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Short communication

Phosphinato(dialkylmethyl)phosphonates as pyrophosphate mimics: synthesis and squalene synthetase inhibitory activity of farnesyl phosphinato(dialkylmethyl)phosphonates*

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Summary — The synthesis and squalene synthetase inhibitory activity of farnesyl phosphinato(dialkylmethyl)phosphonates has been described. The phosphinato(dimethylmethyl)phosphonate group provided a suitable mimic of pyrophosphate.

squalene synthetase / phosphinato(dialkylmethyl)phosphonate / pyrophosphate mimics / cholesterol biosynthesis pathway / enzyme inhibitors

Introduction

Squalene synthetase catalyses the condensation of 2 molecules of farnesyl pyrophosphate (FPP) and reductive rearrangement of the resulting presqualene pyrophosphate to produce squalene [1]. The inhibitors of this enzyme are attractive because of its strategic location in the cholesterol biosynthesis pathway.

Methylenediphosphonate is a known stable isostere of pyrophosphate; however, pK_a values of the 2 differ significantly. Introduction of 2 fluorine atoms at the methylene unit of methylenediphosphonate leads to a difluoromethylenediphosphonate moiety which shows pK_a values very similar to those of pyrophosphate [2]. Analogs of FPP using phosphinatomethylphosphonate as well as phosphinato(difluoromethyl)phosphonate as mimics of pyrophosphate have been reported by the Squibb group [3]. Interestingly, both analogs possessed identical biological activity, suggesting that the adjustment of pK_a values of phosphinatom-ethylphosphonate moiety closer to those of pyrophosphate with 2 fluorine atoms was not essential. Recognizing the inherent problem of transport associated with this class of compounds, we became interested in increasing the lipophilic character of phosphinatomethylphosphonate moiety by introducing 2 alkyl chains at the methylene unit with the possibility of overcoming this problem. The substitution at the methylene unit with 2 alkyl chains would, however, adversely affect the pK_a values [4] and might lead to loss of activity.

In this paper we describe the synthesis and squalene synthetase inhibitory activity of FPP analogs (**2–4**, **6**; fig 1) containing phosphinato(dialkylmethyl)phosphonate as pyrophosphate mimics where one of the oxygens is replaced by a methylene unit to attach the farnesyl chain analogous to the Squibb analogs [3]. The utility of phosphinato(dialkylmethyl)phosphonates as pyrophosphate mimics has not been previously reported.

Chemistry

We envisioned that diethyl ethoxy(methyl)phosphinoylmethylphosphonate **7** would serve as a common

* This study is dedicated to Prof EV Dehmlow, Universitat Bielefeld, on the occasion of his 60th birthday.

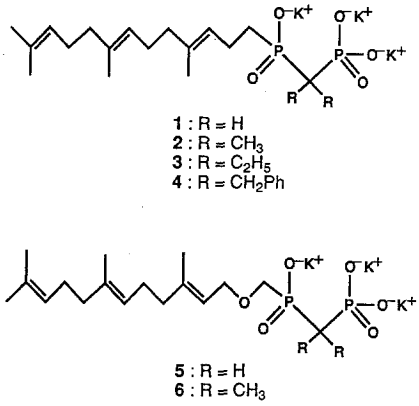

Fig 1. Structure of compounds **1–6**.

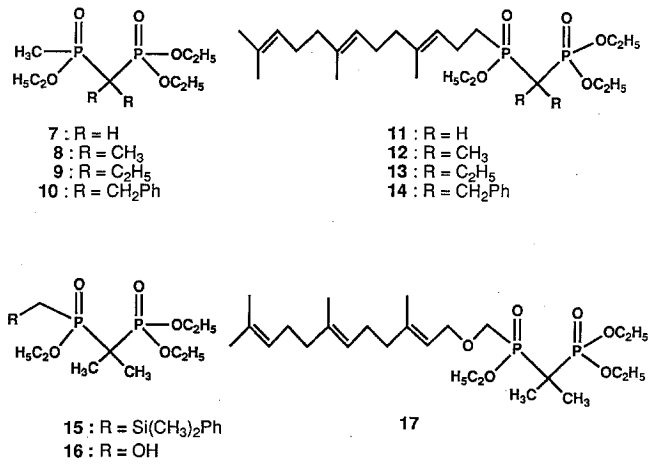

Fig 2. Structure of compounds **7–17**.

synthon for the synthesis of the desired analogs (**2–4**, **6**). Compound **7** was prepared by a known method [5] and modifying the workup conditions. Dialkylation of **7** with appropriate halides in the presence of sodium hydride in tetrahydrofuran yielded the desired intermediates **8–10** (fig 2) in good yields. Treatment of **8** and **9** with *n*-butyllithium in tetrahydrofuran at –78°C followed by alkylation with farnesyl bromide furnished the desired triesters **12** and **13** respectively in good yields. Reaction of **10** under identical conditions failed to yield any desired product **14**. The triester **14** was prepared by an alternate approach. Dianion of **7**, generated [6] by treatment with sodium hydride at room temperature followed by *n*-butyllithium in tetrahydrofuran at–78°C, was alkylated with farnesyl bromide to yield **11.** Dialkylation of **11** with benzyl bromide in the presence of sodium hydride furnished the desired compound **14.**

A recent publication from the Squibb group [7] described compound **5** as being several-fold more active than the analog **1**. This prompted us to prepare the corresponding dimethyl derivative **6** of analog **5** to further evaluate the use of dialkylmethanediphosphonates as a pyrophosphate mimic. In order to synthesize the analog **6** and to further demonstrate the synthetic utility of synthon **7**, we reasoned that alcohol **16** would serve as an intermediate to prepare the desired triester **17**. Thus, treatment of **8** with *n*-butyllithium in tetrahydrofuran at –78°C followed by a reaction with chlorodimethylphenylsilane furnished compound **15** in 55% yield. Oxidation of dimethylphenylsilyl group in **15** with peracetic acid and mercuric acetate utilizing conditions reported by Fleming and Sanderson [8] yielded the desired alcohol **16** in 81% yield. Alkylation of **16** with farnesyl bromide in the presence of sodium hydride furnished the triester **17** in 84% yield.The cleavage of esters in **12–14** and **17** with bromotrimethylsilane in methylene chloride in the presence of 2,4,6-collidine followed by treatment with potassium hydroxide yielded crude products which were purified by reverse phase HPLC on CHP 20P gel [9] to furnish pure **2–4** and **6** respectively. Compounds **1** and **5** were also synthesized for comparison purposes using the chemistry described by the Squibb group [3, 10].

Biological results and discussion

Compounds **1–6** were tested in a rat liver microsomal assay for squalene synthetase inhibitory activity and their IC_{50} values are listed in table I. An examination of table I suggested that compound **1** and its dimethyl analog **2** possessed approximately the same activity. The activity of diethyl analog **3** and the dibenzyl analog **4** was weaker than that of **1**. Interestingly, in the oxygenated series the dimethyl analog **6** was 20-

Table I. Squalene synthetase inhibitory activity of compounds **1–6**.

Compound	*Squalene synthetase inhibitory activity IC_{50} (μm)*
1	7.0*
2	16.0
3	61.0
4	56.0
5	0.02**
6	0.001

* Reported IC_{50} = 31.5 μm [3]; ** reported IC_{50} = 0.05 μm [6].

fold more active than compound **5**. These results suggested that phosphinato(dimethylmethyl)phosphonate is a suitable mimic of pyrophosphate, especially in the etheric series (compound **6**), even though the substitution at methylene unit with 2 methyl groups adversely affects the pK_a values.

Experimental protocols

Chemical synthesis

Farnesyl bromide was commercially available from Aldrich Chemicals. Kieselgel 60 (230-400 mesh) from E Merck was used for silica gel chromatography. NMR spectra were run on a Joel-FX200 FT NMR or Bruker-500 NMR spectrometer. Mass spectra were obtained using a Finnigan 4600 spectrometer.

Diethyl ethoxy(methyl)phosphinoylmethylphosphonate 7
A solution of diethyl methylphosphonate (7.6 g, 50 mmol) in dry THF (15 ml) was cooled to –78°C and with rapid stirring and under an argon atmosphere was added *n*-BuLi (32 ml of 1.6 M solution in hexane) dropwise. After the addition the cooling bath was allowed to warm to room temperature (in ≈ 2.5 h) and the stirring was continued at this temperature for an additional 2 h. The mixture was quenched with 3 N HCl and extracted with methylene chloride. The combined organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The residue was purified by silica gel chromatography using 10% isopropanol in ethyl acetate and then with 15% methanol in methylene chloride as the eluent to furnish pure **7** as an oil. Yield: 91.5%; ^{1}H-NMR ($CDCl_3$, δ): 1.38 (t, 9H), 1.74 (d, 3H), 2.45 (dd, 2H), 4.04–4.38 (m, 6H); ^{31}P-NMR ($CDCl_3$, δ): 20.45 (d), 45.1 (d); ms, *m/z* 259 (MH^+).

General method of dialkylation of 7
To an ice-cooled stirred suspension of sodium hydride (0.48 g of 60% dispersion, 12 mmol) in dry THF (30 ml) was added **7** (1.03 g, 4 mmol) dropwise under an argon atmosphere. The mixture was warmed to room temperature and an appropriate alkyl halide (20 mmol) was added dropwise. The resulting mixture was heated at 60°C (1.5 h for methyl iodide, 4 h for ethyl iodide and 7 h for benzyl bromide) and cooled in an ice bath. It was decomposed with brine and extracted with methylene chloride. The organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The residue was purified by silica gel chromatography using 10% methanol in methylene chloride as the eluent to yield pure products.

Diethyl 1-[ethoxy(methyl)phosphinoyl]-1-methylethylphosphonate 8
Yield: 74%; oil; ^{1}H-NMR ($CDCl_3$, δ): 1.33 and 1.34 (2t, 9H), 1.42 and 1.45 (2dd, 6H), 1.69 (d, 3H), 4.08–4.3 (m, 6H); ^{31}P-NMR ($CDCl_3$, δ): 28.54 (d), 55.2 (d); = ms, *m/z* 287 (MH^+).

Diethyl 1-[ethoxy(methyl)phosphinoyl]-1-ethylpropylphosphonate 9
Yield: 49%, oil; ^{1}H-NMR ($CDCl_3$, δ): 1.05 and 1.1 (2t, 6H), 1.32 and 1.34 (2t, 9H), 1.7 (d, 3H), 1.81–2.2 (m, 4H), 4.04–4.25 (m, 6H); ^{31}P-NMR ($CDCl_3$, δ): 28.28 (d), 55.74 (d); ms, *m/z* 315 (MH^+).

Diethyl 1-benzyl-1-[ethoxy(methyl)phosphinoyl]-2-phenylethylphosphonate 10
Yield: 65%; oil; ^{1}H-NMR ($CDCl_3$, δ): 1.15–1.35 (m, 12H), 3.28–3.5 (m, 4H), 3.98–4.23 (m, 6H), 7.23–7.37 (m, 6H), 7.44–7.6 (m, 4H); ^{31}P-NMR ($CDCl_3$, δ): 24.97 (d), 54.96 (d); ms, *m/z* 439 (MH^+).

Diethyl (3E,7E)-1-[ethoxy-(4,8,12-trimethyl-3,7,11-tridecatrienyl)phosphinoyl]-1-methylethylphosphonate 12
To a solution of **8** (1 mmol) in dry THF (3 ml), cooled to –78°C and under an argon atmosphere, was added *n*-BuLi (1.2 mmol; 0.75 ml of 1.6 M hexane solution) dropwise. After stirring for an additional 35 min farnesyl bromide (0.4 g, 1.4 mmol) was added dropwise. After stirring for another 1 h, the mixture was quenched with acetic acid (0.12 g, 2 mmol), warmed to room temperature and diluted with methylene chloride. This mixture was washed with brine, dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The residue was purified by silica gel chromatography using 10% isopropanol in ethyl acetate and then with 15% isopropanol in ethyl acetate as the eluent to furnish pure **12**. Yield: 78%; oil; ^{1}H-NMR ($CDCl_3$, δ): 1.22–1.4 (m, 15H), 1.53 (s, 6H), 1.55 (s, 3H), 1.6 (s, 3H), 1.85–2.05 (m, 10H), 2.12–2.2 (m, 2H), 4.0–4.2 (m, 6H), 4.98-5.12 (m, 3H); ^{31}P-NMR ($CDCl_3$, δ): 28.90 (d), 56.19 (d); ms, *m/z* 491 (MH^+).

Diethyl (3E,7E)-1-[ethoxy-(4,8,12-trimethyl-3,7,11-tridecatrienyl)phosphinoyl]-1-ethylpropylphosphonate 13
13 was prepared from **9** using the same method as described above for the preparation of **12**. Yield: 46%; oil; ^{1}H-NMR ($CDCl_3$, δ): 1.05 (t, 3H), 1.09 (t, 3H), 1.31, 1.32 (2t, 9H), 1.61, 1.64, 1.69 (3s, 12H), 1.82–2.16 (m, 14H), 2.19–2.51 (m, 2H), 4.05–4.28 (m, 6H), 5.04–5.21 (m, 3H); ^{31}P-NMR ($CDCl_3$, δ): 28.68 (d), 56.76 (d); ms, *m/z* 519 (MH^+).

Diethyl (3E,7E)-1-benzyl-1-[ethoxy-(4,8,12-trimethyl-3,7,11-tridecatrienyl)phosphinoyl]-2-phenylethylphosphonate 14
To a stirred suspension of sodium hydride (0.044 g, 1.1 mmol; 60% dispersion) in dry THF (3 ml) was added **7** (0.258 g, 1 mmol) dropwise under an argon atmosphere. The solution was allowed to stir at room temperature for 1.25 h and then cooled to –78°C. *n*-BuLi (0.69 ml of 1.6 M hexane solution, 1.1 mmol) was then added dropwise and the resulting solution was stirred for an additional 30 min. Farnesyl bromide (0.342 g, 1.2 mmol) was added dropwise and the solution was stirred for an additional 30 min at –78°C. The reaction mixture was quenched with acetic acid (0.157 g, 2.6 mmol), diluted with methylene chloride and washed with brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The residue was purified by silica gel chromatography using 20% isopropanol in ethyl acetate as the eluent to yield pure diethyl (3*E*, 7*E*-[ethoxy-(4,8,12-trimethyl-3,7,11-tridecatrienyl)phosphinoyl]methylphosphonate **11**.Yield: 23%; oil; ^{1}H-NMR ($CDCl_3$, δ): 1.33 (t, 9H), 1.6, 1.63, 1.68 (3s, 12H), 1.97–2.13 (m, 10H), 2.21–2.4 (m, 2H), 2.38 (dd, 2H), 4.03–4.22 (m, 6H), 5.0–5.18 (m, 3H); ms, *m/z* 463 (MH^+).

To a stirred suspension of sodium hydride (0.0242 g, 0.6 mmol; 60% dispersion) in dry THF (1.5 ml) was added **11** (0.1 g, 0.216 mmol) dropwise under an argon atmosphere. This was followed by the addition of benzyl bromide (0.0924 g, 0.54 mmol) and the mixture was heated at 60°C for 1.5 h. The mixture was cooled to room temperature, diluted with ether and washed with brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The residue was purified by silica gel chromatography using ethyl acetate and then 10% isopropanol in ethyl acetate as the eluent to furnish

pure **14**. Yield: 62%; oil; ^{1}H-NMR ($CDCl_3$, δ): 1.13, 1.18, 1.23 (3t, 9H), 1.22–1.5 (m, 2H), 1.57, 1.6, 1.66 (3s, 12H), 1.85–2.1 (m, l0H), 3.19–3.4 (m, 4H), 3.93–4.13 (m, 6H), 4.88 (t, 1H), 5.03–5.12 (m, 2H), 7.18–7.26 (m, 6H), 7.42–7.5 (m, 4H); ^{31}P-NMR ($CDCl_3$, δ): 25.25 (d), 56.16 (d); ms, *m/z* 643 (MH^+).

Diethyl 1-[[dimethyl(phenyl)silylmethyl]ethoxyphosphinoyl]-1-methylethylphosphonate ***15***
A stirred solution of **8** (1.43 g, 5 mmol) in dry THF (15 ml) was cooled to –78°C and to it was added *n*-BuLi (4 ml of 1.6 M solution in hexane, 6.4 mmol) under an argon atmosphere. After stirring at –78°C for 35 min, chlorodimethylphenylsilane (1.2 g, 7 mmol) was added dropwise. The mixture was stirred at –78°C for an additional 1.5 h and then quenched with 3 N HCl. The mixture was extracted with methylene chloride, the combined organic layer was dried over anhydrous magnesium sulfate an concentrated *in vacuo*. The crude product was purified by silica gel chromatography using 20% isopropanol in ethyl acetate as the eluent to give pure **15**. Yield: 55%; oil; ^{1}H-NMR ($CDCl_3$, δ): 0.46 and 0.51 (2s, 6H), 1.12 (t, 3H), 1.3 (t, 6H), 1.37 (d, 3H), 1.42 (d, 3H), 1.79 (dt, 2H), 3.87–4.02 (m, 2H), 4.05–4.23 (m, 4H), 7.33 (m, 2H), 7.57 (m, 2H); ms, *m/z* 421 (MH^+).

Diethyl 1-[ethoxy(hydroxymethyl)phosphinoyl]-1-methylethylphosphonate ***16***
To a stirred solution of **15** (1.15 g, 2.75 mmol) in peracetic acid (16.95 g of 32% weight solution in acetic acid, 71.37 mmol) was added mercuric acetate (1.33 g, 4.17 mmol). The mixture was stirred at room temperature for 2 h. Solid sodium bicarbonate was then added to neutralize the acid and the mixture was cooled in an ice bath. Saturated $Na_2S_2O_3$ solution was added dropwise to the resulting mixture and this was followed by the addition of brine. The mixture was extracted with methylene chloride. The combined organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The crude product was purified by silica gel chromatography using 35% isopropanol in ethyl acetate and then 10% methanol in methylene chloride as the eluent to furnish pure **16**. Yield: 81%; oil; ^{1}H-NMR ($CDCl_3$, δ): 1.3–1.55 (m, 15H), 3.91–4.3 (m, 8H), 5.17 (bm, lH); ^{31}P-NMR ($CDCl_3$, δ): 31.04 (d), 50.56 (d); ms, *m/z* 303 (MH^+).

Diethyl (2E, 6E)-1-[ethoxy-(3,7,11-trimethyl-2,6,10-dodecadienyloxymethyl)phosphinoyl]-1-methylethylphosphonate ***17***
To a stirred suspension of sodium hydride (0.0242 g, 0.6 mmol; 60% dispersion) in dry THF (2 ml), cooled to –80°C, was added a solution of **16** (0.15 g, 0.5 mmol) in dry THF (0.5 ml) dropwise under an argon atmosphere. This was followed by the addition of farnesyl bromide (0.2 g, 0.7 mmol) and tetra-*n*-butylammonium bromide (0.016 g, 0.05 mmol). The mixture was warmed to room temperature and stirred at this temperature for 2 h. The resulting mixture was treated with brine and extracted with methylene chloride. The combined organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The crude product was purified by silica gel chromatography using 10% isopropanol in ethyl acetate and then 20% isopropanol in ethyl acetate as the eluent to yield pure **17**. Yield: 84%; oil; ^{1}H-NMR ($CDCl_3$, δ): 1.32 and 1.33 (2t, 9H), 1.45 and 1.47 (2dd, 6H), 1.6 (s, 6H), 1.68 (s, 6H), 1.93–2.17 (m, 8H), 3.98–4.3 (m, l0H), 5.1 (m, 2H), 5.33 (t, lH); ^{31}P-NMR ($CDCl_3$, δ): 28.31 (d), 47.47 (d); ms, *m/z* 507 (MH^+).

General method of the preparation of tripotassium salts from triesters
To a stirred solution of an appropriate triester (**12**–**14** and **17**, 0.444 mmol) in dry methylene chloride (2 ml) was added bromotrimethylsilane (2.22 mmol) under an argon atmosphere. This was followed by the addition of 2,4,6-collidine (0.888 mmol) and the mixture was stirred at room temperature for 18 h. The mixture was concentrated *in vacuo* and the residue was treated with an aqueous solution of potassium hydroxide (3.39 mmol). The resulting solution was lyophilized. The crude product was purified by reverse-phase HPLC on CHP 20P gel [9] using water and then an acetonitrile/water mixture as the eluent to furnish pure products **2**–**4** and **6.**

Tripotassium (3E, 7E)-1-methyl-1-[(4,8,12-trimethyl-3,7,11-tridecatrienyl)phosphinato]ethylphosphonate ***2***
Yield: 82%; white hygroscopic solid; ^{1}H-NMR (CD_3OD + D_2O, δ): 1.15 and 1.2 (2d, 6H), 1.46, 1.52, 1.55 (3s, 12H), 1.68–1.76 (m, 2H), 1.8–1.86 (m, 4H), 1.9–1.97 (m, 4H), 2.15–2.21 (m, 2H), 4.93, 4.95 (2t, 2H), 5.08 (t, lH); ^{31}P-NMR (CD_3OD + D_2O, δ): 26.4 (d), 47.02 (d); ms (FAB), *m/z* 559 (M+K), 521 (M+H), 483 (M+2H-K).

Tripotassium (3E, 7E)-1-ethyl-1-[(4,8,12-trimethyl-3,7,11-tridecatrienyl)phosphinato]propylphosphonate ***3***
Yield; 72%; white hygroscopic solid; ^{1}H-NMR (CD_3OD + D_2O, δ): 1.1 (t, 6H), 1.59, 1.6, 1.65, 1.67 (4s, 12H), 1.71–1.9 (m, 6H), 1.93–2.0 (m, 4H), 2.03–2.1 (m, 4H), 2.25 (q, 2H), 5.09 (t, 2H), 5.21 (t, 1H); ^{31}P-NMR (CD_3OD + D_2O, δ): 24.79 (d), 47.14 (d); ms (FAB), *m/z* 549 (M+H), 511 (M+2H-K).

Tripotassium (3E, 7E)-1-benzyl-2-phenyl-1-[(4,8,12-trimethyl-3,7,11-tridecatrienyl)phosphinato]ethylphosphonate ***4***
Yield: 87%; white hygroscopic solid; ^{1}H-NMR (CD_3OD + D_2O, δ): 1.53, 1.55, 1.61 (3s, 12H), 1.72–1.81 (m, 2H), 1.88–2.1 (m, 8H), 2.23 (q, 2H), 2.97–3.27 (m, 4H), 5.02, 5.04, 5.14 (3t, 3H), 7.13–7.25 (m, 6H), 7.55 (d, 4H); ^{31}P-NMR (CD_3OD + DO, δ): 21.81 (d), 45.28 (d); ms (FAB), *m/z* 673 (M+H), 635 (M+2H-K).

Tripotassium (2E, 6E)-1-methyl-1-(3,7,11-trimethyl-2,6,10-dodecatrienyloxymethylphosphinato)ethylphosphonate ***6***
Yield: 94%; white hygroscopic solid; ^{1}H-NMR (CD_3OD + D_2O, δ): 1.31 (dd, 6H), 1.6 (s, 6H), 1.66 (s, 3H), 1.7 (s, 3H), 1.92–2.17 (m, 8H), 3.83 (d, 2H), 4.11 (d, 2H), 5.08 (m, 2H), 5.37 (t, lH); ^{31}P–NMR (CD_3OD + D_2O, δ): 25.40 (d), 40.17 (d); ms (FAB), *m/z* 575 (M+K), 537 (M+H), 499 (M+2H-K).

Biological methods

Squalene synthetase assay
Rat liver microsomes (≈ 1 mg/ml protein) were prepared [11] in buffer P (20 mM phosphate buffer containing 0.1 mM EDTA, pH 7.4) and frozen at –80°C until used in the assay.

The solution of test compound (5 µl) in buffer (20 mM, pH 7.4, no EDTA) and 15 µl of microsomes (1 mg/ml protein stock microsomal suspension) were added to 190 µl of buffer and incubated in a shaking water bath at 37°C under nitrogen for 5 min. Twenty µl of an NADPH/$MgCl_2$ solution was added to the sample to yield a final assay concentration of 1.2 mM NADPH and 5 mM $MgCl_2$. The enzymatic reaction was immediately initiated by adding 20 µl tritium-labeled farnesyl pyrophosphate (500 000 dpm, 125 µM) to yield a final concentration of 10 µM farnesyl pyrophosphate in the sample. The samples were then incubated for 10 min at 37°C in a nitrogen atmosphere. The reaction was quenched by adding 20 µl of 190 mM EDTA to each sample. The samples were then removed from the incubator and placed on ice. Ten µl of 5% squalene in *N,N*- dimethylacetamide was then added to each sample.

2.5 ml of 25% toluene in hexane were then added to each sample followed by sonication (Bronson sonicator, micro tip) for 6 s at a setting of 6. The sonicated samples were each poured onto a column (6 cm x 0.7 cm diameter) containing 600 mg silicic acid in 3 ml of 25% toluene in hexane. Another 2.5 ml of 25% toluene in hexane were added to each sample tube. The samples were sonicated again and poured onto the column. A final 2.5-ml rinse of 25% toluene in hexane was poured directly onto the column after the first 2 applications had drained.

2.5 ml of Merit Liquid Scintillation Counter Fluid was then added to each sample and the samples were counted in a scintillation counter. IC_{50} values were determined by linear regression analysis of the combined data. All reactions were run in duplicate. All the inhibitors were tested at several different concentrations in at least 4 independent assays.

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