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Published on Web 05/01/2002

Solid-Phase Synthesis of Lipidated Peptides

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Received January 31, 2002

Lipidated proteins play important roles in numerous biological processes. For instance the correct functioning of the Ras proteins in biological signal transduction and of the Rab proteins in the orchestration of vesicular transport are paramount to regulation of cell growth and differentiation.[1] The Ras proteins embody both acid labile farnesyl thioethers and base-sensitive palmitic acid thioesters and terminate in a cysteine methyl ester. The Rab proteins are *S*-geranylgeranylated and carry either a carboxylic acid or a methyl ester at the C-terminus (Figure 1).

Tailor-made lipidated peptides representing the characteristic lipid-modified partial structures of their parent proteins are efficient tools for the investigation of biological processes in molecular detail.[2] Of paramount importance to the efficient and rapid synthesis of such lipidated peptide conjugates would be the availability of a flexible solid-phase technique. Ideally such a technique would (i) give access to peptides carrying different combinations of acid- and base-labile lipid groups, (ii) allow for release of the peptides from the solid support as either C-terminal carboxylic acid or methyl ester, and (iii) allow for the introduction of additional reporter and/or linking groups required for application of the target peptides in further biological experiments. The synthesis of lipidated peptides requires the application of a set of suitable orthogonally stable blocking groups and a linker to the solid support that are cleavable under the mildest conditions.[2] Currently such a solid-phase technique is not available. In a few notable cases the solid-phase synthesis of exclusively *S*-palmitoylated[3] or *S*-farnesylated[4] peptides has been described. In this paper we report on the development of a solid-phase technique that fulfils the demands raised above. It relies on the combined use of the base labile Fmoc-group for *N*-terminal deprotection of the peptide chain, the application of acid- and reduction-labile protecting groups for the cysteine side chain, as well as *S*-farnesylation and *S*-palmitoylation of the growing peptide chain on the solid support. For attachment to the solid support the oxidation-sensitive hydrazide linker was employed. It can be cleaved by oxidation with Cu^{II} or NBS and allows the target peptides to be released as either carboxylic acid or methyl ester from the polymeric carrier.[5] For the synthesis commercially available 4-Fmoc-hydrazinobenzoyl AM NovaGel **1** (Novabiochem) was employed. The Fmoc group was removed and *S*-trityl protected Fmoc-cysteine was coupled to the hydrazide resin to yield immobilized amino acid **2** (loading 3.5 to 4.3 mmol g^{-1}). Subsequent elongation of the peptide chain by means of standard methodology yielded polymer bound tripeptide **3** (Scheme 1). The *S*-trityl group was removed by treatment with 50% trifluoroacetic acid (TFA) in dichloromethane and then *S*-farnesylation was carried out by treatment of the liberated thiols with farnesyl bromide in the presence of a tertiary amine.

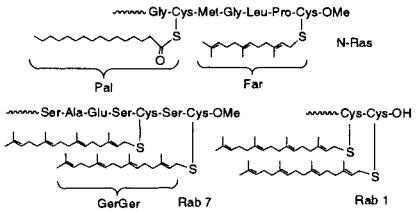
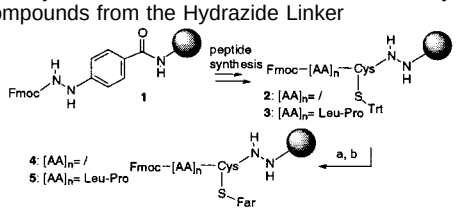


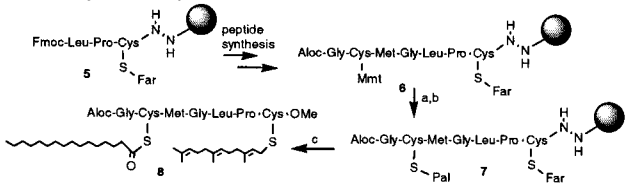
Figure 1. Structure of the lipidated C-terminus of the N-Ras protein and of Rab proteins.

Scheme 1. Synthesis and Oxidative Release of Farnesylated Model Compounds from the Hydrazide Linker



[a] 50% TFA, TES, CH_2Cl_2, 1 h. [b] Far-Br, DIPEA, DMF, 4 h.

Scheme 2. Solid-Phase Synthesis of *S*-Farnesylated and *S*-Palmitoylated Peptide **8**



[a] 1% TFA, TES, CH_2Cl_2, 1 h. [b] Pal-Cl, HOBt, Et_3N, DCM, DMF, 15 h. [c] $Cu(OAC)_2$, acetic acid, pyridine, O_2, CH_2Cl_2, methanol, 2 h, 42% over all steps.

We would like to stress that the described farnesylations are the first examples of peptide farnesylation on a solid support. The peptide chain of polymer-bound and *S*-farnesylated tripeptide **5** was then elongated by successive removal of the *N*-terminal Fmoc group and coupling of the appropriate amino acids (Scheme 2). The mercapto group of the second cysteine required in the sequence was protected as monomethoxytrityl (Mmt) thioether. After completion of peptide assembly, the Mmt group was removed from protected heptapeptide **6** by treatment with 1% TFA in dichloromethane and in the presence of triethylsilane. The farnesyl group remained unattacked under these mild, weakly acidic conditions.[6] *S*-palmitoylation was then carried out by treatment with palmitoyl chloride in the presence of HOBt and triethylamine to yield doubly lipidated polymer-bound peptide **7** (Scheme 2).

The best results were observed when 10 equiv of both reagents were used for 15 h; application of only 4 equiv for 2 h resulted in lower yields and impure product. Finally, the desired N-Ras peptide methyl ester **8** was released from the solid support by oxidation with $Cu(OAc)_2$ in the presence of pyridine and acetic acid. An

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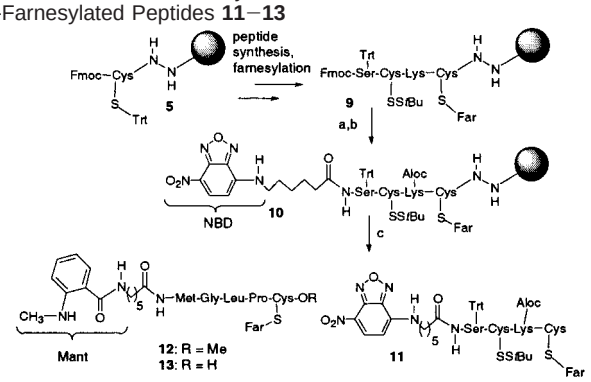
10.1021/ja025768t CCC: $22.00

Scheme 3. Solid-Phase Synthesis of Fluorescent-Labeled and *S*-Farnesylated Peptides **11**−**13**



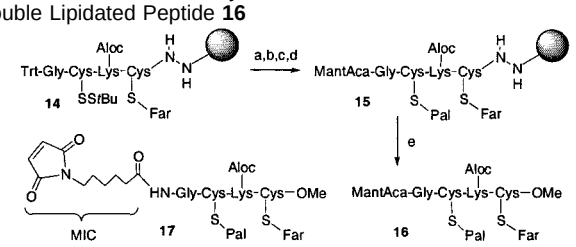
[a] Piperidine, DMF, 2 × 7 min. [b] NBDAca-OH, HBTU, HOBt, DIPEA, DMF, 2 h. [c] $Cu(OAc)_2$, pyridine, O_2, CH_2Cl_2, methanol, 2 h.

undesired attack on the acid-sensitive farnesyl thioether or the base-labile thioester was not recorded and Ras peptide **8** was obtained in an overall yield of 42% (with respect to the amount of the first amino acid bound to the resin). By means of this procedure multimilligram amounts of pure lipopeptide are readily obtained within 1−2 days whereas the synthesis of the same compound by means of solution-phase methods[2e,7] requires more than one week. In addition, purification of the final product is readily achieved by simple column chromatography during which the copper is completely removed. To demonstrate that differently lipidated and additionally tagged peptides are accessible by means of the solid-phase method described above, *S*-farnesylated tetrapeptide **11** embodying the fluorescent NBD group was synthesized (Scheme 3). After assembly of the peptide chain and *N*-terminal deprotection the fluorescent label was coupled to the *N*-terminus and then the desired compound was released from the solid support. It was obtained in 47% overall yield and with high purity after simple flash chromatography. In the course of this synthesis neither the acid-labile trityl protecting group nor the reduction sensitive NBD-label and the S-tBu disulfide were attacked. The same methodology gave access to N-Ras peptide **12** incorporating the fluorescent Mant label in 49% overall yield.

To demonstrate that not only peptide esters but also—as for instance required for Rab-derived peptides, see Figure 1—lipidated peptides with an unmasked carboxylic acid at the C-terminus can be obtained via this solid-phase technique, the activated intermediate formed in the oxidative release of the Mant-labeled peptide from the solid support was alternatively trapped with water instead of methanol to give lipopetide carboxylic acid **13**.

In addition, doubly lipidated peptides carrying a tag for subsequent biological experiments were prepared. To this end, farnesylated tetrapeptide **14** was assembled on the solid phase (Scheme 4). It embodies a reduction-sensitive *tert*-butyl disulfide as masking group for the cysteine thiol, an acid labile trityl blocking function, and a lysine with Aloc-protected side chain amino group. This set of protecting groups and the hydrazide linker are orthogonally stable. The *tert*-butyl disulfide was cleaved by treatment with *n*-Bu_3P and the liberated mercapto group was then palmitoylated. From the formed intermediate the *N*-terminal trityl group was removed under weakly acidic conditions and then the Mant-label was introduced by coupling with Mant-aminocaproic acid to yield

Scheme 4. Solid-Phase Synthesis of Fluorescent-Labeled and Double Lipidated Peptide **16**



[a] PBu_3, H_2O, DMF, CH_2Cl_2, 15 h. [b] Pal-Cl, HOBt, Et_3N, CH_2Cl_2, DMF, 15 h. [c] 1% TFA, TES, DCM, 1 h. [d] MantAca, DIC, HOBt, Et_3N, 2 h. [e] $Cu(OAC)_2$, acetic acid, pyridine, O_2, CH_2Cl_2, methanol, 2 h.

immobilized fluorescent-labeled lipopeptide **15**. Finally, desired lipopeptide methyl ester **16** was released from the solid support by oxidation with Cu^{II} in methanol in the presence of pyridine and acetic acid. After flash chromatography it was obtained in pure form in 29% overall yield for 13 steps on the polymeric carrier. Using the same approach, doubly lipidated peptide **17** was synthesized. It embodies a maleimido group that allows for coupling to mercapto groups of proteins, as was demonstrated in the synthesis of biologically fully functional Ras proteins.[2b,c]

In conclusion we have developed the first generally applicable solid-phase method for the synthesis of differently lipidated and additionally labeled peptides. This new methodology should substantially facilitate the use of lipidated peptides and lipidated proteins accessible from them by combination of organic synthesis and molecular biology techniques[2b] in the study of important biological phenomena like biological signal transduction, localization, and vesicular transport.

Acknowledgment. This research was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

Supporting Information Available: Experimental procedures and characterization data for all new compounds and HPLC traces of compound **11** and **17** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA025768T