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Reversed Approach to *S*-Farnesylation and *S*-Palmitoylation: Application to an Efficient Synthesis of the C-Terminus of Lipidated Human N-Ras Hexapeptide

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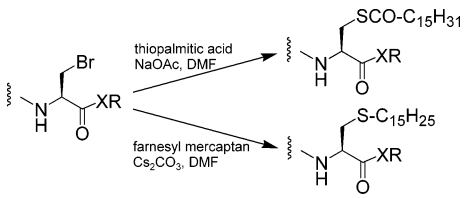

A general reversed approach is described to synthesize *S*-palmitoylated and *S*-farnesylated peptides via S_N2 displacement of bromide by reaction of a thiol group containing lipid as nucleophile with bromoalanine-containing peptides as electrophile. By employing this approach, lipidated peptides, including characteristic partial structures of human Ras peptides, were synthesized in good yields. This method gives access to farnesylated, palmitoylated, and doubly lipidated peptides.

The biological activities of several cellular proteins require association with the inner surface of the plasma membrane, and this membrane localization is dependent on the post-translational attachment of lipid residues such as farnesyl, myristoyl, and palmitoyl moieties to the carboxy terminus of the protein. Covalently modified proteins (for instance, by lipid attachment) play important roles in various biological processes,[1] as signal transduction and cell-cycle control. Among the different types of covalently modified proteins, lipidation is particularly relevant.[2] For example, the transmembrane G proteins are *S*-palmitoylated, the heterotrimeric G proteins are *N*-myristoylated, *S*-palmitoylated, and *S*-farnesylated, and most of the Ras proteins carry *S*-palmitoyl and *S*-farnesyl groups. Further, membrane-associated proteins such as the enzyme NO_x synthetase[3] and various viral envelope proteins are *S*-palmitoylated. The lipid groups are believed to be involved in protein−protein and protein−lipid interactions, and they serve as anchors of the proteins to different membranes.

The wide interest in Ras proteins is largely based on the existence of various Ras oncogenes.[4] It is estimated that oncogenic Ras proteins are present in about 30% of all human tumors.[5] The Ras proteins have both farnesyl thioether and palmitic acid thioester moieties. Protein farnesylation is a stable and irreversible protein modification that plays a critical role in directing the modified protein to the plasma membranes.[2] Unlike farnesylation, palmitoylation is a reversible reaction suggesting that it may be particularly important for regulating protein function.[6] This palmitoylation is required for transformation of cells by H-Ras.

The synthesis of peptide conjugates such as the characteristic C-terminal lipidated hexapeptide of the human N-Ras protein is more complicated due to its pronounced acid and base lability.[7] The pioneering work of Waldmann et al. enabled the synthesis of substantial amounts of such types of peptide conjugates through multistep solution-phase[7] as well as solid-phase method.[8] Recently, van der Donk et al. also synthesized fluorescent farnesylated Ras peptide.[9] In continuation of our work to synthesize peptide conjugates, particularly thio glycopeptides,[10] we became interested in developing a general method to synthesize *S*-farnesylated and *S*-palmitoylated peptides from bromoalanine (BrAla) containing peptides. Herein, we report on this reversed approach to the *S*-farnesylation and *S*-palmitoylation and exploitation of this method to the synthesis of the C-terminus of human N-Ras hexapeptide.

Attachment of lipid moieties such as the palmitoyl or the farnesyl group to the cysteine is generally performed by reacting the sulfhydryl group of cysteine with excess palmitoyl chloride[7] and excess farnesyl bromide under basic conditions.[11] The method reported in this paper is opposite to these literature methods. Reaction of nucleophilic thiopalmitic acid or farnesyl mercaptan with electrophilic bromoalanine containing peptide segments under basic conditions should give the corresponding *S*-palmitoyl and *S*-farnesyl peptides (Scheme 1). For the present investigation, the required starting material thiopalmitic acid **2** was prepared by the reaction of *N*-succinimide ester **1** with H_2S in the presence of Et_3N in dioxane (Scheme 2).

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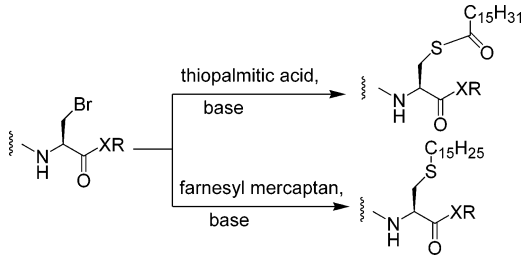
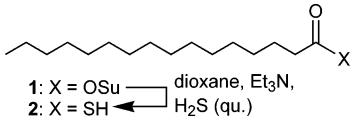
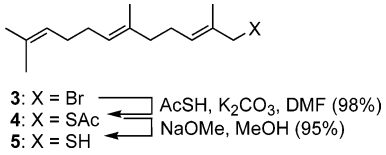
SCHEME 1. Reversed Approach To Synthesize *S*-Palmitoyl and *S*-Farnesylated Cysteine and Lipidated Peptides



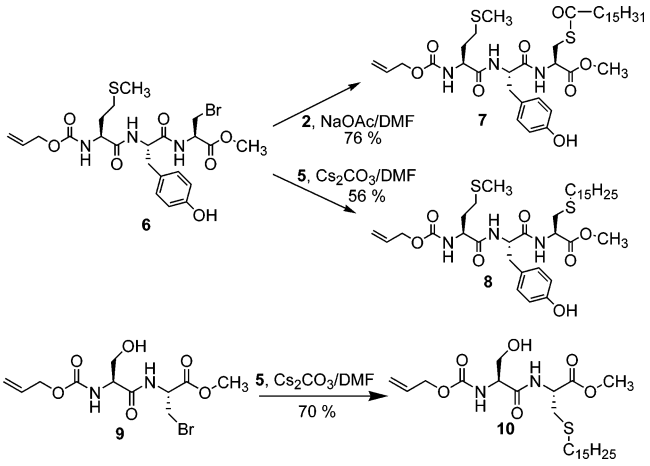
SCHEME 2. Synthesis of Thiopalmitic Acid 2



SCHEME 3. Synthesis of Farnesyl Mercaptan 5



SCHEME 4. Synthesis of *S*-Farnesylated Di- and Tripeptides 7, 8, and 10



Farnesyl mercaptan **5** was prepared by modifying the reported method.[12] We first reacted farnesyl bromide with thioacetic acid in the presence of K_2CO_3 in DMF followed by deacetylation in the presence of catalytic amounts of NaOMe in methanol yielding farnesyl mercaptan **5**, which could be purified by column chromatography (Scheme 3, 93% overall yield).

As a model substrate, a tripeptide AlocMetTyr-BrAlaOMe **6** was synthesized by coupling dipeptide AlocMetTyrOH with bromoalanine methyl ester (BrAlaOMe)[13,14] in the presence of EEDQ in dry dichloromethane (Scheme 4). Reaction of thiopalmitic acid with the tripeptide **6** in the presence of 1.1 equiv of NaOAc in DMF afforded the corresponding *S*-palmitoylated product

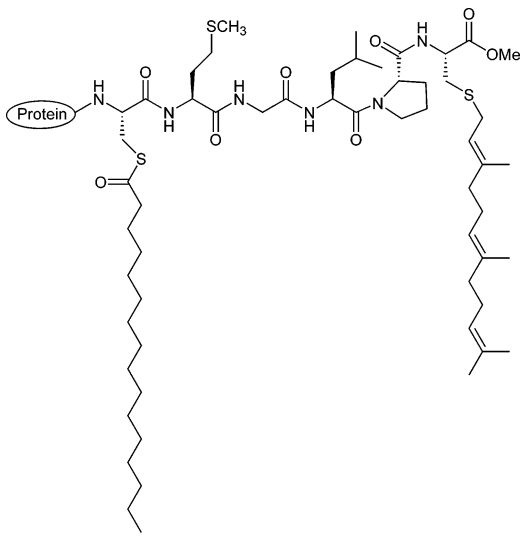
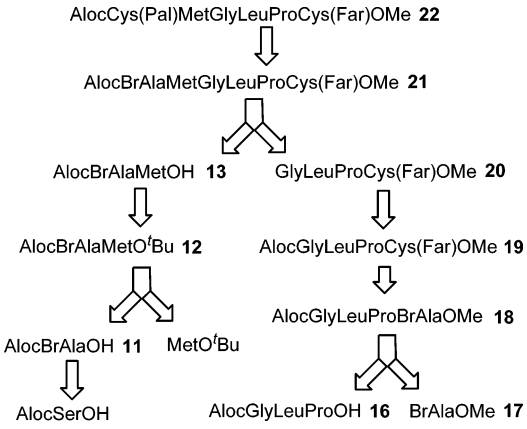


FIGURE 1. Structure of the C-terminus of human *N*-Ras protein.

SCHEME 5. Retrosynthetic Analysis of the C-Terminal Hexapeptide 22



7 in 76% yield. Reaction of the same tripeptide with farnesyl mercaptan **5** in the presence of Cs_2CO_3 as base in DMF gave the corresponding *S*-farnesylated product **8** in 56% yield. In a similar manner, reaction of dipeptide AlocSerBrAlaOMe **9** with farnesyl mercaptan **5** provided the *S*-farnesylated product **10** in 70% yield.

A biologically relevant target compound, the *S*-farnesylated, *S*-palmitoylated C-terminus of human N-Ras hexapeptide was chosen in order to demonstrate the feasibility of the present reaction conditions (Figure 1). The retrosynthetic pathway is depicted in Scheme 5. The target compound **22**[7a] should be available from the S-farnesylated bromoalanine containing hexapeptide **21** which was disconnected into intermediates **13** and **20**.

N-Aloc-protected bromoalanine **11** was synthesized in one pot by (i) heating of *N*-Aloc protected serine with 2.5 equiv of TMSCl in dry THF (ii) followed by the addition of CBr_4 and PPh_3 at 0 °C (Scheme 6). The direct conversion of the hydroxy group of serine to bromide,

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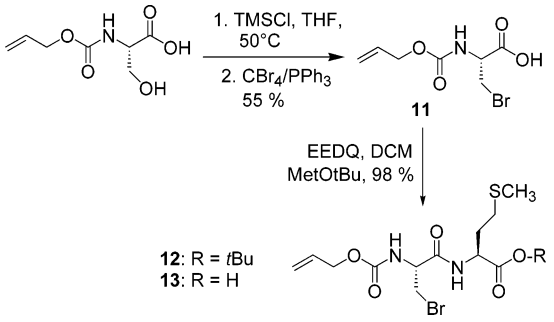
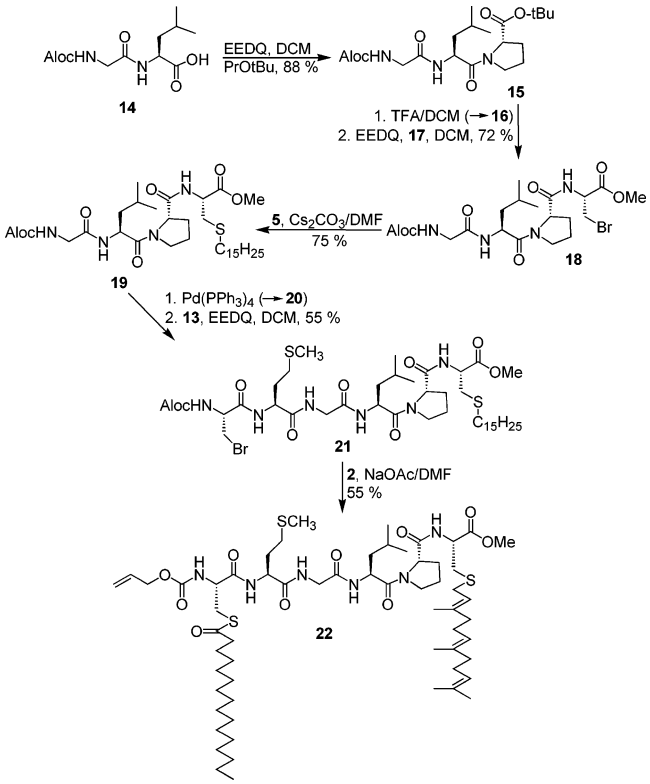
(14) To a stirred solution of *N*-Boc BrAlaOMe (282 mg, 1 mmol) in DCM (4 mL) was added TFA (1 mL) at 0 °C. The reaction mixture was stirred at room temp. for 1 h. Evaporation of the solvent and coevaporation with toluene yielded the corresponding TFA salt of BrAlaOMe and this was used as such for further reaction.

SCHEME 6. Synthesis of AlocBrAlaMetOH 13



SCHEME 7. Synthesis of AlocCys(Pal)MetGlyLeuProCys(Far)OMe 22



without protecting the carboxyl group, was not successful. Compound **12** was obtained by coupling of **11** with commercially available methionine *tert*-butyl ester in the presence of EEDQ in dry dichloromethane in 98% yield. The *tert*-butyl ester was cleaved using 50% TFA in dichloromethane to give the dipeptide **13**.

For the synthesis of **20** (Scheme 7), *N*-Aloc-protected dipeptide **14** was prepared by employing standard reaction conditions.[7a] Coupling of **14** with commercially available proline *tert*-butyl ester in the presence of EEDQ in dry dichloromethane led to tripeptide **15** in 88% yield. Cleavage of the *tert*-butyl group (→ **16**) and reaction with the TFA salt of bromoalanine methyl ester[13] (**17**) in the presence of EEDQ in dry dichloromethane afforded tetrapeptide **18** in 72% yield. Reaction of **18** with farnesyl mercaptan $C_{15}H_{25}$−SH **5** in the presence of Cs_2CO_3 in DMF gave the corresponding *S*-farnesylated product **19** in 75% yield. Cleavage of the *N*-allyloxy group using catalytic amounts of $Pd[PPh_3]_4$ and piperidine as a scavenger led to tripeptide **20**. This compound was coupled with dipeptide **13** in the presence of EEDQ in dichloromethane at room temp. for 12 h yielding the required *S*-farnesylated, bromoalanine containing hexapeptide **21** in 55% yield. EEDQ as coupling reagent gave the best results throughout our studies in peptide segment connections. For instance, coupling of dipeptide **14** with proline *tert*-butyl ester worked well to give tripeptide **15** only when EEDQ was employed as a coupling agent and coupling was not as successful when other coupling reagents such as DCC, DIC, EDC, or PyBOP/DIPEA were used. Finally, reaction of **21** with thiopalmitic acid in the presence of NaOAc afforded the desired *S*-palmitoylated, *S*-farnesylated hexapeptide **22** which had physical data (optical rotation, ^{1}H, and ^{13}C NMR) in excellent agreement with those previously reported.[7a] On incorporation of bromoalanine into peptides, side reactions especially with coupling reagents were not observed. Further, intramolecular alkylation, for instance, of methionine side chains or neighboring carbonyl groups with bromoalanine, was not noticed under the employed reaction conditions.

Conclusion

In conclusion, an efficient reversed approach to the synthesis of *S*-pamitoylated and *S*-farnesylated peptides has been demonstrated where instead of alkylating a peptide cysteine residue with a halogenated lipid, a thiol-containing lipid was used to displace the bromide of a bromoalanine containing peptide. This method was successfully applied to the convergent synthesis of the lipidated C-terminus of human N-Ras hexapeptide. Under the required reaction conditions neither the acid nor the base sensitivity of this molecule was affected, thus pure product could be obtained in good yields. All starting materials can be readily prepared; the synthesis of *N*-Aloc-protected bromoalanine from *N*-Aloc-protected serine could be performed in one-pot.

Experimental Section

***trans,trans*-Farnesyl Thioacetate (4).** To a stirred solution of farnesyl bromide **3** (568 mg, 2 mmol) and thioacetic acid (167 mg, 2.2 mmol) in DMF (10 mL) was added K_2CO_3 (276 mg, 2 mmol) at 0 °C. The reaction mixture was stirred at rt for 12 h. The solution was extracted with ethyl acetate followed by washing with 5% dilute HCl, water, and brine, and the organic layer was dried over anhydrous $MgSO_4$. After concentration of the organic solvent, the crude material was purified by flash column chromatography (9:1, petroleum ether/ethyl acetate) to give the product **4** in 98% yield (555 mg) as a colorless liquid which was immediately used in the next step.

***trans,trans*-Farnesyl Mercaptan (5).** NaOMe (27 mg, 0.5 mmol) was added to the solution of **4** (555 mg, 1.98 mmol) in dry methanol (15 mL), and the reaction mixture was allowed to stir at rt for 6 h. The solvent was evaporated, the solution was extracted with ethyl acetate and washed with 5% dilute HCl, water, and brine, and the combined organic layer was dried over anhydrous $MgSO_4$. Evaporation of the solvent followed by purification by flash column chromatography yielded the product **5** (449 mg, 95%) as a colorless liquid which was identical with previously obtained material.[12]

***N*-Allyloxycarbonyl-L-methionyl-L-tyrosyl-(*S*-palmitoyl)-L-cysteine Methyl Ester (AlocMetTyCys(Pal)OMe) (7).** A solution of **2** (39 mg, 0.14 mmol) in THF (5 mL) was added to a stirred solution of **6** (40 mg, 0.07 mmol) and NaOAc (12 mg, 0.14 mmol) in DMF (5 mL) at 0 °C, and then the reaction mixture was allowed to stir at rt for 12 h. The solution was extracted

with ethyl acetate followed by washing with 5% dilute HCl, water, and brine. The combined organic layer was dried over anhydrous $MgSO_4$, and concentration of the solvent in vacuo yielded the crude product, which was purified by flash column chromatography (petroleum ether/ethyl acetate, 1.2:0.8) to yield **7** (40 mg, 76%) as an amorphous solid: $R_f = 0.6$ (petroleum ether/ethyl acetate, 1:1); $[\alpha]_D = -2.8$ ($c = 1$, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.88 (t, $J = 6.5$ Hz, 3H, CH_3), 1.25 (s, CH_2, 24H, 12 × CH_2), 1.58−1.70 (m, 2H, CH_2), 1.86−2.04 (m, 2H, CH_2), 2.07 (s, 3H, SCH_3), 2.48−2.59 (m, 4H, 2 × CH_2), 2.89−3.08 (m, 2H, CH_2), 3.30 (t, $J = 6.2$ Hz, 2H, CH_2), 3.73 (s, 3H, OCH_3), 4.25−4.34 (m, 1H, CH), 4.56 (br s, 2H, OCH_2), 4.61−4.80 (m, 2H, CH), 5.20−5.34 (m, 2H, CH=C*H*$_2$), 5.64−5.67 (m, 1H, NH), 5.82−6.03 (m, 1H, C*H*=CH_2), 6.71 (d, $J = 8.2$ Hz, 2H, aromatic), 6.85 (br s, 3H, NH, OH), 7.00 (d, $J = 8.2$ Hz, 2H, aromatic); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 14.1, 15.2, 22.7, 25.5, 28.9, 29.2, 29.3, 29.4, 29.6, 29.65, 29.7, 30.1, 31.4, 31.9, 37.3, 44.0, 52.4, 52.7, 54.1, 54.4, 66.1, 68.2, 115.7, 118.1, 128.8, 130.4, 130.8, 132.5, 155.1, 170.0, 170.6, 171.1, 199.0; MS (MALDI) *m/z* 774.5 (M + Na)$^+$.

***N*-Allyloxycarbonyl-L-methionyl-L-tyrosyl-(*S*-farnesyl)-L-cysteine Methyl Ester (AlocMetTyrCys(Far)OMe) (8).** Cs_2CO_3 (33 mg, 0.1 mmol) was added to a stirred solution of **6** (58 mg, 0.1 mmol) and farnesyl mercaptan **5** (26 mg, 0.11 mmol) in DMF (5 mL) at 0 °C, and then the reaction mixture was allowed to stir at rt for 1 h. The solution was extracted with ethyl acetate followed by washing with 5% dilute HCl, saturated $NaHCO_3$, water, and brine. The combined organic layer was dried over anhydrous $MgSO_4$. The ethyl acetate layer was concentrated and purified by flash column chromatography (petroleum ether/ethyl acetate, 1.2:0.8) to give **8** (140 mg, 56%) as colorless viscous oil: $R_f = 0.6$ (petroleum ether/ethyl acetate, 1:1); $[\alpha]_D = -20.0$ ($c = 1$, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.60, 1.70 (s, 12H, CH_3), 1.90−2.06 (m, 13H, SCH_3, CH_2), 2.44−2.58 (m, 2H, CH_2), 2.73−3.20 (m, 6H, CH_2), 3.75 (s, 3H, OCH_3), 4.30−4.35 (m, 1H, CH), 4.60 (br s, 2H, CH_2), 4.70−4.90 (m, 2H, CH), 5.05−5.40 (m, 5H, CH=C*H*$_2$, olefinic(Far)), 5.70−6.00 (m, 2H, C*H*=CH_2, NH), 6.70 (d, $J = 8.2$ Hz, 2H, aromatic), 6.90−7.04 (m, 3H, NH, OH), 7.00 (d, $J = 8.2$ Hz, 2H, aromatic); MS (MALDI) *m/z* 740.6 (M + Na)$^+$. Anal. Calcd for $C_{37}H_{55}N_3O_7S_2$ (717.35): C, 61.90; H, 7.72; N, 5.85. Found: C, 62.20; H, 7.83; N, 5.72.

***N*-Allyloxycarbonylglycyl-L-leucyl-L-prolyl(*S*-farnesyl)-L-cysteine Methyl Ester (AlocGlyLeuProCys(Far)OMe) (19).** To a stirred solution of **18** (60 mg, 0.12 mmol) and thiofarnesol **5** (30 mg, 0.13 mmol) in DMF (5 mL) was added Cs_2CO_3 (38 mg, 0.12 mmol) at 0 °C, and the reaction mixture was allowed to stir at rt for 1 h. The solution was extracted with ethyl acetate followed by washing with dilute HCl, saturated $NaHCO_3$, water, and brine. The combined organic layer was dried over anhydrous $MgSO_4$. The ethyl acetate layer was concentrated and purified by flash column chromatography (ethyl acetate/methanol, 9.9:0.1) to give **19** (61 mg, 75%) as a colorless viscous oil: $R_f = 0.2$ (ethyl acetate/methanol, 9.9:0.1); $[\alpha]_D = -37.3$ ($c = 1$, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.86−0.94 (m, 6H, 2 × CH_3), 1.30−2.30 (m, 15H, 7 × CH_2, CH), 1.56, 1.64 (s, 12H, 4 × CH_3), 2.63−3.21 (m, 4H, 2 × CH_2), 3.51−4.03 (m, 4H, 2 × CH_2), 3.70 (s, 3H, OCH_3), 4.56−4.70 (m, 4H, CH_2, 2 × CH), 4.86−4.88 (m, 1H, CH), 5.03−5.30 (m, 5H, CH=C*H*$_2$, olefinic(Far)), 5.57 (br s, 1H, NH), 5.80−6.00 (m, 1H, C*H*=CH_2), 7.38, 7.50 (br d, 2H, NH); MS (MALDI) *m/z* 713 (M + Na)$^+$. Anal. Calcd for $C_{36}H_{58}N_4O_7S$ (690.40): C, 62.58; H, 8.46; N, 8.11. Found: C, 62.73; H, 8.35; N, 7.89.

***N*-Allyloxycarbonyl-L-bromoalanyl-L-methionylglycyl-L-leucyl-L-prolyl(*S*-farnesyl)-L-cysteine Methyl Ester (AlocBrAlaMetGlyLeuProCys(Far)OMe) (21).** Morpholine (63 mg, 0.73 mmol) and tetrakis(trisphenylphosphine)palladium-(0) (42 mg) were added to a solution of the tetrapeptide **19** (251 mg, 0.364 mmol) in THF (10 mL), and the mixture was stirred under argon and in darkness for 1 h at rt. The solvent was removed in vacuo, and the product was isolated by flash column chromatography (silica gel, ethyl acetate/methanol, 9:1) to give free amine **20** in 80% yield (177 mg) which was used immediately for further reaction.

To a stirred solution of **12** (105 mg, 0.29 mmol) in dichloromethane (4 mL) was added 1 mL of TFA at 0 °C. The solution was stirred for 2 h at rt. Evaporation of the solvent and coevaporation with toluene yielded the compound **13** which was dissolved in dichloromethane (5 mL). To this solution were added compound **20** (177 mg, 0.29 mmol) and EEDQ (108 mg, 0.44 mmol) in dichloromethane (10 mL) at 0 °C. The reaction mixture was stirred at rt for 12 h. Removal of the solvent in vacuo and purification of the crude material by flash column chromatography (silica gel, ethyl acetate/methanol, 17:3) gave pentapeptide **21** (155 mg, 55%) as a waxy solid: $R_f = 0.6$ (ethyl acetate/methanol, 17:3); $[\alpha]_D = -84.1$ ($c = 1.7$, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.92−1.00 (m, 6H, 2 × CH_3), 1.59, 1.65, 1.68 (s, 12H, 4 × CH_3), 1.84−2.30 (m, 17H, 8 × CH_2, CH), 2.08 (s, 3H, SCH_3), 2.50−3.30 (m, 6H, 3 × CH_2), 3.56−3.80 (m, 4H, 2 × CH_2), 3.72 (s, 3H, OCH_3), 4.19 (br s, 2H, CH_2), 4.58−5.46 (m, 12H, OCH_2, CH=C*H*$_2$, 5 × CH, 3 × olefinic), 5.80−6.00 (1H, C*H*=CH_2), 6.40 (br s, 1H, NH), 7.41, 7.80, 7.90, 8.10 (br s, 4H, NH); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 15.3, 16.0, 16.1, 17.6, 21.9, 23.3, 24.7, 24.8, 25.6, 26.5, 26.7, 28.5, 29.5, 30.0, 31.9, 32.8, 33.9, 40.0, 42.2, 43.3, 47.5, 49.0, 52.0, 52.1, 52.3, 55.1, 59.7, 66.2, 118.0, 120.0, 123.8, 124.3, 131.2, 132.5, 135.3, 139.8, 156.1, 168.1, 168.7, 171.2 (2C), 171.3, 171.9. Anal. Calcd for $C_{44}H_{71}BrN_6O_9S_2$ (970.39): C, 54.36; H, 7.36; N, 8.65. Found: C, 54.45; H, 7.48; N, 8.54.

***N*-Allyloxycarbonyl(*S*-palmitoyl)-L-cysteyl-L-methionylglycyl-L-leucyl-L-prolyl(*S*-farnesyl)-L-cysteine Methyl Ester (AlocCys(Pol)MetGlyLeuProCys(Far)OMe) (22).** A solution of **5** (26 mg, 0.094 mmol) in THF (5 mL) was added at 0 °C to a stirred solution of **21** (45 mg, 0.047 mmol) and NaOAc (8 mg, 0.094 mmol) in DMF (5 mL), and the reaction mixture was allowed to stir at rt for 12 h. The solution was extracted with ethyl acetate followed by washing with dilute HCl, water, and brine. The combined organic layer was dried over anhydrous $MgSO_4$, and concentration of the solvent in vacuo yielded the crude product, which was purified by flash column chromatography (petroleum ether/ethyl acetate/methanol, 9.5:9.5:1) to yield **22** (30 mg, 55%) as a waxy solid: $R_f = 0.4$ (petroleum ether/ethyl acetate/methanol, 9.5:9.5:1); $[\alpha]_D = -33.2$ ($c = 0.7$, $CHCl_3$) [lit.[7a] $[\alpha]_D = -34.0$ ($c = 0.7$, $CHCl_3$)]; ^{1}H NMR (250 MHz, $CDCl_3$) δ 0.84−0.98 (m, 9H, 3 × CH_3), 1.24 (s, 24H, 12 × CH_2), 1.56−1.76 (m, 14H), 1.95−2.35 (m, 17H), 2.09 (s, 3H, SCH_3), 2.50−3.39 (m, 10H), 3.63−3.91 (m, 3H), 3.71 (s, 3H, OCH_3), 4.27−4.34 (m, 1H, CH), 4.51−4.95 (m, 7H), 5.08−5.33 (m, 5H, CH=C*H*$_2$, 3 × olefinic(Far)), 5.80−6.04 (m, 2H, NH, C*H*=CH_2), 7.26, 7.36, 7.81, 7.84, (br d, 4H, NH); .^{13}C NMR (62.5 MHz, $CDCl_3$) δ 14.0, 15.1, 16.0, 16.1, 17.6, 21.8, 22.6, 23.3, 24.7, 24.9, 25.6, 25.62, 26.5, 26.7, 28.2, 28.9, 29.2, 29.3, 29.4, 29.6, 29.62, 29.65, 30.2, 30.8, 31.5, 31.9, 32.9, 39.7, 41.7, 43.4, 44.0, 47.3, 49.2, 52.0, 52.3, 52.5, 55.7, 60.0, 66.2, 117.9, 119.7, 123.8, 124.3, 131.2, 132.5, 135.3, 140.0, 156.3, 168.7, 169.8, 171.2, 171.24, 171.4, 172.2, 200.4.

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Supporting Information Available: General experimental details, procedures for the synthesis of compounds **6**, **9**, **11**, **12**, **14**, and **15** and physical data of these compounds, and NMR spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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