with HCl (1 N, 0.3 mL) that contained EGTA (5 mM). After centrifugation, an aliquot (0.5 mL) of the upper aqueous phase was removed and the radioactivity was measured with scintillation counting.

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Solution- and Solid-Phase Synthesis of the Polybasic Lipid-Modified C Termini of Rho A and K-Ras 4B

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KEYWORDS:

lipopetides · lipoproteins · solid-phase synthesis · Ras proteins · Rho proteins

Lipid-modified peptides and semisynthetic lipid-modified proteins have proven to be invaluable chemical tools for the study of biological processes like membrane localization of, and signal transduction through, lipidated proteins.[1] Paramount to the success of this chemical – biological approach is the availability of efficient methods for the synthesis of such peptide conjugates that include the attachment of suitable reporter groups for application in subsequent biological or biophysical experiments.[1, 2–4]

The techniques developed so far have given access to different acid- and base-labile lipidated peptides. However, peptide conjugates that include a farnesyl or geranylgeranyl thioether and a stretch of basic amino acids, in particular a polybasic oligolysine sequence, proved to be, and still are, major challenges to the synthetic chemist. Currently, efficient methods for the synthesis of such conjugates are not available. Combinations of an *S*-lipidated cysteine with polybasic amino acid sequences frequently occur in signal-transducing proteins that belong to the Ras super family, such as the Rho proteins and K-Ras 4B. This structural motif is thought to mediate, for instance, selective intracellular localization and trafficking of the proteins (in particular in the case of K-Ras 4B[5]) and to play a decisive role in protein activation/deactivation (both the Ras and the Rho proteins may be targets of farnesyltransferase inhibitors[6] currently under clinical investigation.)

The problems encountered in the synthesis of polybasic lipidated peptides are highlighted by the synthesis of the C terminus of the K-Ras 4B protein (**2**) by Gelb et al.[7] In this

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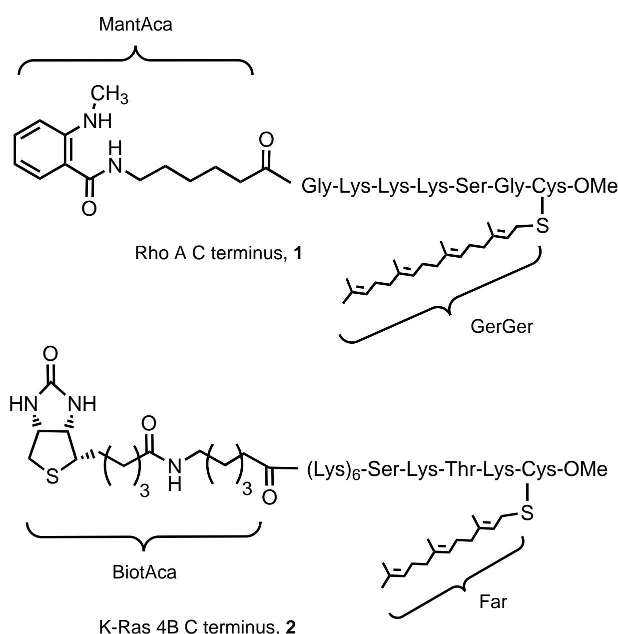
synthesis *S*-farnesylation of a presynthesized and fully deprotected fluorescence-labeled peptide was employed as the key step. However, as a result of competitive *N*-alkylation a complex product mixture was formed that gave access to only small amounts of the desired compound.[8] Although this reaction could be considerably improved by using Lewis acid catalysis (Zn^{2+}) under acidic conditions, it could not approach quantitative yield. Silvius et al.[9] investigated the synthesis of K-Ras peptides that include a hexalysine stretch by employing a lipidated amino acid building block. However, they did not get access to the correct C terminus of K-Ras 4B either and had to resort to a model compound in which the natural farnesyl thioether was replaced by a nonnatural chemically inert undecyl group.

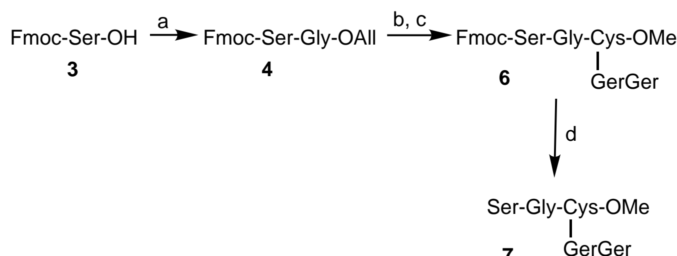


Scheme 1. *Structures of fluorescence- and biotin-labeled lipidated peptides **1** and **2**, the C termini of RhoA and K-Ras 4B. Mant = N-methylanthranyl; Biot = biotin; Aca = aminocaproic acid.*

The purpose of this paper is to report on the development of methods that give rapid and reliable access to fluorescent- or biotin-labeled lipidated peptides that include polybasic stretches of amino acids by means of solution-phase synthesis or solid-phase methodology. The characteristic C termini of Rho A and K-Ras 4B (**1** and **2**, Scheme 1) were chosen as examples for the delineation of the methodology

The synthesis of these peptides requires a protecting group strategy that is compatible with the pronounced acid-lability of the farnesyl group[1–3] and the required C terminal methyl ester. The strategy also has to allow for selective deprotection of the *N*-terminal amino groups and must leave the blocking functions of the lysine ε-amino groups untouched. These requirements were fulfilled for solution-phase synthesis by employment of the base-labile *N*-fluorenylmethoxycarbonyl (Fmoc) urethane as a temporary *N*-terminal protecting group and the Pd^0-sensitive *N*-allyloxycarbonyl (Aloc) urethane to mask the lysine side chains in order to build up the selectively deprotected polybasic core peptide. We planned to couple this peptide to a prenylated cysteine ester building block in solution.

The viability of this strategy was investigated for the synthesis of the fluorescence-labeled *S*-geranylgeranylated C terminus **1** of Rho A. *S*-geranylgeranylated cysteine methyl ester **5**[10] (Scheme 2) was synthesized by *S*-alkylation of geranylgeranyl bromide obtained from geranylgeraniol by treatment with *N*-bromosuccinimide.[11] Dipeptide **4**[12] was obtained by coupling of Fmoc-protected serine **3** with glycine allyl ester. Deprotection of the allyl group and coupling to cysteine derivative **5** yielded fully protected lipopeptide **6**, from which the N-terminal urethane was selectively removed under basic conditions to give lipidated tripeptide ester **7** in high yield. In a separate series of experiments, fluorescence-labeled trilysyl peptide **15** was synthesized. In this synthesis lysine derivative **9**, which carries three orthogonally stable protecting groups, was employed as the key building block (Scheme 3). Compound **9** was obtained from commercially available[13] lysine derivative **8** by esterification with *tert*-butanol. Consecutive removal of the base-labile Fmoc-urethane and carbodiimide-mediated chain elongation with Fmoc/Aloc-protected lysine derivative **8** yielded selectively deprotected trilysyl peptide **10** in 49% overall yield. This compound was acylated with glycine derivative **13**, which carries the fluorescent Mant label, to yield fluorescence-labeled tetrapeptide acid **15** after acidic cleavage of the C-terminal *tert*-butyl ester. This compound was then coupled with geranylgeranylated tripeptide ester **7** and, finally, all Aloc protecting groups were removed simultaneously in the presence of a Pd^0 catalyst, with phenyl silane as an alkyl-trapping nucleophile.[14] The desired fluorescence-labeled lipidated peptide **1**, which contains a highly polar trilysine sequence, was purified by gel filtration on Sephadex LH 20 and obtained in high yield.



Scheme 2. *Synthesis of S-geranylgeranylated tripeptide **7**. a) TosOH · GlyOAll (Tos = tosyl, Gly = glycine, All = allyl), N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide-hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt), Et_3N, CH_2Cl_2, 18 h, 82%. b) N,N-Dimethyl barbituric acid, $Pd(PPh_3)_4$, tetrahydrofuran (THF), 30 min, 97%. c) Cys(GerGer)OMe (**5**), EDC, HOBt, Et_3N, CH_2Cl_2, 18 h, 79%. d) Piperidene, CH_2Cl_2, 2 h, 97%.*

This fragment condensation strategy successfully gave access to Rho A peptide **1** but it could not be applied to the synthesis of the C terminus **2** of K-Ras 4B. In this case the peptide KKKKKKSKTK was synthesized on a chlorotrityl resin and released in a protected form that carried an N-terminal Fmoc group and eight Aloc urethanes attached to the lysine side chains. This

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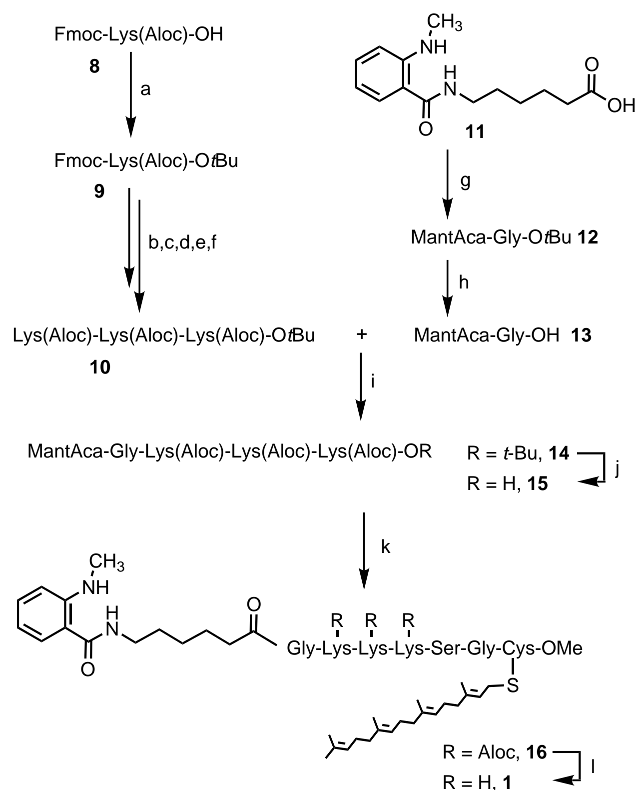
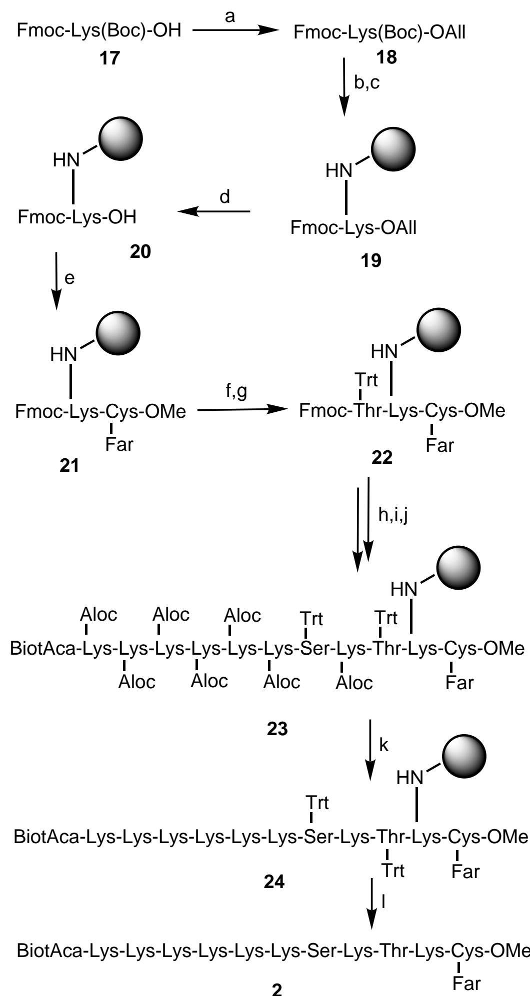


*Scheme 3. Solution-phase synthesis of Rho A peptide **1**. a) tBuOH, $POCl_3$, pyridine, 2 h, 89%. b) Piperidine, CH_2Cl_2, 2 h, 97%. c) Fmoc-Lys(Aloc)-OH (**8**), EDC, HOBt, CH_2Cl_2, 18 h, 76%. d) Piperidine, CH_2Cl_2, 2 h, 95%. e) Fmoc-Lys(Aloc)-OH (**8**), EDC, HOBt, CH_2Cl_2, 18 h, 72%. f) Piperidine, CH_2Cl_2, 2 h, 98%. g) MantAca (**11**), TosOH · Gly-OtBu, EDC, HOBt, Et_3N, CH_2Cl_2, 18 h, 60%. h) Trifluoroacetic acid (TFA), CH_2Cl_2, 1 h, 96%. i) EDC, HOBt, CH_2Cl_2, 18 h, 79%. j) TFA, CH_2Cl_2, 2 h, 99%. k) Ser-Gly-Cys(GerGer)-OMe (**7**), EDC, HOBt, trifluorethanol/$CHCl_3$ 1:3, 18 h, 52%. l) $PhSiH_3$, $Pd(PPh_3)_4$, dimethylformamide (DMF), 2 h, 83%.*



*Scheme 4. Solid-phase synthesis of K-Ras 4B peptide **2**. a) Cs_2CO_3, MeOH, 10 min, then allylbromide, DMF, 1.5 h (87%). b) TFA, CH_2Cl_2, 3 h. c) Trityl (Trt) resin, N,N′-diisopropylethylamine (DIEA), CH_2Cl_2. d) $PhSiH_3$, $Pd(PPh_3)_4$, THF, 12 h. e) Cys(Far)-OMe (Far = farnesyl), PyBOP, NMM, DMF, 3 h. f) Piperidine, DMF, 2 × 4 min. g) Fmoc-Thr(Trt)-OH, HBTU, HOBt, DIEA, DMF, 3 h. h) Protected amino acid or BiotAca-OH, HATU, HOAt, DIEA, DMF 4 h. i) Piperidine, DMF. j) Ac_2O, pyridine. Steps (h), (i), and (j) were repeated to build up peptide backbone **23**. k) $Pd(PPh_3)_4$, piperidine, DMF, 5 h. l) TFA, triethylsilane (TES), CH_2Cl_2.*

compound was only poorly soluble in organic solvents, could hardly be purified, and showed little conversion in attempted subsequent coupling steps. In the light of these unfavourable properties we abandoned the building block strategy for the synthesis of the K-Ras 4B peptide and developed a solid-phase methodology to circumvent the problems encountered in the attempted solution-phase synthesis.

Key elements of the solid-phase synthesis are, the attachment of the first lysine in the peptide sequence to an acid-labile trityl linker, introduction of the farnesylated cysteine methyl ester as a building block (as opposed to *S*-farnesylation on a solid support[14d]), employment of the Aloc group and trityl ethers as masks for the side chains of the lysines and hydroxyamino acids, respectively, and use of the base-labile Fmoc urethane for temporary N-terminal protection.

Lysine derivative **18**, which contains three orthogonally stable blocking groups, was synthesized from the commercially available[15] building block **17** by nucleophilic esterification (Scheme 4). The *N-tert*-butyloxycarbonyl (Boc) group was selectively removed and the amine that resulted was attached to the trityl resin. Typically, a loading of 0.49 $mmol\,g^{-1}$ was achieved (determined by the UV – Fmoc method). The allyl ester was selectively cleaved by treatment with a Pd^0 catalyst in the presence of $PhSiH_3$ (Ph = phenyl) as allyl scavenger. Subsequent coupling with farnesylated cysteine methyl ester yielded polymer-bound dipeptide **21** in quantitative yield when (benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) in 4% *N*-methylmorpholine (NMM) was employed as coupling reagent. Use of *N*,*N*-diisopropylcarbodiimide/HOBt instead of NMM resulted in incomplete conversion even with a 4-hour reaction time. The Fmoc group was removed from intermediate **21** by treatment with piperidine in DMF twice for four minutes each time and then trityl-protected threonine was introduced by means of *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmetanaminium hexafluorophosphate *N*-oxide (HBTU)/HOBt chemistry. All subsequent coupling reactions were carried out in the presence of *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methyl-ene]-*N*-methylmetanaminium hexafluorophosphate *N*-oxide (HATU)/1-hydroxy-7-aza-

benzotriazol (HOAT) as activating reagents and the coupling times were extended to 3 – 4 h to avoid incomplete peptide bond formation.

After the entire biotin-tagged and farnesylated peptide **23** had been assembled on the polymeric support, the seven Aloc groups present were removed simultaneously by treatment with $Pd[PPh_3]_4$ in the presence of piperidine for four hours. Removal of the catalyst was achieved by simple washing, which rendered the troublesome purification of the unmasked oligolysine peptide unnecessary.

Finally, fully unmasked lipidated K-Ras peptide **2** was released from the solid support by treatment with 1% TFA in the presence of 2% TES. Under these conditions both *O*-trityl groups present in **24** were removed as well and the farnesyl group remained unattacked.[16] Purification of the target peptide was readily achieved by means of HPLC on an RP-C18 column to yield the desired biotin-tagged and lipidated oligolysine peptide **2** (Figure 1) in high purity and with 11% overall yield.

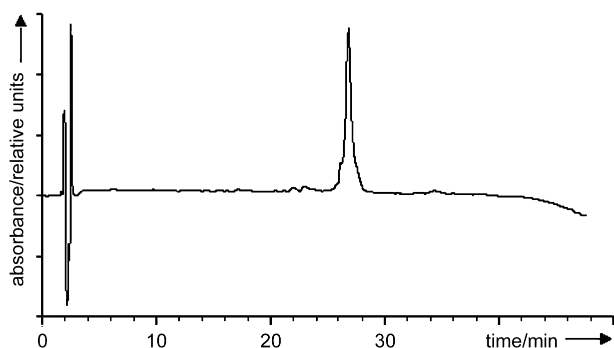


Figure 1. *HPLC analysis of lipidated polybasic Ras-peptide **2**. Detection at 210 nm; RP-C18PPN column; solvent A: water; solvent B: acetonitrile; solvent C: water with 1% TFA; gradient: 0 min 80% A, 10% B, 10% C; 40 min 35% A, 55% B, 10% C; retention time: 26.9 min.*

In summary, we have developed a reliable and practical method for the synthesis of polybasic lipidated peptides in solution or on a solid support. In both cases an *S*-prenylated cysteine methyl ester is introduced as a building block and Fmoc and Aloc urethanes are employed for protection of the *N* terminus and the lysine side-chain amino groups, respectively. These methods give rapid access to the desired deprotected peptides in pure form and thereby open up new opportunities for future biological experiments to address, for instance, selective membrane targeting by Ras, Rho and related lipidated proteins.

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Binding and Docking of Synthetic Heterotrimeric Collagen Type IV Peptides with α1β1 Integrin

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KEYWORDS:

binding affinity · cell adhesion · collagen · integrins · structure elucidation

Collagen type IV, whose major and ubiquitous form consists of one α2 and two α1 chains,[1, 2] forms a network that determines the biomechanical stability and macromolecular organization of the basement membrane and provides a scaffold into which other constituents of the tissue are incorporated[3]. This collagen

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