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A New Motif for Inhibitors of Geranylgeranyl Diphosphate Synthase

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Abstract

The enzyme geranylgeranyl diphosphate synthase (GGDPS) is believed to receive the substrate farnesyl diphosphate through one lipophilic channel and release the product geranylgeranyl diphosphate through another. Bisphosphonates with two isoprenoid chains positioned on the α -carbon have proven to be effective inhibitors of this enzyme. Now a new motif has been prepared with one isoprenoid chain on the α -carbon, a second included as a phosphonate ester, and the potential for a third at the α -carbon. The pivaloyloxymethyl prodrugs of several compounds based on this motif have been prepared and the resulting compounds have been tested for their ability to disrupt protein geranylgeranylation and induce cytotoxicity in myeloma cells. The initial biological studies reveal activity consistent with GGDPS inhibition, and demonstrate a structure-function relationship which is dependent on the nature of the alkyl group at the α -carbon.

Graphical abstract

Keywords

GGDP synthase; inhibition; isoprenoid biosynthesis; bisphosphonate monoester; prodrug

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Supplementary data

Supplementary data associated with this article, including NMR spectra, can be found in the online version, at

1. Introduction

Geranylgeranyl diphosphate synthase (GGDPS) is a key enzyme in the later stages of the mevalonate pathway in humans^{1,2} and other organisms.³ Among other applications, the linear C₂₀ diterpenoid that it affords (geranylgeranyl diphosphate or GGDP) is utilized to convert a variety of proteins to lipoproteins through post-translational modification, and the resulting proteins are central to important signaling and protein transport processes. Clinical drugs such as zoledronate (**1**) and pamidronate (**2**, Figure 1)⁴ that inhibit the upstream enzyme farnesyl diphosphate synthase (FDPS) are used for treatment of osteoporosis, Paget's disease, and malignant bone disease including myeloma.⁵ However, there is evidence that suggests that these drugs exert their effect through depletion of GGDP,² which increases interest in identification of compounds that inhibit GGDPS directly.

One compound that has been found to inhibit GGDPS selectively is digeranyl bisphosphonate (**3**, Figure 2).⁶ The bisphosphonate head group is important to binding with the enzyme through coordination with magnesium cations in the active site. However, early investigation into the nature of substrate binding elucidated the importance of the two nonpolar side chains as well, because they can occupy hydrophobic channels within the enzyme. This led to the hypothesis that binding would be enhanced with a 'V-shaped' inhibitor.¹ Early inhibitors attained a V-shape through placement of two isoprenoid chains on the α -carbon of the bisphosphonate,^{6,7} but some linear compounds also show significant activity. For example, we recently reported that a linear triazole bisphosphonate is also a potent inhibitor of this enzyme (IC₅₀ = 45 nM).⁸ This clearly supports the view that still other strategies for including isoprenoid chains on a methylene bisphosphonate may offer opportunities for potent inhibitors. In this paper, we report the synthesis and initial bioassays of a family of compounds that can achieve a shape similar to digeranyl bisphosphonate by placement of one isoprenoid chain on the α -carbon and a second isoprenoid chain as a phosphonate ester (e.g. **4**). Among other attractive features, this structure could more closely mimic the degree of negative charge found in a diphosphate monoester, and this general structure still allows for incorporation of a third substituent on the α -carbon.

2. Synthesis

At the outset of our work we decided to target a prodrug of structures like compound **4** rather than the salts themselves for a variety of reasons. Highly charged phosphonate salts may have trouble crossing the cell membrane,^{9,10} while more lipophilic prodrugs can penetrate rapidly by simple diffusion and liberate the charged phosphonate once inside the cell.¹¹ Various analyses of cell lysate easily can distinguish between compounds that inhibit GGDPS and those that inhibit geranylgeranyl transferase I and/or II (GGTase I and GGTase II), thus providing information on both activity and selectivity from one assay. The specific prodrug form selected was the pivaloyloxymethyl or POM group, in part because of its long history¹² and in part because it already is in clinical use in both antibiotics¹³ and especially in antiviral agents.¹⁴ The POM group is cleaved by nonspecific esterases within the cell, which liberates pivalic acid, formaldehyde, and the phosphonic acid, while simple alkyl esters of phosphonic acids are far more stable to metabolism.¹¹

The synthetic sequence began with commercial dimethyl methylphosphonate (**5**). After treatment of this phosphonate with *n*-BuLi, reaction with freshly prepared geranyl bromide (**6**) gave the alkyl phosphonate **7**. The mono methyl ester mono acid chloride **8** then could be obtained by reaction with oxalyl chloride in DMF.¹⁵ In principal, reaction of this acid chloride with any alcohol should afford a mixed diester. However, out of concern for the lability of an allylic ester like that derived from geraniol,¹⁶ *S*-(-)-citronellol was employed to provide the mixed ester **9**. Phosphonylation of compound **9** could be accomplished by treatment with strong base and dimethyl chlorophosphate.¹⁷⁻¹⁹ This gave the key intermediate **10** in modest yield.

Compound **10** served as the point of divergence to obtain several products of similar structure. The direct reaction of this trimethyl ester with POMCl in the presence of sodium iodide gave the triPOM compound **11** in low yield,¹⁶ and the larger citronellyl ester appeared to be replaced more slowly than the methyl groups under these conditions. To introduce an additional substituent at the α -carbon, treatment of ester **10** with sodium hydride followed by reaction with an alkylating agent (methyl iodide, or allyl, prenyl, or geranyl bromide) gave the expected products **12–15**. With each of these four compounds, reaction with POMCl and sodium iodide converted the methyl esters to POM groups while preserving the isoprenoid ester, albeit in low yields. Nevertheless, through these parallel reactions the desired products **16–19** were obtained in quantities sufficient for bioassay.

It should be noted that compounds **11** and **16–19** were obtained as mixtures of stereoisomers. While the (*S*)-citronellol employed was a single enantiomer, no effort was expended at this stage to control the stereochemistry at the adjacent phosphorus or at the α -carbon. This is readily apparent in the ³¹P NMR spectra of these compounds (cf. the Supporting Information). In the phosphorus spectrum of compound **9**, two resonances of equal intensity are observed reflecting two stereoisomers at phosphorus. Introduction of the second phosphorus through formation of compound **10** results in a more complex ³¹P NMR spectrum, with two clusters of peaks reflecting both formation of the new stereocenter at the α -carbon and phosphorus-phosphorus coupling. Similar clusters are observed in most of the subsequent products, although introduction of the second geranyl chain in compounds **15** and **19** eliminates the stereocenter at the α -carbon, diminishes the number of diastereomers, and simplifies those phosphorus spectra.

Assuming that the POM groups function as prodrugs as intended,¹¹ the phosphorus stereocenter will be destroyed once the POM group of the mixed phosphonate ester is hydrolyzed within the cell. The stereochemistry at the α -carbon is more permanent, at least in compounds **16–18** and probably in compound **10** at physiological pH, so even after POM hydrolysis the materials tested would be a mixture of two diastereomers. In theory, these diastereomers can be separated but because such separations can prove challenging it was decided to postpone any efforts along these lines until the biological activity of the new structures were determined. While there may be some risks associated with this approach a similar strategy has proven useful with two olefin stereoisomers, where the material first demonstrated activity as a mixture²⁰ and subsequent synthesis of the individual stereoisomers allowed identification of the more active isomer.²¹

After significant activity was observed in the first representatives of this U-shaped series (*vide infra*), we began to investigate the importance of stereochemistry through preparation of an *R*-(+)-citronellol ester of the most active inhibitor, compound **16**. Accordingly, the acid chloride **8** was allowed to react with the *R*-(+)-citronellol enantiomer to afford the mixed ester **20** (Scheme 2). Phosphorylation to the bisphosphonate **21** was achieved under conditions parallel to those used to obtain compound **10**, and methylation also proceeded smoothly to give compound **22**. Transformation of this trimethyl ester to the corresponding POM derivative **23** completed this reaction sequence.

3. Biological results and discussion

To gauge the impact of these new compounds on isoprenoid metabolism, their ability to disrupt geranylgeranylation in myeloma cells was determined. As shown in Figure 3A-B, concentration-dependent effects were observed for all tested compounds within the *S*-(-)-citronellol ester series. Disruption of protein geranylgeranylation was demonstrated via two methods: 1) immunoblot analysis for unmodified Rap1a which is a substrate of GGTase I (Fig 3A); and, 2) ELISA for intracellular lambda light chain which is a marker for disruption of Rab GTPase geranylgeranylation²² (Figure 3B). Of the five compounds examined, the methyl derivative **16** most potently disrupted protein geranylgeranylation. When ranked from most potent to least potent, the order is **16** > **18** > **17** ≈ **19** > **11**. The HMG-CoA reductase inhibitor lovastatin, which inhibits the synthesis of mevalonate and therefore depletes cells of all isoprenoids downstream of mevalonate, was used as a positive control in these studies.²²

The cytotoxic activities of these compounds were tested in three human-derived myeloma cell lines. As shown in Table 1, the structure-function relationship was the same across all tested cell lines with the methyl derivative **16** the most potent and the geranyl derivative **19** the least potent. In general, the cytotoxic activity correlated with the potency of these compounds in disrupting cellular geranylgeranylation with the exception of compound **11**. The limited activity of the geranyl compound **19** is somewhat disappointing, given that the three isoprenoid chains could offer multiple ways to accomplish binding in the FDP and GGDP channels. Even so, the activity associated with compound **16** shows that this template has promise for development of new GGDPs inhibitors.

After identification of compound **16** as the most potent member of this family, add-back experiments were performed with this compound and isoprenoid intermediates. As shown in Figure 4A-B, while addition of either mevalonate or GGDP prevents lovastatin's effects on disrupting protein geranylgeranylation, only GGDP prevents the effects of compound **16**. In aggregate the results of these studies are consistent with these agents inhibiting GGDPs because there is global disruption of geranylgeranylation of both GGTase I and GGTase II substrates and addition of GGDP can prevent these effects.

Finally, bioassays were performed to determine whether the stereochemistry of the citronellyl group has an impact on activity. As shown in Figure 5, the (*R*)-(+)-citronellol analogue **23** was compared directly with the (*S*)-(-) citronellol analogue **16**. In general the potency of the two compounds appeared very similar, with compound **23** displaying slightly

more activity than compound **16** at lower concentrations based on levels of unmodified Rap1a and intracellular light chain.

4. Conclusions

In conclusion, we have developed a synthetic route for preparation of bisphosphonate isoprenoid esters and used this strategy to prepare a small group of new isoprenoid bisphosphonates. These compounds have been examined in cell-based assays for their impact on isoprenoid metabolism, and found to function as inhibitors of GGDPS. The most active compounds in this series, bisphosphonates **16** and **23**, show activity at sub-micromolar activity in both immunoblot and ELISA analyses. Given that both materials are still mixtures of diastereomers at the α -carbon even after hydrolysis of the prodrug groups, additional efforts to obtain the individual isomers appear to be justified. Further synthetic studies along those lines, as well as assays of the corresponding salts with the isolated enzyme, will be reported in due course.

5. Experimental procedures and methods

5.1 General experimental conditions

Tetrahydrofuran was freshly distilled from sodium/benzophenone, while methylene chloride was distilled from calcium hydride prior to use and toluene was dried over molecular sieves. All other reagents and solvents were purchased from commercial sources and used without further purification. All reactions in nonaqueous solvents were conducted in flame-dried glassware under a positive pressure of argon and with magnetic stirring. All NMR spectra were obtained at 300, 400, or 500 MHz for ^1H , and 75, 100, or 125 MHz for ^{13}C , with internal standards of $(\text{CH}_3)_4\text{Si}$ (^1H , 0.00) or CDCl_3 (^1H , 7.27; ^{13}C , 77.2 ppm) for non-aqueous samples or D_2O (^1H , 4.80) and 1,4-dioxane (^{13}C , 66.7 ppm) for aqueous samples. The ^{31}P chemical shifts were 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. Silica gel (60 Å, 0.040–0.063 mm) was used for flash chromatography.

5.2 (*E*)-Dimethyl (4,8-dimethylnona-3,7-dien-1-yl)phosphonate (**7**)

Dimethyl methylphosphonate (2.56 mL, 22.9 mmol) was added to an oven-dried round bottom flask with THF (100 mL). The solution was cooled in a dry ice/acetone bath for 20 minutes, and then *n*-butyl lithium (10.08 mL, 25.2 mmol) was added as a 2.5 M solution in hexanes. After 1.5 hours freshly prepared geranyl bromide (5.47 g, 25.2 mmol) was added dropwise and the reaction was stirred and allowed to reach room temperature overnight (15 h). The reaction was diluted with diethyl ether, quenched by addition of water, and washed with water (3×20 mL), and the organic layer was dried (Na_2SO_4). The mixture was vacuum filtered through celite, and the filtrate was concentrated to give a dark yellow oil. Final purification via flash chromatography (EtOAc, silica gel) gave the desired product (**7**) as a yellow oil (5.67 g, 95%): ^1H NMR (300 MHz, CDCl_3) δ 5.15–5.06 (m, 2H), 3.74 (d, $J_{\text{PH}} = 10.8$ Hz, 6H), 2.34–2.23 (m, 2H), 2.07–1.98 (m, 4H), 1.86–1.72 (m, 2H), 1.73 (s, 3H), 1.71 (s, 3H), 1.67 (s, 3H); ^{31}P NMR (121 MHz, CDCl_3) δ +34.4.

5.3 (*E*)-Methyl (4,8-dimethylnona-3,7-dien-1-yl)phosphonochloridate (8)

Compound **7** (1.93 g, 7.4 mmol) was diluted with anhydrous toluene and then concentrated under reduced pressure (3×3 mL) to remove any residual water. The phosphonate then was dissolved in dichloromethane (25 mL) and dimethyl formamide was added (1 drop). The solution was then cooled on an ice bath for 30 minutes. Oxalyl chloride (1.91 mL, 22.3 mmol) was added, and the solution was allowed to react for 13 hours. The excess oxalyl chloride and solvent were removed *in vacuo* and the resulting deep red oil was utilized without further purification. The ^{31}P NMR spectrum showed nearly complete conversion to product (approximately a 13:1 ratio of product to starting material by integration, or 92% by ^{31}P NMR). ^{31}P NMR (121 MHz, CDCl_3) δ +46.1 (product peak), +34.8 (residual starting material).

5.4 (*S*)-3,7-Dimethyloct-6-en-1-yl methyl ((*E*)-4,8-dimethylnona-3,7-dien-1-yl)phosphonate (9)

Compound **8** (1.963 g, 7.4 mmol) was dissolved in anhydrous toluene (25 mL). After (*S*)-(-)-citronellol (2.71 mL, 14.8 mmol) and freshly distilled triethylamine (2.07 mL, 14.8 mmol) were added sequentially, the solution was allowed to react overnight. Upon addition of triethylamine formation of a white precipitate and a color change from deep red to dark brown was noted. The reaction was quenched by addition of deionized water (5 mL). A cloudy emulsion formed which clarified after washing with brine (3×10 mL). The organic layers were combined, dried (Na_2SO_4), and vacuum filtered through celite, and the filtrate was concentrated *in vacuo* to afford a dark yellow oil. Final purification by flash chromatography (silica gel, 50% EtOAc in hexanes) gave the desired product **9** as a light yellow oil (1.695 g, 59% yield, 64% based on recovered **7**): ^1H NMR (300 MHz, CDCl_3) δ 5.15–5.05 (m, 3H), 4.16–4.01 (m, 2H), 3.73 (d, $J_{\text{PH}} = 10.5$ Hz, 3H), 2.35–2.23 (m, 2H), 2.05–1.91 (m, 6H), 1.82–1.70 (m, 2H), 1.68 (s, 6H), 1.62 (s, 3H), 1.60 (s, 6H), 1.57–1.13 (m, 5H), 0.92 (d, $J = 6.3$ Hz, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 135.9 (d, $J_{\text{CP}} = 1.2$ Hz), 130.8, 130.7, 124.2, 123.8, 122.7 (d, $J_{\text{CP}} = 16.4$ Hz), 63.5, 51.5, 39.2, 37.2 (d, $J_{\text{CP}} = 5.3$ Hz), 36.6, 28.7 (d, $J_{\text{CP}} = 0.9$ Hz), 26.2, 25.1 (d, $J_{\text{CP}} = 137.9$ Hz), 25.3, 25.0, 20.7, 20.5, (d, $J_{\text{CP}} = 4.6$ Hz), 18.9, 17.2, 15.6, 13.8; ^{31}P NMR (121 MHz, CDCl_3) δ +33.11, +33.07; HRMS (ES^+ , m/z) calcd for $(\text{M}+\text{H})^+ \text{C}_{22}\text{H}_{42}\text{O}_3\text{P}$: 385.2872; found: 385.2875.

5.5 (*S*)-3,7-Dimethyloct-6-en-1-yl methyl ((*E*)-1-(dimethoxyphosphoryl)-4,8-dimethylnona-3,7-dien-1-yl)phosphonate (10)

Freshly dried 2,2,6,6-tetramethylpiperidine (5.35 mL, 31.7 mmol) was combined with *n*-butyllithium (12.68 mL, 31.7 mmol as a 2.46M solution in hexanes) and THF (50 mL) at dry ice/acetone temperatures. After 15 minutes, compound **9** (5.54 g, 14.4 mmol) was added slowly to the solution and allowed to react for 20 minutes. Then the solution was transferred by cannula into a solution of dimethyl chlorophosphate (3.42 mL, 31.7 mmol) in THF (15 mL) and the resulting solution was allowed to react and reach room temperature overnight (9 hours). The reaction was then diluted with diethyl ether, quenched by addition of deionized water, dried (Na_2SO_4), and vacuum filtered through celite. The filtrate was concentrated to a yellow oil, which was purified further via flash chromatography (silica gel, 10% EtOH in EtOAc) to give the desired product (2.72 g, 38%, 52% based on recovered **9**): ^1H NMR (300

MHz, CDCl₃) δ 5.28 (t, J = 6.6 Hz, 1H), 5.11–5.06 (m, 2H), 4.20–4.09 (m, 2H), 3.81 (d, J_{PH} = 11.1 Hz, 9H), 2.72–2.56 (m, 2H), 2.36 (tt, J_{PH} = 24.0 Hz, J = 6.2 Hz 1H), 2.08–1.90 (m, 6H), 1.67 (s, 6H), 1.64 (s, 3H), 1.60 (s, 6H), 1.55–1.30 (m, 4H), 1.24–1.12 (m, 1H), 0.91 (d, J = 6.4 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 137.3, 131.5, 131.4, 124.6, 124.2, 121.6 (t, J_{CP} = 7.4 Hz), 65.0, 53.4–53.0 (m, 3 C overlap), 40.1 (t, J_{CP} = 105.8 Hz), 39.8, 38.7, 37.6–37.5 (m), 37.1–36.9 (m), 35.2, 29.1 (d, J_{CP} = 1.4 Hz), 26.7, 25.8, 25.4, 24.0 (m), 19.4, 17.8, 16.3; ³¹P NMR (121 MHz, CDCl₃) δ +26.3–26.2 (m), +24.8–24.6 (m); HRMS (ES⁺, m/z) calcd for (M+H)⁺ C₂₄H₄₇O₆P₂: 493.2848; found: 493.2845.

5.6 (((E)-1((((S)-3,7-Dimethyloct-6-en-1-yl)oxy)((pialoxy)methoxy)phosphoryl)-4,8-dimethylnona-3,7-dien-1-yl)phosphoryl)bis(oxy))bis(methylene) bis(2,2-dimethylpropanoate) (11)

Compound **10** (340 mg, 0.7 mmol) was dissolved in acetonitrile (3 $\times$ 3 mL) and then concentrated under reduced pressure to remove any residual water. Compound **10** was then added to a solution of chloromethyl pivalate (0.31 mL, 2.16 mmol) and sodium iodide (330 mg, 2.2 mmol) in acetonitrile (5 mL). This reaction mixture was then heated at reflux for approximately 5 hours. The acetonitrile was removed under reduced pressure and the resulting material was dissolved in diethyl ether. The solution was washed with brine (4 $\times$ 10 mL) and the organic layer was dried (Na₂SO₄), vacuum filtered through celite, and concentrated to a yellow oil. Final purification by flash chromatography (silica gel, 25% EtOAc in hexanes + 1% TEA) afforded the desired product **11** as a faint yellow oil (60 mg, 11%, 20% based on recovered **10**): ¹H NMR (300 MHz, CDCl₃) δ 5.71–5.7 (m, 6H), 5.28–5.23 (m, 1H), 5.10–5.06 (m, 2H), 4.20–4.12 (m, 2H), 2.70–2.60 (m, 2H), 2.50 (tt, J_{PH} = 24.0 Hz, J = 6.3 Hz, 1H), 2.07–1.95 (m, 6H), 1.68 (s, 6H), 1.62 (s, 3H), 1.60 (s, 6H), 1.54–1.29 (m, 5H), 1.24 (s, 27H), 0.91 (d, J = 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 176.9, 176.8, 176.6, 137.9, 131.5, 131.4, 124.5, 124.0, 120.7, 82.8–81.8 (m, 3 C overlap), 65.2, 39.7, 38.8, 38.7, 37.3–37.2 (m), 37.00, 36.98 29.1, 26.93, 26.91, 26.88, 26.85, 26.81, 26.59, 26.58, 25.70, 25.68, 25.4, 23.71, 23.66, 19.19, 19.14, 17.68, 17.65 16.13, 16.11, 15.3; ³¹P NMR (121 MHz, CDCl₃) δ +23.1 (d, J_{PP} = 2.9 Hz), +23.0 (d, J_{PP} = 2.8 Hz), +22.4–22.3 (m), +22.2–22.1 (m); HRMS (ES⁺, m/z) calcd for (M+H)⁺ C₃₉H₇₁O₁₂P₂: 793.4421; found: 793.4411.

5.7 (S)-3,7-Dimethyloct-6-en-1-yl methyl ((E)-2-(dimethoxyphosphoryl)-5,9-dimethyldeca-4,8-dien-2-yl)phosphonate (12)

Compound **10** (302 mg, 0.6 mmol) was dissolved in THF (5 mL) and the solution was cooled in an ice bath for 15 minutes. After NaH (37 mg, 0.9 mmol, 60% dispersion in mineral oil) was added to the solution, it was allowed to react for 30 minutes. Iodomethane (0.06 mL, 0.9 mmol) was then added and the reaction was allowed to proceed for 1.5 hours. The solution was then diluted with diethyl ether, quenched by addition of deionized water, and washed with water (3 $\times$ 5 mL). The organic layer was dried (Na₂SO₄) and vacuum filtered through celite. The filtrate was concentrated to a yellow oil which was purified by flash chromatography (silica gel, 5% EtOH in EtOAc) yielding the desired compound **12** as a yellow oil (0.30 g, 97%): ¹H NMR (300 MHz, CDCl₃) δ 5.34 (t, J = 7.2 Hz, 1H), 5.12–5.05 (m, 2H), 4.20–4.12 (m, 2H), 3.75 (d, J_{PH} = 10.8 Hz, 3H), 3.74 (d, J_{PH} = 10.5 Hz, 3H), 3.73 (d, J_{PH} = 10.2 Hz, 3H), 2.60 (td, J_{PH} = 15.3 Hz, J = 7.4 Hz, 2H), 2.10–1.93 (m, 6H),

1.73–1.70 (m, 2H), 1.67 (s, 6H), 1.61 (s, 3H), 1.60 (s, 6H), 1.39 (t, $J_{\text{PH}} = 15.6$ Hz, 3H), 1.30–1.12 (m, 3H), 0.92 (d, $J = 6.6$ Hz, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 138.2, 131.4, 131.3, 124.6, 124.3, 118.8 (t, $J_{\text{CP}} = 6.9$ Hz), 65.0, 53.6–53.2 (m, 3C overlap), 41.5 (t, $J_{\text{CP}} = 133.1$ Hz), 40.1, 39.7, 37.6 (t, $J_{\text{CP}} = 3.6$ Hz), 37.1–37.0 (m), 31.1 (t, $J_{\text{CP}} = 6.2$ Hz), 29.0, 26.7, 25.7, 25.4, 19.3, 19.3, 17.7, 17.7, 16.2; ^{31}P NMR (121 MHz, CDCl_3) δ +29.8 (d, $J_{\text{PP}} = 7.6$ Hz), +28.3–28.1 (m); HRMS (ES^+ , m/z) calcd for $(\text{M}+\text{H})^+ \text{C}_{25}\text{H}_{49}\text{O}_6\text{P}_2$: 507.3004; found: 507.3000.

5.8 (S)-3,7-Dimethyloct-6-en-1-yl methyl ((E)-4-(dimethoxyphosphoryl)-7,11-dimethyldeca-1,6,10-trien-4-yl)phosphonate (13)

According to the procedure described for preparation of compound **12**, compound **10** (300 mg, 0.6 mmol) was dried, dissolved in THF (8 mL), and treated with NaH (40 mg, 0.9 mmol, 60% dispersion in mineral oil) and allyl bromide (0.08 mL, 0.9 mmol). Standard work-up and concentration in vacuo gave a light yellow oil. Final purification by flash chromatography (silica gel, 5% EtOH in EtOAc) afforded the desired compound **13** (97 mg, 30% yield, 74% based on recovered **10**) ^1H NMR (300 MHz, CDCl_3) δ 6.03–5.92 (m, 1H), 5.40–5.36 (m, 1H), 5.11–5.07 (m, 4H), 4.17–4.12 (m, 2H), 3.775 (d, $J_{\text{PH}} = 10.5$ Hz, 3H), 3.767 (d, $J_{\text{PH}} = 10.8$ Hz, 3H), 3.760 (d, $J_{\text{PH}} = 10.8$ Hz, 3H), 2.69–2.58 (m, 4H), 2.10–1.96 (m, 6H), 1.74–1.71 (m, 2H), 1.67 (s, 6H), 1.61 (s, 3H), 1.60 (s, 6H), 1.55–1.30 (m, 3H), 0.91 (d, $J = 6.9$ Hz, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 137.9, 133.7 (t, $J_{\text{CP}} = 7.3$ Hz) 131.5, 131.5, 124.7, 124.4, 118.8 (t, $J_{\text{CP}} = 7.7$ Hz), 118.1, 65.1–64.9 (m), 53.6–53.2 (m, 3C overlap), 46.3 (t, $J_{\text{CP}} = 130.7$ Hz), 40.2, 37.7–37.6 (m), 37.2–37.1 (m), 35.3–35.2 (m), 29.2, 27.4, 26.7, 25.8, 25.5, 25.1, 19.4–19.3 (m), 17.8, 17.8, 16.5; ^{31}P NMR (121 MHz, CDCl_3) δ +28.8–28.7 (m), 27.4–27.1 (m); HRMS (ES^+ , m/z) calcd for $(\text{M}+\text{H})^+ \text{C}_{27}\text{H}_{51}\text{O}_6\text{P}_2$: 533.3161; found: 533.3164.

5.9 (S)-3,7-Dimethyloct-6-en-1-yl methyl ((E)-5-(dimethoxyphosphoryl)-2,8,12-trimethyltrideca-2,7,11-trien-5-yl)phosphonate (14)

According to the procedure described for preparation of compound **12**, compound **10** (360 mg, 0.7 mmol) was dried, dissolved in THF (5 mL), and treated with NaH (40 mg, 1.1 mmol, 60% dispersion in mineral oil) and freshly prepared prenyl bromide (0.13 mL, 1.1 mmol). After stirring overnight, standard work-up gave a faint yellow oil purified further by flash chromatography (silica gel, 5% EtOH in EtOAc) to afford the product **14** (270 mg, 65% yield). ^1H NMR (300 MHz, CDCl_3) δ 5.39–5.37 (m, 2H), 5.12–5.10 (m, 2H), 4.14–4.13 (m, 2H), 3.80 (d, $J_{\text{PH}} = 11.1$ Hz, 9H), 2.67–2.57 (m, 4H), 2.08–1.97 (m, 6H), 1.73 (s, 3H), 1.69 (s, 6H), 1.63 (s, 6H), 1.61 (s, 6H), 1.58–1.34 (m, 5H), 0.93 (d, $J = 6.6$ Hz, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 137.7, 133.9, 131.5, 131.4, 124.7, 124.4, 119.3–119.0 (m, 2C overlap), 65.0–64.8 (m), 53.5–53.1 (m, 3C overlap), 46.5 (t, $J_{\text{PH}} = 130.8$ Hz), 40.2, 37.7, 37.7, 37.2, 37.1, 29.3–29.2 (m), 26.8, 26.3, 25.8, 25.5, 19.4, 19.3, 18.0, 18.0, 17.8, 16.4. ^{31}P NMR (121 MHz, CDCl_3) δ +29.3–29.3 (m), 27.8–27.6 (m); HRMS (ES^+ , m/z) calcd for $(\text{M}+\text{H})^+ \text{C}_{29}\text{H}_{55}\text{O}_6\text{P}_2$: 561.3474; found: 561.3479

5.10(S)-3,7-Dimethyloct-6-en-1-yl methyl ((6*E*,11*E*)-9-(dimethoxyphosphoryl)-2,6,12,16-tetramethylheptadeca-2,6,11,15-tetraen-9-yl)phosphonate (15)

According to the procedure described for preparation of compound **12**, compound **10** (290 mg, 0.6 mmol) was dissolved in THF (5 mL), and treated with NaH (40 mg, 0.9 mmol, 60% dispersion in mineral oil), and freshly prepared geranyl bromide (0.18 mL, 0.9 mmol). After the reaction was allowed to stir overnight, standard work-up gave a light yellow oil (0.265 mg, 71%): ¹H NMR (300 MHz, CDCl₃) δ 5.38 (m, 2H), 5.10–5.08 (m, 3H), 4.15–4.13 (m, 2H), 3.78 (d, *J*_{PH} = 10.8 Hz, 9H), 2.66–2.56 (dt, *J*_{PH} = 16.2 Hz, *J* = 8.7 Hz 4H), 2.07–2.04 (m, 10H), 1.67 (s, 9H), 1.62 (s, 6H), 1.60 (s, 9H), 1.48–1.43 (m, 5H), 0.92 (d, *J* = 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 137.6, 131.43, 131.38, 124.7, 124.4, 119.16 (t, *J*_{PC} = 7.6 Hz), 65.0–64.8 (m), 53.4–53.1 (m, 3 C overlap), 46.5 (t, *J*_{PC} = 130.7 Hz), 40.2, 37.8–37.7 (m), 37.2–37.1 (m), 34.4, 29.3, 26.7, 25.8, 25.5, 19.4, 19.3, 17.8, 17.7, 16.4; ³¹P NMR (121 MHz, CDCl₃) δ 29.4–29.3 (m), +27.8–27.7 (m); HRMS (ES⁺, *m/z*) calcd for (M+Na)⁺ C₃₄H₆₂O₆P₂Na: 651.3919; found: 651.3922.

5.11 (((*E*)-2-(((*S*)-3,7-dimethyloct-6-en-1-yl)oxy)((pivaloyloxy)methoxy)phosphoryl)-5,9-dimethyldeca-4,8-dien-2-yl)phosphoryl)bis(oxy))bis(methylene) bis(2,2-dimethylpropanoate) (16)

Compound **12** (0.12 g, 0.2 mmol) was dissolved in acetonitrile (3 × 3 mL) and then concentrated under reduced pressure to remove any residual water. Compound **12** was then added to a solution of chloromethyl pivalate (0.11 mL, 0.76 mmol) and sodium iodide (0.11 g, 0.6 mmol) in acetonitrile (5 mL), and this reaction mixture was heated at reflux for approximately 24 hours. The acetonitrile was removed under reduced pressure and the resulting material was dissolved in diethyl ether. The solution was washed with brine (4 × 10 mL) and the organic layer was dried (Na₂SO₄), vacuum filtered through celite, and concentrated to a yellow oil. Final purification by flash chromatography (silica gel, 20% EtOAc in hexanes + 1% TEA) afforded the final product as a faint yellow oil (0.07 g, 34%): ¹H NMR (300 MHz, CDCl₃) δ 5.77–5.66 (m, 6H), 5.29 (t, *J*_{PH} = 7.5 Hz, 1H), 5.08–5.07 (m, 2H), 4.16–4.11 (m, 2H), 2.60 (dt, *J*_{PH} = 15.7, *J* = 6.9 Hz, 2H), 2.06–1.95 (m, 6H), 1.80–1.71 (m, 2H), 1.70 (s, 3H), 1.67 (s, 6H), 1.59 (s, 6H), 1.54–1.48 (m, 3H), 1.39 (t, *J*_{PH} = 16.5 Hz, 3H), 1.23 (s, 27H), 0.90 (d, *J* = 6.3 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 176.9–176.8 (m, 3C overlap), 139.2, 131.5, 131.5, 124.6, 124.3, 124.3, 83.0–82.1 (m, 3C overlap), 65.1–65.0 (m), 41.5 (t, *J*_{PC} = 133.3 Hz), 40.2, 38.8, 38.8, 37.4–37.3 (m), 37.11–37.06 (m), 30.6, 30.5, 29.22, 29.18, 27.04, 27.00, 26.99, 26.97, 26.7, 25.8, 25.5, 19.35, 19.31, 19.29, 17.80, 17.76, 16.30, 16.27, 15.7; ³¹P NMR (121 MHz, CDCl₃) δ +26.8–26.6 (m), +25.9–25.8 (m); HRMS (ES⁺, *m/z*) calcd for (M+Na)⁺ C₄₀H₇₂O₁₂P₂Na: 829.4397; found: 829.4404.

5.12 (((*E*)-4-(((*S*)-3,7-Dimethyloct-6-en-1-yl)oxy)((pivaloyloxy)methoxy)phosphoryl)-7,11-dimethyldodeca-1,6,10-trien-4-yl)phosphoryl)bis(oxy))bis(methylene) bis(2,2-dimethylpropanoate) (17)

According to the procedure described for preparation of compound **16**, compound **17** (97 mg, 0.2 mmol) was added to a solution of chloromethyl pivalate (0.30 mL, 1.99 mmol), sodium iodide (300 mg, 1.99 mmol), and acetonitrile (5 mL) and the solution was heated at

reflux for 8 hours. Standard workup and purification by flash chromatography (silica gel, 15% EtOAc in hexanes + 1% TEA) gave a clear oil (34 mg, 23%): ^1H NMR (300 MHz, CDCl_3) δ 6.01–5.85 (m, 1H), 5.79–5.66 (m, 6H), 5.35–5.31 (m, 1H), 5.10–5.07 (m, 4H), 4.18–4.08 (m, 2H), 2.69–2.57 (m, 4H), 2.08–1.95 (m, 6H), 1.80–1.70 (m, 2H), 1.67 (s, 6H), 1.59 (s, 9H), 1.53–1.45 (m, 3H), 1.23 (s, 27H), 0.92 (d, $J = 6.3$ Hz, 3H). ^{13}C NMR (75 MHz, CDCl_3) δ 176.8–176.7 (m, 3C overlap), 138.5, 138.5, 134.7, 131.4, 131.3, 124.5, 124.2, 124.2, 82.9, 82.5, 81.8, 64.8, 47.6, 40.1, 40.0, 38.7, 38.4, 37.3, 37.3, 37.0, 36.3, 29.2, 29.2, 27.1, 27.0, 26.9, 26.9, 26.8, 26.7, 25.7, 25.7, 25.4, 19.2, 19.1, 17.9, 17.7, 17.6, 16.2, 16.2; ^{31}P NMR (121 MHz, CDCl_3) δ +25.5–25.4 (m), 24.8–24.7 (m); HRMS (ES^+ , m/z) calcd for $(\text{M}+\text{Na})^+ \text{C}_{42}\text{H}_{74}\text{O}_{12}\text{P}_2\text{Na}$: 855.4553; found: 855.4559.

5.13 (((E)-5-(((S)-3,7-Dimethyloct-6-en-1-yl)oxy) ((pivaloyloxy)methoxy)phosphoryl)-2,8,12-trimethyltrideca-2,7,11-trien-5-yl)phosphoryl)bis(oxy))bis(methylene) bis(2,2-dimethylpropanoate) (18)

According to the procedure described for preparation of compound **16**, compound **18** (267 mg, 0.41 mmol) was added to a solution of chloromethyl pivalate (0.37 mL, 2.55 mmol), sodium iodide (380 mg, 2.55 mmol), and acetonitrile (7 mL) and the solution was heated at reflux for 8 hours. Standard workup and purification by flash chromatography (silica gel, 15% EtOAc in hexanes + 1% TEA) gave a clear oil (133 mg, 32%): ^1H NMR (300 MHz, CDCl_3) δ 5.78–5.62 (m, 6H), 5.35–5.27 (m, 2H), 5.10–5.02 (m, 2H), 4.20–4.03 (m, 2H), 2.66–2.56 (m, 4H), 2.06–1.94 (m, 6H), 1.81–1.76 (m, 2H), 1.71 (s, 3H), 1.66 (s, 6H), 1.59 (s, 12H), 1.52–1.40 (m, 3H), 1.23 (s, 27H), 0.90 (d, 6.3 Hz, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 176.8–176.7 (m, 3C overlap), 138.5, 138.5, 134.7, 131.4, 131.4, 124.5, 124.2, 124.2, 82.9, 82.6, 81.8, 64.7, 47.6, 40.1, 40.1, 38.7, 38.4, 37.3, 37.2, 37.0, 36.3, 29.2, 29.2, 27.1, 26.9, 26.9, 26.9, 26.9, 26.7, 26.1, 25.7, 25.7, 25.4, 19.2, 19.1, 17.9, 17.7, 17.6, 16.2, 16.2; ^{31}P NMR (121 MHz, CDCl_3) δ +26.0–25.9 (m), 25.3–25.1 (m); HRMS (ES^+ , m/z) calcd for $(\text{M}+\text{Na})^+ \text{C}_{44}\text{H}_{78}\text{O}_{12}\text{P}_2\text{Na}$: 883.4866; found: 883.4872.

5.14 (((6E,11E)-9-(((S)-3,7-Dimethyloct-6-en-1-yl)oxy) ((pivaloyloxy)methoxy)phosphoryl)-2,6,12,16-tetramethylheptadeca-2,6,11,15-tetraen-9-yl)phosphoryl)bis(oxy))bis(methylene) bis(2,2-dimethylpropanoate) (19)

According to the procedure described for preparation of compound **16**, compound **19** (265 mg, 0.42 mmol) was added to a solution of chloromethyl pivalate (0.30 mL, 2.08 mmol), sodium iodide (312 mg, 2.08 mmol), and acetonitrile (5 mL) and the solution was heated at reflux for 10 hours. Standard workup and flash chromatography (silica gel, 10% EtOAc in hexanes + 1% TEA) gave a yellow oil (36 mg, 9%): ^1H NMR (500 MHz, CDCl_3) δ 5.78–5.65 (m, 6H), 5.36–5.31 (m, 2H), 5.10–5.08 (m, 3H), 4.22–4.05 (m, 2H), 2.68–2.57 (m, 4H), 2.09–1.95 (m, 10H), 1.80–1.70 (m, 2H), 1.68 (s, 9H), 1.62 (s, 6H), 1.60 (s, 9H), 1.52–1.40 (m, 3H), 1.23 (s, 27H), 0.91 (d, $J = 5.7$ Hz, 3H). ^{13}C NMR (125 MHz, CDCl_3) δ 176.8–176.7 (m, 3C overlap), 138.4, 138.4, 131.4, 131.3, 124.5, 124.2, 82.9, 82.6, 81.8, 64.8, 46.3 (t, $J_{\text{PC}} = 130.6$ Hz), 40.2, 40.1, 38.7, 37.5, 37.4, 37.3, 37.3, 37.0, 29.7, 29.2, 29.2, 28.8, 26.9, 26.9, 26.9, 26.7, 25.7, 25.6, 25.4, 19.3, 19.2, 17.7, 17.6, 16.3, 16.2; ^{31}P NMR (202 MHz, CDCl_3) δ +26.0 (d, $J_{\text{PP}} = 9.9$ Hz), +25.3 (d, $J_{\text{PP}} = 9.9$ Hz); HRMS (ES^+ , m/z) calcd for $(\text{M}+\text{Na})^+ \text{C}_{49}\text{H}_{86}\text{O}_{12}\text{P}_2\text{Na}$: 951.5492; found: 951.5507.

5.15 (*R*)-3,7-Dimethyloct-6-en-1-yl methyl ((*E*)-4,8-dimethylnona-3,7-dien-1-yl)phosphonate (20)

According to the procedure described for preparation of compound **9**, the acid chloride **8** (2.63 g, 9.9 mmol) was allowed to react with (*R*)-(+)-citronellol (1.55 g, 9.9 mmol). Parallel workup and purification provided the mixed ester **20** (2.21 g, 58%): ¹H NMR (400 MHz, CDCl₃) δ 5.12–5.08 (m, 3H), 4.10–4.05 (m, 2H), 3.74 (d, *J*_{PH} = 11.2 Hz, 3H), 2.30–2.26 (m, 2H), 2.07–1.96 (m, 6H), 1.80–1.70 (m, 2H), 1.67 (s, 6H), 1.61 (s, 3H), 1.60 (s, 6H), 1.59–1.44 (m, 2H), 1.37–1.21 (m, 2H), 1.20–1.17 (m, 1H), 0.92 (d, *J* = 6.3 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 136.4, 131.4, 131.6, 124.5, 124.1, 122.9 (d, *J*_{CP} = 17.0 Hz), 64.0 (d, *J*_{CP} = 6.6 Hz), 52.0 (d, *J*_{CP} = 6.5 Hz), 39.5, 37.5 (d, *J*_{CP} = 6.7 Hz), 36.9, 29.0 (d, *J*_{CP} = 2.5 Hz), 26.5, 25.3 (d, *J*_{CP} = 137.2 Hz), 25.6, 25.3, 20.9, 20.9, 19.2, (d, *J*_{CP} = 1.7 Hz), 19.2, 17.6, 15.9; ³¹P NMR (161 MHz, CDCl₃) δ +33.25, +33.20; HRMS (ES⁺, *m/z*) calcd. for (M + H)⁺ C₂₂H₄₂O₃P: 385.2872; found: 385.2864.

5.16 (*R*)-3,7-Dimethyloct-6-en-1-yl methyl ((*E*)-1-(dimethoxyphosphoryl)-4,8-dimethylnona-3,7-dien-1-yl)phosphonate (21)

According to the procedure described for preparation of compound **10**, the mixed ester **9** (1.02 g, 2.6 mmol) was treated with a solution of LTMP, formed from 2,2,6,6-tetramethylpiperidine (0.99 mL, 5.8 mmol) and *n*-butyl lithium (2.32 mL, 5.8 mmol), followed by reaction with dimethyl chlorophosphate (0.65 mL, 5.8 mmol). Standard work-up and purification gave the desired bisphosphonate **21** (0.46 g, 36%): ¹H NMR (400 MHz, CDCl₃) δ 5.20 (t, *J* = 6.0 Hz, 1H), 5.03–5.00 (m, 2H), 4.10–4.05 (m, 2H), 3.73 (d, *J*_{PH} = 14.8 Hz, 9H), 2.62–2.50 (m, 2H), 2.29 (tt, *J*_{PH} = 23.6 Hz, *J* = 6.0 Hz 1H), 2.01–1.85 (m, 6H), 1.60 (s, 6H), 1.57 (s, 3H), 1.53 (s, 6H), 1.50–1.35 (m, 2H), 1.32–1.23 (m, 2H), 1.18–1.06 (m, 1H), 0.85 (d, *J* = 6.4 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 137.1, 131.4, 131.2, 124.5, 124.1, 121.5 (t, *J*_{CP} = 7.0 Hz), 65.0–64.9 (m), 53.2–52.9 (m, 3 C overlap), 39.6, 36.8 (t, *J*_{CP} = 132.2 Hz), 37.5–37.4 (m), 37.0, 36.9, 28.9 (d, *J*_{CP} = 2.0 Hz), 26.5, 25.7, 25.6, 25.3, 23.9 (t, *J*_{CP} = 5.1 Hz), 19.2, 17.6, 16.0; ³¹P NMR (121 MHz, CDCl₃) δ +26.3, +24.7–24.6 (m); HRMS (ES⁺, *m/z*) calcd for (M + H)⁺ C₂₄H₄₇O₆P₂: 493.2848; found: 493.2850.

5.17 (*R*)-3,7-Dimethyloct-6-en-1-yl methyl ((*E*)-2-(dimethoxyphosphoryl)-5,9-dimethyldeca-4,8-dien-2-yl)phosphonate (22)

According to the procedure described for methylation of compound **12**, the bisphosphonate **21** (0.160 g, 0.3 mmol) was treated with NaH (20 mg, 0.5 mmol) and methyl iodide (0.05 mL, 0.8 mmol) to give the expected product **22** (152 mg, 92%): ¹H NMR (300 MHz, CDCl₃) δ 5.26 (t, *J* = 6.8 Hz, 1H), 5.03–4.99 (m, 2H), 4.12–4.03 (m, 2H), 3.75 (d, *J*_{PH} = 10.8 Hz, 3H), 3.74 (d, *J*_{PH} = 10.8 Hz, 3H), 3.73 (d, *J*_{PH} = 10.8 Hz, 3H), 2.53 (td, *J*_{PH} = 15.5 Hz, *J* = 7.5 Hz, 2H), 2.03–1.86 (m, 6H), 1.69–1.63 (m, 2H), 1.60 (s, 6H), 1.54 (s, 3H), 1.52 (s, 6H), 1.32 (t, *J*_{PH} = 16.7 Hz, 3H), 1.26–1.07 (m, 3H), 0.85 (d, *J* = 6.9 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 138.3, 131.5, 131.4, 124.7, 124.3, 118.8 (t, *J*_{CP} = 7.3 Hz), 65.1 (m), 53.7–53.3 (m, 3C overlap), 41.6 (t, *J*_{CP} = 133.3 Hz), 40.2, 38.9, 37.7–37.6 (m), 37.2–37.1 (m), 31.1 (t, *J*_{CP} = 5.2 Hz), 29.1, 26.7, 25.8, 25.5, 19.4, 19.3, 17.8, 17.7, 16.3; ³¹P NMR (121 MHz, CDCl₃) δ +29.8 (d, *J*_{PP} = 7.9 Hz), +28.3–28.2 (m); HRMS (ES⁺, *m/z*) calcd for (M + H)⁺ C₂₅H₄₉O₆P₂: 507.3004; found: 507.3006.

5.18 ((((*E*)-2-(((*R*)-3,7-dimethyloct-6-en-1-yl)oxy)((pivaloyloxy)methoxy)phosphoryl)-5,9-dimethyldeca-4,8-dien-2-yl)phosphoryl)bis(oxy))bis(methylene) bis(2,2-dimethylpropanoate) (23)

According to the procedure described for preparation of compound **16**, compound **22** (165 mg, 0.3 mmol) was treated with POMCl (0.15 mL, 1.0 mmol) and NaI (151 mg, 1.0 mmol) in acetonitrile at reflux. Standard work-up and purification provided the desired POM ester **23** (108 mg, 41%): ¹H NMR (300 MHz, CDCl₃) δ 5.79–5.66 (m, 6H), 5.29 (t, *J*_{PH} = 7.5 Hz, 1H), 5.09–5.06 (m, 2H), 4.19–4.08 (m, 2H), 2.61 (dt, *J*_{PH} = 15.8, *J* = 7.4 Hz, 2H), 2.08–1.91 (m, 6H), 1.77–1.70 (m, 2H), 1.67 (s, 6H), 1.59 (s, 9H), 1.56–1.47 (m, 3H), 1.39 (t, *J*_{PH} = 17.3 Hz, 3H), 1.24 (s, 9H), 1.23 (s, 18H), 0.90 (d, *J* = 6.3 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 176.8–176.7 (m, 3C overlap), 139.1, 131.5, 131.5, 124.5, 124.3, 118.0, 83.1–81.8 (m, 3C overlap), 65.2–64.9 (m), 41.6 (t, *J*_{PC} = 133.4 Hz), 40.1, 38.8, 38.8, 37.4–37.3 (m), 37.1–37.0 (m), 30.6, 30.5, 29.21, 29.18, 27.1, 27.00, 26.98, 26.97, 26.6, 25.7, 25.5, 19.4, 19.32, 19.28, 17.79, 17.77, 16.30, 16.27, 15.7; ³¹P NMR (121 MHz, CDCl₃) δ +26.8–26.6 (m), +25.9–25.8 (m); HRMS (ES⁺, *m/z*) calcd for (M + Na)⁺ C₄₀H₇₂O₁₂P₂Na: 829.4397; found: 829.4395.

5.19 Immunoblot analysis

RPMI-8226 (ATCC, Manassas, VA) cells were incubated (37 °C and 5% CO₂) with test compounds for 48 hrs in RPMI-1640 media containing 10% fetal bovine serum and penicillin-streptomycin. Whole cell lysate was obtained using RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton (v/v) X-100, 0.05 M Tris HCl) containing protease and phosphatase inhibitors. Protein content was determined using the bicinchoninic acid (BCA) method (Pierce Chemical, Rockford, IL). Equivalent amounts of cell lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, probed with the appropriate primary antibodies, and detected using HRP-linked secondary antibodies and Amersham Pharmacia Biotech ECL Western blotting reagents per manufacturer's protocols.

5.20 Lambda light chain ELISA

Human lambda light chain kit (Bethyl Laboratories, Montgomery, TX) was used to quantify intracellular monoclonal protein levels of whole cell lysate. Lambda light chain levels were normalized to total protein levels (as determined by BCA).

5.21 MTT cytotoxicity assay

RPMI-8226, MM.1S (ATCC, Manassas, VA), or ALMC-2 (obtained from Dr. Diane Jelinek, Mayo Clinic, Rochester, MN) cells were plated (2.5 × 10⁴ cells/100 μL/well) in 96-well plates in the presence or absence of the novel compounds and incubated for 48 hrs. MTT solution (35 μL/well; 5 mg/mL PBS) was subsequently added and cells were incubated for an additional 4 hrs. MTT solubilizing solution (0.01 M HCl/10% SDS) was then added (100 μL/well). After overnight incubation at 37 °C, plates were analyzed on a microplate spectrophotometer at 540 nm. Compounds were tested in quadruplicate and three independent experiments were performed. The absorbance for control cells was defined as an MTT activity of 100%.

5.22 Statistics

Two-tailed *t*-testing was used to calculate statistical significance. An α of 0.05 was set as the level of significance. CalcuSyn software (Biosoft, Cambridge, UK) was used to analyze the concentration response curves and determine the EC₅₀ for the MTT cytotoxicity assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1 (zoledronate) **2** (pamidronate)

Figure 1.
Some bisphosphonates in current clinical use.

Figure 2.
V- and U-shaped isoprenoid bisphosphonates.

Scheme 1.
Synthesis of bisphosphonate mixed esters.

Scheme 2.
 Synthesis of an *R*-(+)-citronellol ester (**23**).

Figure 3. The new bisphosphonates disrupt protein geranylgeranylation in human myeloma cells RPMI-8226 cells were incubated for 48 hours in the presence or absence of lovastatin (*Lov*, 5 μ M) or varying concentrations of the test compounds. A) Immunoblot analysis of Rap1a (antibody detects only unmodified protein) and β -tubulin (as a loading control). B) Intracellular lambda light chain concentrations were determined via ELISA. Data are expressed as a percentage of control (mean $\pm$ SD, n=3). The * denotes $p < 0.05$ per unpaired two-tailed t -test.

Fig 4. The novel bisphosphonate 16 disrupts protein geranylgeranylation by depletion of GGDP RPMI-8226 cells were incubated for 48 hours in the presence or absence of lovastatin (*Lov*, 5 μ M) or **16** (1 μ M) along with either mevalonate (*Mev*, 1 mM), FDP (10 μ M), or GGDP (10 μ M). A) Immunoblot analysis of unmodified Rap1a and β -tubulin (loading control). B) Intracellular lambda light chain concentrations were determined via ELISA. Data are expressed as a percentage of control (mean $\pm$ SD, n=3). The * denotes $p < 0.05$ per unpaired two-tailed t -test comparing combination treatment to either lovastatin or **16** alone.

Fig 5. Effect of citronellol stereochemistry on activity

RPMI-8226 cells were incubated for 48 hours in the presence or absence of lovastatin (*Lov*; 10 μM) or varying concentrations of compounds **16** or **23**. A) Immunoblot analysis of unmodified Rap1a and β-tubulin (loading control). B) Intracellular lambda light chain concentrations were determined via ELISA. Data are expressed as a percentage of control (mean ± SD, n=3). The * denotes $p < 0.05$ per unpaired two-tailed t -test comparing treatments to untreated control.

Table 1

Cytotoxic activity of the tested compounds in human myeloma cell lines. The EC₅₀'s were determined from MTT assays are shown as “mean ± standard deviation”.

	RPMI-8226	MM.1S	ALMC-2
11	7.1 ± 1.6 µM	5.7 ± 1.9 µM	12.6 ± 2.8 µM
16	1.6 ± 0.5 µM	2.7 ± 0.7 µM	5.4 ± 0.6 µM
17	8.4 ± 0.4 µM	10.7 ± 1.5 µM	25.4 ± 9.8 µM
18	7.3 ± 3.3 µM	25.3 ± 7.0 µM	28.2 ± 3.9 µM
19	26.7 ± 1.6 µM	48.2 ± 6.6 µM	>100 µM