Prenylated Proteins. A Convenient Synthesis of Farnesyl Cysteinyl Thioethers

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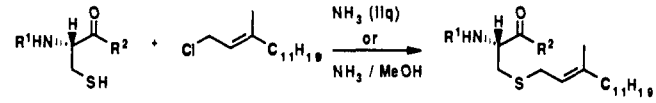
From recent discoveries it is abundantly clear that posttranslational modification of proteins with isoprene residues is an essential feature of several critical signal transduction systems in eukaryotic cells. The modifications occur at C-terminal cysteines and target the proteins to membranes.[1-5] C_{15} farnesyl moieties were first discovered in yeast a-factor mating hormones[6-8] and subsequently found in nuclear lamins,[9] *ras*-encoded proteins,[1,2,10,11] and the γ subunit of transducin, a retinal heterotrimeric G protein.[12] More recently, C_{20} geranylgeranyl units were reported in γ subunits of rat[13] and bovine[14] G proteins. Geranylgeranylated proteins also appear to be the major prenylated species in HeLa[15] and Chinese hamster ovary[16] cells. The consensus sequence for prenylation is ---Cys-Axx-Axx-Xxx (Axx, aliphatic amino acids; Xxx, general amino acids).[1,4,5,17] Additional processing involves proteolytic removal of Axx-Axx-Xxx to generate a C-terminal thioether and, in some instances, methylation of the cysteinyl carboxylate.[3,4,18] The *ras*-encoded proteins and heterotrimeric G proteins associate with the inner face of plasma membrane, where they form a biological link between membrane-spanning receptors and intracellular effectors. For human c-H-*ras*^{Val12} protein, inhibition of farnesylation results in cytosolic inactive protein.[1]

In conjunction with their pioneering studies on yeast mating factors, Kamiya et al. synthesized *S*-farnesylcysteine and prenylated peptides by alkylation of the sulfhydryl groups with farnesyl bromide in alcohol/water mixtures.[6,7,19] Recent discoveries of isoprene residues in signal transduction proteins have rekindled interest in the synthesis of cysteinyl thioethers.[11,14-16,18] The modest yields of purified material we obtained with the Kamiya procedure, especially when the substrates were expensive peptides, prompted us to explore other conditions for the alkylation of cysteine. This communication reports an improved synthesis of farnesyl cysteinyl thioethers.

A major problem in the prenylation of cysteine or peptides containing cysteinyl residues is the selection of a solvent that solubilizes the substrates without reacting with isoprenyl halides. We found liquid ammonia and 4 M ammonia in methanol to be suitable for small peptides and cysteine, respectively. In a typical reaction, 13 μmol of the pentapeptide Lys-Cys-Val-Leu-Ser (KCVLS)[20] was dissolved in 5 mL of liquid ammonia, and 23 μmol of farnesyl chloride[21] was added. The mixture was refluxed for 2 h, solvent was allowed to evaporate, and the residue was dissolved in 5:50:1 MeCN/H_2O/HOAc. The solution was washed with



cysteine	R^1 = H, R^2 = OH
cysteine methyl ester	R^1 = H, R^2 = OCH_3
KCVLS	R^1 = Lys, R^2 = Val-Leu-Ser

hexane, and solvent was removed at reduced pressure to give farnesylated KCVLS (>95%) as the sole peptide along with 1 equiv of ammonium chloride. For analytical purposes, residual salt was removed by HPLC on a Vydac C_{18} reversed-phase column, although the material obtained after evaporation of solvent was suitable for use as standards in biological assays. FABMS showed an M + 1 peak at m/z 753, expected for addition of a single farnesyl residue. Comparisons of ^1H and ^{13}C NMR[22] spectra with those of *S*-farnesylcysteine and *S*-farnesylcysteine methyl ester (see below) indicated that alkylation occurred at the sulfhydryl moiety.[23] The absence of ^{13}C peaks for diastereomers suggests that the reaction proceeded without racemization.

Although farnesylation of cysteine or cysteine methyl ester in liquid ammonia gave 40-60% yields, better results were obtained with an anhydrous 4 M solution of ammonia in methanol. In a standard experiment, 0.8 mmol of cysteine was allowed to react with 0.8 mmol of farnesyl chloride in 6 mL of ammonia/methanol at 0 °C for 3 h and 20 °C for 1 h. Solvent was removed at reduced pressure, and the residue was partitioned between 1-butanol and water. The butanol layer was dried over $MgSO_4$, and solvent was removed at reduced pressure. The residue was dissolved in methanol and washed with hexane. Evaporation of methanol gave a white waxy solid (R_f = 0.70; cellulose; 4:1 MeCN/H_2O), which was >95% pure as judged by HPLC (Vydac C_{18}; 50:50:0.1 MeCN/H_2O/TFA) and NMR spectroscopy, in 80% yield.[24] Cysteine methyl ester was farnesylated in 72% yield under similar conditions. In this case, the workup involved evaporation of solvent and partitioning of the residue into methyl *tert*-butyl ether (MTBE) and water. Chromatography of the extract on silica gel (R_f = 0.31, 0.1% triethylamine/MTBE) gave a pale yellow oil.[25]

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(21) Davisson, V. J.; Woodside, A. B.; Poulter, C. D. *Methods Enzymol.* **1985**, *110*, 130-144. We found that filtering a hexane solution of the product through a pad of silica gel removed colored impurities and gave better results in the farnesylation reaction.
(22) ^{13}C NMR (75 MHz, 2:2:1 $CD_3CN/D_2O/CD_3CO_2D$): δ 174.1, 173.0, 172.8, 172.2, 169.7, 141.1, 136.2, 131.8, 125.0, 124.6, 120.0, 62.3, 60.2, 55.6, 54.1, 53.7, 52.8, 40.9, 40.2, 40.1, 40.0, 33.2, 31.4, 31.3, 30.3, 27.2, 27.2, 26.9, 25.9, 25.3, 23.3, 22.2, 21.8, 19.4, 18.3, 17.8, 16.3, and 16.2 ppm.
(23) The C(1) farnesyl and β cysteinyl carbons were assigned to peaks at 30.3 ± 0.2 and 33.5 ± 0.2 ppm, respectively, in farnesylated cysteine and KCVLS. These assignments were based on a single frequency decoupling experiment with *S*-(dimethylallyl)cysteine, where irradiation at 2.73 ppm caused the ^{13}C multiplet at 33.5 ppm to collapse to a singlet, while the signal at 30.3 ppm narrowed but remained a triplet. *S*-(Dimethylallyl)cysteine was prepared in 4 M NH_3/CH_3OH as described for the farnesyl derivative: ^1H NMR (300 MHz, CD_3OD) δ 1.62 (s, 3 H), 1.66 (s, 3 H), 2.73 (dd, 1 H, J = 9.1, 14.6 Hz), 3.05 (dd, 1 H, J = 3.8, 14.6 Hz), 3.16 (m, 2 H), 3.58 (dd, 1 H, J = 3.8, 9.1 Hz), and 5.18 (m, 1 H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 171.2, 137.0, 121.1, 55.2, 33.5, 30.3, 26.5, and 25.8 ppm.
(24) Mp: 143-146 °C dec (lit.[6] mp 148-150 °C). ^{13}C NMR (75 MHz, CD_3OD): δ 172.5, 140.9, 136.2, 132.0, 125.3, 125.0, 121.0, 55.2, 40.9, 40.8, 33.6, 30.3, 27.8, 27.5, 26.0, 17.8, 16.3, and 16.2 ppm.

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Ammonia/methanol was also used as a solvent to farnesylate KCVLS; however, yields were lower and the peptide was less pure than for ammonia.

Du Vigneaud et al.[26] first described alkylation of cysteine in liquid ammonia. They generated the sodium thiolate by in situ reduction of cystine with sodium and then added electrophilic alkyl halides. We found cysteine to be readily alkylated by farnesyl chloride in ammonia by simply mixing the amino acid and the electrophile. The use of ammonia or methanol/ammonia[27] affords a mild regioselective procedure (note that KCVLS contains free amino, hydroxyl, and carboxylate moieties) for formation of farnesyl cysteinyl thioethers. We have used this procedure to construct farnesylated peptides needed for assays of protein prenyltransferase activity in yeast[5] and as authentic samples for structure determinations of prenylated proteins.[16] We are currently extending the study to other isoprene units and peptides.

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(25) ^{13}C NMR (75, MHz, $CDCl_3$): δ 168.4, 140.8, 135.3, 131.2, 124.2, 123.5, 118.7, 53.5, 52.4, 39.7, 39.7, 31.2, 29.5, 26.8, 26.5, 25.8, 17.8, 16.2, and 16.1 ppm.

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Efficient Method for Regioselective Isoprenylation of Cysteine Thiols in Unprotected Peptides

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Isoprenylation, a recently discovered posttranslational modification of proteins,[1] has been found to be crucial for both membrane association and transforming activity of oncogenic *ras* proteins[2-4] in which the isoprene unit is located at the C-terminal cysteine.[5,6] The isoprene moiety on yeast a-factor mating hormone was identified unambiguously as the C_{15} farnesyl group by ^1H NMR spectroscopy and mass spectrometry,[7] and more recently, proteins of HeLa cells were reported to be geranylgeranylated (C_{20} unit).[8]

The chemical synthesis of farnesylated peptides by alkylation of the sulhydryl group with farnesyl bromide in 50% DMF in the presence of MgO has been documented in the literature;[9,10] however, we have had little success in preparing various isoprenylated peptides by the reported procedure. In this communication, we describe an improved and efficient method for regioselective isoprenylation of cysteine thiols in unprotected peptides.

Scheme I

2	R^1 = H	R^2 = OCH_3	**2_p**	R^1_p = H	R^2_p = OCH_3
3	R^1 = dansylWDPA	R^2 = VIA	**3_p**	R^1_p = dansylWDPA	R^2_p = VIA
4	R^1 = YIIKGVFWDPA	R^2 = VIA	**4_p**	R^1_p = YIIKGVFWDPA	R^2_p = VIA
5	R^1 = DESGPGSMSSK	R^2 = VLS	**5_p**	R^1_p = DESGPGSMSSK	R^2_p = VLS

The model system, cysteine methyl ester (**2**) containing an amino and a thiol nucleophile, in acetonitrile (ACN) and dimethylformamide (DMF) was treated with 4 equiv of farnesyl bromide[11] (**1**) (Scheme I) at 0 °C in the presence of diisopropylethylamine (DIEA). The resulting S-farnesylated cysteine methyl ester ($\mathbf{2}_p$) (Scheme I), obtained in 90% yield, appeared as a single regioisomer by ^1H NMR analysis,[12] displaying the reported chemical shift for the C-1 allylic protons at 3.2 ppm.[3] In addition, a positive quantitative ninhydrin result supports the structure of $\mathbf{2}_p$. The farnesylation reaction was found to be very dependent on the quality of the farnesyl bromide,[13] which is sensitive to decomposition upon silica gel purification and, therefore, was used directly without further purification.

Successful mono- and regioselective farnesylation of unprotected peptides with farnesyl bromide depends on the solvent system, temperature, pH, amount of the farnesyl bromide, and peptide concentration. Among various solvents,[14] we have found that use of a mixed solvent system, DMSO/DMF/ACN, provided the maximal solubility for most peptides. Although several competing nucleophiles are present in the unprotected peptide, such as carboxylate anion, free amino group, phenolic anion, etc., we have found that farnesyl bromide reacts exclusively with the sulfhydryl anion of the unprotected peptide at pH 10-12 at 0 °C to afford the desired S-alkylated adduct. The pH of the reaction mixture was maintained by addition of 6-9 equiv of DIEA (measured on moist pH paper). Three to four equivalents of farnesyl bromide with 0.01-0.02 M concentration of the peptide was found to represent optimal conditions. Use of greater amounts of farnesyl bromide (>10 equiv) and higher peptide concentrations (>0.06 M) often generate multifarnesylated adducts. The formation of the disulfide dimer was significantly suppressed by degassing of the reaction solution followed by sparging with argon.

In a typical reaction, the peptide (dansyl)WDPACVIA[15] (**3**) (Scheme I) was treated with 3 equiv of farnesyl bromide in the mixed solvent DMSO/DMF/ACN (3:3:1) at a concentration of 0.01 M in the presence of 8 equiv of DIEA at 0 °C under an argon atmosphere for 3 h, to afford the desired *S*-farnesyl regioisomer (dansyl)WDPAC(far)VIA ($\mathbf{3}_p$) (Scheme I), in 86% yield. The reaction mixture was quenched with acetic acid at 0 °C to bring the pH to around 4 and then lyophilized followed by reversed-phase (C_{18}) HPLC purification. The reaction is monitored by reversed-phase (C_{18}) HPLC with photodiode array detection[16] from which the characteristic UV spectrum of each individual peak in the chromatogram is used to determine the progress of the reaction and to identify the mono- and multifarnesylated adducts. The structure of peptide $\mathbf{3}_p$ was characterized by amino acid analysis

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