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A Photoactivatable Prenylated Cysteine Designed to Study Isoprenoid Recognition

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Abstract: Protein prenylation, involving the alkylation of a specific C-terminal cysteine with a C_{15} or C_{20} isoprenoid unit, is an essential posttranslational modification required by most GTP-binding proteins for normal biological activity. Despite the ubiquitous nature of this modification and numerous efforts aimed at inhibiting prenylating enzymes for therapeutic purposes, the function of prenylation remains unclear. To explore the role the isoprenoid plays in mediating protein−protein recognition, we have synthesized a photoactivatable, isoprenoid-containing cysteine analogue (**2**) designed to act as a mimic of the C-terminus of prenylated proteins. Photolysis experiments with **2** and RhoGDI (GDI), a protein which interacts with prenylated Rho proteins, suggest that the GDI is in direct contact with the isoprenoid moiety. These results, obtained using purified GDI as well as *Escherichia coli* (*E. coli*) crude extract containing GDI, suggest that this analogue will be an effective and versatile tool for the investigation of putative isoprenoid binding sites in a variety of systems. Incorporation of this analogue into peptides or proteins should allow for even more specific interactions between the photoactivatable isoprenoid and any number of isoprenoid binding proteins.

Introduction

Prenylated GTP-binding proteins (G-proteins) play a key role in the progression of cancer with Ras oncoproteins being present in roughly 30% of human tumors.[1] For both normal and cancerous activity, Ras proteins require addition of a C_{15} (farnesyl) isoprenoid to a specific C-terminal cysteine via a thioether linkage,[2] yet most members of the Ras-like superfamily of G-proteins undergo geranylgeranylation (C_{20} addition) for biological function.[3] Other Ras superfamily members have also been implicated in tumor development and require prenylation for this activity as well.[4] Efforts to prevent prenylation have been pursued by developing inhibitors of the prenyltransferases which catalyze isoprenoid addition.[5] Another means of halting oncogenic signal transduction may involve disruption of the interaction between prenylated proteins and other proteins involved in signaling processes. Whereas prenyltransferase inhibition affects all suitable G-protein substrates, disruption of prenylated protein−protein interactions may be of greater therapeutic interest since it may be possible to design compounds to interfere with key contacts within a specific prenylated protein−protein system.

Elimination of C-terminal prenylation, addition of an incorrect isoprenoid, or substitution of the prenyl group with various alternatives have been shown to either eliminate or alter normal

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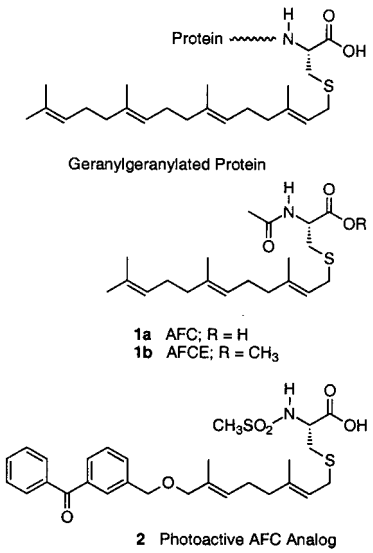
biological activity in a wide variety of cases: Ras proteins,[2,6] the γ-subunits of heterotrimeric G-proteins,[7] and the yeast a-factor mating dodecapeptide pheromone.[8] These results and other studies[9−11] support the idea that isoprenoid binding sites exist within proteins which interact with prenylated substrates. The presence of such a pocket would impart a specificity requirement for the types of proteins and molecules a G-protein effector, activator, or receptor protein recognizes, thereby paving the way for synthetic analogues of pharmaceutical relevance to be produced. With the goal of understanding how prenylated proteins are recognized by other proteins, we have developed a photoaffinity labeling reagent designed to probe the interaction between prenylated proteins and their cognate receptors.

To examine the effectiveness of using a photoaffinity labeling compound for isoprenoid binding site investigation, photolysis experiments were performed using Rho GDP-dissociation inhibitor (GDI). This is a cytosolic protein which performs a variety of regulatory activities on Rho proteins, including their solubilization from membranes.[12,13] Rho proteins are members of the Ras superfamily of proteins which are farnesylated or, more commonly, geranylgeranylated, and have been shown to be necessary for oncogenic Ras transformation.[4,14] The proposed mechanism of Rho protein membrane extraction involves first a binding step between GDI and Rho followed by a slower isomerization step where the geranylgeranyl moiety moves from the lipid membrane into an isoprenoid binding pocket of GDI.[13] NMR studies of an amino-terminally truncated GDI suggest that *N*-acetyl farnesylated cysteine (AFC, **1a**) binds to the isoprenoid binding site in GDI,[10a,b] and this type of interaction was recently confirmed with an X-ray crystal structure, showing the geranylgeranyl portion of Cdc42 (a geranylgeranylated Rho protein) buried in a hydrophobic pocket of GDI.[11] Given these results, we thought GDI was an appropriate choice for evaluating the binding capabilities of our synthetic, photoactivatable isoprenoid mimic.



Experimental Section

Materials and Methods. All synthetic reactions were conducted under a nitrogen atmosphere, stirred magnetically, and carried out at room temperature unless otherwise noted. Analytical TLC was performed on precoated (250 μm) silica gel 60 F-254 plates from E. Merck. Visualization of plates was done under UV irradiation or by staining with an ethanolic phosphomolybdic acid solution followed by heating. Flash chromatography silica gel (60−120 mesh) was obtained from E. M. Science or Scientific Adsorbents, Inc. CH_2Cl_2 was distilled from CaH_2. Deuterated NMR solvents were used as obtained from Cambridge Isotope Laboratories, Inc. NMR spectra were obtained at 300 MHz (^{1}H) or 75 MHz (^{13}C) on Varian instruments. Chemical shifts are reported in ppm, and *J* values are given in Hertz. Protein concentrations were determined by the Biorad method, which employs the Bradford procedure,[15] using BSA as a standard. Molecular masses of GDI and GST−GDI were determined by comparison with high-molecular weight standards (GIBCO BRL Life Technologies: myosin (200 kDa), phosphorylase b (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa)) using SDS-polyacrylamide gel electrophoresis (PAGE) analysis. Unless otherwise noted, HPLC analyses were carried out using a Beckman model 127/166 instrument equipped with a diode array UV detector and a Varian Dynamax C_{18} column (8.0 μm, 4.6 × 250 mm) equipped with a 5 cm guard column (flow rate: 1.0 mL/min, 500 μL injection loop, 5 to 100% B in 40 min. Solvent A: 95% H_2O, 5% CH_3CN, 0.2% TFA; solvent B: 100% CH_3CN, 0.2% TFA) with monitoring at 220 and 260 nm. Phosphorimaging analysis was performed with either a Molecular Dynamics STORM 840 instrument and ImageQuaNT version 4.1 software or with a Bio-Rad Molecular Imager FX instrument using Quantity One version 4.1.0 software.

(*E*,*E*)-8-*O*-(3-Benzoylbenzyl)-3,7-dimethyl-2,6-octadiene-1-bromide (4). This method follows the basic published procedure which starts from 3-methylbenzophenone and arrives at compound **4**.[16] However, the following changes were made to generate the bromide from the alcohol precursor ((*E*,*E*)-8-*O*-(3-benzoylbenzyl)-3,7-dimethyl-2,6-octadiene-1-ol, **3**): polymer-supported PPh_3 beads[17] (Aldrich; 2 equiv) were soaked in 3 mL of dry CH_2Cl_2 along with **3** (1 equiv) for 30 min to allow the beads to swell. CBr_4 (1.2 equiv) was then added and the reaction stirred for 5 h. TLC analysis (5:2 toluene/EtOAc, R_f = 0.88) showed the reaction was complete at this time, and the beads were removed by filtration. The filtrate was evaporated, and purification was not necessary; product was present in >90% purity as determined by ^{1}H NMR. ^{1}H NMR ($CDCl_3$) δ 1.65 (s, 3H), 1.71 (s, 3H), 2.07−2.21 (m, 4H), 3.91 (s, 2H), 3.95 (d, J = 8.4, 2H), 4.48 (s, 2H), 5.38 (t, J = 6.5, 1H), 5.51 (t, J = 8.4, 1H), 7.25−7.80 (m, 9H). ^{13}C-DEPT NMR ($CDCl_3$) δ 14.1, 16.0 (primary), 25.8, 29.6, 39.1, 71.1, 76.4 (secondary), 120.9 (worth 2 C), 127.6, 128.3, 128.4, 129.2, 129.3, 130.1, 130.4, 131.7, 132.7 (tertiary), 137.6, 137.7, 139.1, 143.1, 196.6 (quarternary). HR-FAB-MS calcd for $C_{24}H_{28}O_2Br$ $[M + H]^+$ 427.1265, found 427.1251.

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Cysteine (*S*-(*E*,*E*)-8-*O*-(3-Benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) Methyl Ester (5). Following literature precedent,[18] bromide **4** (85.1 mg, 0.20 mmol, 1 equiv) was dissolved in 180 μL of DMF and allowed to stir. Cysteine methyl ester hydrochloride (51.1 mg, 0.30 mmol, 1.5 equiv) was then added followed by 55.8 μL of DIEA (0.32 mmol, 1.6 equiv). The reaction continued to stir for roughly 20 h at which time it was diluted with ethyl acetate and washed twice with water. The organic layers were combined and dried over anhydrous Na_2SO_4, and the solvent was evaporated. After flash chromatography (1:1 toluene/EtOAc, $R_f = 0.23$), product was recovered in 40% yield (38.5 mg). ^{1}H NMR ($CDCl_3$) δ 1.66 (s, 6H), 2.07−2.18 (m, 4H), 2.67 (dd, $J = 13.5, 7.8$, 1H), 2.87 (dd, $J = 15.0, 4.5$, 1H), 3.16 (m, 2H), 3.65 (m, 1H), 3.73 (s, 3H), 3.91 (s, 2H), 4.49 (s, 3H), 5.23 (t, $J = 7.5$, 1H), 5.40 (m, 1H) 7.43−7.50 (m, 3H), 7.57−7.59 (m, 2H), 7.68−7.71 (m, 1H), 7.77−7.80 (m, 3H). ^{13}C-DEPT NMR ($CDCl_3$) δ 14.0, 14.2, 52.2 (primary), 26.1, 29.8, 36.5, 39.2, 70.9, 76.5 (secondary), 120.3, 128.0, 128.3 (worth 2 C), 129.1, 129.3, 130.3 (worth 2 C), 131.7, 132.5 (tertiary), 132.2, 137.6, 137.7, 139.0, 139.1, 174.6, 196.6 (quaternary). HR-FAB-MS calcd for $C_{28}H_{36}NO_4S$ $[M + H]^+$ 482.2356, found 482.2362.

***N*-Methanesulfonyl Cysteine (*S*-(*E*,*E*)-8-*O*-(3-Benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) Methyl Ester (6).** The following procedure is a modification of a published protocol.[19] Cysteine (*S*-(*E*,*E*)-8-*O*-(3-benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) methyl ester **5** (189.9 mg, 0.39 mmol, 1 equiv), was dissolved in 200 μL of freshly distilled CH_2Cl_2 followed by addition of Et_3N (109 μL, 0.78 mmol, 2 equiv). Methanesulfonyl chloride was then added slowly (51.3 μL, 0.66 mmol, 1.7 equiv) over a period of 1 min, and the reaction quickly became more yellow. Additional CH_2Cl_2 (0.5 mL) was added, and TLC analysis showed that the reaction was essentially complete in 10 min. After 1 h, the reaction was diluted with CH_2Cl_2 and washed two times with saturated $NaHCO_3$. The aqueous layers were combined and washed one time with CH_2Cl_2, and all organic layers were combined, dried over anhydrous Na_2SO_4, and evaporated. Flash chromatography (5:2 toluene/EtOAc, $R_f = 0.42$) was performed, and 154.8 mg of product were recovered, a 71% yield. ^{1}H NMR ($CDCl_3$) δ 1.67 (s, 6H), 2.03−2.21 (m, 4H), 2.83 (dd, $J = 13.8, 6.3$, 1H), 2.90 (dd, $J = 13.9, 5.2$, 1H), 3.00 (s, 3H), 3.15 (dd, $J = 13.1, 7.6$, 1H), 3.22 (dd, $J = 13.2, 7.8$, 1H), 3.76 (s, 3H), 3.91 (s, 2H), 4.32 (dt, $J = 8.6, 5.8$, 1H), 4.51 (s, 2H), 5.21 (t, $J = 7.7$, 1H), 5.39 (t, $J = 6.3$, 1H), 5.48 (d, $J = 8.4$, 1H), 7.42−7.49 (m, 3H), 7.55−7.60 (m, 2H), 7.68−7.70 (m, 1H), 7.78−7.80 (m, 3H). ^{13}C-DEPT NMR ($CDCl_3$) δ 14.0, 16.2, 41.9, 53.0 (primary), 26.0, 30.0, 34.4, 39.2, 71.0, 76.5 (secondary), 55.8, 119.8, 127.9, 128.3 (worth 2 C), 129.2, 129.4, 130.1 (worth 2 C), 131.8, 132.5 (tertiary), 132.3, 137.6, 137.7, 139.1, 139.9, 171.2, 196.8 (quaternary). HR-FAB-MS calcd for $C_{29}H_{38}NO_6S_2$ $[M + H]^+$ 560.2131, found 560.2106. Purity analysis by analytical scale reversed-phase HPLC indicated >85% pure material when the reaction was performed at this scale ($t_R = 40.5$ min). An extinction coefficient (254 nm) value of 14 800 M^{-1} cm^{-1} was determined in CH_3CN.

***N*-Methanesulfonyl Cysteine (*S*-(*E*,*E*)-8-*O*-(3-Benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) (2).** *N*-Methanesulfonyl cysteine (*S*-(*E*,*E*)-8-*O*-(3-benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) methyl ester **6** (132.7 mg, 0.24 mmol, 1 equiv) was dissolved in a 0.5 M LiOH solution consisting of 1:1 *i*PrOH/H_2O (4.75 mL, 2.4 mmol LiOH, 10 equiv). After stirring for 24 h, the reaction was acidified to pH 5 with 10% HCl and then dissolved in EtOAc, followed by three washings with water. The organic layer was dried over anhydrous Na_2SO_4 and then evaporated, leaving the product as 81.6 mg of a thick, dark yellow-colored oil (60% yield). ^{1}H NMR (CD_3OD) δ 1.64 (s, 3H), 1.67 (s, 3H), 2.13 (m, 4H), 2.73 (dd, $J = 13.8, 7.2$, 1H), 2.88 (dd, $J = 13.8, 5.1$, 1H), 2.96 (s, 3H), 3.18 (dd, $J = 12.9, 7.8$, 1H), 3.24 (dd, $J = 12.9, 8.0$, 1H), 3.91 (s, 2H), 4.05 (m, 1H), 4.47 (s, 2H), 5.22 (t, $J = 7.8$, 1H), 5.39 (t, $J = 7.0$, 1H), 7.46−7.70 (m, 9H). ^{13}C-DEPT NMR (CD_3OD) δ 12.8, 14.9, 39.9 (primary), 25.6, 29.3, 34.4, 38.9, 70.2, 76.1 (secondary), 57.4, 120.7, 128.1, 128.2 (worth 2 C), 128.8, 128.9, 129.7 (worth 2 C), 131.7, 132.5 (tertiary), 131.9, 137.5 (worth 2 C), 138.6, 139.2, 174.1, 197.1 (quaternary). HR-FAB-MS calcd for $C_{28}H_{36}NO_6S_2$ $[M + H]^+$ 546.1975, found 546.1975; for $[M + Na]^+$: calcd 568.1795, found 568.1781. Purity analysis by analytical scale reversed-phase HPLC indicated a purity of >90% when the reaction was performed at this scale ($t_R = 37.0$ min). An extinction coefficient (256 nm) value of 17 700 M^{-1} cm^{-1} was determined in MeOH.

[^{35}S]*N*-Methanesulfonyl Cysteine (*S*-(*E*,*E*)-8-*O*-(3-Benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) Methyl Ester. The title compound was prepared using the procedure of Dean et al.[19] A solution of [^{35}S]methanesulfonyl chloride (24 mCi, 1100 Ci/mmol) in anhydrous CH_2Cl_2 (9 mL) was dried over Na_2SO_4 for 1 h, filtered, then concentrated by atmospheric distillation to 120 μL. This [^{35}S]MsCl concentrate was added to a solution of cysteine (*S*-(*E*,*E*)-8-*O*-(3-benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) methyl ester (4.8 mg, 9.9 μmol) **5** and Et_3N (5 μL) in anhydrous CH_2Cl_2 (100 μL). After stirring 1 h, the reaction was quenched by the addition of saturated aqueous $NaHCO_3$ (2 mL) and diluted with CH_2Cl_2 (2 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (4 × 2 mL). The combined organic layers were concentrated, and the residue was dissolved in MeOH (2.5 mL) and the amount of radioactivity determined using a liquid scintillation counter (7.9 mCi). Analysis of the solution by analytical HPLC (Zorbax RX-C18 4.6 × 250 mm column, 1 mL/min, 30 °C, 210/254 nm, Packard flow monitor, A = 0.1% aqueous TFA, B = CH_3CN, 50A/50B linear gradient to 0A/100B over 30 min, t_R amine = 8.5 min, t_R [^{35}S]sulfonamide = 19.5 min) showed the radiochemical purity of the [^{35}S]sulfonamide to be 37%. The identity of the product was confirmed by coelution with an authentic sample of unlabeled sulfonamide. The crude [^{35}S]*N*-methanesulfonyl cysteine (*S*-(*E*,*E*)-8-*O*-(3-benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) methyl ester was used without further purification in the next step.

[^{35}S]*N*-Methanesulfonyl Cysteine (*S*-(*E*,*E*)-8-*O*-(3-Benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) ([^{35}S]2). To the above methanolic solution of [^{35}S]*N*-methanesulfonyl cysteine (*S*-(*E*,*E*)-8-*O*-(3-benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) methyl ester was added THF (3.0 mL), followed by a solution of $LiOH\cdot H_2O$ (30 mg) in H_2O (1.0 mL). The reaction mixture was stirred for 3.5 h at which stage HPLC indicated that reaction was complete. The reaction mixture was acidified with 0.15 M HCl (20 mL) and diluted with EtOAc (20 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (4 × 5 mL). The combined organic layers were concentrated, and the residue was dissolved in CH_3CN (5 mL), counted (4.46 mCi), and analyzed by HPLC (Zorbax RX-C18, 4.6 × 250 mm, 5 μm, 1 mL/min, 30 °C, 210/254 nm, Packard flow monitor, A = 0.1% aqueous TFA, B = CH_3CN, 50A/50B linear gradient to 0A/100B over 30 min, t_R [^{35}S]sulfonamide ester = 19.5 min, t_R [^{35}S]**2** = 14.9 min, 38% radiochemical purity). Purification was effected by RP-HPLC (Zorbax SB−Phenyl, 9.4 × 250 mm, 5 μm, 5 mL/min, 20 °C, 210/254 nm, Packard flow monitor, A = 0.1% aqueous TFA, B = CH_3CN, 52A/48B isocratic, t_R [^{35}S]**2** = 27 min). The purified [^{35}S]**2** was isolated by SepPak, concentrated, taken up in 2:1 CH_3CN/H_2O (1.5 mL), counted (390 μCi), and analyzed by HPLC (Zorbax SB-Phenyl, 4.6 × 250 mm, 5 μm, 1 mL/min, 30 °C, 210/254 nm, Packard flow monitor, A = 0.1% aqueous H_3PO_4, B = CH_3CN, 46A/54B isocratic, t_R [^{35}S]**2** = 16.5 min, 97.5% radiochemical purity). The identity of the [^{35}S]*N*-methanesulfonyl cysteine (*S*-(*E*,*E*)-8-*O*-(3-benzoylbenzyl)-3,7-dimethyl-2,6-octadiene) was confirmed by coelution with an authentic sample of unlabeled **2**.

Ac-N-KKSRRC(*S*-Geranylgeranyl)-OH. The unmodified peptide was synthesized and cleaved from the resin as a C-terminal carboxylic acid at the Microchemical Facility at the University of Minnesota, Minneapolis, MN. Alkylation, using geranylgeranyl bromide, was performed as previously described.[20] Purity analysis by analytical scale reversed-phase HPLC (Vydac C_4 column (8.0 μm, 4.6 × 250 mm) equipped with a 5 cm guard column (flow rate: 1.0 mL/min, 500 μL injection loop, 5 to 100% B in 40 min. Solvent A: 95% H_2O, 5% CH_3CN, 0.2% TFA; solvent B: 100% CH_3CN, 0.2% TFA) with monitoring at 220 and 260 nm) indicated a purity of >85% ($t_R = 27.1$ min). MALDI-TOF-MS calcd for $C_{52}H_{95}N_{14}0_9S$ $[M + H]^+$ 1091.71,

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found 1091.44. ESI-MS for $[M + H]^+$ found 1091.6; $[M + 2H]^{2+}$ calcd 546.3, found 546.6; $[M + 3H]^{3+}$ calcd 364.6, found 364.8. ESI-MS-MS resulted in the prenyl group fragmenting first, leaving the original peptide (calcd $[M + H]^+$ 819.5, found 819.4). The entire peptide was sequenced using multiple MS−MS (MS^3, MS^4) fragmentation methods (see Supporting Information).

Preparation of GST−GDI *Escherichia coli* (*E. coli*)/pGEX-KG Crude Cell Lysates; Purification of GDI. GST−GDI was expressed in *E. coli* using a modified protocol,[21] and the following represents a typical procedure (cell growth time and final cell densities varied with each preparation; see text). A culture plate, supplemented with ampicillin at 100 μg/mL, was streaked with a frozen culture of JM-109 cells containing the pGEX-KG GDI plasmid and incubated overnight at 37 °C. A 2 mL overnight culture was grown from a selected colony, and following overnight incubation at 37 °C with vigorous shaking (275 rpm), the saturated culture was diluted into 100 mL of LB media (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract) containing 100 μg/mL ampicillin. This culture was incubated at 37 °C with vigorous shaking (275 rpm) until the OD_{560} reached 0.7 at which time one-half of the material was removed for later experiments and termed the "uninduced sample." Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μM, and the incubation was continued for an additional 2−3 h. Uninduced and induced cells were harvested by centrifugation (10 min at 5000*g* and then resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.2 mM phenylmethanesulfonyl chloride (PMSF), 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μM benzamidine), followed again by centrifugation. Cells of both types not being immediately used were flash frozen with N_2 (l) and stored at −80 °C until further use. Cell lysis of both types was carried out by sonication (3 × 30 s) in fresh lysis buffer followed by centrifugation (10 min at 12000*g*, thus producing the crude soluble protein extract.

Purification of the fusion protein, when desired, was performed using Glutathione Sepharose 4B resin (Amersham Pharmacia Biotech; 0.5 mL) equilibrated first with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) and then TEDA buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM NaN_3) plus 100 mM NaCl and 0.2 mM PMSF. Crude induced soluble extract (250−500 μL) was applied to the column resin, and undesired proteins were eluted by washing with TEDA, 100 mM NaCl, and 0.2 mM PMSF. The fusion protein was then eluted using TEDA, 100 mM NaCl, 0.2 mM PMSF, and 10 mM glutathione.

In cases where isolation of GDI was desired, thrombin (Amersham Pharmacia Biotech; 1 unit/μL activity) was used to cleave GDI while GST−GDI was still bound to the glutathione affinity resin after unwanted proteins were removed by washing with TEDA. Cleavage was monitored by SDS-PAGE. To further purify GDI, literature precedent was followed,[21] employing a Mono-Q HR 5/5 ion-exchange column (Amersham Pharmacia Biotech).

Preparation of *E. coli* HB101 Crude Cell Lysate. *E. coli* HB101 competent cells were purchased from Transfinity, BRL Life Technologies, Inc. Colony development, culture growth, centrifugations, cell lysis, and preparation of the crude extract for photolysis were performed in the same manner as described for uninduced GST−GDI except for the removal of ampicillin from the procedure.

General Photolysis Procedure. All reactions (50 μL final volume unless otherwise noted) were performed in silinized quartz test tubes covered with Parafilm at 4 °C in a UV Rayonet minireactor equipped with 8 RPR-3500° lamps and a circulating platform that allowed for irradiation of up to eight samples simultaneously. The amount of [^{35}S]**2** added to photolysis mixtures, unless otherwise noted, came from stock solutions in DMSO to yield 2.9 pmol of material (58 nM in 50 μL). Competitor stock solutions were also prepared as either DMSO or GDI buffer solutions (20 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 1 mM NaN_3, 100 mM NaCl) and added to the photolysis mixtures as needed. The percent DMSO in all photolysis reactions remained under 10%. GDI buffer was used for reactions. All reagents (buffer, protein, [^{35}S]**2**, competitor as applicable) were combined, mixed gently, and immediately photolyzed. Irradiation times up to 10.7 h resulted in no decomposition of GDI as detected by SDS-PAGE, and cross-linking only occurred when the mixtures were irradiated. Following photolysis, unless otherwise indicated, loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was added to each sample, and samples were heated for 2 min at 100 °C followed by analysis by SDS-PAGE using Tris-glycine gels (12 or 15% acrylamide). Unincorporated [^{35}S]**2** passed through the gel and did not interfere with analysis of most cross-linked protein products, but upper and lower portions of each gel were removed before autoradiography in order to facilitate analysis. Gels were then stained with Coomassie brilliant blue or SYPRO stain (Bio-Rad), dried, and subjected to autoradiography. Intensities of radiolabeled products were determined by phosphorimaging analysis of the dried gels.

Immunoprecipitation of Cross-Linked GST−GDI. Two samples of crude GST−GDI-induced cell lysate described above (30−40 μg each) were combined with the typical amount of [^{35}S]**2** (2.9 pmol) in total volumes ranging from 30 to 50 μL using RIPA buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 600 mM KCl, 5 mM EDTA, 3 mM PMSF, 1 μg/mL aprotinin, 2% Triton X-100 (v/v)) as desired. After photolysis (0.5 h), an equivolume amount of loading buffer was added to one sample, and following heating of the sample for 2 min at 100 °C, the material was stored at −20 °C. The second sample was transferred to a small microcentrifuge tube, and a small magnetic stir bar was added. Anti-GST antibodies (Amersham Pharmacia Biotech; 4 μL at 5 mg/mL) were added, and the material was slowly stirred for 6 h at 4 °C.

During this time, Gammabind Sepharose (Amersham Pharmacia Biotech) resin (100−200 μL as a 50/50 (v/v) slurry) was washed sequentially with the following: 0.5 M AcOH, pH 3.0; 1.0 M AcOH, pH 2.5; binding buffer (0.01 M NaH_2PO_4, 0.15 M NaCl, 0.01 M EDTA, pH 7.0) and then equilibrated in RIPA buffer. Equilibrated resin was then added to the crude lysate/antibody mixture. Stirring then continued for an additional 16 h at 4 °C at which time the stir bar was removed, and the vessel was centrifuged (10 min at 12000*g*), leaving the immunoprecipitated GST−GDI on resin as a pellet. Resin was washed with RIPA buffer, and the material was centrifuged again, followed by removal of the supernatant.

Photolysis of Commercially Available Proteins. A mixture of commercially available proteins (GIBCO BRL Life Technologies) was prepared at either 0.1 or 0.4 mg/mL concentrations and photolyzed in the presence of [^{35}S]**2** (58 nM) in GDI buffer (50 μL total volume) for 0.5 h. The samples were treated as described in the General Photolysis Procedure although cutting of the upper and lower portions of the gel removed the following proteins and prevented their analysis by autoradiography: myosin, phosphorylase b, and lysozyme (see Supporting Information).

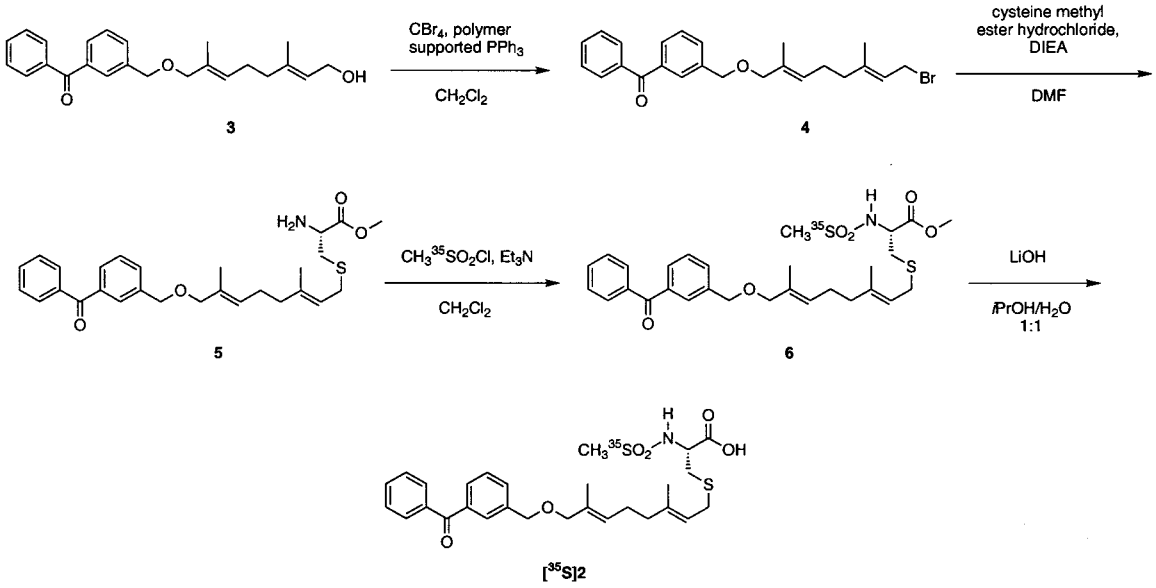
Determination of [^{35}S]2 Percent Incorporation Following Photolysis with GDI. A solution of unlabeled **2** was prepared in DMSO and added to 2.9 pmol of [^{35}S]**2** (the typical amount used for photolysis; see General Photolysis Procedure) such that the final specific activity was 8 Ci/mmol. This solution was used to prepare a photolysis reaction mixture containing an equimolar ratio of GDI to total compound **2** (each at 4.0 μM). Following the General Photolysis Procedure, the phosphorimaged gel was compared to a phosphorimaged standard which consisted of 2.9 pmol of [^{35}S]**2**, unincubated with protein and unphotolyzed, applied to a separate gel of the same percentage acrylamide. This unincorporated material was allowed to run partway through the gel and, by phosphorimaging analysis, migrated as a single spot. Imaging of this gel in the same manner (i.e., the same cassette) as the gel containing the photolyzed protein allowed for direct comparisons to the standard by phosphorimaging analysis.

Results

Design and Synthesis. Prenylated cysteine analogues including AFC (**1a**) have been used to examine prenylated protein−

(21) We used a truncated GDI whose first 25 amino-terminal residues had been removed. This modification increases the stability of the protein (G. R. Hoffman, personal communication) and behaves similarly to the wild-type protein (Platko, J. V.; Leonard, D. A.; Adra, C. N.; Shaw, R. J.; Cerione, R. A.; Lim, B. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2974−2978). For expression and purification of GST−GDI and GDI alone, see: Leonard, D.; Hart, M. J.; Platko, J. V.; Eva, A.; Henzel, W.; Evans, T.; Cerione, R. A. *J. Biol. Chem.* **1992**, *267*, 22860−22868.

Scheme 1



protein behavior[10] and in several cases, have been shown to significantly alter these interactions.[22] These results, along with the desire to create a universal probe which could be used with a wide variety of proteins thought to interact with prenylated proteins, led us to design compound **2**. Benzophenone-containing isoprenoid analogues have been shown to be effective mimics of the isoprenoidal portion of prenyl diphosphates;[23] recently, a crystal structure of such an analogue bound in the farnesyl diphosphate binding site of farnesyltransferase has been obtained.[24] Thus, we incorporated this photoactivatable group, with a C_{10} isoprenoid spacer, into a cysteine residue via a thioether linkage. Cross-linking between **2** and a putative isoprenoid binding protein should provide evidence for protein−isoprenoid interactions and may also allow for the eventual identification of amino acid residues important for isoprenoid binding.[25] To facilitate identification of these cross-linked products, an ^{35}S-labeled sulfonamide was introduced in lieu of the *N*-acetyl group used in many analogues.[10,22] Compound **2** was prepared using a geranyl bromide derivative (**4**) containing the benzoylbenzyl moiety linked to it via a stable ether linkage (see Scheme 1).[16] This linkage has the advantage of withstanding a variety of conditions, an important feature not only for the purposes of generating **2** and compounds similar to it, but also for the eventual process of identifying cross-linked residues. Compound **3** was prepared as previously reported[16] and converted to **4** using a modified procedure employing resin-bound PPh_3.[17] We have found this reagent extremely useful for the preparation of allylic bromides since the only major impurity, resin-bound triphenylphosphine oxide, is easily removed by filtration in lieu of chromatography. Cysteine methyl ester was alkylated with the allylic bromide **4**, followed by sulfonamidation of the free amine and saponification of the methyl ester. Using methane[^{35}S]sulfonyl chloride to form the sulfonamide linkage,[19] [^{35}S]**2** was synthesized with a specific activity of 1100 Ci/mmol from the free amine precursor and purified using reversed-phase HPLC chromatography. The product was obtained in 97.5% radiochemical purity and matched the retention time of unlabeled probe as determined by reversed-phase HPLC.

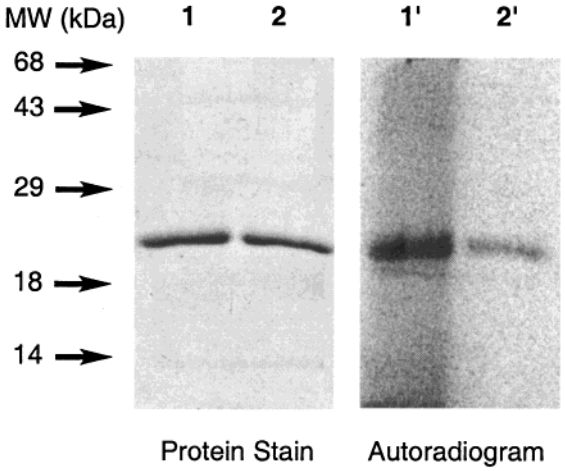


Figure 1. Analysis of photolabeling of GDI by [^{35}S]**2** by SDS-polyacrylamide gel electrophoresis (PAGE). Lanes 1 and 2 show protein staining, while lanes 1′ and 2′ show the radiolabeled proteins. Lanes 1 and 1′ contain a sample of GDI (4.0 μM) irradiated in the presence of [^{35}S]**2** (116 nM); lanes 2 and 2′ contain a sample of GDI (4.0 μM) irradiated in the presence of **1a** (10 mM) and [^{35}S]**2** (116 nM). Irradiation time was 0.5 h.

Preliminary Photolabeling Experiments with AFC Analogue [^{35}S](2). To investigate the cross-linking ability of our probe to GDI, [^{35}S]**2** was first photolyzed in the presence of a purified sample of GDI.[21] Photolysis experiments using 58−116 nM probe in the presence of 4.0 μM GDI resulted in successful cross-linking (Figure 1, lanes 1 and 1′). Inclusion of 10 mM AFC (**1a**) in the photolysis reaction (Figure 1, lanes 2, 2′) resulting in a 5−7-fold decrease in the amount of radiolabeling (ca. 15% of original, lanes 1, 1′). AFC was initially chosen as a competitor with [^{35}S]**2** due to its perturbation of putative prenyl binding site residues of GDI in the NMR study

(22) (a) Scheer, A.; Gierschik, P. *Biochemistry* **1995**, *34*, 4952−4961. (b) Parish, C. A.; Brazil, D. P.; Rando, R. R. *Biochemistry* **1997**, *36*, 2686−2693. (c) Kilic, F.; Dalton, M. B.; Burrell, S. K.; Mayer, J. P.; Patterson, S. D.; Sinensky, M. *J. Biol. Chem.* **1997**, *272*, 5298−5304.

(23) (a) Gaon, I.; Turek, T. C.; Weller, V. A.; Edelstein, R. L.; Singh, S. K.; Distefano, M. D. *J. Org. Chem.* **1996**, *61*, 7738−7745. (b) Turek, T. C.; Gamache, D.; Distefano, M. D. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2125−2130. (c) Marecak, D. M.; Horiuchi, Y.; Arai, H.; Shimonaga, M.; Maki, Y.; Koyama, T.; Ogura, K.; Prestwich, G. D. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1973−1978. (d) Zhang, Y.-W.; Koyama, T.; Marecak, D. M.; Prestwich, G. D.; Maki, Y.; Ogura, K. *Biochemistry* **1998**, *37*, 13411−13420.

(24) Turek, T. C.; Gaon, I.; Distefano, M. D.; Strickland, C. L. *J. Org. Chem.* In press.

(25) Dorman, G.; Prestwich, G. D. *Biochemistry* **1994**, *33*, 5661−5673.

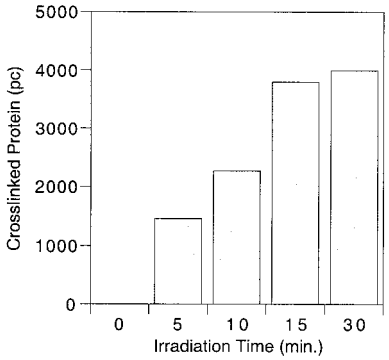


Figure 2. Analysis of the effect of irradiation time on the photolabeling of GDI by [^{35}S]**2**. Reactions were performed using GDI (4.0 μM) and [^{35}S]**2** (58 nM), and the extent of cross-linking is expressed as the amount of radioactivity determined by phosphorimaging analysis (phosphorimage counts, pc).

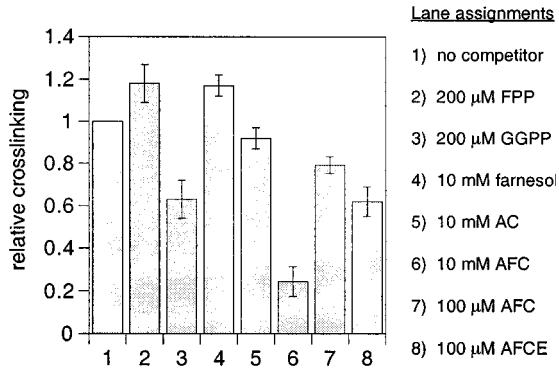


Figure 3. Phosphorimaging analyses following photolabeling of GDI by [^{35}S]**2** in the presence of various potential competitors. Reactions, irradiated for 0.5 h, were performed using GDI (4.0 μM) and [^{35}S]**2** (58 nM). Lane assignments, from left to right, are as follows: no competitor, 200 μM FPP, 200 μM GGPP, 10 mM farnesol, 10 mM AC, 10 mM AFC, 100 μM AFC, 100 μM AFCE.

by Rosen and co-workers.[10a] Thus, it appears that **1a** and **2** bind to a common site on GDI, suggesting that [^{35}S]**2** is a good probe to study isoprenoid binding by GDI. Initially, photolysis was performed for 2.7 h but later it was found that at 0.5 h, cross-linking appears to reach a maximum, as shown in Figure 2.

It should be noted that no cross-linking occurred in the absence of light and that the amount of cross-linking was reduced upon addition of unlabeled **2**. However, we were unable to completely block radiolabeling by [^{35}S]**2** because addition of higher concentrations of unlabeled probe ($\geq$100 μM) created problematic degradation of the protein and prevented us from completely suppressing the cross-linking by [^{35}S]**2** (data not shown). Finally, photoaffinity labeling of GDI with [^{35}S]**2** is efficient. Photolysis of an equimolar ratio (4.0 μM) of GDI and [^{35}S]**2**, followed by electrophoretic separation and quantitation via phosphorimaging, indicates that 21% of the GDI is labeled.

Competition Studies. Structural determinants important for decreasing the cross-linking of [^{35}S]**2** were examined next in a series of competition experiments. Photolysis of GDI and [^{35}S]**2** in the presence of the natural prenyl diphosphates, geranylgeranyl diphosphate (GGPP) and farnesyl diphosphate (FPP), showed that GGPP was a more effective competitor than FPP, suggesting a preference in the binding site for the longer prenyl group (Figure 3). High levels (10 mM) of farnesol or *N*-acetyl cysteine (AC) resulted in no significant decrease in cross-linking, indicating that these fragments bind with markedly less avidity than **1a** (Figure 3). Because of the possibility of detergent-like

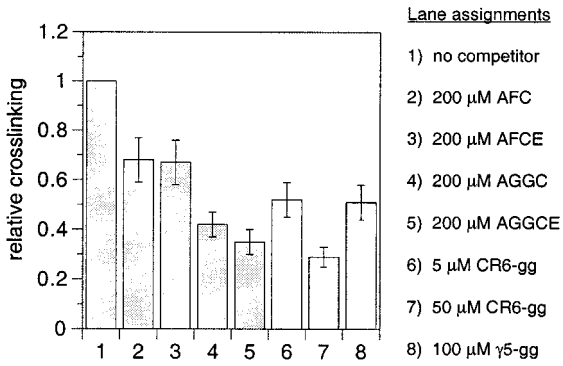


Figure 4. Phosphorimaging analyses following photolabeling of GDI by [^{35}S]**2** in the presence of various potential competitors. Reactions, irradiated for 0.5 h, were performed using GDI (4.0 μM) and [^{35}S]**2** (58 nM). Shaded bars represent cross-linking obtained with 200 μM competitor (aside from lane 1), while white bars represent cross-linking obtained using the peptides indicated. Lane assignments, from left to right, are as follows: no competitor, 200 μM AFC, 200 μM AFCE, 200 μM AGGC, 200 μM AGGCE, 5 μM Ac-KKSRRC(*S*-geranylgeranyl) (CR6-gg), 50 μM CR6-gg, 100 μM VSSSTNPFRPQKVC(*S*-geranylgeranyl) (γ5-gg).

effects occurring with AFC at high concentrations,[22b] we performed additional competitions at lower concentrations. Interestingly, photolysis employing the methyl ester of **1a** as a competitor (AFCE, **1b**) produced somewhat less cross-linking than was obtained in the presence of **1a** at the same concentration (100 μM, Figure 3). Rando and co-workers recently found similar results with GDI: carboxymethylated cysteine derivatives bound with higher affinity to GDI than their acid counterparts.[26]

Examining this phenomenon further, GDI and [^{35}S]**2** were photolyzed in the presence of equimolar concentrations of AFC, AFCE, and two other prenylated cysteine derivatives: geranylgeranylated *N*-acetyl cysteine and its methyl ester, AGGC and AGGCE, respectively (Figure 4). Of the four prenylated cysteine derivatives, AGGCE was the most effective competitor, in keeping with Rando's observed trend. Their studies utilized a fluorescently labeled geranylgeranylated cysteine methyl ester probe to determine dissociation constants for the binding of various prenylated cysteines to GDI.[26] AGGCE bound with greater affinity than AGGC, and AFCE exhibited an improvement in binding over AFC. Similarly, our data show that differences in the amount of competition between acid and ester is more pronounced in the geranylgeranyl system than in the farnesyl system as noted by Rando and co-workers.[26] These results may have biological significance since many (but not all) prenylated proteins are esterified in subsequent posttranslational events.[27]

Also shown in Figure 4 are the competition results with geranylgeranylated peptides. The 14-mer, VSSSTNPFRPQKVC-(*S*-geranylgeranyl) (γ5-gg), mimics the C-terminal residues of a γ-subunit of a geranylgeranylated heterotrimeric protein known to interact with the M2 muscarinic receptor and terminates in a carboxylic acid.[28] At 100 μM, the peptide does not compete as well as AGGC or AGGCE, but is a more effective competitor than AFC (Figure 4). At 200 μM peptide, cross-linking is reduced to roughly 30% that of GDI alone, a value comparable to the competition seen with AGGCE at the same concentration.

(26) Mondal, M. S.; Wang, Z.; Seeds, A. M.; Rando, R. R. *Biochemistry* **2000**, *39*, 406−412.

(27) For a review of prenylation and further processing events, see: Zhang, F. L.; Casey, P. J. *Annu. Rev. Biochem.* **1996**, *65*, 241−269.

(28) Azpiazu, I.; Cruzblanca, H.; Li, P.; Linder, M.; Zhuo, M.; Gautam, N. *J. Biol. Chem.* **1999**, *274*, 35305−35308.

While the peptide γ5-gg clearly competed with [^{35}S]**2** for the GDI isoprenoid binding site, this peptide does not contain the same C-terminal sequence present in typical Rho proteins. To address this issue, a peptide, acetyl-KKSRRC(*S*-geranylgeranyl) (CR6-gg), a C-terminal mimic of Rho proteins,[10a] was prepared. A farnesylated version of this peptide (CR6-f) was previously used by Rosen and co-workers in their NMR study of GDI.[10a] In their work performed at mM concentrations of CR6-f, GDI was fully folded and several specific NOEs between CR6-f and GDI were reported. At 5 μM CR6-gg, some competition with [^{35}S]**2** was observed, while at 50 μM, the cross-linking by [^{35}S]**2** was reduced to 30% of that obtained in the absence of any competitor (Figure 4). Clearly, CR6-gg is the most effective competitor used in this study.

In summary, the results of the competition experiments with prenyl cysteine derivatives and short prenylated peptides demonstrate that the majority of cross-linking of GDI by [^{35}S]**2** can be suppressed using ligands known to bind to the isoprenoid binding site of GDI. Particularly in the case of CR6-gg, a biologically relevant competitor,[10a] the reduction in cross-linking occurs at a concentration of CR6-gg (50 μM) too low to be ascribed as a nonspecific detergent effect in which the competitor is reducing the cross-linking either by denaturing the protein or by sequestering [^{35}S]**2** in a micellular phase.[29] As noted from the structural work cited above, GDI remains folded both in the presence of mM concentrations of CR6-f and saturating concentrations of AFC (**1a**).[10a] In light of this, our results suggest that [^{35}S]**2** does bind with significant specificity to GDI. However, some residual cross-linking of GDI does occur even in the presence of a variety of competitors. This may be due to low levels of nonspecific protein binding (see specificity studies described below) or may reflect the rapid on/off kinetics of competitor binding to GDI;[30] in all likelihood, both of these phenomena are occurring. Consistent with the kinetic explanation, it is interesting to note that many of the competitors employed here have only modest affinities for GDI. For example, the reported dissociation constant of AFC for GDI is >20 μM.[26] Thus, the bound and unbound states are likely to be in rapid equilibrium enabling irreversible cross-linking to occur even in the presence of excess competitor. It is also noteworthy that while some nonspecific labeling is probably occurring in these experiments, it is still possible to clearly differentiate between high versus low affinity binding of [^{35}S]**2**. As reported above, high concentrations of AFC (10 mM) are necessary to reduce labeling of GDI by [^{35}S]**2**; these high levels also decrease the background labeling (compare lanes 1′ and 2′, Figure 1). However, a similar same extent of protection can be accomplished using a much lower concentration (50 μM) of a more specific competitor, CR6-gg. Under those conditions, there is no reduction in background labeling (compare lanes 1′ and 3′, Figure 5).

Specificity Studies. To evaluate the binding selectivity of our isoprenoid mimicking probe, [^{35}S]**2** was photolyzed in the presence of a mixture of commercially available proteins: myosin, phosphorylase b, bovine serum albumin (BSA), ovalbumin, carbonic anhydrase, β-lactoglobulin, and lysozyme. These proteins are not known to bind isoprenoids and were collectively irradiated with a concentration of [^{35}S]**2** comparable to that used in the GDI labeling experiments above (58 nM). Two experiments were performed using this protein mixture,

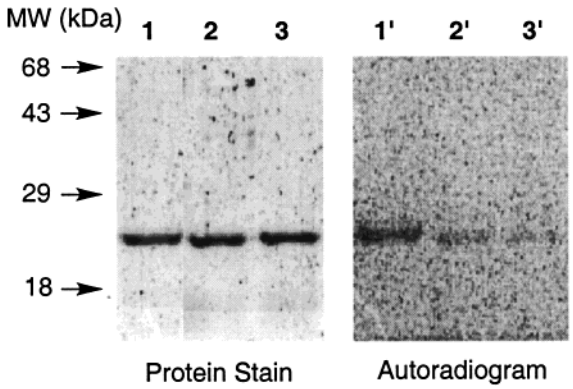


Figure 5. Analysis of photolabeling of GDI by [^{35}S]**2** by SDS-polyacrylamide gel electrophoresis (PAGE). Lanes 1, 2, and 3 show protein staining while lanes 1′, 2′, and 3′ show the radiolabeled proteins. Lanes 1 and 1′ contain a sample of GDI (4.0 μM) irradiated in the presence of [^{35}S]**2** (58 nM); lanes 2 and 2′ contain a sample of GDI (4.0 μM) irradiated in the presence of Ac-KKSRRC(*S*-geranylgeranyl) (CR6-gg) (5 μM) and [^{35}S]**2** (58 nM); lanes 3 and 3′ contain a sample of GDI (4.0 μM) irradiated in the presence of CR6-gg (50 μM) and [^{35}S]**2** (58 nM). Irradiation time was 0.5 h.

and the results were, in both cases, compared to an amount of photolyzed GDI also identical to that used in the competition experiments above (4.0 μM, 0.1 mg/mL GDI; 58 nM [^{35}S]**2**). When the individual test proteins were present at concentrations comparable to GDI, no detectable labeling of the random proteins occurred. Increasing the amount of proteins 3.5−5.4-fold over GDI resulted observable amounts of cross-linking in the protein mix ranging from 1% (BSA) to a high of 12% (ovalbumin) of that of GDI. These results suggest that [^{35}S]**2** has an affinity 10−100 fold greater for GDI than for the other proteins. Thus, probe **2** shows specificity for interacting with isoprenoid binding proteins, even in the presence of higher concentrations of other proteins.

Application of [^{35}S]2 to Crude Extracts. After establishing that the labeling of GDI is preferential to that of several known nonisoprenoid binding proteins, we next examined the ability of [^{35}S]**2** to label GDI in the presence of a complex mixture of other, unknown cellular proteins: compound [^{35}S]**2** was photolyzed in a crude *E. coli* cell extract expressing GDI as a glutathione-*S*-transferase (GST)−GDI fusion protein under the control of an inducible promoter.[21] The products of those photolysis reactions after fractionation via SDS-polyacrylamide gel electrophoresis (PAGE) are shown in Figure 6. Lane 1 illustrates that large a number of proteins are present in the crude extract. Despite this plethora of targets, [^{35}S]**2** effectively labels GST−GDI (lane 1′).[31] Additionally, the labeling of GST−GDI can be suppressed by the inclusion of **1a** (lane 2′), analogous to results obtained with purified GDI (Figure 1).

To obtain a more quantitative view of the effectiveness of [^{35}S]**2** as a probe, photolysis reactions were performed on crude cellular extracts obtained from *E. coli* lacking the expression plasmid for GST−GDI and samples of *E. coli* harboring this plasmid before and after induction with IPTG. An important difference between the initial experiments with crude extracts

(29) As a comparison, a recent study has shown that the critical micellular concentration of AGGC is 340 μM: Desrosiers, R. R.; Gauthier, F.; Lanthier, J.; Béliveau, R. *J. Biol. Chem.* **2000**, *275*, 14949−14957.

(30) Chowdry, V.; Westheimer, F. H. *Annu. Rev. Biochem.* **1979**, *48*, 293−325.

(31) It should be noted that cleavage of the GST−GDI fusion protein with thrombin either before or after photolysis with [^{32}S]**2** yielded GDI and GST fragments that were both radiolabeled. This is not surprising given the affinity of GST for hydrophobic compounds (Wang, J.; Bauman, S.; Colman, R. F. *Biochemistry* **1998**, *37*, 15671−15679). While we were able to determine that the level of GST cross-linking by [^{32}S]**2** is less than that of GDI, for technical reasons it was not possible to determine a precise value for this ratio. This was one of the reasons we photolyzed [^{32}S]**2** in the presence of several nonisoprenoid binding proteins as noted above.

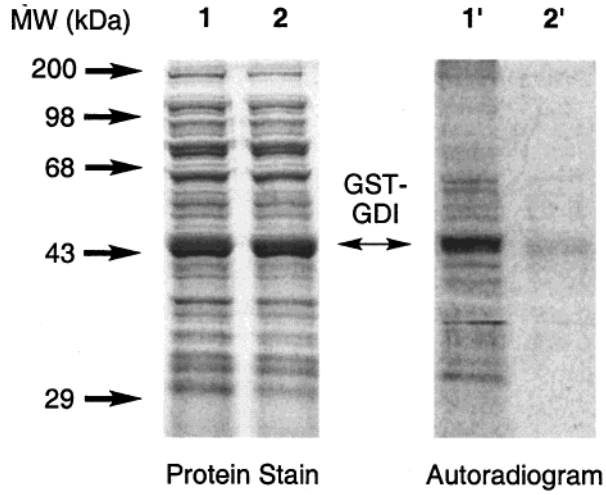


Figure 6. Analysis of photolabeling of a crude *E. coli*/pGEX-KG cell extract of GST−GDI after induction with IPTG by [^{35}S]**2** by SDS-polyacrylamide gel electrophoresis (PAGE). Lanes 1 and 2 show protein staining, while lanes 1′ and 2′ show the radiolabeled proteins. Lanes 1 and 1′ contain a sample of crude extract (26 μg) irradiated in the presence of [^{35}S]**2** (58 nM); lanes 2 and 2′ contain a sample of GDI (26 μg) irradiated in the presence of **1a** (10 mM) and [^{35}S]**2** (58 nM). Irradiation time was 0.5 h.

described above and subsequent ones is that the crude lysates in the latter experiments were isolated after shorter growth periods. This was done to decrease the levels of proteolytic degradation of GST−GDI[32] and to decrease the expression of GST−GDI to render its concentration closer to other proteins in the extract. Densitometric analyses of these reactions following SDS−PAGE are given in Figure 7. Photolysis of a crude lysate generated from cells containing no plasmid for the GST−GDI protein (HB101) resulted in only very low levels of protein labeling. Similarly, prior to induction with IPTG, only faint levels of cross-linked products were observed. However, photolysis of [^{35}S]**2** in extracts obtained from cells after induction of expression resulted in the marked appearance of a significantly radiolabeled protein attributable to GST−GDI.[33]

To unambiguously identify the predominant cross-linked species,[34] an immunological approach was employed. Following irradiation of the crude induced lysate with [^{35}S]**2**, addition of anti-GST antibodies reduced the protein amount of putative GST−GDI as well as the amount of corresponding radioactivity similarly (ca. 50% less protein and 50% less labeling). A densitometric analysis of the cross-linked products separated by SDS−PAGE before and after immunoprecipitation is given in Figure 8. This analysis clearly shows that the major cross-linked product is GST−GDI.

Concluding Remarks

The results reported here illustrate the utility of **2** as a probe for investigating the interaction between prenylated proteins and other proteins. The ability of **2** to cross-link to GDI suggests the presence of an isoprenoid binding site on GDI which is consistent with recent structural studies of this protein.[10a,b,11] Given the results described here with purified proteins and crude cellular extracts, it should be possible to use **2** in many types of studies involving pure or partially pure proteins. Experiments employing **2** with membrane proteins that are refractory to conventional methods of structural analysis may be particularly fruitful. However, for experiments targeting proteins at low abundance present in crude extracts, it is unlikely that **2** manifests sufficient specificity. This may not be a significant limitation of this approach since the photoactivatable isoprenoid substructure present in **2** could also be incorporated into peptides via chemical synthesis using conditions similar to those employed here for the alkylation of cysteine. This would undoubtedly result in more selective probes that could be used to study specific subclasses of prenylated protein−protein interactions.

Important examples of prenylated protein−protein interactions that are currently under study include the prenyl-specific protease[27,35] and methyltransferase;[27,36] both of these are possible

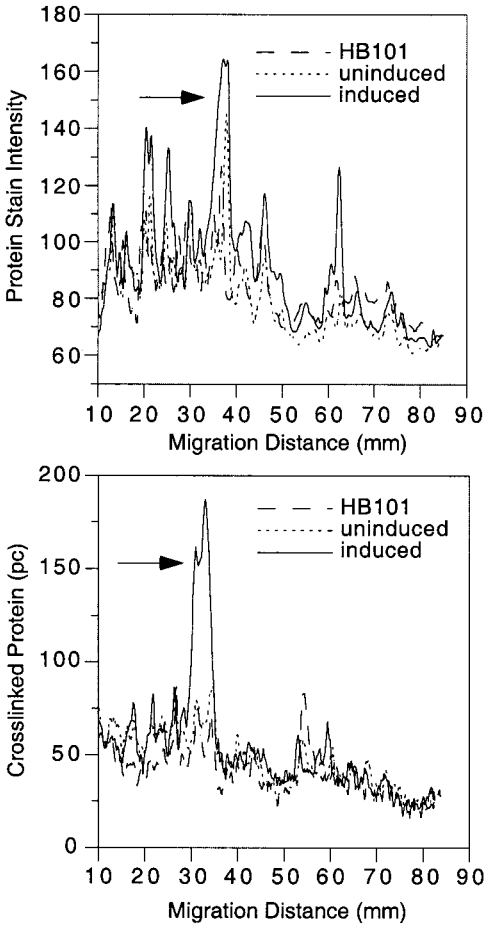


Figure 7. Densitometric representations of protein staining (above) and radiolabeling (below) by [^{35}S]**2** of three crude cell extracts after fractionation by SDS-polyacrylamide gel electrophoresis (PAGE): *E coli*. HB101 (no plasmid control) (— — —), uninduced GST−GDI *E. coli*/pGEX-KG (contains plasmid for GST−GDI expression) (- - -), and induced GST−GDI *E. coli*/pGEX-KG (solid line). The arrow indicates the band corresponding to GST−GDI. The extent of cross-linking is expressed as the amount of radioactivity determined by phosphorimaging analysis (phosphorimage counts, pc). Data has been normalized to represent equivalent amounts of protein for each extract; 58 nM [^{35}S]**2**, 0.5 h irradiation. See Supporting Information for a more detailed analysis of these data.

(32) Various homologues of GDIs are proteolytically sensitive: footnote 10a and G. R. Hoffman, personal communication.

(33) Sheffield, P.; Garrard, S.; Derewenda, Z. *Protein Expression Purif.* **1999**, *15*, 34−39.

(34) In addition to the major cross-linked product, several less pronounced products at both higher and lower mass than GST−GDI were observed in Figures 7 and 8. All of these species must contain portions of GST since they react with the anti-GST antibodies (Figure 8). As noted above, the smaller cross-linked products are likely to be proteolytic fragments of GST−GDI (see footnote 32). The identity of the higher mass species is somewhat less clear. One possibility is that they are incompletely reduced disulfide-linked dimers of GST−GDI or fragments thereof. It is noteworthy that the amount of these species appears to vary in different experiments (compare Figure 7, lower panel with Figure 8).

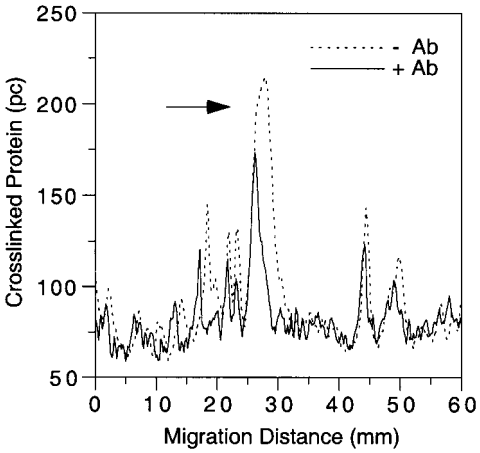


Figure 8. Densitometric autoradiographic representation of the SDS-polyacrylamide gel electrophoretic fractionation of crude *E. coli*/pGEX-KG cell lysate overexpressing [^{35}S]**2**-labeled GST−GDI before (dashed line) and after (solid line) treatment with anti-GST antibodies (Ab). The extent of cross-linking is expressed as the amount of radioactivity determined by phosphorimaging analysis (phosphorimage counts, pc). Data has been normalized and is representative of equal amounts of protein for both sets of conditions; 58 nM [^{35}S]**2**, 0.5 h irradiation.

targets for cancer therapy. Additional examples involving possible prenylated protein−protein interactions include heterotrimeric G-protein receptor complexes,[7,28,36] a variety of proteins that interact with the smaller GTP-binding proteins including Ras,[6,9e,35,36] and others.[9a−d,f,37,38] The use of **2** or compounds containing the photoactivatable moiety present in **2** may also reveal previously unknown proteins that interact with prenylated proteins. Moreover, the effectiveness of **2** as an isoprenoid mimic suggests that it may be possible to prepare inhibitors of prenylated protein−protein interactions by combining modified isoprenoid units with peptides or other peptidomimetic moieties. Benzophenone-containing compounds appear to be good structural mimics of isoprenoids[39] and are effective inhibitors of farnesyltransferase with K_I's as low as 45 nM.[16] As a final point, it is important to emphasize the benefits of the ^{35}S radiolabel in these experiments. The [^{35}S]CH_3SO_2Cl reagent developed by Dean and co-workers[19] allows for the facile introduction of a high specific activity radiolabel into amine-containing molecules that provides greater sensitivity than ^{3}H and ^{14}C without the health risks associated with ^{125}I. Thus, the novel probe presented here and variations thereof offer a versatile and sensitive means of obtaining structural information about the isoprene binding region of virtually any protein thought to contain such a site.

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Supporting Information Available: Synthesis and characterization of compounds used in this study and additional figures and details concerning the photolysis reactions with [^{32}S]**2** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org

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(39) Crystal structures of farnesyltransferase complexed with a benzophenone-containing farnesyl diphosphate analogue (footnote 24) and a geranylgeranyl diphosphate analogue (M. D. Distefano, unpublished results) demonstrate that the benzophenone unit in each case is within the isoprenoid binding site of the protein.