

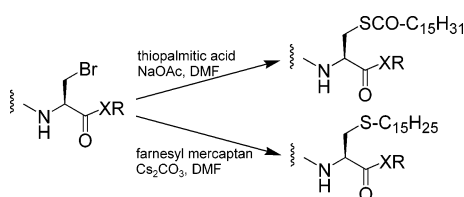
Reversed Approach to S-Farnesylation and S-Palmitoylation: Application to an Efficient Synthesis of the C-Terminus of Lipidated Human N-Ras Hexapeptide

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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 synthetase³ 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.

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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-phase⁷ 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 chloride⁷ 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 H₂S in the presence of Et₃N in dioxane (Scheme 2).

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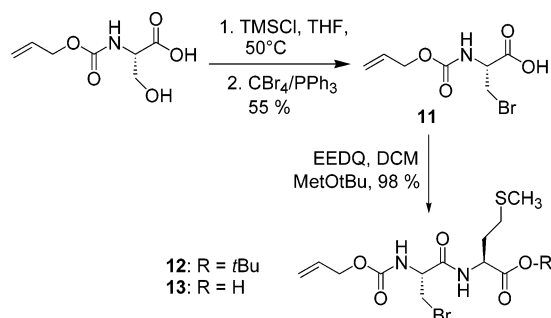
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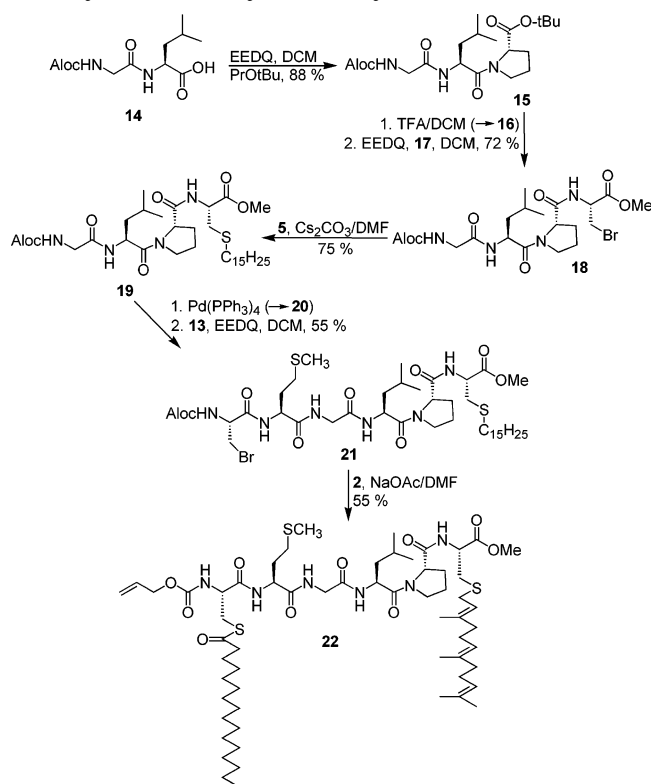
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SCHEME 6. Synthesis of AlocBrAlaMetOH 13



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 ester¹³ (**17**) in the presence of EEDQ in dry dichloromethane afforded tetrapeptide **18** in 72% yield. Reaction of **18** with farnesyl mercaptan C₁₅H₂₅-SH **5** in the presence of Cs₂CO₃ in DMF gave the corresponding *S*-farnesylated product **19** in 75% yield. Cleavage of the *N*-allyloxy group using catalytic amounts of Pd[PPh₃]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, ¹H, and ¹³C 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*-palmitoylated 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 K₂CO₃ (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 MgSO₄. 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 MgSO₄. 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 MgSO₄, 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); $[\alpha]_D = -2.8$ ($c = 1$, CHCl₃); ¹H NMR (250 MHz, CDCl₃) δ 0.88 (t, $J = 6.5$ Hz, 3H, CH₃), 1.25 (s, CH₂, 24H, 12 \times CH₂), 1.58–1.70 (m, 2H, CH₂), 1.86–2.04 (m, 2H, CH₂), 2.07 (s, 3H, SCH₃), 2.48–2.59 (m, 4H, 2 \times CH₂), 2.89–3.08 (m, 2H, CH₂), 3.30 (t, $J = 6.2$ Hz, 2H, CH₂), 3.73 (s, 3H, OCH₃), 4.25–4.34 (m, 1H, CH), 4.56 (br s, 2H, OCH₂), 4.61–4.80 (m, 2H, CH), 5.20–5.34 (m, 2H, CH=CH₂), 5.64–5.67 (m, 1H, NH), 5.82–6.03 (m, 1H, CH=CH₂), 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); ¹³C NMR (62.5 MHz, CDCl₃) 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). Cs₂CO₃ (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 NaHCO₃, water, and brine. The combined organic layer was dried over anhydrous MgSO₄. 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); $[\alpha]_D = -20.0$ ($c = 1$, CHCl₃); ¹H NMR (250 MHz, CDCl₃) δ 1.60, 1.70 (s, 12H, CH₃), 1.90–2.06 (m, 13H, SCH₃, CH₂), 2.44–2.58 (m, 2H, CH₂), 2.73–3.20 (m, 6H, CH₂), 3.75 (s, 3H, OCH₃), 4.30–4.35 (m, 1H, CH), 4.60 (br s, 2H, CH₂), 4.70–4.90 (m, 2H, CH), 5.05–5.40 (m, 5H, CH=CH₂, olefinic(Far)), 5.70–6.00 (m, 2H, CH=CH₂, 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 C₃₇H₅₅N₃O₇S₂ (717.35): C, 61.90; H, 7.72; N, 5.85. Found: C, 62.20; H, 7.83; N, 5.72.

N-Allyloxycarbonyl-glycyl-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 Cs₂CO₃ (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 NaHCO₃, water, and brine. The combined organic layer was dried over anhydrous MgSO₄. 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); $[\alpha]_D = -37.3$ ($c = 1$, CHCl₃); ¹H NMR (250 MHz, CDCl₃) δ 0.86–0.94 (m, 6H, 2 \times CH₃), 1.30–2.30 (m, 15H, 7 \times CH₂, CH), 1.56, 1.64 (s, 12H, 4 \times CH₃), 2.63–3.21 (m, 4H, 2 \times CH₂), 3.51–4.03 (m, 4H, 2 \times CH₂), 3.70 (s, 3H, OCH₃), 4.56–4.70 (m, 4H, CH₂, 2 \times CH), 4.86–4.88 (m, 1H, CH), 5.03–5.30 (m, 5H, CH=CH₂, olefinic(Far)), 5.57 (br s, 1H, NH), 5.80–6.00 (m, 1H, CH=CH₂), 7.38, 7.50 (br d, 2H, NH); MS (MALDI) m/z 713 (M + Na)⁺. Anal. Calcd for C₃₆H₅₈N₄O₇S (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); $[\alpha]_D = -84.1$ ($c = 1.7$, CHCl₃); ¹H NMR (250 MHz, CDCl₃) δ 0.92–1.00 (m, 6H, 2 \times CH₃), 1.59, 1.65, 1.68 (s, 12H, 4 \times CH₃), 1.84–2.30 (m, 17H, 8 \times CH₂, CH), 2.08 (s, 3H, SCH₃), 2.50–3.30 (m, 6H, 3 \times CH₂), 3.56–3.80 (m, 4H, 2 \times CH₂), 3.72 (s, 3H, OCH₃), 4.19 (br s, 2H, CH₂), 4.58–5.46 (m, 12H, OCH₂, CH=CH₂, 5 \times CH, 3 \times olefinic), 5.80–6.00 (1H, CH=CH₂), 6.40 (br s, 1H, NH), 7.41, 7.80, 7.90, 8.10 (br s, 4H, NH); ¹³C NMR (62.5 MHz, CDCl₃) 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 C₄₄H₇₁BrN₆O₉S₂ (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 MgSO₄, 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); $[\alpha]_D = -33.2$ ($c = 0.7$, CHCl₃) [lit.^{7a} $[\alpha]_D = -34.0$ ($c = 0.7$, CHCl₃)]; ¹H NMR (250 MHz, CDCl₃) δ 0.84–0.98 (m, 9H, 3 \times CH₃), 1.24 (s, 24H, 12 \times CH₂), 1.56–1.76 (m, 14H), 1.95–2.35 (m, 17H), 2.09 (s, 3H, SCH₃), 2.50–3.39 (m, 10H), 3.63–3.91 (m, 3H), 3.71 (s, 3H, OCH₃), 4.27–4.34 (m, 1H, CH), 4.51–4.95 (m, 7H), 5.08–5.33 (m, 5H, CH=CH₂, 3 \times olefinic(Far)), 5.80–6.04 (m, 2H, NH, CH=CH₂), 7.26, 7.36, 7.81, 7.84, (br d, 4H, NH); ¹³C NMR (62.5 MHz, CDCl₃) 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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