Peptide Synthesis

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Efficient Solid-Phase Lipopeptide Synthesis Employing the Ellman Sulfonamide Linker**

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Dedicated to Professor H. Kunz on the occasion of his 65th birthday

Lipid-modified proteins are major determinants in the regulation of important biological processes, such as vesicular transport and cell signaling, growth, and differentiation.[1]

Tailor-made lipidated peptides that embody the characteristic lipidated amino acid sequences of their parent proteins have proven to be efficient reagents and chemical tools for chemical-biological, biochemical, biophysical, structural-biological, and cell-biological studies.[2,3]

For the efficient and rapid synthesis of these peptide conjugates, a flexible solid-phase technique is required. Such a technique must feature very mild, preferably neutral, conditions as the characteristic lipidated peptides often

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embody acid- and base-labile farnesyl thioethers and palmitic acid thioesters, allow for the introduction of additional reporter groups (e.g., fluorophores, biotin) and tags (e.g., for coupling to expressed proteins), and allow for the release of the peptides from the solid support as carboxylic acids or esters (depending on the precise structure of the natural blueprint, see below), or equipped with a different functional group (e.g., tags for surface immobilization) at the C terminus. Importantly, it must proceed with preparatively viable overall yields, which is particularly true for long multiply lipidated peptides that represent fully lipid-modified parts of lipoproteins. These protein conjugates are often not accessible in their completely lipidated form from gene-technological methods but need to be prepared by semisynthesis from suitably functionalized synthetic lipopeptides and expressed protein parts.[3] A prototypical example for such a lipidated sequence is the N-myristoylated and doubly S-palmitoylated N terminus of endothelial nitric oxide (NO) synthase (eNOS; see below).

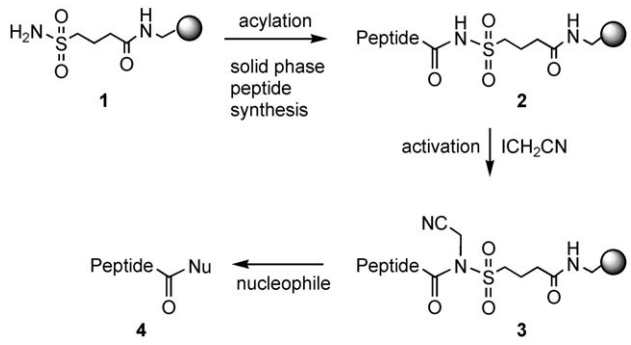
Currently, only the hydrazide linker meets the majority of these criteria.[4,5] However, although it gives access to differently modified lipopeptides, the oxidative cleavage of this anchoring group is accompanied by undesired side reactions,[6] thus resulting in product loss and low overall yields of typically 15–35%. Consequently, lipidated peptides with 10–15 amino acids have become available by means of this technique, but numerous attempts to break this barrier, in particular for multiply lipidated peptides, failed in our hands.

Herein, we describe the successful development of a solid-phase method that meets the demands described above. It employs pre-lipidated amino acid building blocks[4b] together with the Ellman alkyl sulfonamide linker for anchoring to the solid support.

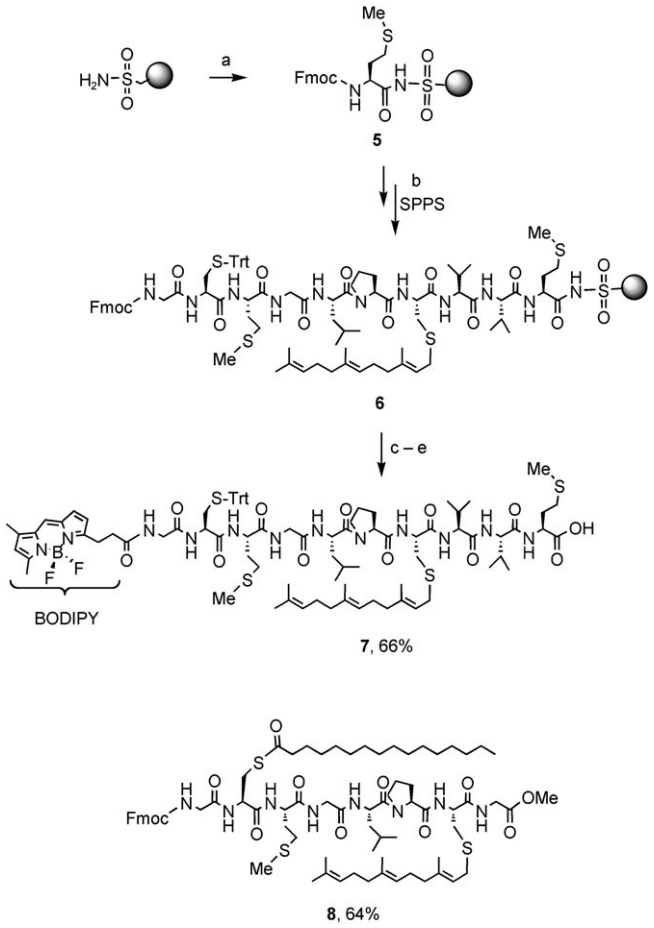
The alkyl sulfonamide linker developed by Ellman et al.[7] is stable to treatment with acid or base. The target compounds are released under very mild conditions by selective N-alkylation of the *N*-acyl sulfonamide and attack of different nucleophiles on the intermediary formed *N*-alkyl acyl sulfonamide (Scheme 1). The linker has been used successfully in the synthesis of different peptide derivatives and other compound classes on solid support.[8]

The lipopeptide solid-phase synthesis method was established by employing the C termini of the Ras proteins as targets. These proteins serve as central molecular switches in biological signaling cascades and are among the most important human oncogenes.[1] They are biosynthesized as precursor proteins with a C-terminal "CAAX-box" sequence (A = amino acid; X = Ser, Met), which is processed to the mature form with an S-farnesylated cysteine methyl ester at the C terminus (H-, K-, and N-Ras) and S-palmitoylated cysteines (H- and N-Ras) upstream.

In an initial sequence, N-Ras-derived CAAX-box peptide **7** was synthesized. To this end, FmocMet (Fmoc = 9-fluorenylmethoxycarbonyl) was coupled to the resin by using PyBOP for activation[7c] (Scheme 2), and after removal of



Scheme 1. Solid-phase peptide synthesis employing the Ellman sulfonamide linker.



Scheme 2. Solid-phase synthesis of N-Ras-derived CAAX box peptide **7** and peptide **8** employing the Ellman sulfonamide linker. a) Fmoc-Met-OH (4 equiv), PyBOP (4 equiv), DIPEA (8 equiv), $CHCl_3$, 8 h, −20 °C; b) 20% piperidine/DMF; standard solid-phase peptide synthesis (SPPS): 1. Fmoc-AA-OH (5 equiv), HBTU/HOBt (5 equiv), DIPEA (10 equiv), DMF, 2 h (for the incorporation of lipidated cysteines: AA (4 equiv), HBTU/HOBt/TMP (4 equiv), CH_2Cl_2/DMF (1:1), 4 h); 2. 20% piperidine/DMF; c) 1. 20% piperidine/DMF; 2. BODIPY-FL (5 equiv), HBTU/HOBt (5 equiv), DIPEA (10 equiv), DMF, 4 h; d) ICH_2CN (25 equiv), DIPEA (10 equiv), NMP, 24 h; e) H_2O (30 equiv), DMAP (0.8 equiv), THF, 24 h. DMF: dimethylformamide, PyBOP: benzotriazole-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate, HOBt: 1-hydroxybenzotriazole, HBTU: *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide, DIPEA: *N*,*N*-diisopropylethylamine, TMP: trimethylpyridine, NMP: 1-methyl-2-pyrrolidinone, DMAP: dimethylaminopyridine, BODIPY FL: 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-(*S*)-indacene-3-propionic acid.

the Fmoc group the peptide chain was elongated to yield immobilized decapeptide **6**. The farnesylated cysteine building block[4b] was attached to the solid support using HBTU/TMP in CH_2Cl_2/DMF (1:1) to avoid cysteine racemization.[9]

After removal of the N-terminal Fmoc group, the BODIPY FL fluorophore was attached to the lipopeptide. For release from the solid support, the acyl sulfonamide was converted into the cyanomethylsulfonamide by N-alkylation with 25 equivalents of iodoacetonitrile and DIPEA in NMP, followed by treatment with H_2O and DMAP. Thereby, the lipidated CAAX-box peptide was obtained in >85% purity, and after simple extraction and washing was isolated in 66% yield. To investigate whether the introduction of a base-labile S-palmitoylated cysteine and functionalization at the C terminus are also feasible by means of this technique, doubly lipidated lipopeptide methyl ester **8** was synthesized by analogy and obtained in 64% overall yield. The palmitoylated cysteine building block[4b] was coupled under the conditions described above for the farnesylated cysteine. Selective removal of the Fmoc group and chain elongation without an undesired S→N acyl shift of the palmitic acid in the N-terminally deblocked cysteine peptide were achieved by treatment with 1% DBU in DMF twice for 30 seconds followed by immediate acylation of the liberated amino group with preactivated FmocGly (preactivation carried out with 5 equivalents of HATU and 20 equiv of DIPEA) in CH_2Cl_2/DMF (7:1) as described before.[4]

Notably, in both syntheses competing S-alkylation of methionine or farnesylated cysteine and competing thioester cleavage were not detected.[10]

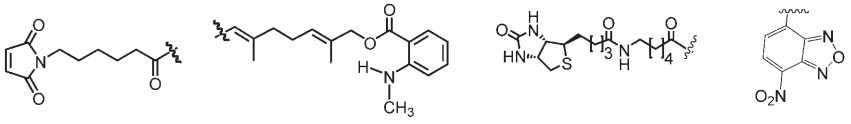
For the synthesis of lipidated Ras peptides terminating in a S-farnesylated cysteine methyl ester, which is characteristic for the fully matured proteins, a viable method for the racemization-free coupling of the bulky FmocCys(Far) building block to the sulfonamide linker had to be developed. While the use of PyBOP and related activation reagents did not yield satisfactory results, in situ formation of the amino acid fluoride by treatment of the carboxylic acid with tetramethylfluoroformamidinium hexafluorophosphate (TFFH) introduced by Carpino et al.[11] provided an advantageous solution to the problem. Preactivation with 3 equivalents of TFFH and 6 equivalents of DIPEA in CH_2Cl_2/DMF (1:1) for 10 min and double or triple coupling for 1.5 h gave high loading levels, which gratifyingly also translated into high overall yields for different Ras peptides (see Table 1). After deprotection of the amino acid side chains,[12] peptides **9**–**13**, which represent the C termini of N-Ras (**9** and **10**), H-Ras (**11** and **12**), K-Ras 4B (**13**), were obtained in 60–75% yield and in multi-milligram amounts.

Considered together, the synthetic Ras peptides shown in Table 1 provide examples that embody both the acid-labile farnesyl thioether and the base-sensitive palmitic acid thioester; different fluorophores (NBD, BODIPY, *N*-methylanthraniloyl (Mant)) attached to the N terminus,[13] to an amino acid side chain, or incorporated into a lipid group; a maleimide suitable for covalent coupling to expressed proteins by means of conjugate addition; and an N-terminally deprotected yet S-disulfide-masked cysteine suitable for coupling to proteins by expressed protein ligation.

Table 1: Lipidated peptides synthesized on a solid phase employing the Ellman sulfonamide linker as an anchoring group.

Entry	No.	Peptide structure	Parent protein	Yield [%]
1	**9**	NBD-Aca-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe	N-Ras	65
2	**10**	MIC-Gly-Cys(Pal)-Met-Gly-Leu-Pro-Cys(Far)-OMe	N-Ras	66
3	**11**	Ac-Met-Ser-Cys(Pal)-Lys-Cys(GerMant)-OMe	H-Ras	60
4	**12**	H_2N-Cys(*t*BuSS)-Met-Ser-Cys(*t*BuSS)-Lys-Cys(Far)-OMe	H-Ras	67
5	**13**	NH_2-Lys-Ser-Lys-Thr(O*t*Bu)-Lys-Cys(Far)-OMe	K-Ras 4B	75
6	**14**	Ac-Lys(NBD)-Ser-Gly-Ser-Gly-Gly-Cys(Pal)-Cys(Far)-OMe	Ras 2 from *Saccharomyces cerevis*	44
7	**15**	Biotin-Aca-Leu-Asn-Cys(Pal)-Cys(Pal)-Gly-Gln-Arg-Ala-Cys-Tyr-Arg-Trp-Ser-Lys(NBD)-Arg-Trp-Leu-OH	Phospholipase D	39

MIC= GerMant= Biotin-aca= NBD=



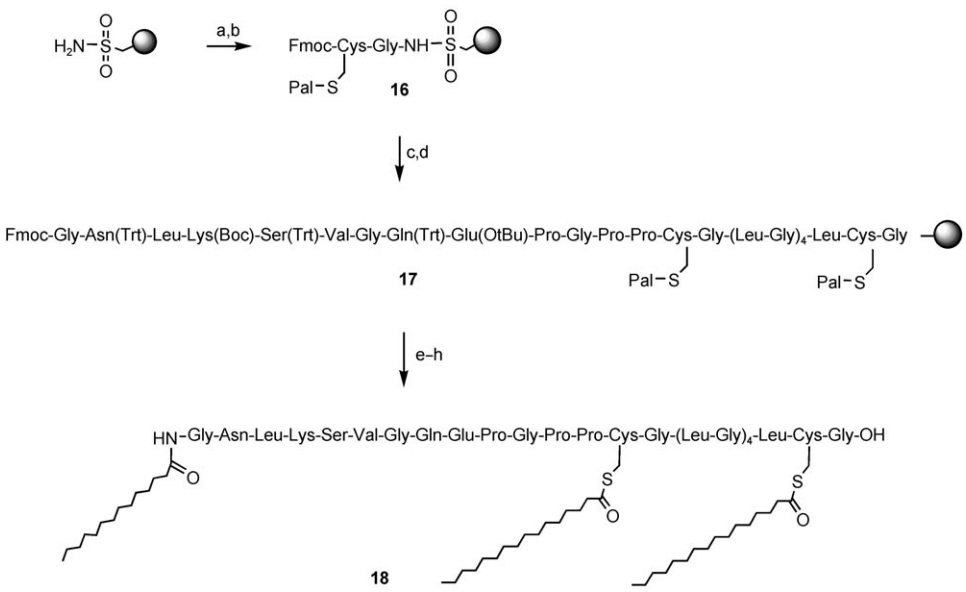
Remarkably, after release from the resin, the purity of these peptides is already fairly high (see also below), so that further purification to homogeneity is achieved readily by straightforward extraction and washing steps and without need for further chromatographic separation.

To determine whether the C-terminal cysteine partially racemizes under the coupling conditions described above, FmocProCys(Far)OMe was synthesized on the solid support and compared with reference compounds incorporating optically pure L and D amino acids synthesized in solution. Examination of the peptides by means of reverse-phase HPLC did not detect any racemization of the cysteine.

To investigate the scope of the method, the synthesis of heptadecapeptide **15**, which represents a characteristic partial structure of phospholipase D,[14] was attempted. This peptide carries two neighboring S-palmitoylated cysteines and embodies a fluorophore as well as a biotin tag. Phospholipase D peptide **15** was obtained in multi-milligram amounts and again in high purity (see Figure 1 and the Supporting Information) after release from the resin and deprotection of the amino acid side chains[14] by treatment with trifluoroacetic acid (TFA)/triethylsilane/H_2O/ethanedithiol (94:1:2.5:2.5) for 2 hours; furthermore, it was isolated in 39% overall yield and characterized with ^{1}H NMR spectroscopic and mass-spectrometric analysis (ESI MS: *m*/*z* calcd for $[M+2Na+H]^{3+}$: 1055.88; found: 1055.67).

Encouraged by this result, the synthesis of the N-terminal triply lipidated hexacosapeptide of eNOS, an enzyme that could not be expressed and isolated in its fully lipidated form, was attempted. This lipopeptide had been prepared by us before by means of an extended and very laborious multistep solution-phase synthesis, which required more than a year of development and execution, was riddled with severe isolation problems, and finally delivered the desired peptide with an overall yield of less than 1%.[15] All attempts to synthesize multiply lipidated peptides of comparable length by means of the hydrazide linker (see above) completely failed in our hands.

Much to our delight, the desired 26-mer peptide could be obtained in 25 mg and 24% yield of isolated product within three weeks by means of the methodology described herein (Scheme 3). This peptide was characterized by ^{1}H NMR spectroscopic and mass-spectrometric analysis (ESI MS: *m*/*z* calcd for $[M+H+K]^{2+}$: 1559.44; found: 1559.50; see also the Supporting Information).



Scheme 3. Solid-phase peptide synthesis of the N-myristoylated and doubly S-palmitoylated N-terminal 26-mer peptide **18** of endothelial NO synthase employing the Ellman sulfonamide linker. a) Fmoc-Gly-OH (8 equiv), DIC (8 equiv), 1-MIM (8 equiv), CH_2Cl_2/DMF (4:1), 24 h, RT; b) 1. 20% piperidine/DMF; 2. Fmoc-Cys(Pal)-OH (4 equiv), HBTU/HOBt/TMP (4 equiv), CH_2Cl_2/DMF (1:1), 4 h; c) 1. 1% DBU in DMF for 2×30 s; 2. DMF (2×15 s); 3. HATU (5 equiv), Fmoc-Leu-OH (5 equiv), DIPEA (20 equiv), CH_2Cl_2/DMF (4:1)—preactivated for 20 min—, 3 h; d) SPPS: 1. 1% DBU in DMF (2×30 s); 2. Fmoc-AA-OH (5 equiv), HATU (5 equiv), DIPEA (10 equiv), DMF, 3 h; repeat (b and c) for Fmoc-Cys(Pal)-OH coupling; e) 1% DBU/DMF (2×30 s); f) DIPEA (8 equiv), $H_3C(CH_2)_{12}COCl$ (4 equiv), NMP; g) 1. ICH_2CN (25 equiv), DIPEA (10 equiv), NMP, 24 h; 2. H_2O (30 equiv), DMAP (0.8 equiv), THF, 24 h; h) CF_3COOH/ethanedithiol/H_2O/TIS (96:2:1:1), 24% yield. DIC: 1,3-diisopropylcarbodiimide, 1-MIM: 1-methylimizadole, DBU: 1,8-diazobicyclo[5.4.0]undec-7-ene, HATU: *N*-[(dimethylamino)-1-*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium; TIS: triisopropylsilane.

In the synthesis of NO-synthase-derived peptide **18**, couplings of the amino acid building blocks and release from the solid support were carried out as described above. The N-terminal glycine was introduced as the Fmoc derivative to give intermediate **17**, and after removal of the Fmoc group the N terminus was acylated with myristoyl chloride. All acid-labile side-chain protecting groups were removed simultaneously after release of the peptide from the resin. The N-myristoylated and doubly S-palmitoylated hexacosa eNOS lipopeptide represents the longest lipidated peptide synthesized on a solid support so far.

The successful and preparatively viable syntheses of this lipopeptide and the peptides shown in Scheme 2 and Table 1 prove that the use of the Ellman sulfonamide linker provides a major advance in terms of efficiency and practicability in methodology development for the synthesis of lipopeptide conjugates and, in extension, to the synthesis of tailor-made semisynthetic lipidated proteins[2,3] for research in chemical biology.

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