Expedited Articles

Geranylgeranyl Diphosphate-Based Inhibitors of Post-Translational Geranylgeranylation of Cellular Proteins

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A novel series of stable analogs of geranylgeranyl diphosphate (GGdP) are described in which the biologically labile diphosphate moiety of GGdP is replaced by portions that can act as stable isosters. The compounds inhibited the geranylgeranyltransferase activity in whole PC-3 prostate cancer cells, as determined by the inhibition of post-translational isoprenylation of the small GTP-binding protein p21*rap* 1 and the accumulation of unprocessed p21*rap* 1 in the cytosolic fraction. However, the compounds did not affect the farnesylation of p21*ras*, as shown by protein immunoprecipitation after whole cell labeling with [^{3}H]-(*R*,*S*)-mevalonolactone. Despite the absence of effects on post-translational processing of p21*ras*, these compounds proved to be cytotoxic for prostate cancer cells, with half-maximal inhibition of cell growth obtained in the range 18.5−35.1 μM. The GGdP analogs described in the this study are novel, non-peptidic inhibitors of geranylgeranylation that may be active as antitumor agents.

Introduction

The biological function of certain cellular proteins, including members of the *ras* superfamily of small GTP-binding proteins, such as p21*ras*, p21*rap* 1, p21*rho*, p21*rac*, and Cdc42, depends on their post-translational isoprenylation.[1] This process includes the attachment of isoprenoids, such as farnesyl and geranylgeranyl moieties, to a cysteine residue contained in a carboxy-terminal tetrapeptide signal sequence, frequently referred to as a CAAX motif, where C is cysteine, A is an aliphatic amino acid and X is any amino acid. Farnesyl diphosphate (FdP) and geranylgeranyl diphosphate (GGdP) are intermediates of the cholesterol biosynthetic pathway and serve as isoprenoid group donors in this post-translational modification.[2] The enzymes involved in the attachment of the isoprenoid groups to the target proteins are the farnesyl protein transferase (FPTase) and geranylgeranyl protein transferases (GGPTases).[2] The product of the *ras* gene is known to be farnesylated, while the closely related p21*rap* 1, -*rho*, -*rac*, and Cdc42 proteins are geranylgeranylated.[1]

Proteins of the *ras* superfamily play a crucial role in the regulation of cell proliferation.[3] In particular, proteins encoded by mutated *ras* genes are able to trasform cells to a malignant phenotype.[4] For this reason, recent research has focused on the synthesis of a large number of inhibitors of p21*ras* farnesylation, to suppress the oncogenic potential of the transformed protein and to provide a specific approach to anti-cancer therapy. Compounds with proven efficacy in inhibiting p21*ras* farnesylation include pseudopeptide and farnesyl diphosphate-based inhibitors of FPTase.[5]

However, recent research has also pointed out the essential role for geranylgeranylated proteins, including p21*rho*, p21*rac*, and Cdc42, in cell cycle progression and mitosis.[6] On the basis of these premises, it is conceivable that inhibitors of GGPTases may well function as effective antiproliferative agents in cancer chemotherapy. However, specific inhibitors of GGPTases in cells have not been reported in literature so far.

As part of a project aimed to the design of inhibitors of geranylgeranylation of cellular proteins, we report here the synthesis and preliminary pharmacological evaluation of compounds **1**−**3**. These compounds have been designed as stable analogs of GGdP, in which the biologically labile diphosphate moiety of GGdP is replaced by portions that can act as stable isosters, i.e. the [[(aminosulfonyl)amino]carbonyl]oxy (**1**), (phosphonoacetamido)oxy (**2**) and [(*O*-ethylphosphono)acetamido]-oxy groups (**3**). The [[(aminosulfonyl)amino]carbonyl]-oxy group of **1** has already been reported by us to be an isosteric replacement for the diphosphate moiety in an analog of 5-iodo-2′-deoxyuridine 5′-diphosphate, which showed an appreciable antiviral activity.[7] The (phosphonoacetamido)oxy group of **2** is structurally related to phosphonoacetic acid (PPA), a known antiherpes virus agent which can mimic the action of pyrophosphate.[8] The [(*O*-ethylphosphono)acetamido]oxy group of **3** should make it possible to verify whether the esterification of an OH residue of the phosphonic terminal portion of the (phosphonoacetamido)oxy group of **2** affects the ability to mimic the diphosphate moiety of GGdP.

Chemistry

Compounds **1**−**3** were synthesized as outlined in Scheme 1. Reaction of geranylgeraniol with chlorosulfonyl isocyanate followed by treatment with ammonia gave compound **1**. Treatment of geranylgeraniol with *N*-hydroxyphthalimide in the presence of triphenylphos-

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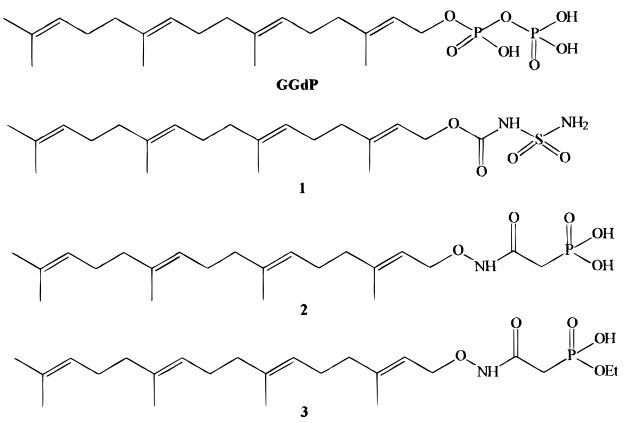
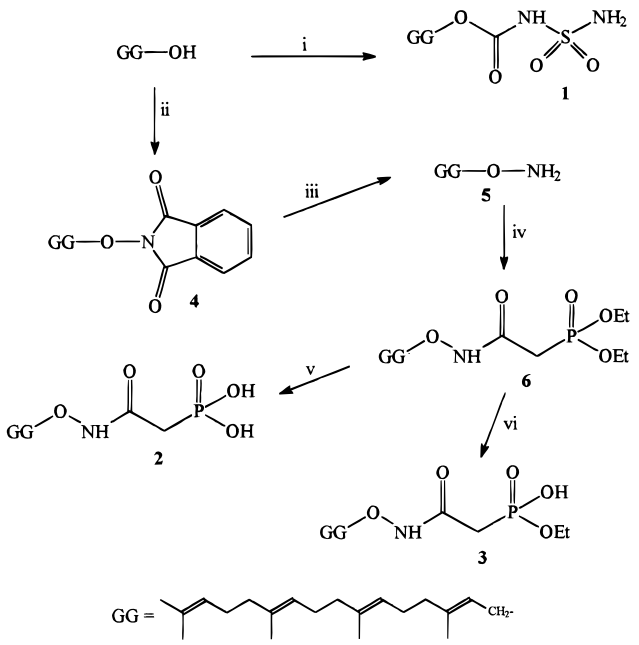


phine and diethyl azodicarboxylate afforded the phthalimido derivative **4**. Hydrazinolysis of **4** gave intermediate **5**, which, after condensation with diethylphosphonoacetic acid[7] in the presence of 1-hydroxybenzotriazole and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, yielded compound **6**. Reaction of **6** with bromotrimethylsilane in methylene chloride in the presence of 2,4,6-collidine followed by treatment with potassium hydroxide yielded compound **2** as a dipotassium salt. Treatment of **6** with 1 N aqueous sodium hydroxide gave product **3** as a sodium salt.

Pharmacological Results and Discussion

In order to test whether compounds **1**−**3** were able to specifically inhibit the geranylgeranylation rather than the farnesylation of cellular proteins in whole cells, we analyzed their effects on the post-translational lipid modification of two target proteins, p21*rap* 1 and p21*ras*, in the human prostate cancer cell line PC-3. These proteins undergo a differential processing, since they are modified by geranylgeranyl (p21*rap* 1) or farnesyl (p21*ras*) moieties. The post-translational isoprenylation of p21*rap* 1 was evaluated by immunoblotting analysis of detergent-solubilized cells to document the accumulation of the immature non-geranylgeranylated precursor in PC-3 cells. Unprocessed members of the *ras* family migrate slower compared to their fully processed counterparts when subjected to SDS−PAGE,[9] and this property has been exploited to demonstrate the inhibition of GGPTase activity in whole cells by compounds **1**−**3**. The immunoblot analysis demonstrated the presence of the mature, geranylgeranylated *rap* 1 protein as a single band of 21 kDa molecular mass in untreated PC-3 cells. As a result of the exposure of cells to the compounds **1**−**3**, it was possible to detect an additional protein band of slower electrophoretic motility corresponding to the immature, non-isoprenylated p21*rap* 1 (Figure 1), indicating that geranylgeranylation was indeed inhibited. In addition to the demonstration that the inhibition of GGPTase activity led to the accumulation of unprocessed *rap* 1 protein, the immunoprecipitation analysis of cells labeled with [^{3}H]-(*R*,*S*)-mevalonolactone was performed. The results showed that treatment with compounds **1**−**3** resulted in the inhibition of the incorporation of the geranylgeranyl moiety in the p21*rap* 1 protein, as demonstrated by the reduction of the protein band density in the fluorogram (Figure 1). The analysis of the fluorogram signals indicated that the effect was dose-responsive, with the IC_{50} of compounds **1**, **2**, and **3** corresponding to 28.2 ± 4.7, 20.5 ± 3.1, and 40.2 ± 6.2 μM, respectively.

Scheme 1[a]



[a] Reagents and conditions: (i) chlorosulfonyl isocyanate, NH_3, CH_3CN, −20 °C, 7 h; (ii) N-hydroxyphthalimide, triphenylphosphine, diethyl azodicarboxylate, THF, room temperature, 42 h; (iii) NH_2NH_2, EtOH, room temperature, 18 h; (iv) (diethylphosphono)acetic acid, 1-hydroxybenzotriazole, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, THF, room temperature, 8 h; (v) bromotrimethylsilane, 2,4,6-collidine, CH_2Cl_2, 18 h, KOH; (vi) 1 N NaOH, dioxane, 40 °C, 16 h.

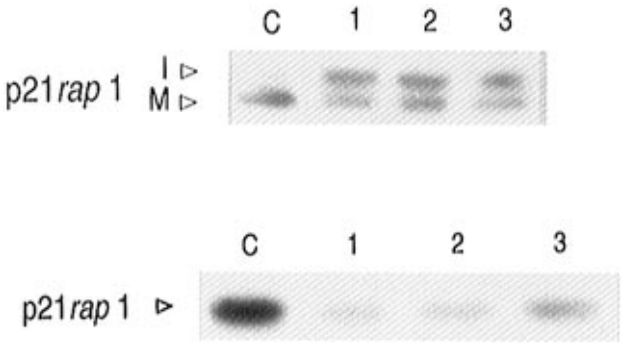


Figure 1. Inhibition of p21*rap* 1 geranylgeranylation in PC-3 prostate cancer cells treated with compounds **1**−**3** at 75 μM for 24 h. (Upper) Immunoblotting analysis of *rap* 1 protein. Lane C: untreated control, lanes 1−3, compounds **1**, **2**, and **3**, respectively. The inhibition of geranylgeranylation is demonstrated by the appearance of a protein band (I) representing the immature, non-geranylgeranylated p21*rap* 1, while the control sample contains only the mature, isoprenylated protein (M). (Lower) Immunoprecipitation analysis of p21*rap* 1. Lane C: untreated control, lanes 1−3, compounds **1**, **2**, and **3**, respectively. The GGdP analogs markedly inhibited the incorporation of the geranylgeranyl moiety in p21*rap* 1, as demonstrated by the presence of faint protein bands in treated samples. The analysis of fluorogram signals of cells treated with compounds **1**−**3** (75 μM) gave the following results, expressed as percent relative to control samples: 10.8 ± 2.3, 8.7 ± 2.4, and 19.7 ± 3.9, respectively.

The effect of compounds **1**−**3** on the farnesylation of p21*ras* was tested by immunoprecipitation analysis of cells labeled with [^{3}H]-(*R*,*S*)-mevalonolactone, and the results are reported in Figure 2. p21*ras* appeared as a single radioactive band of 21 kDa molecular mass in the fluorogram of control cells. After treatment with compounds **1**−**3**, p21*ras* labeling was not inhibited (Figure

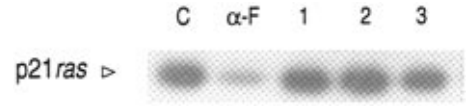


Figure 2. Lack of inhibition of p21*ras* farnesylation in PC-3 prostate cancer cells treated with compounds **1**−**3** at 75 μM for 24 h. The post-translational processing of *ras* protein was analyzed by immunoprecipitation. Lane C: untreated control, lane α-F, α-(hydroxyfarnesyl)-phosphonic acid, lanes 1−3, compounds **1**, **2**, and **3**, respectively. The lack of inhibition of p21*ras* farnesylation by the GGdP analogs is demonstrated by the presence of a radioactive band similar to the control one, while α-(hydroxyfarnesyl)phosphonic acid markedly reduced the incorporation of the radioactive farnesyl moiety in p21*ras*, and a very faint radioactive band was detected.

2). On the contrary, the specific farnesylation inhibitor α-(hydroxyfarnesyl)phosphonic acid 20 μM was able to almost completely suppress p21*ras* isoprenylation (Figure 2). These findings indicate that compounds **1**−**3** do not affect the farnesylation reaction in whole cells.

The cytotoxicity of the GGdP analogs was also tested on PC-3 prostate cancer cells. The analysis of cell proliferation demonstrated that the compounds under study exihibited a dose-dependent effect, with a calculated IC_{50}s for compounds **1**−**3** of 22.2 ± 4.5, 18.5 ± 2.6, and 35.1 ± 4.6 μM, respectively. The effects of the GGdP analogs on protein geranylgeranylation were not associated with significant cell differentiating activity on PC-3 cells. On the contrary, cells appeared vacuolated and easily detached from the surface of culture dishes at the end of treatment with compounds **1**−**3** at 25−75 μM. It should be pointed out that based on the IC_{50}s of the GGdP analogs, the relative potency of compounds **1**−**3** at inhibiting *rap* 1 protein isoprenylation corresponded to that on cell growth, and compound **3** was found to be the most active analog synthesized. This finding supports the correlation between geranylgeranylation and cell proliferation obtained with the GGdP analogs under study.

Finally, the results of the present study indicate that compounds **1**−**3** are inhibitors of geranylgeranylation and they do not affect farnesylation. Furthermore, the present study provides the first evidence that the inhibition of geranylgeranylation indeed results in cytotoxicity, hence supporting the hypothesis that geranylgeranylated proteins play an important role in promoting cell proliferation. Interestingly, the GGdP analogs are able to inhibit geranylgeranylation in whole cells, thus indicating that compounds **1**−**3** are indeed able to cross the cell membrane and exert their pharmacological activity in an intact cell system, a prerequisite for *in vivo* activity.

On the basis of the present results, it is also possible to conclude that the ability of compounds **1**−**3** to inhibit geranylgeranylation indicates that the [[(aminosulfonyl)amino]carbonyl]oxy, (phosphonoacetamido)oxy and [(*O*-ethylphosphono)acetamido]oxy groups of compounds **1**, **2**, and **3**, respectively, are able to behave as stable isosters of the diphosphate moiety of GGdP.

Experimental Section

Chemistry. ^{1}H NMR spectra of all compounds were obtained with a Varian CFT-20 instrument operating at 80 MHz in a ca. 2% solution of $CDCl_3$, CD_3OD, or D_2O, using Me_4Si or $Me_3Si(CH_2)_3SO_3Na$ as the internal standard. The proton magnetic resonance assignments were established on the basis of the expected chemical shifts and the multiplicity of the signals. IR spectra for comparison of compounds were taken as paraffin oil mulls or as liquid films on a Mattson 1000 Series FTIR spectrometer. Analytical TLCs were carried out on 0.25 mm layer silica gel plates containing a fluorescent indicator (Macherey-Nagel Alugram SilG/UV254 Art. No. 81813) and on 0.15 mm layer silica gel C_{18} plates containing a fluorescent indicator (Macherey-Nagel Alugram RP-18W/UV254 Art. No. 818144); spots were detected under UV light (254 nm) and by phosphomolybdic acid. All compounds were homogeneous by TLC. Column chromatographies were performed using both 70−230 mesh silica gel (Macherey-Nagel silica gel 60 Art. No. 81538) and reverse phase silica gel (Macherey-Nagel Polygosil 60-4063 C_{18}). Analytical RP-HPLCs of compounds **1**−**3** were performed using a Beckman System Gold apparatus with diode array detector in the following conditions: Vydac C_{18} column (0.46 × 15 cm); eluant A, 0.1% TFA/water; eluant B, 0.1% TFA/acetonitrile; gradient from 40% to 85% B over 15 min (product **1**) or from 45% to 85% B over 20 min (products **2** and **3**); flow 1 mL/min. Compounds **1**−**3** were homogeneous by RP-HPLC. Mass spectra were recorded on a VG 70-250S mass spectrometer or a HP-5988 A spectrometer. Elemental analysis of compound **4** was carried out by our analytical laboratory and was consistent with theoretical values to within ±0.4%. The melting point of compound **4** was determined on a Kofler hot-stage apparatus and is uncorrected. Evaporations were made *in vacuo* (rotating evaporator).

Synthesis of (*E*,*E*,*E*)-*O*-(3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl)-*N*-(aminosulfonyl)urethane (1). A solution of geranylgeraniol (0.5 g, 1.72 mmol) in anhydrous CH_3CN (15 mL) at −20 °C under nitrogen was treated with chlorosulfonyl isocyanate (0.15 mL, 1.72 mmol), and the mixture was stirred at −20 °C for 4 h. The mixture was then treated dropwise at −20 °C with a saturated solution of NH_3 in CH_3CN (7 mL) and left at −20 °C for 3 h under stirring. The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography on reverse phase silica gel, eluting with CH_3CN-H_2O (6:4) and collecting 6 mL fractions. The appropriate fractions were combined, evaporated, lyophilized, and pump-dried to give **1** (0.4 g, 56%) as a very hygroscopic white lyophilate: TLC silica gel C_{18} CH_3CN-H_2O (6:4) $R_f = 0.13$; HPLC t_R 14.32; ^{1}H-NMR ($CDCl_3$, 80 MHz) δ 1.60 (s, 9H, 3 × CH_3), 1.68 (s, 3H, CH_3), 1.71 (s, 3H, CH_3), 2.03 (m, 12H, 6 × CH_2), 4.30 (br, 3H, D_2O exchangeable, NH + NH_2), 4.68 (d, 2H, J = 7.2 Hz, CH_2), 5.10 (br, 3H, 3 × CH), 5.38 (t, 1H, J = 7.2 Hz, CH); MS (FAB^-) m/e 411 $(M - H)^-$.

Synthesis of Compound 4. A solution of *N*-hydroxyphthalimide (1.12 g, 6.88 mmol), triphenylphosphine (1.81 g, 6.88 mmol), and diethyl azodicarboxylate (1.19 mL, 7.57 mmol) in anhydrous THF (60 mL) was treated with geranylgeraniol (2.0 g, 6.88 mmol), and the resulting mixture was stirred for 18 h at room temperature. After addition of triphenylphosphine (0.905 g, 3.44 mmol) and diethyl azodicarboxylate (0.595 mL, 3.78 mmol), the mixture was stirred at room temperature for another 24 h. The solvent was evaporated, and the residue was purified on 70−230 mesh silica gel eluting with CH_2Cl_2−hexane (6:4) and collecting 10 mL fractions. The appropriate fractions were combined and evaporated to give the intermediate **4** (2.63 g, 88%) as a white solid: mp 40−41 °C; ^{1}H-NMR ($CDCl_3$, 80 MHz) δ 1.58 (s, 9H, 3 × CH_3), 1.67 (s, 3H, CH_3), 1.71 (s, 3H, CH_3), 2.03 (m, 12H, 6 × CH_2), 4.71 (d, 2H, J = 7.2 Hz, CH_2), 5.07 (br, 3H, 3 × CH), 5.52 (t, 1H, J = 7.2 Hz, CH), 7.73 (m, 4H, Ar). Anal. Calcd for $C_{28}H_{37}NO_3$ (MW 435.3): C, 77.26; H, 8.50; N, 3.22. Found: C, 77.38; H, 8.38; N, 3.04.

Synthesis of Compound 5. Hydrazine monohydrate (0.44 mL, 9.06 mmol) was added to a solution of **4** (2.63 g, 6.04 mmol) in EtOH (130 mL), and the resulting mixture was stirred at room temperature for 18 h. After filtration of a white solid, the solution was evaporated and the resulting oily crude residue was purified by column chromatography on reverse phase silica gel eluting with CH_3CN-H_2O (7:1) and collecting 10 mL fractions. The appropriate fractions were combined, evaporated, and pump-dried to give the intermediate **5** (1.07 g, 57%) as an oil: ^{1}H-NMR ($CDCl_3$, 80 MHz) δ 1.60 (s, 9H, 3 × CH_3), 1.69 (s, 6H, 2 × CH_3), 2.04 (m, 12H, 6 × CH_2), 4.17

(d, 2H, J = 7.2 Hz, CH_2), 5.08 (br, 3H, 3 × CH), 5.29 (t, 1H, J = 7.2 Hz, CH); MS m/e 306 $(M + H)^+$.

Synthesis of Compound 6. A solution of compound **5** (0.200 g, 0.65 mmol), (diethylphosphono)acetic acid (0.139 g, 0.71 mmol), and 1-hydroxybenzotriazole (0.131 g, 0.98 mmol) in anhydrous THF (10 mL) was treated with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.150 g, 0.78 mmol). The mixture was stirred at room temperature for 8 h, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography on 70−230 mesh silica gel eluting with AcOEt−hexane (1:1) and collecting 3 mL fractions. The appropriate fractions were combined, evaporated, and pump-dried to give intermediate **6** (0.208 g, 66%) as an oil: ^{1}H-NMR ($CDCl_3$, 80 MHz) δ 1.33 (t, 6H, J = 7.2 Hz, 2 × CH_3CH_2), 1.60 (s, 9H, 3 × CH_3), 1.67 (s, 3H, CH_3), 1.71 (s, 3H, CH_3), 2.04 (m, 12H, 6 × CH_2), 2.81 (d, 2H, J = 20 Hz, CH_2P), 4.13 (q, 4H, J = 7.2 Hz, 2 × CH_3CH_2), 4.41 (d, 2H, J = 7.2 Hz, CH_2), 5.07 (br, 3H, 3 × CH), 5.38 (t, 1H, J = 7.2 Hz, CH); MS (FAB$^+$) m/e 484 $(M + H)^+$.

Synthesis of the Dipotassium Salt of (*E,E,E*)-{2-Oxo-2-[[(3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl)oxy]amino]ethyl}phosphonic Acid (2). Bromotrimethylsilane (0.320 mL, 3 mmol) was added to a stirred solution of compound **6** (0.290 g, 0.6 mmol) and 2,4,6-collidine (0.158 mL, 1.2 mmol) in anhydrous CH_2Cl_2 (6 mL); the resulting mixture was stirred at room temperature for 18 h. After evaporation of the solution, the residue was treated with an aqueous solution of 0.9 N KOH (1.7 mL) and then stirred at room temperature for 3 h. The solution was evaporated, and the resulting crude residue was purified by column chromatography on reverse phase silica gel eluting with MeOH−H_2O (2.5:3) and collecting 2 mL fractions. The appropriate fractions were combined, evaporated, lyophilized, and pump-dried to give **2** (0.125 g, 41%) as a very hygroscopic white lyophilate: TLC silica gel C_{18} MeOH−H_2O (3:1) R_f = 0.16; HPLC t_R 13.21; ^{1}H-NMR (D_2O, 80 MHz) δ 1.56 (s, 9H, 3 × CH_3), 1.62 (s, 3H, CH_3), 1.72 (s, 3H, CH_3), 2.02 (m, 12H, 6 × CH_2), 2.48 (d, 2H, J = 20 Hz, CH_2P), 4.40 (d, 2H, J = 7.2 Hz, CH_2), 5.07 (br, 3H, 3 × CH), 5.40 (t, 1H, J = 7.2 Hz, CH); MS (FAB$^+$) m/e 504 $(M + H)^+$.

Synthesis of the Monosodium Salt of the Monoethyl Ester of (*E,E,E*)-{2-Oxo-2-[[(3,7,11,15-tetramethyl-2,6,10,-14-hexadecatetraenyl)oxy]amino]ethyl}phosphonic Acid (3). An aqueous solution of 1 N NaOH (1.6 mL) was added to a stirred solution of **6** (0.193 g, 0.4 mmol) in dioxane (4 mL); the resulting mixture was stirred at 40 °C for 4 h, treated with another 2 mL of 1 N NaOH, and then stirred at 40 °C for 12 h. The solvent was removed under reduced pressure, and the crude residue was purified by column chromatography on reverse phase silica gel eluting with MeOH−H_2O (3:2) and collecting 2 mL fractions. The appropriate fractions were combined, evaporated, lyophilized, and pump-dried to give **3** (0.137 g, 72%) as a very hygroscopic white lyophilate: TLC silica gel C_{18} MeOH−H_2O (3:1) R_f = 0.22; HPLC t_R 16.42; ^{1}H-NMR (CD_3OD, 80 MHz) δ 1.24 (t, 3H, J = 7.2 Hz, CH_3CH_2), 1.59 (s, 9H, 3 × CH_3), 1.66 (s, 3H, CH_3), 1.69 (s, 3H, CH_3), 2.03 (m, 12H, 6 × CH_2), 2.19 (d, 2H, J = 20 Hz, CH_2P), 3.93 (q, 2H, J = 7.2 Hz, CH_3CH_2), 4.34 (d, 2H, J = 7.2 Hz, CH_2), 5.08 (br, 3H, 3 × CH), 5.42 (t, 1H, J = 7.2 Hz, CH); MS (FAB$^+$) m/e 478 $(M + H)^+$, 500 $(M+Na)^+$.

Immunoblotting Analysis of p21*rap* 1 Geranylgeranylation. The human androgen-independent prostate cancer cell line PC-3 (American Type Culture Collection, Rockville, MD) was maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine (Gibco-Brl, Gaithersburg, MD) and 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). Cells were routinely subcultivated when 75% confluent at a ratio of 1:15 by treatment with 1.5 mM EDTA and plated in 75 cm^2 culture flasks; cultures were incubated in 5% CO_2 /95% air at 37 °C. PC-3 cells were plated in 60 mm culture dishes in serum-supplemented medium and treated for 24 h with compounds **1**−**3** at 25, 50, and 75 μM. Cells were harvested with 1.5 mM EDTA and centrifuged, and the cell pellet was solubilized for 30 min at 4 °C in lysis buffer containing 10 μL/mL NP-40, 50 mM Tris, pH 7.6, 2 mM EDTA, 100 mM NaCl, 20 μg/mL phenylmethanesulfonyl fluoride, and 5 μg/mL each of pepstatin, antipain, and aprotinin (Sigma, St. Louis, MO). Lysates were centrifuged for 30 min at 14 000 rpm, and the protein concentration in the detergent-solubilized cells was measured with the bicinchoninic acid (Pierce, Rockford, IL) using bovine serum albumin as the standard. Aliquots of 100 μg of proteins were boiled in sodium dodecyl sulfate (SDS)−sample buffer (50 mM Tris, pH 6.8, 20 mg/mL SDS, 100 mM dithiothreitol, 100 μL/mL glycerol, and 0.25 mg/mL bromophenol blue), separated by 12.5% SDS−polyacrylamide gel electrophoresis (PAGE), and blotted onto Immobilon-P (Millipore, Bedford, MA) by a Multiphor II NovaBlot cell (Pharmacia, Piscataway, NJ). Blots were probed with the antibody to p21*rap* 1 (1:1000) and detected by an alkaline phosphatase-linked secondary antibody (Western-Light, Tropix, Bedford, MA). The anti-*rap* 1/Krev-1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) specifically recognizes p21*rap* 1 but does not exhibit detectable cross reactivity with neither *rap* 2 nor *ras*-encoded proteins.

Immunoprecipitation Analysis of p21*rap* 1 Geranylgeranylation and p21*ras* Farnesylation. The small GTP-binding proteins *ras* and *rap* 1 have been previously used to document the effects of isoprenylation inhibitors in whole cells.[9,10] PC-3 cells were plated in 60 mm culture dishes in serum-supplemented medium and treated for 24 h with compounds **1**−**3** (25, 50, and 75 μM). Separate cell cultures were treated with the FPTase inhibitor 20 μM α-(hydroxyfarnesyl)phosphonic acid (Biomol, Plymouth Meetings, PA). In each case the HMG-Co A reductase inhibitor lovastatin (Biomol, Plymouth Meetings, PA) was added at 20 μM to suppress endogenous production of mevalonic acid, thus preventing the dilution of the labeled compound that penetrates the cell membrane. After 8 h of incubation at 37 °C each plate received 100 μCi/mL of [^{3}H]-(*R,S*)-mevalonolactone (50−60 Ci/mmol, American Radiolabeled Chem., St. Louis, MO) in 100 μL of cell culture medium, and incubation was continued for 16 h. Cells were harvested with 1.5 mM EDTA and centrifuged, and the cell pellet was solubilized for 30 min at 4 °C in lysis buffer. Detergent-solubilized cells were centrifuged for 30 min at 14 000 rpm, and the protein concentration was measured. Cell extracts (3 mg/mL proteins) were mixed with 20 μL/mL of the anti-*rap* 1/Krev-1 or the anti-v-Ha-*ras* antibodies at 4 °C for 6 h. The rat monoclonal antibody to v-Ha-*ras* (clone Y13-259, Oncogene Science, Uniondale, NY) specifically reacts with the 21 kDa translational products of the H-, K-, and N-*ras* human oncogenes. Immune complexes were precipitated overnight at 4 °C on a rotating platform by addition of 70 μL of a 15% suspension of GammaBind Plus protein G-Sepharose beads (Pharmacia, Uppsala, Sweden) in lysis buffer. The immunoprecipitates were collected by centrifugation at 14 000 rpm at 4 °C for 15 min and washed four times with 1 mL each of ice-cold buffer used for cell lysis. The pellets were dissolved in 20 μL of SDS−sample buffer and the antigens released by heating at 95 °C for 5 min before electrophoresis. Samples were size-fractionated on 12.5% SDS−PAGE. Gels were stained with Coomassie blue G-250 to document equal sample loading, equilibrated for 30 min with the Enlightning fluorography enhancer (NEN-Dupont, Boston, MA), dried under a vacuum at 80 °C for 2 h, and finally fluorographed at −70 °C.

Analysis of Cell Proliferation. PC-3 cells (1 × 10^3) were seeded in 96-well plates and treated with compounds **1**−**3** (5−75 μM) for 72 h. Cell growth was assessed by the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-[3-(carboxymethoxy)phenyl]-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS, Promega, Madison, WI) following the technical manual. The formazan metabolite released by living cells was measured at 490 nm using the Titertek Multiskan reader (ICN, Irvine, CA).

Analysis of Data. The effect of compounds **1**−**3** on isoprenylation was quantified by video imaging densitometry of protein fluorograms using the Kontron Imaging System KS300 (Kontron Elektronik, Eching, Germany). The half-maximal inhibitory concentration (IC_{50}) ± SE of the mean on protein prenylation and cell growth was calculated by nonlinear regression fit of the experimental data obtained in triplicate experiments.

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