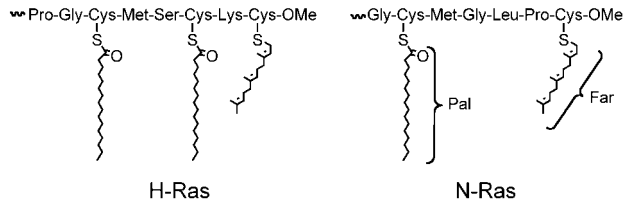

Scheme 1. Structure of the C-termini of the N-Ras and the H-Ras protein.

Protein Synthesis

Solid-Phase Synthesis of Lipidated Peptides**

*Goran Kragol, Maria Lumbierres, Jose M. Palomo, and Herbert Waldmann**

The Ras proteins serve as central molecular switches in biological signal transduction cascades regulating cell growth and differentiation.[1] They incorporate both acid-labile farnesyl thioethers and base-sensitive palmitic acid thioesters, which are required for biological activity and terminate in a cysteine methyl ester (Scheme 1).

For the efficient and rapid synthesis of tailor-made lipidated peptides representing the characteristic partial structures of their parent proteins[2,3] a flexible solid-phase technology is required. Ideally such a technique would:

1. give access to peptides carrying different combinations of acid- and base-labile lipid groups; this requires the application of a set of suitable orthogonally stable protecting groups and a linker to the solid support, all of which can be cleaved under the mildest conditions;[3]
2. allow for the introduction of additional reporter and/or linking groups required for application of the target peptides in further biological investigations;
3. allow for release of the peptide as a methyl ester, or—if required—equipped with a different functional group, for example, a fluorophore at the C-terminus.[2d]

The only method currently available for this purpose[4] requires a large excess of lipidation reagent, is not readily automatable, and is not suitable for the preparation of longer peptides. Thus attempts to prepare peptides with >10 amino acids by this method failed in our hands.

Here we describe the successful development of a solid-phase synthesis method that meets the demands and overcomes the drawbacks described above. It employs pre-lipidated amino acid building blocks together with the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group as a blocking function for the N-terminus, and the oxidation-labile hydrazide linker for anchoring to the solid support.

The lipidated building blocks required for the new solid-phase method were synthesized in high overall yields as shown in Scheme 2 employing in part transformations described earlier.[5] Notably only one equivalent of farnesyl chloride and, in particular, of the *N*-methylanthraniloyl (Mant)-functionalized farnesyl analogue GerMantCl were employed.

Building blocks **2** and **4** were then used in the solid-phase synthesis of H- and N-Ras peptides **9** and **10** (Scheme 3). The hydrazide unit[6] was employed as a linker to the solid support since it can be cleaved under mild oxidative conditions and gives access to lipopeptide esters and acids.[4] Fmoc-hydrazinobenzoic acid functionalized aminomethyl polystyrene resin is commercially available (NovaBioChem). In order to avoid racemization of cysteines DIC/HOBt or HBTU/HOBt/TMP cocktails in CH_2Cl_2/DMF 1:1 were used for coupling the cysteine building blocks.[7]

Coupling of Fmoc-Cys(Far)OH and Fmoc-Cys(Pal)OH proceeded with $>90\,\%$ efficiency if a coupling time of 4 h was allowed and if for Fmoc-Cys(Pal)OH a double coupling was performed (monitored by Fmoc determination employing the established UV-based method). After oxidative cleavage with $Cu(OAc)_2$ and release from the resin by treatment with

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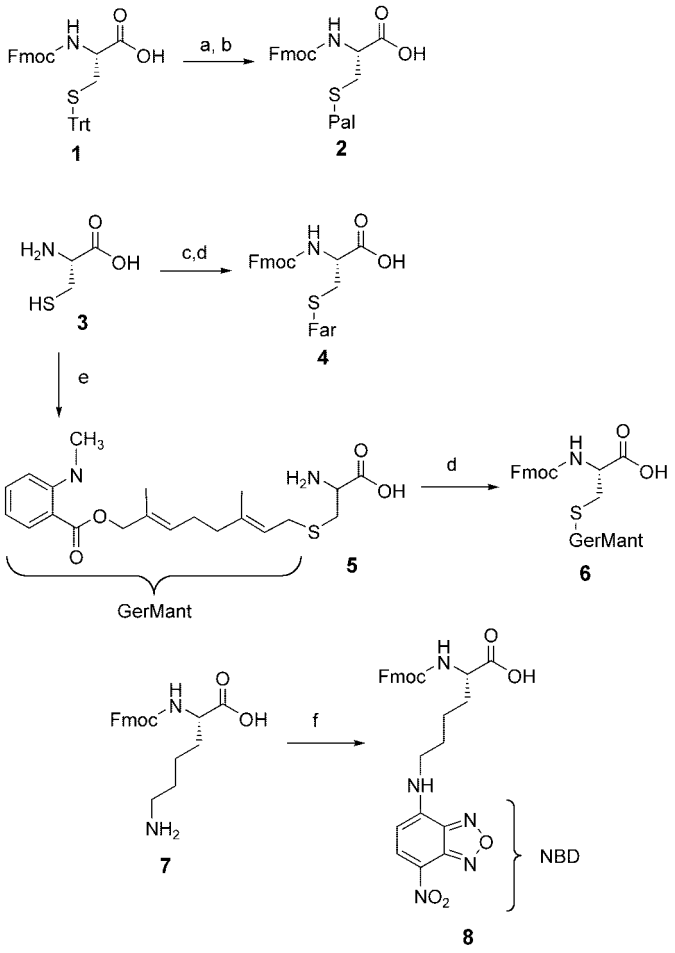
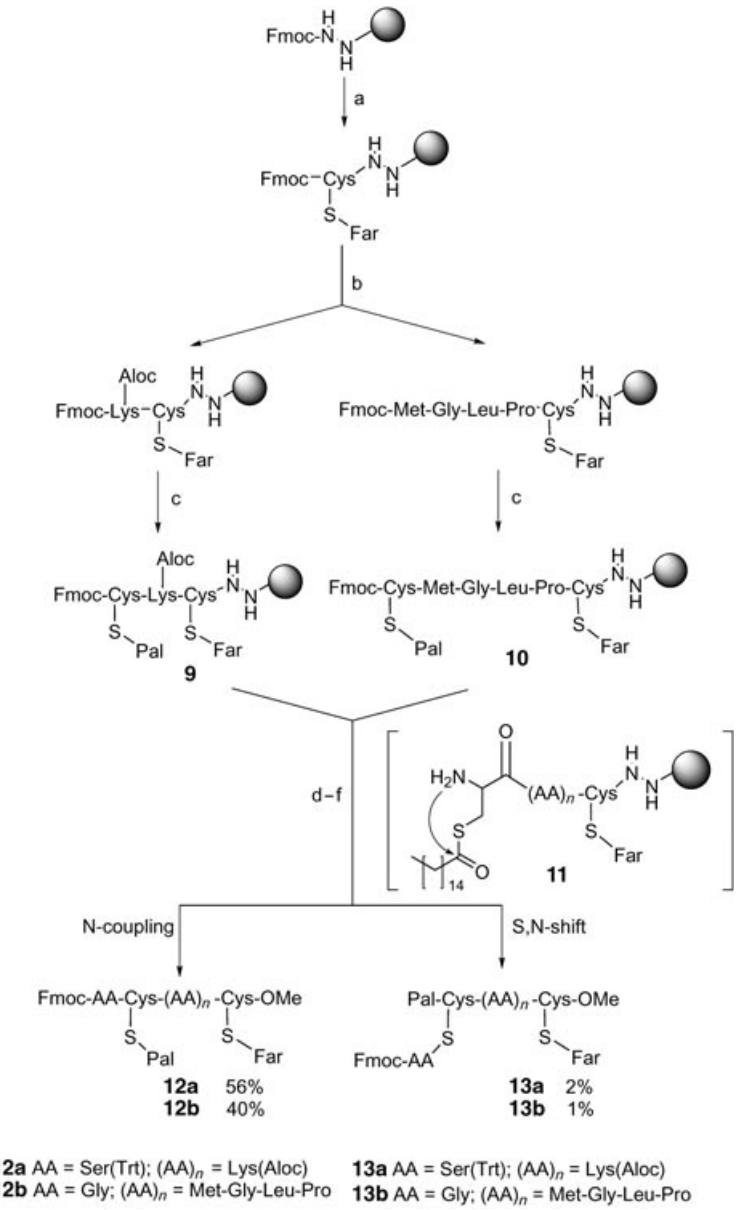


Scheme 2. Synthesis of lipidated building blocks **2**, **4**, **6**, and **8**. a) 5% TFA, 3% TES, CH_2Cl_2, 1 h, RT; b) TMSCl (1.1 equiv), 2 h, reflux, CH_2Cl_2, then PalCl (3 equiv), Et_3N (1.5 equiv) dropwise 3 h, CH_2Cl_2, RT; c) FarCl (1 equiv), 4 N NH_3/methanol, 3 h, 0 °C, 1 h, RT;[5a] d) FmocOSu (1.1 equiv), Et_3N (1.1 equiv), CH_2Cl_2, 2 h, RT; e) GerMantCl (1 equiv), 4 N NH_3/methanol, 3 h, 0 °C, 1 h, RT; f) NBDCl, THF/methanol, 65 °C, 2 h.[5b] Far = farnisyl, Fmoc = 9-fluorenylmethyloxycarbonyl, NBD = 4-nitrobenzo-2-oxa-1,3-diazole, Pal = palmitoyl, RT = room temperature, TES = triethylsilane, TFA = trifluoroacetic acid, TMS = trimethylsilyl.



12a AA = Ser(Trt); $(AA)_n$ = Lys(Aloc)
12b AA = Gly; $(AA)_n$ = Met-Gly-Leu-Pro
13a AA = Ser(Trt); $(AA)_n$ = Lys(Aloc)
13b AA = Gly; $(AA)_n$ = Met-Gly-Leu-Pro

Scheme 3. Solid-phase synthesis of lipidated peptides employing pre-lipidated amino acid building blocks. a) 1. 20% piperidine/DMF, 2. Fmoc-Cys(Far)-OH (4 equiv), DIC/HOBt or HBTU/HOBt/TMP, CH_2Cl_2/DMF (1:1), 4 h; b) Standard solid-phase synthesis: 1. 20% piperidine/DMF, 2. Fmoc-AA-OH (4 equiv), DIC/HOBt or HBTU/HOBt/DIPEA, CH_2Cl_2/DMF (1:1) or DMF, 2 h; c) 1. 20% piperidine/DMF, 2. Fmoc-Cys(Pal)-OH (4 equiv), DIC/HOBt or HBTU/HOBt/TMP, CH_2Cl_2/DMF (1:1), 4 h for **10**, overnight for **9**; d) 1% DBU/DMF, 2 × 30 s; e) Fmoc-AA-OH (5 equiv), HATU (5 equiv), DIPEA (20 equiv), CH_2Cl_2/DMF (4:1), 2 h; f) $Cu(OAc)_2$, pyridine, acetic acid, methanol, CH_2Cl_2, oxygen atmosphere, 3 h. DIC = 1,3-diisopropylcarbodiimide, DIPEA = *N*,*N*-diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HBTU = *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide, HOBt = 1-hydroxybenzotriazole, TMP = tetramethylpiperidide.

CH_3OH followed by filtration of the crude product through a short silica cartridge, the peptide methyl esters corresponding to immobilized peptides **9** and **10** were obtained in overall yields of 69% and 60%, respectively, and with > 90% purity.

For further elongation of the peptide chain the central problem of the entire method had to be solved. After removal of the Fmoc group from the N-terminal S-palmitoylated cysteine a rapid S→N acyl shift had to be feared,[8] and, in addition, the base-labile Fmoc group had to be removed under conditions that leave the thioester intact, although this group is very sensitive to nucleophilic attack.[2]

Substantial experimentation revealed that the desired elongated and S-palmitoylated peptides can be obtained in a very practical and efficient manner if the Fmoc group is cleaved by treatment with a solution of 1% DBU in DMF[9] for 2 × 30 s followed each time by fast washing with DMF (6 × 5 s) and immediate coupling for 2 h employing five equivalents of preactivated (10 min) amino acid building block, five equivalents of HATU, and twenty equivalents of diisopropyl-

ethylamine in CH_2Cl_2/DMF 4:1 as solvent. Under these conditions the desired elongated peptides **12** and the analogue **13** resulting from S→N acyl shift were formed in a ratio of 95:5.

In order to demonstrate the scope of the method, the lipid-modified peptides shown in Table 1 were prepared. In general, a resin with a loading of 0.35–0.37 mmol g^{-1} was employed, and the peptides were prepared in 5- to 20-mg amounts. Immediately after release from the resin and filtration through a silica cartridge the peptides were >80% pure (determined by HPLC analysis; see the Supporting Information).

The peptides shown in Table 1 display different lipidation patterns; in other words, they are mono- or dilipidated and correspond to the C-termini of the H- or N-Ras protein. In addition to the lipid groups they incorporate a fluorescent label (NBD, Mant) or a photoactivatable group (benzophenone) and/or a maleimidocaproyl group (MIC), and a biotin group or an unmasked N-terminal cysteine which can be employed for coupling to expressed proteins by expressed protein ligation.[2e] Three peptides (**18**–**20**) incorporating the GerMant analogue of the farnesyl group were synthesized. Due to the laborious preparation of the GerMant residue (see above) in the coupling steps employing Fmoc-Cys(GerMant)OH only 1.5 equivalents of the building block were employed to ensure efficient use.

Finally, the applicability of the method to the synthesis of long lipidated peptides was investigated. To this end, decapeptide **27** and tetradecapeptide **28** were prepared, which both mimic the C-terminus of N-Ras. Both were obtained in milligram amounts and characterized by ^{1}H NMR spectroscopy and mass spectrometry (**27**: ESI-MS: m/z_{calcd} for $[M+H]^+ = 1887.0$, $m/z_{found} = 1887.3$; **28**: MALDI-MS (DHB): m/z_{calcd} for $[M+Na]^+ = 2481.3$, $m/z_{found} = 2481.8$; m/z_{calcd} for $[M+K]^+$: 2497.3, $m/z_{found} = 2497.9$). These peptides represent the longest S-palmitoylated and S-farnesylated peptides synthesized so far. Both were not accessible by the previously reported solid-phase method,[4] which demonstrates the superiority of the method reported in this article.

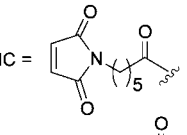
In conclusion we have developed an efficient solid-phase method for the synthesis of differently lipidated and additionally modified peptides. This methods meets the requirements of a widely applicable

Table 1: Lipidated peptides synthesized by means of the solid-phase method.

Entry	Cmpd.	Structure[a]	Yield [%]
1	**14**	NBD Aloc-Cys-Met-Ser-Cys-Lys-Cys–OMe S-tBu, Pal, Far	28
2	**15**	NBD H-Cys-Met-Lys-Leu-Pro-Cys-OMe S-tBu, Far	38
3	**16**	MIC-Gly-Cys-Met-Gly-Leu-Pro-Cys–OMe Pal, Far	30
4	**17**	NBD MIC-Gly-Cys-Met-Lys-Leu-Pro-Cys–OMe Pal, Far	28
5	**18**	MIC-Gly-Cys-Met-Gly-Leu-Pro-Cys-OMe Pal, GerMant	31
6	**19**	MIC-Gly-Cys-Met-Gly-Leu-Pro-Cys-OMe S-tBu, GerMant	23
7	**20**	Ac-Met-Ser-Cys-Lys-Cys-OMe Pal, GerMant	25
8	**21**	Aloc Ac-D-Met-D-Ser-D-Cys-D-Lys-D-Cys-OMe Pal, Far	45
9	**22**	BP MIC-Gly-Cys-Met-Lys-Leu-Pro-Cys-OMe Pal, Far	25
10	**23**	NBD-Aca-Gly-Cys-Met-Gly-Leu-Pro-Cys-OMe Pal, Far	32
11	**24**	BP Biotin-Aca-Gly-Cys-Met-Lys-Leu-Pro-Cys-OMe Pal, Far	20
12	**25**	NBD-Aca-Gly-Cys-Met-Gly-Leu-Pro-Cys-OH S-tBu, Far	37
13	**26**	NBD-Aca-Gly-Ser-Met-Gly-Leu-Pro-Cys-Val-Val-Met-OH Far	25
14	**27**	Fmoc-Gly-Thr-Gln-Gly-Cys-Met-Gly-Leu-Pro-Cys-OMe NTrt, Pal, Far	30
15	**28**	OtBu, OtBu Fmoc-Ser-Ser-Asp-Asp-Gly-Thr-Gln-Gly-Cys-Met-Gly-Leu-Pro-Cys-OMe OtBu, NTrt, Pal, Far	12

[a] Structures to define the abbreviations used:

MIC =



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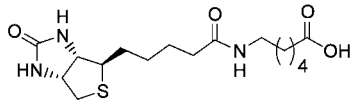
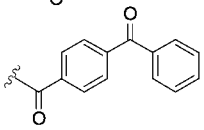


BP =



potentially automatable methodology and overcomes the limitations of earlier procedures. In terms of efficiency and flexibility this solid-phase method is superior to the solution-phase synthesis. It gives pure peptides more quickly and with superior overall yield.

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