 37 °C in the presence of 5% CO_2 in RPMI-1640 media containing 10% heat-inactivated fetal bovine serum. Lovastatin and/or the bisphosphonates were added at the concentrations indicated. Where appropriate, 0.1% ethanol was used as a vehicle control.

5.16. [^{3}H]-Thymidine assay

K562 cells (2×10^5 cells/200 μL) were incubated in a 96-well plate in the presence of test compounds for 24 h. During the last four hours, cells were labeled with [^{3}H]-thymidine (0.04 μCi/well). Cells were harvested, and the amount of [^{3}H]-thymidine incorporated into cellular DNA was determined by liquid scintillation counting as described previously.[18]

5.17. 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.[15] Pan-Ras antibody was obtained from InterBiotech Corporation (Tokyo, Japan). Unmodified Rap1a antibody was purchased from Santa Cruz Biotech (CA).

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