Articles

Identification of Prenylcysteines and Prenylated Proteins by Formation of Substituted Naphthopyrans

William W. Epstein,* Zhaolin Wang, Lilian M. Leining, and David C. Lever

Department of Chemistry, University of Utah, Salt Lake City, Utah 84112

Received February 22, 1996[⊗]

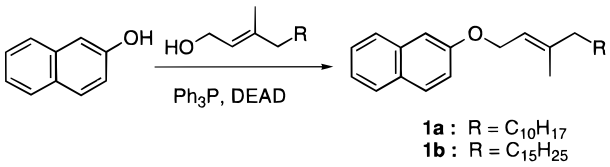
Posttranslational modification by prenylation is important in the activation of a diverse group of proteins. The C_{15} and C_{20} prenylated proteins have been identified by metabolic labeling with radioisotopic mevalonate, but many organisms do not incorporate this precursor readily. We have shown that the thioether bond of prenylated proteins can be cleaved by 2-naphthoxide to yield substituted naphthopyrans **2b** and **2c**. These products are readily resolved by HPLC due to their strongly absorbing chromophore. Thus, this reaction is suitable for qualitative identification of prenylated proteins. The results of model studies and a proposed reaction mechanism for the formation of **2b** and **2c** are discussed.

Introduction

Posttranslational modification by prenylation is important in the activation of a diverse group of proteins.[1,2] These proteins contain farnesyl or geranylgeranyl groups covalently bonded via a thioether linkage to cysteines in the carboxyl terminal region. This modification is common in a variety of proteins.[3] The prenylated proteins have usually been identified by metabolic labeling with radioisotopic mevalonate.[4] Metabolic labeling is limiting because the relative amount of prenyl modification observed depends to some extent on the mevalonate available to the cells and many organisms such as yeast and plants do not incorporate this precursor readily. Acid hydrolysis, a standard procedure for amino acid analysis, cannot be used for proteins bearing prenyl groups because of the instability of the isoprenoid double bonds to strong acid. Alkaline hydrolysis followed by cleavage of the prenyl groups from the cysteine residues with iodomethane[5] or Raney nickel[6] and subsequent mass spectral analysis of the cleaved products has been the method of choice but the sensitivity of radioisotopic labeling is required.

In prenylated proteins the allylic thioether bond is unique. As part of our studies we were interested in removing prenyl groups from proteins by nucleophilic cleavage. A number of nucleophiles were considered, but 2-naphthoxide was found to be the best.[7] In this paper the identification of the products, prenyl-substituted naphthopyrans, are reported as well as the results of model studies.

Scheme 1



1a : R = $C_{10}H_{17}$
1b : R = $C_{15}H_{25}$

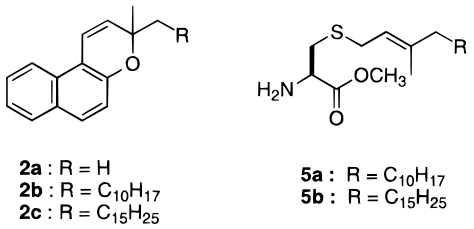
Results and Discussion

Initially the anticipated products from cellular materials with naphthoxide were 2-naphthyl prenyl ethers **1a**,**b** from a simple S_N2 reaction. Thus we required standard samples for HPLC coinjection studies. The farnesyl and geranylgeranyl naphthyl ethers were synthesized using a modified Mitsunobu method[8] as shown in Scheme 1. When protein from CHO (Chinese hamster ovary) cells were reacted with potassium naphthoxide in dioxane at 100 °C, an unexpected product was formed different by HPLC coinjection from the synthetic prenyl naphthyl ethers. Although the amount of product generated from CHO cells with potassium naphthoxide was insufficient to get NMR spectral data for structure determination, a UV spectrum of this product showed a significant shift to higher wavelength (360 nm) when compared to the standard farnesyl (**1a**) and geranylgeranyl (**1b**) naphthyl ethers (330 nm). This indicated an extension of the conjugated system. A literature search revealed that dimethylchroman[9] (**2a**) (Figure 1) has the same UV spectrum as our unknown compound, suggesting that it could be a prenyl-substituted chroman. The synthesis of appropriate chroman systems was carried out in two steps as shown in Scheme 2. Treatment of 2-naphthol with sodium metal in ether followed by addition of the prenyl halides **3a**,**b** gave carbon-alkylated[10] prenyl naphthols **4a**,**b**, which were then oxidatively cyclized with DDQ in refluxing benzene to yield naphthopyrans **2b**,**c**. With the synthetic naphthopyrans **2b** and **2c** in hand,

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1996.

(1) Clarke, S. *Annu. Rev. Biochem.* **1992**, *61*, 355.
(2) Casey, P. J. *J. Lipid Res.* **1992**, *33*, 1731.
(3) Schafer, W. R.; Rine, J. *Annu. Rev. Genet.* **1992**, *30*, 209.
(4) Farnsworth, C, C.; Gelb, M. H.; Glomset, J. A. *Science* **1990**, *247*, 320.
(5) Anderegg, R. J.; Betz, R.; Carr, S. A.; Crabb, J. W.; Duntze, W. *J. Biol. Chem.* **1988**, *263*, 18236.
(6) Rilling, H. C.; Bruenger, E.; Epstein, W. W.; Crain, P. F. *Science* **1990**, *247*, 318.
(7) Epstein, W. W.; Lever, D. C.; Leining, L. M.; Bruenger, E.; Rilling, H. C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 9668.
(8) Mitsunobu, O. *Synthesis* **1981**, 1.
(9) Buckle, D. R.; Waight, E. S. *J. Chem. Soc., Chem. Commun.* **1969**, 922. Iwai, I.; Ide, J. *Chem. Pharm. Bull. Jpn.* **1962**, *10*, 926.
(10) Yamada, S.; Takeshita, T.; Tanka, J. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 2901.

S0022-3263(96)00375-1 CCC: $12.00

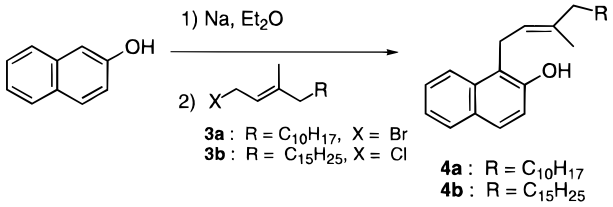


2a : R = H
2b : R = $C_{10}H_{17}$
2c : R = $C_{15}H_{25}$

5a : R = $C_{10}H_{17}$
5b : R = $C_{15}H_{25}$

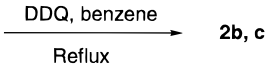
Figure 1.

Scheme 2

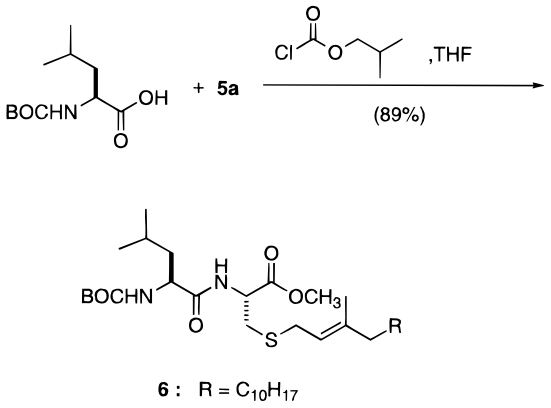


3a : R = $C_{10}H_{17}$, X = Br
3b : R = $C_{15}H_{25}$, X = Cl

4a : R = $C_{10}H_{17}$
4b : R = $C_{15}H_{25}$



Scheme 3



6 : R = $C_{10}H_{17}$

we were able to show by HPLC coinjection that the products formed in the reaction of CHO cells with potassium naphthoxide were indeed identical to the synthetic materials.

Model studies using C_{15} and C_{20} prenylated cysteines **5a**,**b** as well as dipeptide **6** were undertaken. The prenylated cysteines **5a**,**b** (Figure 1) required for the synthesis were prepared from L-cysteine methyl ester and farnesyl chloride or geranylgeranyl chloride.[11] The method of isobutylcarbonic acid mixed anhydrides[12] was then used to prepare the prenylcysteine-containing dipeptide **6** as outlined in Scheme 3. Treatment of these model compounds **5b** and **6** with potassium naphthoxide (**7**) in dioxane under identical conditions[7] to those used for CHO cell cleavage gave prenyl-substituted naphthopyrans **2c** and **2b** as shown in Scheme 4. To further test this method, we treated a tissue sample of mouse brain which is known to contain farnesylated and geranylgeranylated proteins with potassium naphthoxide. Isolation and purification of the cleavage products by HPLC and HRMS analysis confirmed that geranyl (**2b**) and farnesyl (**2c**) naphtholpyrans were formed. In addition, a control experiment using the nonprenylated protein, albumin

Scheme 4

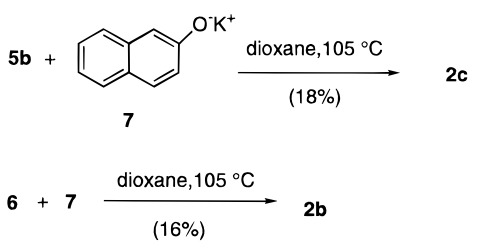
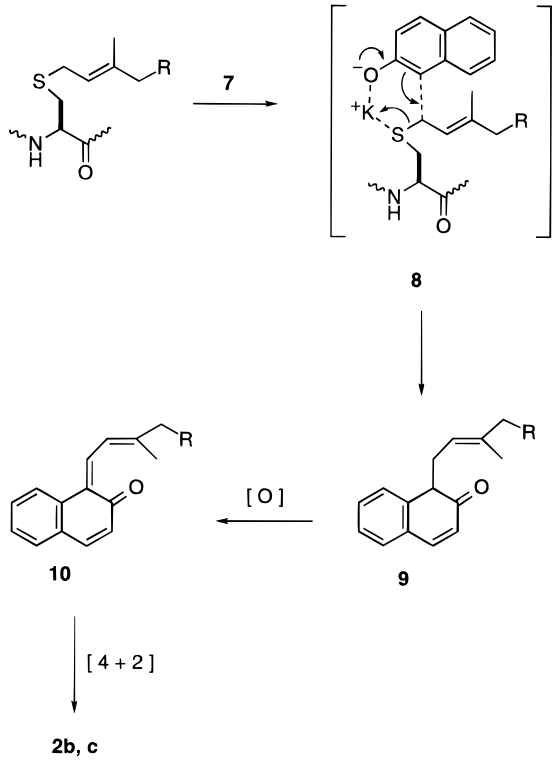


Table 1. Influence of Oxygen on the Formation of Naphthopyrans

entry	compd	conditions	yield (%)
1	**1a**	air	0 (**2b**)
2	**4a**	air	32 (**2b**)
3	**5b**	argon	4 (**2c**)
4	**5b**	air	18 (**2c**)
5	**3b**	argon	10 (**2c**)
6	**3b**	air	43 (**2c**)

Scheme 5



(bovine, fraction V powder), was run and no prenylsubstituted naphthopyrans were found.

We allowed both farnesyl-2-naphthol (**4a**) and farnesyl naphthyl ether (**1a**) to react with potassium naphthoxide under the conditions of cleavage and found that only farnesyl -2-naphthol (**4a**) can be converted to the naphthopyran product (entries 1 and 2, Table 1). Thus the reaction of the prenylated cysteine with naphthoxide first requires C-allylation and not O-allylation to give the cleaved product. The effect of the counterion was also studied. The reaction is dependent on the size of the cation since the small ions Li^+ and Na^+ gave little or no product while K^+ and Cs^+ worked well. These facts led us to propose an intermediate such as **8** shown in Scheme 5 where cation size might be an important factor in the success of the reaction. The dehydrogenation step required to form **2b** or **2c** probably occurs after allylation since treatment of **4a** or **b** with DDQ in refluxing benzene gave prenylnaphtholpyran **2b** or **2c** (Scheme 2). In order

(11) Brown, M. J.; Milano, P. D.; Lever, D. C.; Epstein, W. W.; Poulter, C. D. *J. Am. Chem. Soc.* **1991**, *113*, 3176.
(12) Vaughan, J. R. Jr.; Osato, R. L. *J. Am. Chem. Soc.* **1952**, *74*, 676.

to test this possiblity, we treated geranylgeranyl cysteine (**5b**) and geranylgeranyl chloride (**3b**) with potassium naphthoxide under both oxidative and nonoxiditative conditions. The results are summarized in Table 1, where it can be seen that the presence of air increases the yield of the prenylnaphthopyran. Thus the reaction might proceed by C-allylation of the naphthoxide nucleophile via **8**. The allylated product **9** could then be dehydrogenated to produce the quinone methide system **10**, which is known to undergo a 4 + 2 cycloaddition to form pyrans[13] as outlined in Scheme 5.

Naphthoxide cleavage of prenylcysteines has enabled us to examine different tissues for the presence of this modification. Previous results clearly indicated that prenylation of proteins is very nearly universal.[7] Recently, we treated a trichloroacetic acid precipitated *Escherichia coli* sample with potassium naphthoxide and found small amounts of geranylnaphthopyran (**2b**) by HPLC and identified by mass spectroscopy, indicating that something is farnesylated in *E. coli*. In the same way we found small amounts of farnesylnaphthopyran (**2c**) from the reaction of trichloroacetic acid precipitated human serum with potassium naphthoxide, indicating that human serum may contain geranylgeranylated proteins.

In conclusion, we have demonstrated that naphthoxide cleavage provides a method for determination of prenylated proteins. This technique has a number of distinct advantages in spite of the low yields. The naphthopyrans that are formed are readily isolated and quantified by HPLC. The extinction coefficients of the naphthopyrans (66 070 and 4450 M^{-1} cm^{-1} at 246 and 360 nm, respectively) are large enough for reasonably sensitive analysis. Since naphthopyrans are fluorescent, an even more sensitive method should become available to examine different tissues for the presence of this protein modification. The tissue to be analyzed does not need to be labeled with mevalonate, and this has made it possible to survey organisms, such as bacteria, fungi, and plants, which do not incorporate this metabolite readily.

Experimental Section

General. Commercial reagents were used without further purification. Dioxane and THF were distilled from sodium or potassium immediately before use. Column chromatography was performed with EM SCIENCE silica gel (230−400 mesh) using hexanes:ethyl acetate as eluent. CHO (Chinese hamster ovary) cells were kindly furnished by Genentech. Mouse brain acetone power, albumin (bovine, fraction V powder 96-99%), *E. coli* (lyophilized cells of strain B), and human serum (type AB) were from Sigma. All tissue samples except albumin were precipitated by addition of 20% trichloroacetic acid to a final concentration of 10%. The solutions were kept at 0 °C for 1 h and then delipidated by extraction with ethanol and diethyl ether.

The potassium salt of 2-naphthol was prepared by dissolving KOH in ethanol and adding a molar equivalent of 2-naphthol. Most of the ethanol was removed under vacuum and the product crystallized on the addition of toluene. Lithium, sodium, and cesium naphthoxides were prepared in the same way from their hydroxides and 2-naphthol.

(2*E*,6*E*)-2-Naphthyl-3,7,11-trimethyl 2,6,10-Dodecatrienyl Ether (1a). To a stirred solution of 116 mg (0.50 mmol) of farnesol, 80 mg (0.55 mmol) of 2-naphthol, and 159 mg (0.60 mmol) of Ph_3P in 10 mL of hexanes was added a solution of 104 mg (0.60 mmol) of DEAD in 5 mL of hexanes dropwise over 30 min. The reaction mixture was stirred for 3 h, filtered through a plug of Florisil, and concentrated. The residue was chromatographed on silica gel using 3:97 EtOAc/hexanes to give 89 mg (51%) of **1a**: ^{1}HMR (300 MHz, $CDCl_3$) δ 7.70−7.77 (m, 3H), 7.15−7.45 (m, 4H), 5.57 (bt, 1H), 5.05−5.15 (m, 2H), 4.65 (d, J = 6.6 Hz, 2H), 1.9−2.2 (m, 8H), 1.78 (s, 3H) 1.68 (s, 3H), 1.60 (s, 3H); ^{13}C NMR (75MHz, $CDCl_3$) δ 156.58, 141.13, 135.23, 134.33, 131.09, 129.10, 128.69, 127.41, 126.50, 126.07, 124.12, 123.48, 123.29, 119.21, 118.94, 106.59, 64.78, 39.63, 39.53, 26.68, 26.02, 25.66, 17.67, 16.71, 16.01. Anal. Calcd for $C_{25}H_{32}O$: C, 86.15; H, 9.25. Found: C, 86.11; H, 9.27.

(2*E*,6*E*,10*E*)-2-Naphthyl-3,7,11,15-tetramethyl 2,6,10,14-Hexadecatetraenyl Ether (1b). To a stirred solution of 232 mg (0.80 mmol) of geranylgeraniol, 125 mg (0.87 mmol) of 2-naphthol, and 250 mg (0.95 mmol) of Ph_3P in 15 mL of hexanes was added a solution of 165 mg (0.95 mmol) of DEAD in 10 mL of hexanes as before to yield 111 mg of **1b** (34%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70−7.76 (m, 3H), 7.15−7.45 (m, 4H), 5.58 (bt, 1H), 5.05−5.20 (m, 3H), 4.60−4.66 (m, 2H), 2.25−1.90 (m, 12H), 1.83 (br, 3H), 1.67 (bs, 3H), 1.62 (bs, 3H), 1.58 (bs, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.76, 141.23, 135.38, 134.85, 134.49, 131.16, 129.24, 128.85, 127.56, 126.65, 126.20, 124.34, 124.14, 123.64, 123.42, 119.37, 119.08, 106.70, 64.85, 39.75, 39.70, 39.61, 26.80, 26.65, 26.28, 25.73, 17.72, 16.76, 16.08, 16.05. Anal. Calcd for $C_{30}H_{40}O$: C, 86.48; H, 9.68. Found: C, 86.34; H, 9.66.

1-(3,7,11-Trimethyl-2,6,10-dodecatrienyl)-2-naphthol (4a). To a solution of 355 mg (2.46 mmol) of 2-naphthol in 5 mL of Et_2O was added 56 mg (2.44 mmol) of sodium metal with stirring. The reaction mixture was stirred for 3 h, and a solution of 700 mg (2.45 mmol) of farnesyl bromide in 5 ml of Et_2O was added. The reaction mixture was refluxed for 2 h, the reaction was quenched with 10 mL of a 0.1 N HCl solution, and the solution was extracted with Et_2O (2 × 10 mL). The combined extracts were dried over $MgSO_4$ and concentracted to give 1.1 g of brown liquid, which was used without further purification. An analytical sample was obtained by chromatography with 5:95 EtOAC/hexanes on silica gel to give an orange oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.92 (d, J = 8.5 Hz, 1H), 7.78 (d, J = 8.3 Hz, 1H), 7.64 (d, J = 8.5 Hz, 1H), 7.48 (t, J = 7.7 Hz, 1H), 7.33 (t, J = 7.5 Hz, 1H), 7.09 (d, J = 8.8 Hz, 1H), 5.48 (s, 1H), 5.28 (br t, 1H), 5.07 (br t, 2H), 3.79 (d, J = 6.6 Hz, 2H), 1.95−2.2 (m, 8H), 1.92 (s, 3H), 1.68 (s, 3H), 1.59 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.23, 137.58, 135.24, 132.97, 131.12, 129.26, 128.45, 127.75, 126.17, 124.24, 123.59, 122.87, 121.96, 118.55, 118.03, 39.70, 39.67, 26.74, 26.48, 25.78, 24.43, 17.79, 16.55, 16.13; MS (EI) *m*/*z* (rel intensity) 348 (M^+ 46), 212 (10), 195 (41), 157 (100), 81 (25), 69 (63), 41 (34); HRMS (EI) calcd for $C_{25}H_{32}O$ 348.2453, found 348.2455.

3-Methyl-3-[(3*E*)-4,8-dimethyl-3,7-nonadienyl]-3*H*-naphtho[2,1-b]pyran (2b) (Method 1). To a stirred solution of 500 mg (1.43 mmol) of 1-farnesyl-2-naphthol (**4a**) in 30 mL of benzene was added 340 mg (1.50 mmol) of DDQ, and the mixture was refluxed for 4 h and filtered through Celite. The filtrate was chromatographed on silica gel with 5:95 EtOAC/hexanes to give 227 mg (46%) of **2b** as a colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.94 (d, J = 8.4 Hz, 1H), 7.73 (d, J = 8.4 Hz, 1H), 7.66 (d, J = 8.7 Hz, 1H), 7.46 (t, J = 7.7 Hz, 1H), 7.31 (t, J = 7.5 Hz, 1H), 7.05 (d, J = 9.2 Hz, 2H), 5.69 (d, J = 9.9 Hz, 1H), 5.14−5.04 (m, 2H), 1.7−2.2 (m, 8H), 1.66 (d, J = 1.2 Hz, 3H), 1.57 (d, J = 1.8 Hz, 6H), 1.45 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.38, 135.57, 131.56, 130.05, 129.45, 129.27, 128.71, 128.67, 126.64, 124.53, 124.15, 123.44, 121.36, 118.85, 118.61, 113.69, 78.61, 41.03, 39.89, 26.88, 26.27, 25.91, 22.88, 17.90, 16.19. Anal. Calcd for $C_{25}H_{30}O$: C, 86.66; H, 8.73. Found: C, 86.53; H, 8.68.

2b (Method 2). A solution of **6** (160 mg, 0.28 mmol) and potassium naphthoxide (153 mg, 0.84 mmol) in 0.4 mL of dioxane was heated in a sealed tube at 105 °C for 14 h. The reaction mixture was cooled, diluted with CH_2Cl_2, and chromatographed with 1: 99 EtOAc:hexanes to yield 15 mg of **2b** (16%). Spectral characteristics were identical to those reported above.

3-Methyl-3-[(3*E*,7*E*)-4,8,12-trimethyl-3,7,11-tridecatrienyl]-3*H*-naphtho[2,1-*b*]pyran (2c) (Method 1). To a solution of 703 mg (1.7 mmol) of 1-(geranylgeranyl)-2-naphthol (**4b**) in 40 mL of benzene was added 384 mg (1.7 mmol) of DDQ.

(13) Desimoni, G.; Tacconi, G. *Chem. Rev.* **1975**, *75*, 651.

The reaction mixture was refluxed with stirring for 4 h and filtered through Celite, and the solvent was removed to yield 583 mg (83%) of a dark oil which was chromatographed with difficulty on silica gel using 1:99 EtOAc/hexanes to yield 110 mg (16%) of colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (d, J = 8.4 Hz, 1H), 7.73 (d, J = 8.1 Hz, 1H), 7.63 (d, J = 8.7 Hz, 1H), 7.46 (t, J = 7.7 Hz, 1H), 7.31 (t, J = 7.4 Hz, 1H), 7.05 (d, J = 9.7 Hz, 2H), 5.68 (d, J = 10.2 Hz, 1H), 5.09 (m, 3H), 1.8−2.3 (m, 12H), 1.68 (s, 3H), 1.59−1.61 (s, 9H), 1.45 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.10, 135.26, 134.86, 131.16, 129.78, 129.18, 129.00, 128.43,128.34, 126.35, 124.34, 124.13, 123.91, 123.15, 121.08,118.58, 118.33, 113.40, 78.35, 40.87, 39.73, 39.68, 26.78, 26.66, 26.59, 26.09, 25.74, 22.70, 17.73, 16.03. Anal. Calcd for $C_{30}H_{38}O$: C, 86.90; H, 9.24. Found: C, 86.68; H, 9.29.

2c (Method 2). A solution of **5b** (110 mg, 0.27 mmol) and 147 mg (0.809 mmol) of potassium naphthoxide in 0.4 mL of dioxane was heated in a sealed tube at 105 °C for 14 h. The reaction mixture was cooled, diluted with CH_2Cl_2 (10 mL) and chromatographed with 1:99 EtOAC/hexanes to yield 21 mg (18%) of **2c** as a slightly yellow oil. Spectral characteristics are identical to those reported above.

Boc-Leu-*S*-farnesyl-Cys-OCH$_3$ (6). A solution of *N*-methylmorpholine (45 mg, 0.44 mmol) in THF (2.0 mL), isobutylchloroformate (60 mg, 0.44 mmol) in THF (1.0 mL), and Boc-Leu-OH (110 mg, 0.44 mmol) in THF (2.0 mL) at −20 °C was allowed to stand 5 min, and a mixture of **5a** (100 mg, 0.30 mmol) and *N*-methylmorpholine (30 mg, 0.30 mmol) in THF (1.5 mL) was added. The solution was stirred for 3 h at −20 °C. The reaction mixture was treated with 5% $NaHCO_3$ (3 mL) and allowed to stand for 15 min at room temperature, and EtOAc (60 mL) was added. The organic phase was washed successively with 5% $NaHCO_3$ (2 × 20 mL), 1 M HCl (2 × 20 mL), and H_2O. The organic phase was dried (Na_2SO_4) and filtered, and the evaporated residue was chromatographed with 20:80 EtOAc/hexanes to give 147 mg(89%) of **6**: $[a]^{20}_D$ −20.99° (*c* 4.0, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.88 (m, 1H), 5.20 (m, 1H), 5.09 (m, 1H), 4.97 (m, 1H), 4.76 (m, 1H), 3.76 (s, 3H), 3.16 (m, 2H), 2.90 (m, 2H), 2.05 (m, 8H), 1.68 (d, J = 0.9 Hz, 3H), 1.67 (d, J = 1.2 Hz, 3H), 1.60 (s, 6H), 1.45 (s, 9H), 0.95 (dd, J = 6.6 Hz , 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.6, 171.3, 155.7, 140.1, 135.5, 131.4, 124.4, 123.9, 119.7, 80.0, 53.2, 52.7, 51.8, 41.4, 39.8, 39.7,33.4, 30.0, 28.4, 26.8, 26.6, 25.9, 24.8, 23.1, 22.1, 17.8, 16.3, 16.2. Anal. Calcd for $C_{30}H_{52}N_2O_5S$: C, 65.18; H, 9.48; N, 5.07. Found: C, 65.02; H, 9.50; N, 5.06.

Acknowledgment. We thank Dr. Elliot Rachlin (Chemistry Department, University of Utah) for mass spectral analysis. The mass spectrometry facility is funded by NSF Grant CHE-9002690 and the University of Utah Institutional Funds Committee. This research was supported by National Institutes of Health Grant DK 43055.

Supporting Information Available: ^{1}H NMR spectra for compound **4a** (1 page). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from ACS; see any current masthead page for ordering information.

JO960375W