Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 390–399
Bioorganic & Medicinal Chemistry

Mono- and dialkyl isoprenoid bisphosphonates as geranylgeranyl diphosphate synthase inhibitors

Andrew J. Wiemer,[a] Jose S. Yu,[b] Kimberly M. Lamb,[c] Raymond J. Hohl[a,c,d] and David F. Wiemer[b,d,*]

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

Received 1 June 2007; revised 13 September 2007; accepted 13 September 2007
Available online 18 September 2007

Abstract—Nitrogenous bisphosphonates are used clinically to reduce bone resorption associated with osteoporosis or metastatic bone disease, and are recognized as inhibitors of farnesyl diphosphate synthase. Inhibition of this enzyme decreases cellular levels of both farnesyl diphosphate and geranylgeranyl diphosphate which results in a variety of downstream biological effects including inhibition of protein geranylgeranylation. Our lab recently has prepared several isoprenoid bisphosphonates that inhibit protein geranylgeranylation and showed that one selectively inhibits geranylgeranyl diphosphate synthase. This results in depletion of intracellular geranylgeranyl diphosphate and impacts protein geranylgeranylation but does not affect protein farnesylation. To clarify the structural features of isoprenoid bisphosphonates that account for their geranylgeranyl diphosphate synthase inhibition, we have prepared a new group of isoprenoid bisphosphonates. The complete set of compounds has been tested for in vitro inhibition of human recombinant geranylgeranyl diphosphate synthase and cellular inhibition of protein geranylgeranylation. These results show some surprising relationships between in vitro and cellular activity, and will guide development of clinical agents directed at geranylgeranyl diphosphate synthase.

1. Introduction

Nitrogenous bisphosphonates, including pamidronate (**1**), alendronate (**2**), risedronate (**3**), and zoledronate (**4**), are used clinically for treatment of human skeletal-associated diseases including osteoporosis and metastatic bone disease (Fig. 1).[1,2] These drugs also have been shown to have direct growth inhibitory effects on malignant cells in the laboratory,[3,4] as well as activity in animal models of parasitic infections.[5,6] Methylene bisphosphonic acid (**5**), the parent compound, can be viewed as an analog of pyrophosphoric acid (**6**) where the central carbon imparts metabolic stability and a template for additional substituents. The number and nature of these substituents defines the biological activity of the specific analog.

The clinical bisphosphonates deplete cells of isoprenoid diphosphates leading to inhibition of protein isoprenylation, a modification necessary for activation of many small GTPases.[7] While the clinical bisphosphonates inhibit farnesyl diphosphate synthase (FDPS),[8–14] their cellular effects may result from geranylgeranyl diphosphate (GGPP) depletion.[15–19] Indeed, it has been reported that inhibition of osteoclast-mediated bone resorption by bisphosphonates can be fully reversed by addition of exogenous geranylgeraniol.[15] If GGPP levels are of critical importance, development of bisphosphonates that inhibit geranylgeranyl diphosphate synthase (GGDPS), and specifically deplete cellular GGPP without depleting farnesyl diphosphate (FPP), may be beneficial (see Fig. 2).

There have been few published attempts at designing bisphosphonate inhibitors of GGDPS. One study from the Oldfield laboratory identified saturated 1-alkyl

Keywords: Bisphosphonate; Geranylgeranyl diphosphate; Geranylgeranyl diphosphate synthase; Ras; Rap1a; Prenylation; Geranylgeranylation; Farnesyl diphosphate; Farnesylation.
* Corresponding author. Tel.: +1 319 335 1365; fax: +1 319 335 1270; e-mail: david-wiemer@uiowa.edu

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.09.029

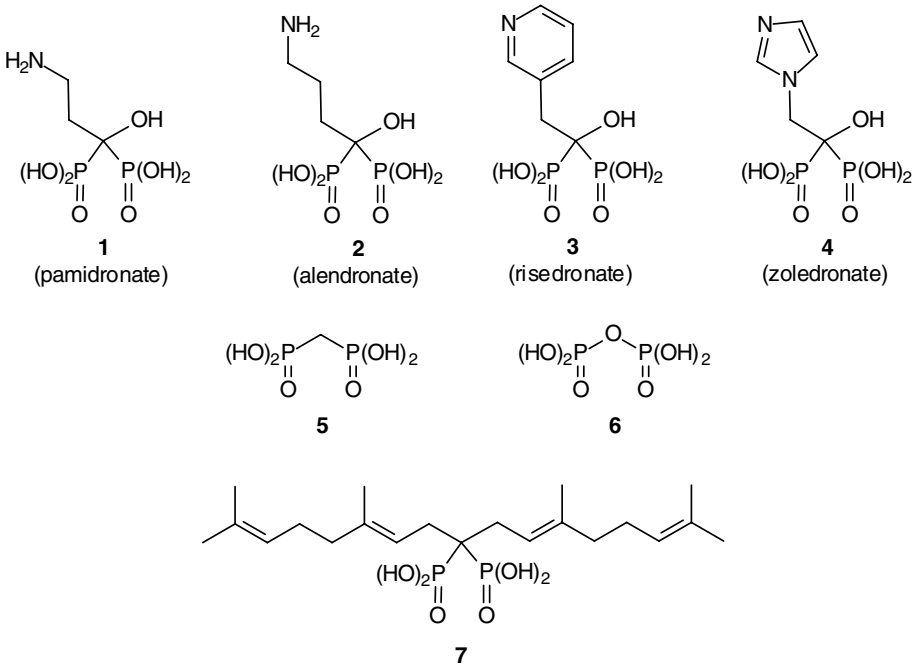


Figure 1. Representative nitrogenous bisphosphonates of clinical usefulness and related compounds.

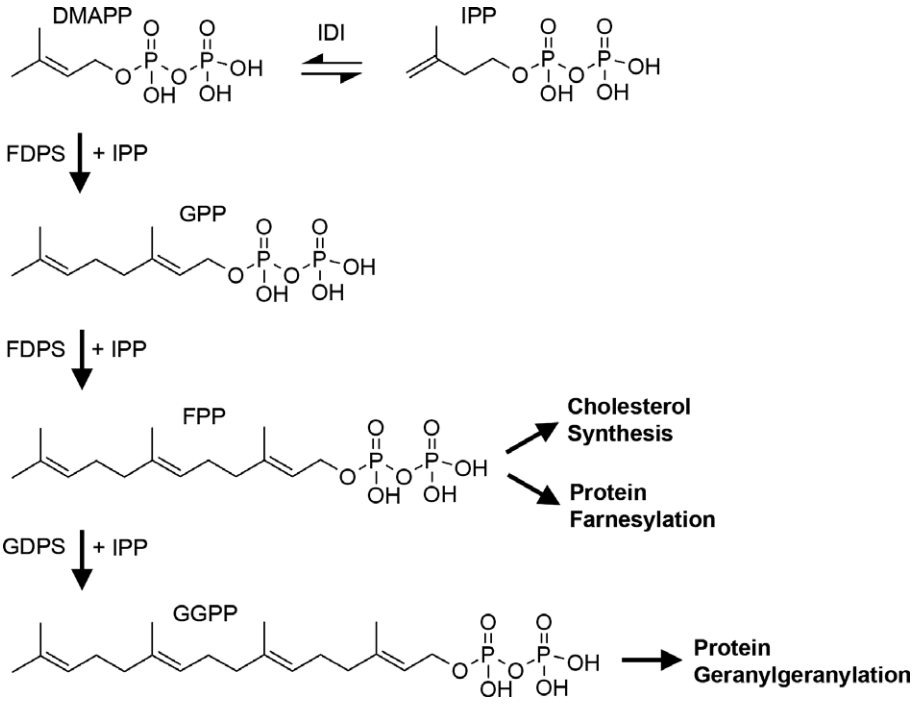


Figure 2. The biosynthesis of farnesyl diphosphate and geranylgeranyl diphosphate from smaller isoprenoids.

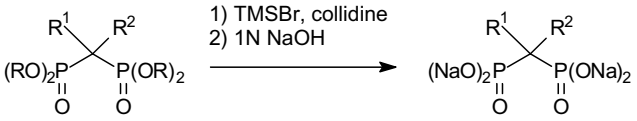
1-hydroxy bisphosphonates as inhibitors in vitro.[20] Another report from Ciosek et al. showed that some mono-alkyl isoprenoid bisphosphonates including 2*E*,6*E*-farnesyl bisphosphonate are capable of potent in vitro inhibition of squalene synthase,[21] but the activity of these compounds against GGDPS was not reported. However, we have reported that 2*E*,6*E*-farnesyl bisphosphonate inhibits protein geranylgeranylation in K562 leukemia cells.[22] These past studies prompted our group to design and prepare two series of dialkyl isoprenoid bisphosphonates of varying chain lengths, and we have demonstrated that some can specifically inhibit protein geranylgeranylation and not farnesylation.[23,24] We also have shown that one of these isoprenoid bisphosphonates, digeranyl bisphosphonate (**7**), is a potent and selective inhibitor of GGDPS and not FDPS in vitro.[25,26] To clarify the structural features of isoprenoid bisphosphonates that account for their GGDPS inhibition, we have prepared a new group of isoprenoid bisphosphonates and tested them in enzyme

and cellular assays. This study describes for the first time the inhibition of GGDPS by this set of isoprenoid bisphosphonates.

2. Results and discussion

As shown in Scheme 1, synthesis of two new neryl bisphosphonates[27] began with the alkylation of tetraethyl methylenebisphosphonate with neryl bromide under basic conditions, to afford both the mono alkylated product **8** (29%) and the dialkylated product **9** (24%). The dineryl bisphosphonate **9** subsequently was converted to the sodium salt **20** via a standard bromotrimethylsilane (TMSBr) reaction followed by treatment with sodium hydroxide. The mononeryl bisphosphonate **8** was converted to the dialkyl bisphosphonate **10** via a typical alkylation procedure with geranyl bromide under basic conditions (Scheme 2). Subsequent hydrolysis upon treatment with TMSBr and NaOH afforded the geranyl-neryl bisphosphonate salt (**19**).

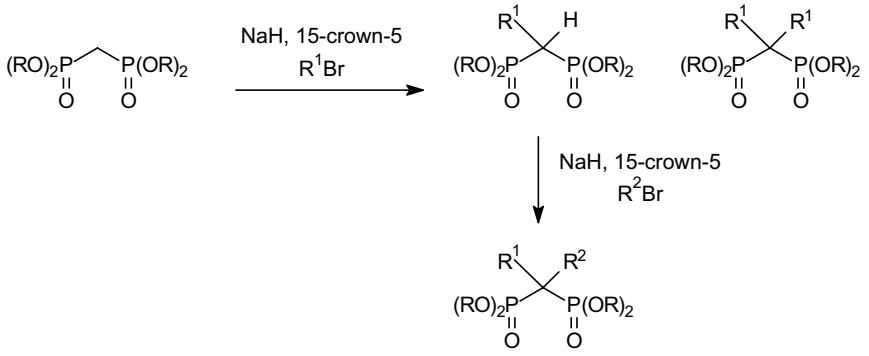
For the next series, tetramethyl methylenebisphosphonate was allowed to react with geranyl bromide to obtain the known compound **11**,[28] or with neryl bromide to give the monoalkyl product **12**. Further alkylation of compound **12** with prenyl bromide under standard basic conditions gave bisphosphonate **13**. In all three cases, phosphonate ester hydrolysis and treatment with NaOH resulted in formation of the required bisphosphonate salts **17**,[29] **18**, and **21** (Scheme 2).



	R^1	R^2	Yield (%)
17	geranyl	H	50
18	neryl	H	24
19	neryl	geranyl	96
20	neryl	neryl	93
21	neryl	prenyl	92
22	2*E*,6*Z*-farnesyl	H	94
23	2*Z*,6*E*-farnesyl	H	97
24	2*Z*,6*Z*-farnesyl	H	90

Scheme 2. Hydrolysis of bisphosphonate esters.

2*E*,6*E*-Farnesyl bisphosphonate (**29**) also is a known compound,[21,29] and the other three farnesyl bisphosphonate isomers were prepared in a straightforward fashion. The farnesyl bromide isomers were obtained via a typical PBr_3 reaction of synthetic and isomerically pure farnesols.[30,31] In each case, a standard alkylation reaction of tetramethyl methylenebisphosphonate afforded the desired monoalkyl bisphosphonates **14**, **15**, and **16** in modest yields (Scheme 1), accompanied by some of



	R	R^1	R^2	Yield (%)
8	Et	neryl	H	29
9	Et	neryl	neryl	24
10	Et	neryl	geranyl	90
11	Me	geranyl	H	32
12	Me	neryl	H	24
13	Me	neryl	prenyl	27
14	Me	2*E*,6*Z*-farnesyl	H	38
15	Me	2*Z*,6*E*-farnesyl	H	35
16	Me	2*Z*,6*Z*-farnesyl	H	32

Scheme 1. General synthesis of bisphosphonate ester isomers.

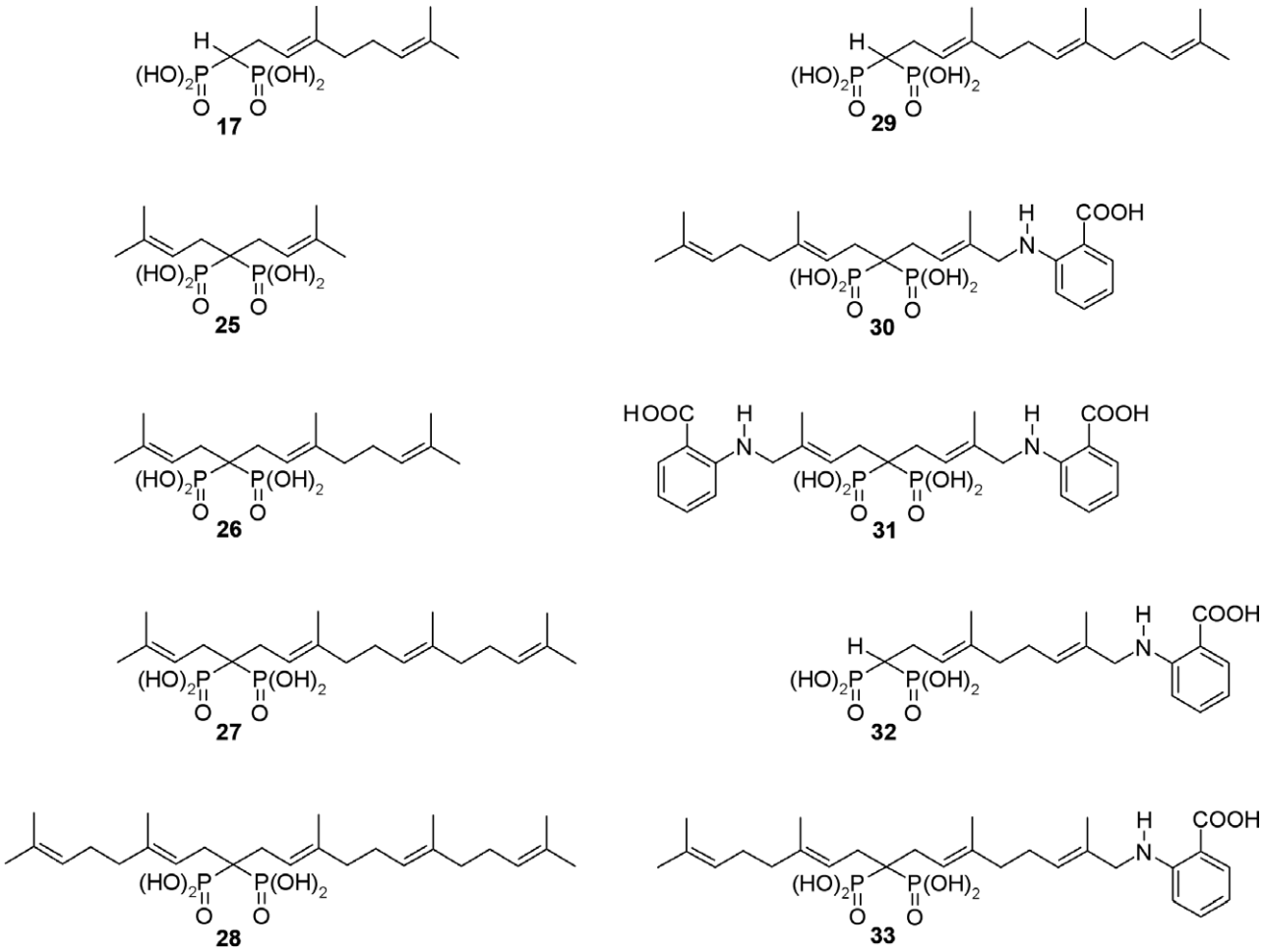


Figure 3. Chemical structures of previously synthesized isoprenoid bisphosphonates.

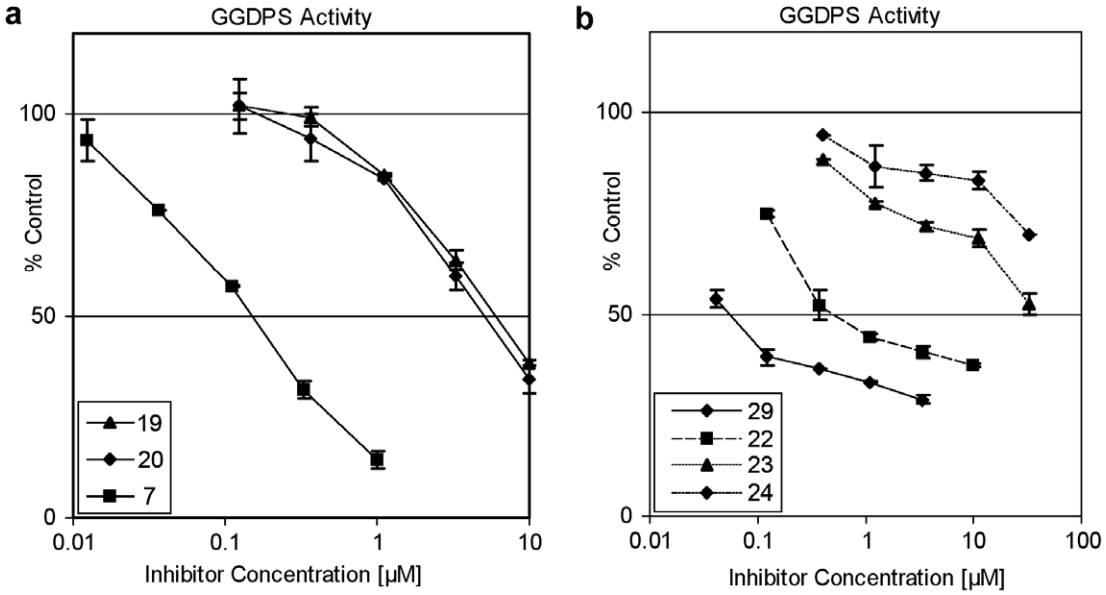


Figure 4. Inhibition of GGDPS by selected bisphosphonates. In vitro enzyme assays were performed as described in Section 4. GGDPS activity as a percentage of control is shown for (a) digeranyl bisphosphonate (**7**) and its isomers geranyl-neryl (**19**) and neryl-neryl bisphosphonate (**20**); and (b) 2*E*,6*E*-farnesyl bisphosphonate (**29**) and its 2*E*,6*Z*- (**22**), 2*Z*,6*E*-(**23**), and 2*Z*,6*Z*- (**24**) isomers.

the corresponding dialkyl compounds. After purification by column chromatography, subsequent hydrolysis through reaction with TMSBr followed by treatment with NaOH provided the respective sodium salts **22**, **23**, and **24** (Scheme 2).

Once the isoprenoid bisphosphonates were prepared, they were examined in enzyme assays in vitro and in whole cell assays. Our previous studies have shown that digeranyl bisphosphonate (**7**) is a potent and specific inhibitor of the enzyme geranylgeranyl diphosphate synthase (GGDPS, EC 2.5.1.29),[26] and that some isoprenoid bisphosphonates, including digeranyl bisphosphonate (**7**), could inhibit cellular protein geranylgeranylation.[23,24] In order to determine the relative potency for GGDPS inhibition for the compound library, dose–response curves for GGDPS inhibition were generated for each of these compounds as described in Section 4. Dose–response curves that reflect inhibition of GGDPS by digeranyl bisphosphonate (**7**) and its isomers (**19, 20**), as well as for 2*E*,6*E*-farnesyl bisphosphonate (**29**) and its isomers (**22–24**), are shown in Figure 4. As reported previously, digeranyl bisphosphate (**7**) is a potent inhibitor of GGDPS. The new isomers of digeranyl bisphosphonate, compounds **9** and **10**, also inhibit GGDPS although they are less potent.

Interestingly, 2*E*,6*E*-farnesyl bisphosphonate (**29**), which has been shown by others to inhibit squalene synthase[21] and in our lab to inhibit farnesyl transferase weakly in vitro but not cellular farnesylation,[29] also is a potent inhibitor of GGDPS. Our previous study[22] had failed to detect inhibition of GGDPS, most likely as a result of testing these compounds with partially purified bovine brain homogenate prior to our successful preparation of recombinant human GGDPS. Each of the other three stereoisomers of farnesyl bisphosphonate (**22–24**) also inhibits GGDPS, although potency is decreased with each successive incorporation of a *Z*-olefin. These results show for the first time that non-nitrogenous bisphosphonates bearing only one isoprene chain inhibit GGDPS in vitro. They also indicate that while *cis*-isoprenoid bisphosphonate isomers are capable of inhibiting GGDPS, they are less potent inhibitors than all *trans*-isoprenoid bisphosphonates. This result may not be surprising given that the primary substrate of GGDPS is 2*E*,6*E*-farnesyl diphosphate, but it does indicate the importance of using isoprenoids of defined and pure olefin stereochemistry.[29]

As with the previous representatives of this family, the compounds reported here also were tested for their ability to inhibit protein prenylation in K562 leukemia cells (Fig. 5). Western blots were conducted for total Ras and for unmodified Rap1a as described in Section 4. Inhibition of farnesylation results in a more slowly migrating or wider band for Ras, which is demonstrated by addition of lovastatin in lane 2 (Fig. 5). As with the other compounds in our library, none of the novel bisphosphonate isomers inhibit protein farnesylation. Because the Rap1a antibody is specific for the non-geranylgeranylated form of Rap1a, diminished protein geranylgeranylation results in the appearance of the unmodified Rap1a band as shown by addition of lovastatin. 2*E*,6*E*-Farnesyl bisphosphonate (**29**, Fig. 5) displays potent inhibition of cellular geranylgeranylation, its 2*E*,6*Z*-isomer (**22**) displays comparatively mild inhibition, and the 2*Z*,6*E*-and 2*Z*,6*Z*-isomers (**23** and **24**, respectively) are inactive up to 100 μM (data not shown). Parallel to our findings with digeranyl bisphosphonate (**7**), both the geranyl-neryl (**19**) and dineryl (**20**) compounds inhibited cellular geranylgeranylation

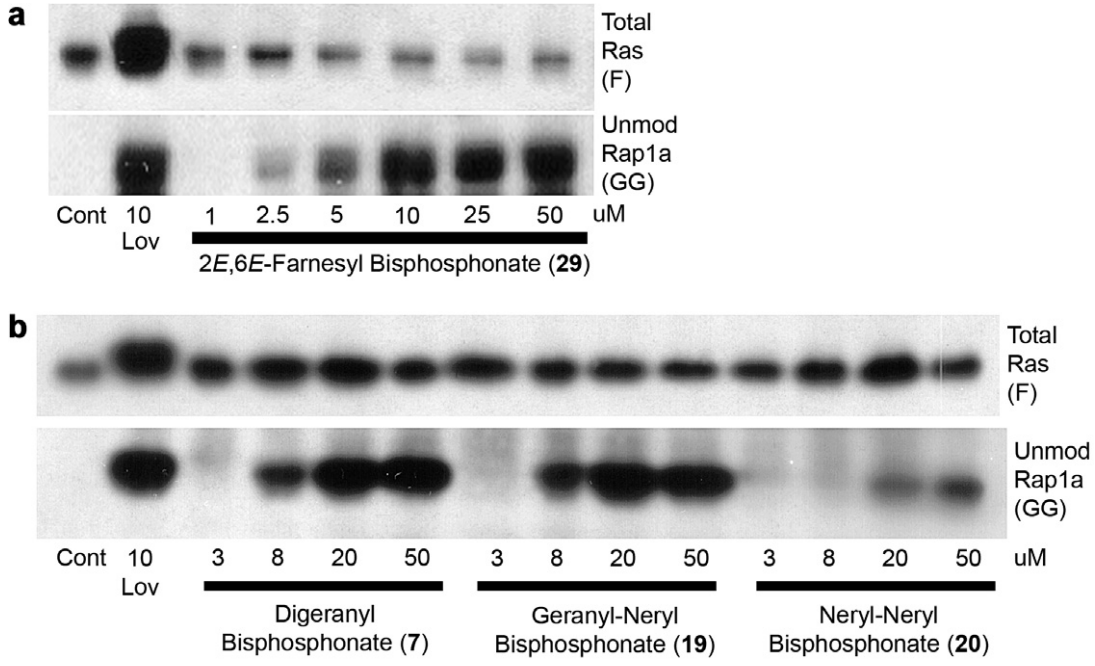


Figure 5. Cellular inhibition of prenylation by selected bisphosphonates. Western blot analysis was performed as described in Section 4. Inhibition of Rap1a geranylgeranylation is shown by the appearance of a band caused by (a) 2*E*,6*E*-farnesyl bisphosphonate (**29**) or (b) digeranyl bisphosphonate (**7**) and its geranyl-neryl (**19**) and neryl-neryl (**20**) isomers.

Table 1. Bisphosphonate concentrations required to inhibit 50% of GGDPS activity in vitro and to inhibit cellular Rap1a geranylgeranylation equivalent to 10 μM lovastatin

Compound	R^1	R^2	GGDPS in vitro IC_{50} (μM)	Inhibition of Rap1a modification in intact cells (μM)
Dialkyl isoprenoid bisphosphonates				
7	Geranyl	Geranyl	0.2	25
28	Geranyl	2*E*,6*E*-farnesyl	0.3	25
30	Geranyl	Fluorescent prenyl	0.9	25
33	Geranyl	Fluorescent geranyl	0.9	25
27	Prenyl	2*E*,6*E*-farnesyl	1	50
26	Geranyl	Prenyl	3	25
20	Neryl	Neryl	6	25
19	Geranyl	Neryl	7	25
21	Neryl	Prenyl	60	>50
31	Fluorescent prenyl	Fluorescent prenyl	>100	>100
25	Prenyl	Prenyl	>100	>100
Monoalkyl isoprenoid bisphosphonates				
29	2*E*,6*E*-farnesyl	H	0.1	10
22	2*E*,6*Z*-farnesyl	H	0.6	10
17	Geranyl	H	10	25
23	2*Z*,6*E*-farnesyl	H	40	>50
24	2*Z*,6*Z*-farnesyl	H	70	>50
32	Fluorescent geranyl	H	>100	>100
18	Neryl	H	>100	>100
4	(zoledronate)		>100	100

of Rap1a but did not affect farnesylation of Ras. The cellular potency of geranyl-neryl bisphosphonate (**19**) is similar to that of digeranyl bisphosphonate (**7**), but the dineryl bisphosphonate (**20**) is less potent. While the new bisphosphonate isomers are generally less potent in vitro inhibitors of GGDPS, their in vitro efficacy is sufficiently potent to affect clearly cellular geranylgeranylation.

Because it was established that compound **7** impaired protein geranylgeranylation before it was determined that inhibition of GGDPS was the actual cellular mechanism, our previous studies did not directly test compounds for GGDPS inhibition. For the present study we generated in vitro dose–response curves for all of the new compounds (Scheme 2) as well as those previously synthesized (Fig. 3). These curves were used to calculate IC_{50} values for GGDPS inhibition and these values are listed in Table 1. The compounds can be divided into two classes, the dialkyl and monoalkyl isoprenoid bisphosphonates, and then the members of these two classes are ranked by their in vitro activity as GGDPS inhibitors. The IC_{50} values span four orders of magnitude. As expected, all of the bisphosphonates (**7**, **26**, **28**, **29**, **30**, **33**) we have shown to inhibit cellular geranylgeranylation also are potent inhibitors of GGDPS in vitro. 2*E*,6*E*-Farnesyl bisphosphonate (**29**) is the most potent inhibitor of GGDPS that we have identified in vitro; however, its usefulness as a cellular inhibitor of this enzyme may be limited because it also has been shown to inhibit squalene synthase[21] and farnesyltransferase.[29] Consistent with our previous conclusion, bisphosphonates that contain at least one geranyl chain all are potent inhibitors of GGDPS. Two of the fluorescent analogs first described in Maalouf et al., [24] compounds **30** and **33**, also are potent inhibitors of GGDPS. This data indicates that structural modifications at the end of one isoprenoid chain do not dramatically affect inhibition of GGDPS, given that even addition of one aromatic group leads to only a fivefold decrease of in vitro potency.

In order to study the correlation between in vitro and cellular potency, we generated cellular dose–response curves for inhibition of geranylgeranylation and analyzed these as described above for inhibition of Rap1a processing. These Western blots were examined to approximate the concentration at which the individual isoprenoid bisphosphonate inhibits cellular geranylgeranylation to a similar extent to 10 μM lovastatin (Table 1). As expected, the compounds that are the most potent in vitro inhibitors of GGDPS are generally the most potent cellular inhibitors of geranylgeranylation. Notably, isoprenoid bisphosphonates with in vitro GGDPS IC_{50} values of up to ~7 μM were able to inhibit cellular geranylgeranylation with similar efficacy. This may indicate that generation of more potent in vitro GGDPS inhibitors may be less important than efforts to improve the ability of these compounds to enter the cell, because the compounds in hand are already sufficiently potent to dramatically affect cellular geranylgeranylation. Furthermore, 2*E*,6*E*-farnesyl bisphosphonate and its 2*E*,6*Z*-isomer both appear to have somewhat greater cellular potency than more active dialkyl analogs (Table 1). Taken together, these results indicate that further structural modifications designed to make these compounds more effective drugs may be beneficial.

3. Conclusions

In summary, a new series of isoprenoid bisphosphonate olefin isomers were synthesized and the ability of these compounds to inhibit GGDPS and diminish protein

geranylgeranylation was established. We now have developed two groups of GGDPS inhibitors—compounds with two isoprenoid chains such as digeranyl bisphosphonate (**7**) and those with a single isoprenoid chain such as 2*E*,6*E*-farnesyl bisphosphonate (**29**). At least eight isoprenoid bisphosphonates are now shown to exhibit sub-micromolar inhibition of GGDPS. These compounds are also all potent inhibitors of cellular geranylgeranylation which may be useful for further studies on the biological consequences of GGPP depletion.

4. Experimental

4.1. General experimental conditions

Tetrahydrofuran (THF) was distilled from sodium/benzophenone immediately prior to use, while CH_2Cl_2 was freshly distilled from CaH_2. All reactions in non-aqueous solvents were conducted in oven-dried glassware under a positive pressure of argon and with magnetic stirring. 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 were reported in ppm relative to 85% H_3PO_4 (external standard). Silica gel (60 Å, 0.040–0.063 mm) was used for flash chromatography. Yields refer to pure compounds after chromatography. High resolution mass spectra were obtained at the University of Iowa Mass Spectrometry Facility.

4.2. General procedure for formation of mono- and dialkyl bisphosphonates esters

To a stirred suspension of NaH (1.1 equiv) and 15-crown-5 (0.1 equiv) in THF was added either tetramethyl or tetraethyl methylenebisphosphonate (1.0 equiv) slowly over 3 min via syringe at 0 °C. Once hydrogen gas evolution had ceased, the corresponding alkyl bromide (1.1 equiv) was added slowly via syringe as a neat liquid at 0 °C. The reaction mixture was stirred an additional 3 h while it warmed to room temperature. The white reaction mixture was quenched via addition of NH_4Cl (satd) and extracted with ether. The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. Purification by flash chromatography (silica gel, 2% methanol in ether) afforded two yellow oils, the mono- and dialkyl bisphosphonates esters.

4.3. (*Z*)-Tetraethyl 4,8-dimethylnona-3,7-diene-1,1-diyldiphosphonate (8)

Yield: 1.02 g, 29%; ^1H NMR δ 5.23 (t, J = 7.2 Hz, 1H), 5.17–5.06 (m, 1H), 4.17 (m, 8H), 2.72–2.51 (m, 2H), 2.30 (tt, J_{HP} = 24 Hz, J = 6 Hz, 1H), 2.15–2.05 (m, 4H), 1.71 (s, 3H), 1.68 (s, 3H), 1.61 (s, 3H), 1.33 (td, J = 7.2 Hz, J_{HP} = 1.2 Hz, 12H); ^{13}C NMR δ 136.9, 131.7, 124.2, 122.7 (t, J_{CP} = 7.3 Hz), 62.7–62.3 (m, 4C), 37.8 (t, J_{CP} = 132.1Hz), 31.9, 26.4, 25.7, 23.9 (t, J_{CP} = 4.7 Hz), 23.4, 17.7, 16.6–16.3 (m, 4C); ^{31}P NMR 24.1 ppm.

4.4. Tetraethyl (6*Z*,11*Z*)-2,6,12,16-tetramethylheptadeca-2,6,11,15-tetraene-9,9-diyldiphosphonate (9)

Yield: 527 mg, 24%; ^1H NMR δ 5.42 (t, J = 6.9 Hz, 2H), 5.17–5.07 (m, 2H), 4.16 (m, 8H), 2.62 (td, J_{HP} = 16.5 Hz, J = 7.2 Hz, 4H), 2.18–2.00 (m, 8H), 1.78 (s, 3H), 1.72 (s, 6H), 1.68 (s, 3H), 1.61 (s, 6H), 1.32 (td, J = 7.2 Hz, J_{HP} = 1.8 Hz, 12H); ^{13}C NMR δ 137.1 (2C), 131.6 (2C), 124.4 (2C), 120.1 (t, J_{CP} = 6.2 Hz, 2C), 62.7–62.1 (m, 4C), 45.8 (t, J_{CP} = 130.4 Hz), 32.1 (2C), 29.0 (q, J_{CP} = 4.0 Hz, 2C), 26.5 (2C), 25.8 (2C), 23.9 (2C), 17.7 (2C), 16.5 (t, J_{CP} = 2.3 Hz, 4C); ^{31}P NMR 27.1 ppm.

4.5. (*Z*)-Tetramethyl 4,8-dimethylnona-3,7-diene-1,1-diyldiphosphonate (12)

Yield: 4.76 g, 24%; ^1H NMR δ 5.21 (t, J = 6.9 Hz, 1H), 5.10–4.99 (m, 1H), 3.74 (d, J_{HP} = 11.1 Hz, 6H), 3.73 (d, J_{HP} = 10.8 Hz, 6H), 2.63–2.43 (m, 2H), 2.29 (tt, J_{HP} = 24 Hz, J = 6 Hz, 1H), 2.04–1.96 (m, 4H), 1.65 (d, J = 1.2 Hz, 3H), 1.61 (s, 3H), 1.54 (s, 3H); ^{13}C NMR δ 137.6, 131.9, 124.2, 122.2 (t, J_{CP} = 7.1 Hz), 53.5–53.0 (m, 4C), 37.0 (t, J_{CP} = 132.3 Hz), 32.0, 26.5, 25.8, 23.7 (t, J_{CP} = 4.6 Hz), 23.5, 17.7; ^{31}P NMR 26.6 ppm.

4.6. Tetramethyl (3*E*,7*Z*)-4,8,12-trimethyltrideca-3,7,11-triene-1,1-diyldiphosphonate (14)

Yield: 1.20 g, 38%; ^1H NMR δ 5.31–5.21 (m, 1H), 5.16–5.04 (m, 2H), 3.81 (d, J_{HP} = 11.1 Hz, 6H), 3.80 (d, J_{HP} = 11.1 Hz, 6H), 2.74–2.52 (m, 2H), 2.37 (tt, J_{HP} = 23.7 Hz, J = 6.3 Hz, 1H), 2.15–1.90 (m, 8H), 1.68 (s, 3H), 1.64 (s, 3H), 1.63 (3H), 1.60 (d, J = 2.1 Hz, 3H); ^{13}C NMR δ 137.6, 135.0, 131.4, 124.1, 123.5, 121.4 (t, J_{CP} = 6.0 Hz), 53.6–53.0 (m, 4C), 39.7, 36.8 (t, J_{CP} = 132.4 Hz), 32.1, 27.1, 26.3, 25.8, 24.0 (t, J_{CP} = 4.8 Hz), 23.5, 17.6, 16.0; ^{31}P NMR 26.7 ppm.

4.7. Tetramethyl (3*Z*,7*E*)-4,8,12-trimethyltrideca-3,7,11-triene-1,1-diyldiphosphonate (15)

Yield: 949 mg, 35%; ^1H NMR δ 5.27 (t, J = 7.2 Hz, 1H), 5.17–5.05 (m, 2H), 3.81 (d, J_{HP} = 11.1 Hz, 6H), 3.80 (d, J_{HP} = 11.1 Hz, 6H), 2.79–2.54 (m, 2H), 2.36 (tt, J_{HP} = 23.7 Hz, J = 6.0 Hz, 1H), 2.15–1.95 (m, 8H), 1.72 (s, 3H), 1.68 (s, 3H), 1.61 (s, 6H); ^{13}C NMR δ 137.7, 135.6, 131.5, 124.4, 124.0, 122.1 (t, J_{CP} = 6.9 Hz), 53.5–53.1 (m, 4C), 39.8, 37.0 (t, J_{CP} = 132.8 Hz), 32.0, 26.8, 26.4, 25.8, 23.8 (t, J_{CP} = 4.8 Hz), 23.6, 17.8, 16.1; ^{31}P NMR 26.6 ppm.

4.8. Tetramethyl (3*Z*,7*Z*)-4,8,12-trimethyltrideca-3,7,11-triene-1,1-diyldiphosphonate (16)

Yield: 614 mg, 32%; ^1H NMR δ 5.28 (t, J = 6.6 Hz, 1H), 5.20–5.05 (m, 2H), 3.81 (d, J_{HP} = 10.8 Hz, 6H), 3.80 (d, J_{HP} = 11.1 Hz, 6H), 2.80–2.52 (m, 2H), 2.36 (tt, J_{HP} = 24 Hz, J = 6.3 Hz, 1H), 2.14–1.94 (m, 8H), 1.71 (s, 3H), 1.69 (s, 6H), 1.61 (s, 3H); ^{13}C NMR δ 137.6, 135.7, 131.7, 124.9, 124.4, 122.2 (t, J_{CP} = 7.4 Hz), 53.3 (t, J_{CP} = 6.2 Hz, 4C), 37.0 (t, J_{CP} = 132.2 Hz), 32.2,

32.0 26.7, 26.2, 25.8, 23.8 (t, J_{CP} = 4.7 Hz), 23.5 (2C), 17.8; ^{31}P NMR 26.6 ppm.

4.9. General procedure for formation of unsymmetrical dialkyl bisphosphonates esters

To a stirred solution of the neryl bisphosphonate **10** or **12** (1.0 equiv) and 15-crown-5 (0.1 equiv) in THF at 0 °C was added NaH (1.1 equiv). After 30 min, the appropriate alkyl bromide (geranyl or prenyl, 1.1 equiv) was added as a neat liquid at 0 °C and the reaction was stirred for an additional 3 h, while it was allowed to warm to room temperature. The white reaction mixture was quenched via 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 (silica gel, 3% methanol in ether) afforded the desired unsymmetrical dialkyl bisphosphonate as a yellow oil.

4.10. Tetraethyl (6*Z*,11*E*)-2,6,12,16-tetramethylheptadeca-2,6,11,15-tetraene-9,9-diyldiphosphonate (10)

Yield: 1.11 g, 90%; ^{1}H NMR δ 5.42 (t, J = 6.6 Hz, 2H), 5.15–5.06 (m, 2H), 4.17 (m, 8H), 2.62 (td, J_{HP} = 16.2, J = 6.9 Hz, 4H), 2.14–1.94 (m, 8H), 1.71 (d, J = 1.2 Hz, 3H), 1.68 (s, 6H), 1.64–1.58 (m, 9H), 1.32 (t, J = 6.9 Hz, 12H); ^{13}C NMR δ 137.2, 137.1, 131.7, 131.4, 124.4 (2C), 120.1 (t, J_{CP} = 6.5 Hz), 119.4 (t, J_{CP} = 7.4 Hz), 62.7–62.3 (m, 4C), 45.9 (t, J_{CP} = 130.2 Hz), 40.3, 32.2, 29.4 (t, J_{CP} = 4.4 Hz), 29.0 (t, J_{CP} = 3.9 Hz), 26.8, 26.5, 25.8 (2C), 24.0, 17.8 (2C), 16.8–16.5 (m, 4C), 16.4; ^{31}P NMR 27.3 ppm.

4.11. (*Z*)-Tetramethyl 2,8,12-trimethyltrideca-2,7,11-triene-5,5-diyldiphosphonate (13)

Yield: 242 mg, 27%; ^{1}H NMR δ 5.42–5.28 (m, 2H), 5.16–5.07 (m, 1H), 3.79 (d, J_{HP} = 10.8, 12H), 2.61 (td, J_{HP} = 15.9 Hz, J = 6.9 Hz, 4H), 2.08–2.01 (m, 4H), 1.73 (s, 3H), 1.72 (s, 3H), 1.68 (s, 3H), 1.65–1.59 (m, 6H); ^{13}C NMR δ 137.8, 134.3, 131.7, 124.4, 119.6 (t, J_{CP} = 7.3 Hz), 119.0 (t, J_{CP} = 7.3 Hz), 53.5–53.0 (m, 4C), 46.4 (t, J_{CP} = 130.8 Hz), 32.1, 29.4 (t, J_{CP} = 4.1 Hz), 29.0 (t, J_{CP} = 4.4 Hz), 26.4, 26.2, 25.8, 23.9, 18.0, 17.7; ^{31}P NMR 29.6 ppm.

4.12. General procedure for bisphosphonate ester cleavage

The starting material (1.0 equiv) was dissolved in CH_2Cl_2 at 0 °C, while 2,4,6-collidine (10 equiv) and TMSBr (10 equiv) were added as neat liquids.[32] After 2 h, the reaction mixture was allowed to gradually warm to room temperature and stirred overnight. Toluene was added, and then the crude mixture was concentrated in vacuo, NaOH was added (11 equiv, 1 M), and the mixture was stirred for an additional 3 h. This white reaction mixture was poured into acetone and held at 4 °C for 24 h. The resulting solid, located between the two layers, was removed by filtration, re-dissolved in water and extracted with CH_2Cl_2 and ether successively. The aqueous layer was lyophilized to afford the desired bisphosphonate salts as white flocculent powders.

4.13. Sodium (*Z*)-4,8-dimethylnona-3,7-diene-1,1-diyldiphosphonate (18)

Yield: 122 mg, 45%; ^{1}H NMR (D_2O) δ 5.47 (t, J = 5.4 Hz, 1H), 5.20–5.12 (m, 1H), 2.50–2.32 (m, 2H), 2.14–1.96 (m, 5H), 1.61 (s, 6H), 1.55 (s, 3H); ^{13}C NMR (D_2O) δ 135.4, 134.2, 128.1, 125.6, 42.0 (t, J_{CP} = 115.8 Hz), 31.8, 26.6, 26.2 (t, J_{CP} = 2.9 Hz), 25.5, 23.2, 17.7; ^{31}P NMR (D_2O) 21.6 ppm; HR-MS (neg. ion ESI) *m*/*z* calcd for $(M-H)^-$ $C_{11}H_{22}O_6P_2$: 311.0813. Found: 311.0828.

4.14. Sodium (6*Z*,11*E*)-2,6,12,16-tetramethylheptadeca-2,6,11,15-tetraene-9,9-diyldiphosphonate (19)

Yield: 1.03 g, 96%; ^{1}H NMR (D_2O) δ 5.64 (t, J = 6.0 Hz, 2H), 5.24–5.14 (m, 2H), 2.46 (td, J = 14.1, 6.3 Hz, 4H), 2.13–1.95 (m, 8H), 1.72–1.64 (m, 9H), 1.60 (s, 6H), 1.56 (s, 3H); ^{13}C NMR (D_2O) δ 135.9, 135.5, 133.7, 133.6, 125.6, 125.5, 125.3 (t, J_{CP} = 7.2 Hz), 124.6 (t, J_{CP} = 7.8 Hz), 45.0 (t, J_{CP} = 113.3 Hz), 40.5, 32.3, 31.4–30.6 (m, 2C), 27.1, 26.7, 25.7 (2C), 23.7, 17.8 (2C), 16.2; ^{31}P NMR (D_2O) 25.2 ppm; HR-MS (neg. ion ESI) *m*/*z* calcd for $(M-H)^-$ $C_{21}H_{38}O_6P_2$: 447.2065. Found: 447.2080.

4.15. Sodium (6Z,11Z)-2,6,12,16-tetramethylheptadeca-2,6,11,15-tetraene-9,9-diyldiphosphonate (20)

Yield: 488 mg, 93%; ^{1}H NMR (D_2O) δ 5.81–5.72 (m, 2H), 5.01–4.92 (m, 2H), 2.45–2.27 (m, 4H), 2.00–1.80 (m, 8H), 1.56 (s, 6H), 1.51 (s, 6H), 1.45 (s, 6H); ^{13}C NMR (D_2O) δ 137.7 (2C), 135.9 (2C), 127.8 (2C), 127.7 (t, J_{CP} = 8.6 Hz, 2C), 47.3 (t, J_{CP} = 114.8 Hz), 34.2 (2C), 30.5 (2C), 28.7 (2C), 27.7 (2C), 25.7 (t, J_{CP} = 3.4 Hz, 2C), 19.9 (2C); ^{31}P NMR (D_2O) 26.5 ppm; HR-MS (neg. ion ESI) *m*/*z* calcd for $(M-H)^-$ $C_{21}H_{38}O_6P_2$: 447.2065. Found: 447.2080.

4.16. Sodium (*Z*)-2,8,12-trimethyltrideca-2,7,11-triene-5,5-diyldiphosphonate (21)

Yield: 70 mg, 92%; ^{1}H NMR (D_2O) δ 5.62–5.43 (m, 2H), 5.20–5.10 (m, 1H), 2.50–2.10 (m, 4H), 2.05–1.95 (m, 4H), 1.61 (s, 9H), 1.56 (s, 3H), 1.52 (s, 3H); ^{13}C NMR (D_2O) δ 138.3, 136.2, 134.8, 127.7, 126.6 (t, J_{CP} = 7.0 Hz), 125.8 (t, J_{CP} = 6.8 Hz), 46.4 (t, J_{CP} = 110.5 Hz), 34.0, 33.7 (t, J_{CP} = 2.9 Hz), 33.3 (t, J_{CP} = 3.0 Hz), 28.6, 28.0, 27.6, 25.6, 20.0, 19.7; ^{31}P NMR (D_2O) 24.4 ppm; HR-MS (neg. ion ESI) *m*/*z* calcd for $(M-H)^-$ $C_{16}H_{30}O_6P_2$: 379.1439. Found: 379.1448.

4.17. Sodium (3*E*,7*Z*)-4,8,12-trimethyl-1-phosphonitotrideca-3,7,11-trienylphosphonate (22)

Yield: 408 mg, 94%; ^{1}H NMR (D_2O) δ 5.60–5.48 (m, 1H), 5.27–5.11 (m, 2H), 2.63 (t, J = 7.5 Hz, 1H), 2.46 (tt, J = 15.3, 6.6 Hz, 2H), 2.20–1.90 (m, 8H), 1.67 (s, 3H), 1.63 (s, 6H), 1.61 (s, 3H); ^{13}C NMR (D_2O) δ 139.1, 137.2, 136.1, 130.3 (t, J_{CP} = 6.3 Hz), 127.6, 127.1, 44.4 (t, J_{CP} = 117.7 Hz), 41.7, 34.0, 29.4–29.0 (m, 2C), 28.2, 27.6, 25.3, 19.7, 18.2; ^{31}P NMR (D_2O)

22.3 ppm; HR-MS (neg. ion ESI) *m/z* calcd for $(M-H)^-$ $C_{16}H_{30}O_6P_2$: 379.1439. Found: 379.1434.

4.18. Sodium (3*Z*,7*E*)-4,8,12-trimethyl-1-phosphonitotrideca-3,7,11-trienylphosphonate (23)

Yield: 340 mg, 97%; ^{1}H NMR (D_2O) δ 5.50 (t, J = 5.7 Hz, 1H), 5.28–5.19 (m, 1H), 5.19– 5.10 (m, 1H), 2.56–2.39 (m, 3H), 2.15–1.95 (m, 8H), 1.68 (s, 3H), 1.65 (s, 3H), 1.60 (s, 3H), 1.59 (s, 3H); ^{13}C NMR (D_2O) δ 139.1, 137.7, 135.9, 129.9 (t, J_{CP} = 7.7 Hz), 127.7, 127.2, 43.9 (t, J_{CP} = 114.2 Hz), 41.7, 34.1, 28.8, 28.7, 28.0 (t, J_{CP} = 3.4 Hz), 27.7, 25.6, 19.8, 18.1; ^{31}P NMR (D_2O) 21.9 ppm; HR-MS (neg. ion ESI) *m/z* calcd for $(M-H)^-$ $C_{16}H_{30}O_6P_2$: 379.1439. Found: 379.1443.

4.19. Sodium (3*Z*,7*Z*)-4,8,12-trimethyl-1-phosphonitotrideca-3,7,11-trienylphosphonate (24)

Yield: 310 mg, 90%; ^{1}H NMR (D_2O) δ 5.54 (t, J = 6.0 Hz, 1H) 5.30–5.15 (m, 2H), 2.58–2.40 (m, 3H), 2.17–2.04 (m, 8H), 1.68 (s, 9H), 1.63 (s, 3H); ^{13}C NMR (D_2O) δ 139.5, 137.1, 136.2, 130.9 (t, J_{CP} = 7.6 Hz), 128.5, 127.2, 44.4 (t, J_{CP} = 117.0 Hz), 34.2, 34.1, 29.0–28.5 (m, 2C), 28.4, 27.6, 25.4 (2C), 19.7; ^{31}P NMR (D_2O) 22.1 ppm; HR-MS (neg. ion ESI) *m/z* calcd for $(M-H)^-$ $C_{16}H_{30}O_6P_2$: 379.1439. Found: 379.1442.

4.20. GGDPS assays

Plasmids containing GST-tagged recombinant human GGDPS were expressed in BL21 gold bacteria by induction with IPTG. Proteins were purified by column chromatography or batch centrifugation with glutathione agarose. All GGDPS assays were performed as follows. The GGDPS reaction mixtures contained 20 μM FPP and 40 μM ^{14}C-IPP in 35 μL buffer (50 mM imidazole pH 7.5, 0.5 mM $MgCl_2$, 0.5 mM $ZnCl_2$). Following a 10-min pre-incubation with the indicated bisphosphonates, reactions were initiated by simultaneous addition of ^{14}C-IPP and FPP. Reactions proceeded for 1 h at 37 °C, and then the longer isoprenoids were extracted with 1 mL saturated butanol and the extract was washed three times with 300 μL saturated water. The amount of ^{14}C incorporation into GGPP was detected by liquid scintillation counting.

4.21. Cell culture and drug incubations

K562 leukemia cells were obtained from ATCC (Manasas, VA) and cultured according to ATCC protocol. Asynchronous suspension cultures (1×10^6 cells/mL) in fresh media were incubated for 24 h in the presence or absence of the indicated concentrations of bisphosphonates. Western blot analyses required 5 million cells per treatment.

4.22. Western blot analysis

Protein concentrations were determined using BCA method (Pierce). Proteins were resolved by electrophoresis on a 12% gel and transferred to a PDVF membrane. Primary and secondary antibodies were added sequentially for 60 min, and then the proteins were visualized using an ECL chemiluminescence detection kit (Amersham). Anti pan-Ras was obtained from InterBiotechnology (Tokyo, Japan). The Rap1a (sc-1482) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-mouse secondary was obtained from Amersham and HRP-conjugated anti-goat was obtained from Santa Cruz Biotechnology.

Acknowledgments

We thank Mr. Larry W. Shull, Dr. Mona A. Maalouf, and Dr. Sarah A. Holstein for preparation of the bisphosphonates shown in Figure 3. This project was supported by the Roy. J. Carver Charitable Trust as a Research Program of Excellence and the Roland W. Holden Family Program for Experimental Cancer Therapeutics.

References and notes

1. Licata, A. A. *Ann. Pharmacother.* **2005**, *39*, 668.
2. Body, J. J.; Bartl, R.; Burckhardt, P.; Delmas, P. D.; Diel, I. J.; Fleisch, H.; Kanis, J. A.; Kyle, R. A.; Mundy, G. R.; Paterson, A. H.; Rubens, R. D. J. *Clin. Oncol.* **1998**, *16*, 3890.
3. Shipman, C. M.; Rogers, M. J.; Apperley, J. F.; Graham, R.; Russell, G.; Croucher, P. I. *Br. J. Haematol.* **1997**, *98*, 665.
4. Kunzmann, V.; Bauer, E.; Feurle, J.; Weissinger, F.; Tony, H. P.; Wilhelm, M. *Blood* **2000**, *96*, 384.
5. Martin, M. B.; Grimley, J. S.; Lewis, J. C.; Heath, H. T., 3rd; Bailey, B. N.; Kendrick, H.; Yardley, V.; Caldera, A.; Lira, R.; Urbina, J. A.; Moreno, S. N.; Docampo, R.; Croft, S. L.; Oldfield, E. *J. Med. Chem.* **2001**, *44*, 909.
6. Yardley, V.; Khan, A. A.; Martin, M. B.; Slifer, T. R.; Araujo, F. G.; Moreno, S. N.; Docampo, R.; Croft, S. L.; Oldfield, E. *Antimicrob. Agents Chemother.* **2002**, *46*, 929.
7. Reszka, A. A.; Rodan, G. A. *Mini Rev. Med. Chem.* **2004**, *4*, 711.
8. Rogers, M. J.; Xiong, X. J.; Ji, X. H.; Monkkonen, J.; Russell, R. G. G.; Williamson, M. P.; Ebetino, F. H.; Watts, D. J. *Pharm. Res.* **1997**, *14*, 625.
9. van Beek, E. P. E.; Cohen, L.; Lowik, C.; Papapoulos, S. *Biochem. Biophys. Res. Commun.* **1999**, *255*, 491.
10. Bergstrom, J. D.; Bostedor, R. G.; Masarachia, P. J.; Reszka, A. A.; Rodan, G. *Arch. Biochem. Biophys.* **2000**, *373*, 231.
11. Dunford, J. E.; Thompson, K.; Coxon, F. P.; Luckman, S. P.; Hahn, F. M.; Poulter, C. D.; Ebetino, F. H.; Rogers, M. J. *J. Pharmacol. Exp. Ther.* **2001**, *296*, 235.
12. Ebetino, F. H.; Roze, C. N.; McKenna, C. E.; Barnett, B. L.; Dunford, J. E.; Russell, R. G. G.; Mieling, G. E.; Rogers, M. J. *J. Organomet. Chem.* **2005**, *690*, 2679.
13. Rondeau, J. M.; Bitsch, F.; Bourgier, E.; Geiser, M.; Hemming, R.; Kroemer, M.; Lehmann, S.; Ramage, P.; Rieffel, S.; Strauss, A.; Green, J. R.; Jahnke, W. *Chem. Med. Chem.* **2006**, *1*, 267.
14. Kavanagh, K. L.; Guo, K.; Dunford, J. E.; Wu, X.; Knapp, S.; Ebetino, F. H.; Rogers, M. J.; Russell, R. G.; Oppermann, U. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 7829.

15. van beek, E.; Lowik, C.; van der Pluijm, G.; Papapoulos, S. *J. Bone Miner. Res.* **1999**, *14*, 722.
16. Fisher, J. E.; Rogers, M. J.; Halasy, J. M.; Luckman, S. P.; Hughes, D. E.; Masarachia, P. J.; Wesolowski, G.; Russell, R. G.; Rodan, G. A.; Reszka, A. A. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 133.
17. Coxon, F. P. H. M. H.; Van't Hof, R.; Sebti, S.; Ralston, S. H.; Hamilton, A.; Rogers, M. J. *J. Bone Miner. Res.* **2000**, *15*, 1467.
18. Van Beek, E. R.; Lowik, C. W.; Papapoulos, S. E. *Bone* **2002**, *30*, 64.
19. Goffinet, M.; Thoulouzan, M.; Pradines, A.; Lajoie-Mazenc, I.; Weinbaum, C.; Faye, J. C.; Seronie-Vivien, S. *BMC Cancer* **2006**, *6*, 60.
20. Szabo, C. M.; Matsumura, Y.; Fukura, S.; Martin, M. B.; Sanders, J. M.; Sengupta, S.; Cieslak, J. A.; Loftus, T. C.; Lea, C. R.; Lee, H. J.; Koohang, A.; Coates, R. M.; Sagami, H.; Oldfield, E. *J. Med. Chem.* **2002**, *45*, 2185.
21. Ciosek, C. P., Jr.; Magnin, D. R.; Harrity, T. W.; Logan, J. V.; Dickson, J. K., Jr.; Gordon, E. M.; Hamilton, K. A.; Jolibois, K. G.; Kunselman, L. K.; Lawrence, R. M., et al. *J. Biol. Chem.* **1993**, *268*, 24832.
22. Holstein, S. A.; Wohlford-Lenane, C. L.; Wiemer, D. F.; Hohl, R. J. *Biochemistry* **2003**, *42*, 4384.
23. Shull, L. W.; Wiemer, A. J.; Hohl, R. J.; Wiemer, D. F. *Bioorg. Med. Chem.* **2006**, *14*, 4130.
24. Maalouf, M. A.; Wiemer, A. J.; Kuder, C. H.; Hohl, R. J.; Wiemer, D. F. *Bioorg. Med. Chem.* **2007**, *15*, 1959.
25. Wiemer, D. F.; Hohl, R. J. U.S. Patent Application #20060052347, **2006**.
26. Wiemer, A. J.; Tong, H.; Swanson, K. M.; Hohl, R. J. *Biochem. Biophys. Res. Commun.* **2007**, *353*, 921.
27. Yu, J. S.; Wiemer, A. J.; Lamb, K. M.; Hohl, R. J.; Wiemer, D. F. *Abstr. Pap. Am. Chem. Soc.* **2007**, 233.
28. Shull, L. W.; Wiemer, D. F. *J. Organomet. Chem.* **2004**, *690*, 2521.
29. Holstein, S. C. D. M.; Wiemer, D. F.; Lewis, K.; Hohl, R. J. *Bioorg. Med. Chem.* **1998**, *6*, 687.
30. Yu, J. S.; Kleckley, T. S.; Wiemer, D. F. *Org. Lett.* **2005**, *7*, 4803.
31. Yu, J. S.; Wiemer, A. J.; Hohl, R. J.; Wiemer, D. F. *Abstr. Pap. Am. Chem. Soc.* **2005**, *230*, U2754.
32. McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M. C. *Tetrahedron Lett.* **1977**, *18*, 155.