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Solid-Phase Synthesis of Lipidated Ras Peptides Employing the Ellman Sulfonamide Linker

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Abstract: A detailed study on the solid-phase synthesis of lipidated peptides of the Ras family employing the Ellman sulfonamide linker is reported. Using the C-terminal N-Ras sequence, critical issues such as lipidated amino acid resin loading, peptide elongation in the presence of labile groups and optimized conditions for release of the peptides were investigated. A versatile methodology for the synthesis of peptides with diverse lipid motifs and C-terminal methyl esters has accordingly been established.

Keywords: lipopeptides · peptides · racemization · solid-phase synthesis · sulfonamide linker

Introduction

Intracellular signaling and transport strongly depend on peripheral membrane proteins specifically anchored to one face of a given membrane. The functioning of these membrane proteins relies on the correct protein localization and affinity for specific internal cell membranes and this is governed by post-translational lipidations.[1–3] Representative examples of this process can be found in proteins of the Ras superfamily. These small guanosine triphosphatases (GTPases) play critical roles in controlling cellular processes, such as signal transduction, cell proliferation and differentiation, and transport.[4] As a result, mutations in Ras proteins can lead to uncontrolled cell growth and cancer.[5,6] Typically the small GTPase is lipidated via *S*-alkylation and/or *S*-acylation of cysteines in its C terminus. Post-translational prenylation (farnesylation or geranylgeranylation) and palmitoylation together with C-terminal methylation and specific phosphorylations are the typical fine-tuning mechanisms that are required for controlled and reversible membrane association of the Ras proteins.[7]

The importance of the membrane affinity of the post-translationally modified Ras proteins is balanced by an elaborate biochemical generation of these fully functionalized and modified lipidated proteins. Their biological synthesis and purification are challenging, time-consuming and in many cases not practical or applicable. Studies with Ras proteins lacking the post-translationally modified C terminus, are significantly easier performed, but lack the important information dictated by membrane association. Therefore chemical biology approaches have been developed that provide access to fully functional lipidated peptides and proteins. Together with the insertion of additional non-natural modifications, these chemical biology approaches have enabled the study of the complete functional proteins in the context of cellular membranes.[3,7–13]

We have previously shown that fully lipidated and modified Ras proteins can be generated by a combination of biological and chemical processes based on the connection of a truncated GTPase core to a synthetic lipidated peptide C terminus.[8,14] The development and application of solid-phase lipidated peptide synthesis techniques has played a major role in the synthesis of these proteins.[7,15,16] The major challenges of a fully solid-supported synthesis of lipidated peptides are the identification of orthogonally stable

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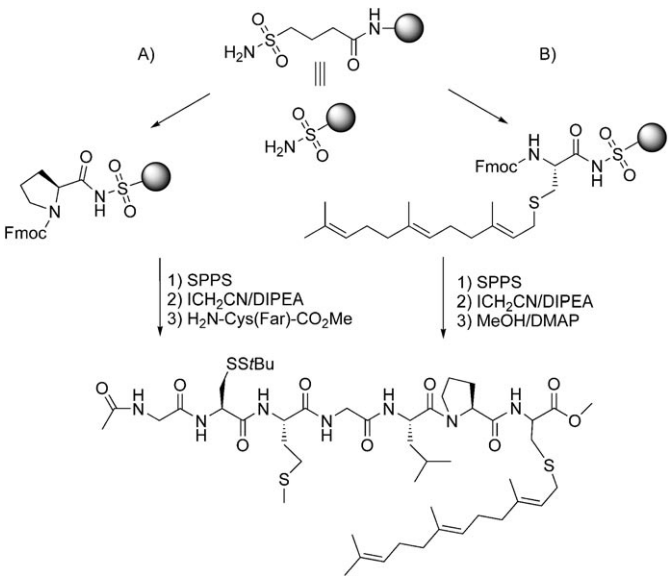
protecting groups, a solid-support linker that allows selective introduction of lipid functionalities as well as additional functional groups for tracing such peptides in biological systems (fluorescence and photoactivatable tags), specific C-terminal modifications, and functional groups for ligation to the protein core. Additionally, specific conditions typical for normal peptide synthesis have to be avoided due to incompatibilities with the lipid functionalities. Examples are the incompatibility of high acid concentrations, typically used to remove Boc-protecting groups, with the acid-labile prenyl groups, and of strong nucleophiles, used, for example, in the removal of the Fmoc-protecting group, with labile lipid thioesters. The development of new linker strategies for solid-phase lipidated peptide synthesis has therefore been attracting significant attention.[7,17] Ideally, these linkers provide a flexible and rapid entry for a wide variety of different types of lipidated peptides. The hydrazide linker and the sulfonamide linker are the two most versatile linkers that currently meet the majority of these criteria.[17–19]

The hydrazide linker has been extensively studied and applied.[20] It provides a flexible tool for the synthesis of several differently modified lipopeptides.[19,21] An alternative for solid-phase lipidated peptide synthesis is the safety-catch sulfonamide linker.[19] This linker was originally developed by Kenner[22] and later modified by Ellman,[23] who improved the loading efficiency and the cleavage conditions by using haloacetonitriles for activation and release. This linker has been successfully used in the synthesis of different peptide derivatives and other compound classes on solid support,[24] and has been shown by us in an initial report to also provide an advantageous platform for lipidated peptide synthesis.[19] The acyl sulfonamide functionality is stable to treatment with acid or base, but can be activated for release of the lipidated peptide under very mild conditions by selective *N*-alkylation of the *N*-acyl sulfonamide, and by subsequent attack of different types of nucleophiles. In general, the alkyl sulfonamide linker thus meets the requirements listed above for the synthesis of a variety of lipidated peptides.

Here we report in detail on the use of the alkyl sulfonamide linker for the synthesis of lipidated Ras peptides. The N-Ras sequence was used as a suitable platform to investigate the scope and limitations for this linker, as it embodies both a prenyl functionality and a palmitoyl group in its C terminus and additionally is post-translationally modified to a C-terminal methyl ester. Using the C-terminal N-Ras sequence critical issues such as lipidated amino acid resin loading, peptide elongation in the presence of labile groups and optimized cleavage conditions are reported. Two general approaches have been investigated to generate the C-terminal N-Ras sequences, differing in concept by the incorporation of the C-terminal functionality via peptide cleavage from the resin. On the one hand the characteristics of the cleavage of the complete peptide with methanol were explored (route B, Scheme 1), while on the other hand, the cleavage of the peptide from the resin with the completely post-translationally modified C-terminal cysteine was studied for the formation of several lipidated peptides (Scheme 1, route A).



Scheme 1. Alternative concepts (general scheme) for the synthesis of lipidated N-Ras peptides with C-terminal modifications using the acyl sulfonamide linker.

Results and Discussion

Loading of the acyl sulfonamide linker: It has previously been shown that a high acyl sulfonamide loading with minimal racemization may pose a problem for the acyl sulfonamide linker strategy.[24] Ellman et al. screened different loading conditions and reported the low reactivity of this linker and the concomitant racemization occurring during the loading step. Coupling conditions such as *N,N′*-diisopropylcarbodiimide (DIC) with HOBt failed and the addition of a catalytic amount of DMAP to a symmetric anhydride typically led to 10% racemization.[23] The optimal conditions found for loading of for example Boc-Phe-OH, being PyBOP with DIPEA in CH_2Cl_2 or $CHCl_3$ at −20°C, are, however, relatively unpractical when using a solid-phase peptide synthesis reactor. As an alternative, Fmoc-Leu-OH has been, for example, loaded on the alkyl sulfonamide linker using DIC and methylimidazole (MeIm) leading to low levels of racemization.[25] Fmoc-protected amino acid fluorides have been extensively used, in solution and solid-phase synthesis, especially for the coupling of hindered amino acids.[26,27] *N*-Protected (Boc, Z or Fmoc) acyl fluorides can be prepared in solution by reaction of the corresponding free acid with DAST or cyanuric fluoride and subsequently they can be purified by recrystallisation in good enantiopurities.[26–30] In situ conversion of the acid into the fluoride and subsequent coupling has also been reported by using TFFH and DIPEA.[31] These conditions were, for example, successfully applied for the synthesis of Z-phenylglycine fluoride (Z-Phg-F) and coupling to H-Pro-OH without

racemization. However, it has also been reported that Bz-Phe-OH was fully racemized when coupled to NH_2-Ala-CO_2Me using these conditions, thus indicating that this methodology finds its limitations, depending on the protected amino acid.[31] Recently, acyl fluorides have been used for loading the acyl sulfonamide linker.[29]

Most proteins of the Ras superfamily feature a C-terminal lipidated cysteine. Protected cysteines are one of the most critical amino acids regarding racemization and thus can pose a limitation to solid-phase peptide synthesis. Systematic studies performed by Barany et al.,[32] showed that most of the standard coupling protocols led to unacceptable epimerization levels for cysteines. In the same work, several improved methods for the incorporation of cysteines into peptides were reported. Basically, the use of DIC in combination with HOBt or HOAT, the use of BOP (or HATU) in combination with HOBt (or HOAT) and trimethylpyridine (TMP) as a base in a 4:4:4 ratio in CH_2Cl_2/DMF (1:1), or preformed pentafluorophenyl (Pfp) esters, led to minimal racemization levels. Racemization of cysteines had, however, not been studied for the loading conditions described above for the acyl sulfonamide linker. Only for the preparation of Fmoc-cysteine fluoride in solution with cyanuric fluoride, it was shown that synthesis and recrystallization did not affect cysteine enantiopurity. Loading of Fmoc-cysteine fluoride to the sulfonamide linker and nucleophilic displacement with H_2N-Phe-CO_2Me, led to a tolerable level of 3% of epimerization.[29] However, loading of lipidated cysteines and other loading conditions had not been studied.

To investigate the characteristics of the linker loading required for typical lipidated peptides, we focused on the loading of cysteine derivatives as these frequently constitute the C-terminal amino acid in fully post-translational modified Ras proteins. First the loading of Fmoc-Cys(Far)-OH to the linker via the in situ generation of Fmoc-Cys(Far)-fluoride by using TFFH and DIPEA (1:1:2), linker activation with iodoacetonitrile, and cleavage using methanol as a nucleophile was investigated. Diastereomeric dipeptide Ac-Pro-Cys(Far)-CO_2Me (**1**) (Scheme 2), corresponding to the C terminus of N-Ras, was therefore prepared on the sulfonamide linker using the conditions described above. To probe for racemization, two dipeptide diastereomers (L,L and L,D) were prepared in enantiomerically pure form on solid support using the hydrazine linker strategy. Analysis of the dipeptides obtained from the sulfonamide linker by ^{1}H NMR and chiral HPLC measurements showed that significant racemization of the cysteine had occurred. ^{1}H NMR spectra of L,L- and L,D-peptides synthesized on the hydrazine linker show characteristic signals corresponding to the methyl ester singlet as well as the NH proton. The doubling of the peaks of the enantiomerically pure samples results from rotamers. The spectra corresponding to the dipeptide synthesized on the acyl sulfonamide linker showed a mixture of two diastereomers, comparable to the spectra obtained with a known mixture of both diastereomers (Scheme 2A).

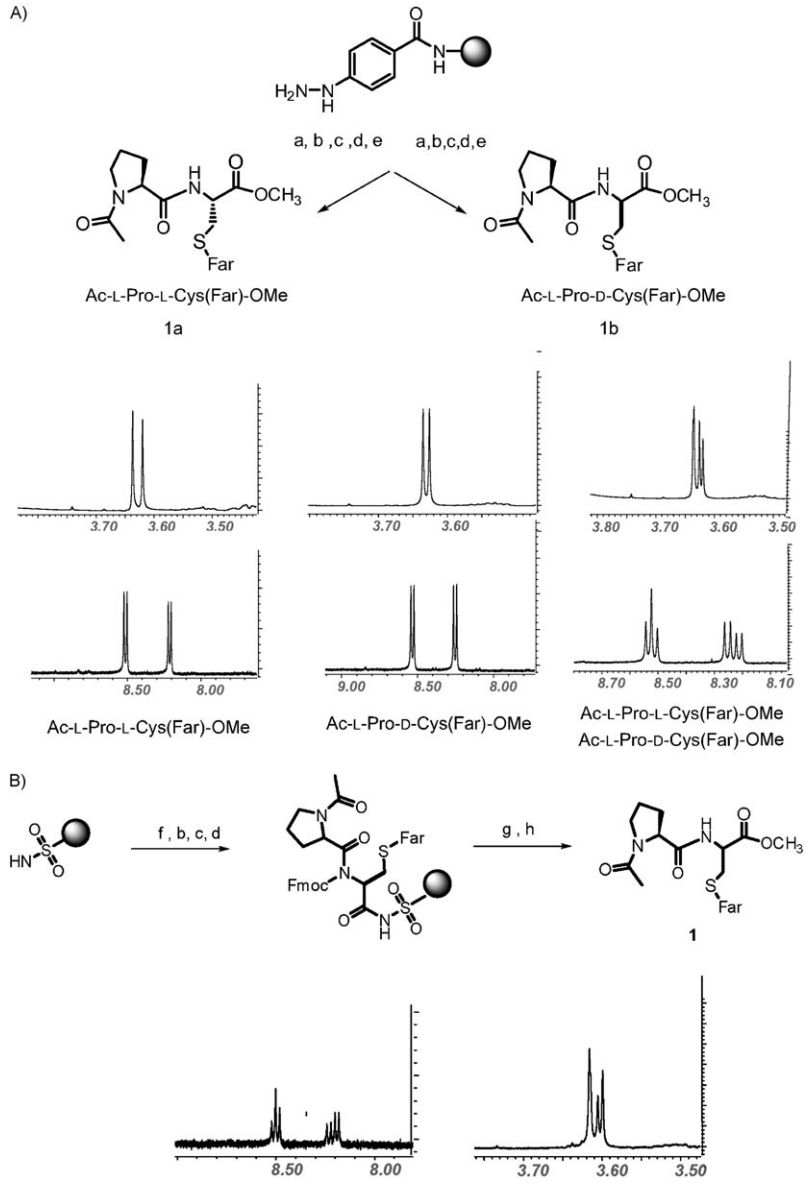
In order to determine the exact contribution of both the loading and the cleavage conditions to the observed racemization of the cysteine, both Fmoc-D- and Fmoc-L-Cys(Trt)-OH were loaded on the acyl sulfonamide linker using the TFFH/DIPEA (1:2). To avoid a possible racemization of the activated cysteine during cleavage with the weaker nucleophile methanol, release of the cysteine was performed using H_2N-Phe-CO_2Me as nucleophile, yielding dipeptide **2** (Scheme 3). Even though a high loading of the Fmoc-Cys(Trt)-OH amino acid was obtained, these conditions resulted in approximately 25% of racemization, as could be detected using normal-phase chiral HPLC (Table 1, entry 1). Clearly the in situ generation of the cysteine fluoride leads to epimerization.

Table 1. Loading conditions, loading efficiency and cysteine racemization detected by cleavage with H_2N-Phe-CO_2Me and subsequent chromatographic evaluation (n.d. = not determined).

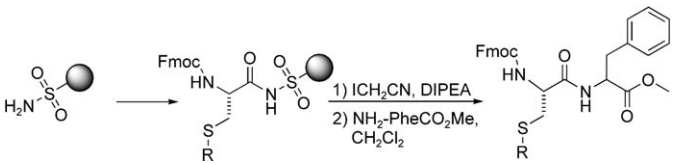
Entry	Conditions	*t*	Loading [%]	D-Cys [%]
1	H-L-Cys(Trt)-H/TFFH/DIPEA	4 h	70	25
2	H-L-Cys(Trt)-H/DIC/MeIm	16 h	60	30
3	H-L-Cys(Trt)-F/DIPEA	1 h	30	2.5
4	H-L-Cys(Far)-F/DIPEA	1 h	30	3
5	H-L-Cys(Far)-F/DIPEA	4 h	70	3
6	H-L-Cys(Far)-OH/PyBOP/DIPEA	8 h	11	n.d.
7	H-L-Cys(Far)-OH/DAST/pyridine	40 min	2	n.d.

Different approaches for the loading of cysteine derivatives to the acyl sulfonamide linker were therefore investigated. The use of DIC with MeIm resulted in a similar degree of racemization (Table 1, entry 2). Loading of Fmoc-Cys(Far)-OH was not efficient when using PyBOP/DIPEA (Table 1, entry 6) or DIC/HOBt (not shown). Stronger activation using DAST as a fluorinating agent resulted in side reactions with the farnesyl moiety and led to unsatisfying low loading efficiencies (Table 1, entry 7). Fmoc-Cys(Trt)-F could, however, be prepared by reaction of cyanuric fluoride and pyridine.[29] Loading of the sulfonamide linker was achieved in 30% and after cleavage only 3% of D-cysteine could be detected (Table 1, entry 3). These conditions could also be successfully applied to the synthesis of farnesylated Fmoc-cysteine fluoride, without side-reactions with the farnesyl chain, in 70% loading yield after longer reaction times (Table 1, entry 5). Cleavage with H_2N-Phe-CO_2Me resulted in dipeptide **3** (Scheme 3) with only low racemization.

Peptide chain elongation: Initial peptide synthesis protocols on the sulfonamide linker employed HBTU/HOBt/DIPEA (1:1:2) for coupling of model N-Ras peptides yielded only incomplete couplings. Gluszok et al. recently studied the reaction between a sulfonamide and HBTU and found that under basic conditions tetramethylsulfonylguanidines are formed.[33] The interception of HBTU through this pathway might explain the inefficient couplings observed. In our hands the combination of DIC/HOBt or PyBOP/HOBt for the elongation of the peptide sequence was more effective.



Scheme 2. A) Characteristic ^{1}H NMR signals of NH and methyl ester signals of Ac-L-Pro-L-Cys(Far)-CO_2Me (**1a**) and Ac-L-Pro-D-Cys(Far)-OCH_3 (**1b**) after cleavage from the hydrazide linker and of a mixture of **1a** and **1b** (NMR spectra represents rotamers). B) ^{1}H NMR signals of **1** after cleavage from acyl sulfonamide linker. a) Fmoc-L- or Fmoc-D-Cys(Far)-OH, HOBt, HBTU, DIPEA in DMF/CH_2Cl_2, b) 20% piperidine in DMF; c) Fmoc-Pro-OH, DIC, HOBt in DMF; d) acetic acid, DIC, HOBt in DMF; e) $Cu(OAc)_2$, MeOH, Pyridine, acetic acid in CH_2Cl_2; f) Fmoc-L-Cys(Far)-OH, TFFH, DIPEA in DMF/CH_2Cl_2; g) ICH_2CN, DIPEA in NMP; h) MeOH, DMAP in CH_2Cl_2/THF.



Scheme 3. Resin loading with cysteine fluorides and cleavage with H_2N-Phe-CO_2Me to afford dipeptides **2** and **3**.

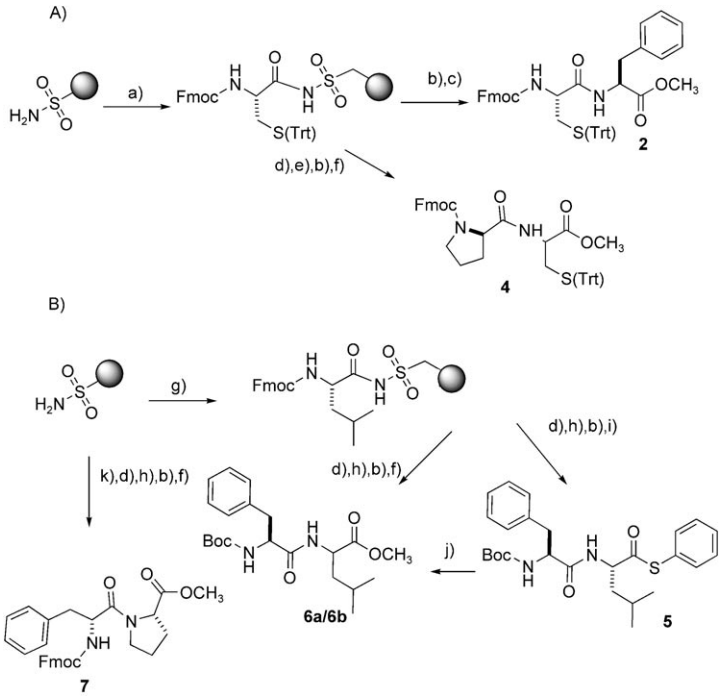
Because of the higher cost of PyBOP and lower stability, the DIC/HOBt combination was chosen for all further peptide chain elongations. Hence, peptide synthesis was performed using four equivalents of the corresponding amino acid and DIC/HOBt in a ratio 1:1:1 and proceed with complete coupling. The same conditions were applied when performing the synthesis in an automated microwave peptide synthesizer (Liberty, CEM) and gave similar results.

Peptide release from the sulfonamide linker: The safety-catch principle incorporated into the sulfonamide linker is based on the high stability of the N-acyl sulfonamides under acidic and basic conditions. This stability is lost when the NH-acyl sulfonamides are alkylated, which allows for a more efficient attack by a nucleophile at the C-terminal acyl functionality of the peptide and release of the peptide from the resin. Alkylation of the *N*-acyl sulfonamide has been reported with trimethylsilyldiazomethanes or haloacetonitriles under basic conditions. Although Flavell et al. proved by NMR that ε-cyanomethylation of methionine occurred under these conditions,[34] Backes and Ellman already reported in 1999 that protected cysteine and methionine were not methylated during the release.[23] Similarly, we did not observe any *S*-alkylation of methionine or protected cysteines during the synthesis of the N-Ras sequences.[19] The second step of the cleavage is the nucleophilic attack on the *N*-alkylated sulfonamide. Different nucleophiles (amines, amino acid methyl esters and thiols) have been successfully used for this purpose.[23,24,35] As mentioned above, N-Ras proteins are post-translationally modified and this includes the methylation of the C-terminal cysteine. For that reason peptide release from the solid support using methanol, thus providing the peptide methyl ester, would be highly desirable. Alcohols can be used for the nucleophilic attack after activation of the linker, but due to their low nucleophilicity a catalytic amount of DMAP should be added. This approach has already proven suitable for the release of the peptides from

the solid support as glycine ester derivatives.[36] Racemization was not an issue in this case as glycine, the C-terminal amino acid in this study bears no stereogenic center.

In order to determine the influence of different cleavage conditions on cysteine racemization Fmoc-L- or Fmoc-D-Cys(Trt)-F was generated using the cyanuric fluoride approach (see above) to avoid racemization and loaded on the acyl-sulfonamide linker. Two dipeptide diastereomers (L,L and L,D Fmoc-Pro-Cys) were then assembled on the solid support. The linker was subsequently activated with ICH_2CN and DIPEA, and finally a solution of methanol with catalytic amounts of DMAP was used for the nucleophilic cleavage of the dipeptides **4a**/**4b**, that were obtained in 80% yield (Scheme 4A). The released dipeptides **4a**/**4b** were analyzed by normal-phase chiral HPLC what showed that 35% of epimerization had occurred under these cleavage conditions (Scheme 3 and Table 2, entry 2). Similar epimerization results were obtained by loading Fmoc-Leu-OH using either DIC/MeIm or Ellman's conditions (PyBOP, DIPEA in CH_2Cl_2 at −20°C). In both cases a Boc-Phe-Leu dipeptide was assembled and after cleavage with a solution of methanol and DMAP, dipeptide **6** was obtained in 44% yield and with 35% of epimerization (Table 2, entry 4). In contrast, no epimerization was observed in the loading of Fmoc-Pro and in the release of dipeptides LL/LD-Leu-Pro (**7**) with MeOH and catalytic amounts of DMAP (Table 2, entry 5). Release of the Boc-Phe-Leu dipeptide with thiophenol as activated ester **5** (Table 2, entry 3) and subsequent transesterification with methanol to give compound **6** also led to racemization after stirring **5** in methanol. Thus, this procedure is not suitable for further use.

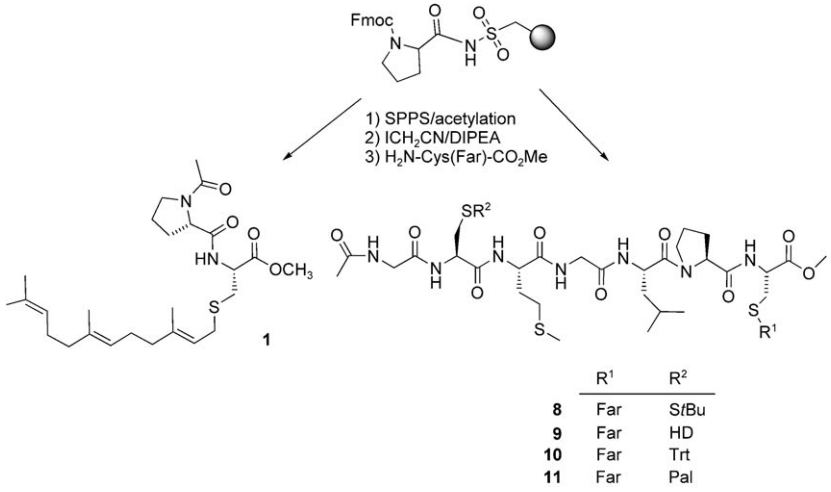
These results demonstrate that release of the peptides with methanol leads to racemization of amino acids, including the important cysteines. In order to obtain access to N-Ras peptides with a C-terminal methyl ester, a different strategy was therefore followed. This strategy was based on the release of the peptide from the resin using the amine functionality of the C-terminal amino acid already functionalized as methyl ester. To establish this approach first Fmoc-Pro-OH was loaded onto the resin and after Fmoc deprotection and acetylation was subsequently released with the farnesylated cysteine methyl ester H_2N-Cys(Far)-CO_2Me (Scheme 5). Following reported conditions, that is, 24 h shaking for activation with ICH_2CN and subsequent shaking for 6 h with the corresponding amine, the dipeptide product was obtained in low yields only. The yield could not be improved using longer reaction times in the cleavage step. Microwave-assisted chemistry, however, al-



Scheme 4. Dipeptides synthesized using the Ellman sulfonamide linker. A) Synthesis of dipeptides **2** and **4**; B) synthesis of dipeptides **5**, **6** and **7**. a) H-Cys(Trt)-F, DIPEA in CH_2Cl_2; b) ICH_2CN, DIPEA in NMP; c) H_2N-Phe-CO_2Me in CH_2Cl_2; d) 20% piperidine in DMF; e) Fmoc-Pro-OH, DIC; HOBt in DMF; f) MeOH, DMAP in CH_2Cl_2; g) Fmoc-Leu-OH, DIC, MeIm, in CH_2Cl_2/DMF; h) Boc-Phe-OH, DIC; HOBt in DMF; i) thiophenol in CH_2Cl_2; j) MeOH; k) Fmoc-Pro-OH, DIC, MeIm in CH_2Cl_2/DMF.

Table 2. Racemization of the model dipeptides upon release from the Ellman linker.

Entry	Cleavage	Yield [%]	D-AA [%]	Peptide
1	$PheCO_2Me$	47	2.5	**2**
2	MeOH/DMAP	80	35	**4**
3	thiophenol	85	<1	**5**
4	MeOH/DMAP	44	35	**6**
5	MeOH/DMAP	31	<1	**7**



Scheme 5. Peptides corresponding to N-Ras termini synthesized using this strategy.

lowed to accelerate the release reaction significantly. Using five equivalents of the farnesylated cysteine methyl ester in a solution of CH_2Cl_2/THF (1:1), the reaction was fast and the desired dipeptide **1** could be obtained after 10 min microwave irradiation at 50 °C with an initial microwave power of 40 W. The dipeptide **1** was isolated from the reaction mixture in 40 % yield, high purity, and without racemization by preparative HPLC (Scheme 5).

Lipidated Ras peptide synthesis employing the sulfonamide linker: After having established a suitable protocol for amino acid loading, peptide elongation, and peptide release, the applicability of the synthesis of the N-Ras sequences **8**–**11** was evaluated. The peptides correspond to the mono- and double-lipidated N-Ras C terminus, featuring for example labile palmitoylated cysteines **11** and stable thioether mimics thereof **9**. These peptides were obtained following the procedure detailed above in high purities and without racemization. Farnesylated N-Ras **8** could be obtained in high purity and in 18 % overall yield after preparative HPLC purification. The same strategy was used for the synthesis of the double lipidated N-Ras peptide **9**, bearing a farnesyl and a hexadecyl moiety as a non-hydrolysable mimic of the fully lipidated N-Ras sequence (farnesylated and palmitoylated), and was obtained in 16 % yield. Palmitoyl groups are known to be highly sensitive to nucleophiles. Therefore, a modified strategy was used for the synthesis of the fully lipidated N-Ras sequence **11**, bearing a farnesyl and a palmitoyl group. In this case, the release step was performed in a buffered mixture containing farnesylated cysteine methyl ester as well as acetic acid and pyridine. Peptide **11** could be obtained following this protocol as determined by LCMS of the crude mixture, but unfortunately separation of **11** from excess amine could not be achieved by reverse-phase preparative HPLC, probably due to the high hydrophobicity of the product. Therefore, trityl-protected peptide **10** was synthesized and isolated, and palmitoylation was performed in solution, affording peptide **11** in 7 % overall yield.

Conclusion

Cysteine is one of the amino acids most prone to racemization. This puts restraints on the synthetic methodology for peptide synthesis in general, and for synthesis of lipidated peptides with C-terminal cysteine esters in particular. The sulfonamide linker in principle offers an attractive linker system to generate lipidated peptide esters, due to its orthogonality to the different functional groups in the peptide, and thus is a good alternative to the hydrazide linker. The direct generation of C-terminal cysteine methyl esters, however, by direct release from solid support turned out to be challenging. Optimization of amino acid loading, peptide elongation and peptide release conditions was required to successfully generate lipidated N-Ras peptides. Loading of either trityl protected or farnesylated cysteines could be achieved using prior formation of acyl fluoride in solution via cyanuric acid fluoride. Elongation of the peptide on the solid support is preferably done with the DIC/HOBt combination. Peptide cleavage from the sulfonamide linker with alcohols and catalytic amounts of DMAP leads to racemization for cysteines. A racemization free cleavage could be obtained by release with the C-terminal amino acid of the peptide via nucleophilic attack of its amino group under microwave conditions. Purification by preparative HPLC afforded the desired peptides in higher purity and yields in the range of those previously reported.[17] Via this protocol differently lipidated C-terminal N-Ras peptides could be obtained in high purity and without racemization.

Experimental Section

General protocol for the synthesis of lipopeptides 8–11: The resin was loaded with Fmoc-L-Pro-OH (DIC, MeIm). In general a resin with an initial loading of 0.40–0.45 mmol $g^{-1}$was employed. Peptide elongation was performed using DIC/HOBt on an automated microwave peptide synthesizer. After washing several times with DMF/CH_2Cl_2/DMF, 0.1–0.15 mmol of the resin were activated with ICH_2CN/DIPEA and nucleophilic displacement was carried out with a solution of NH_2-Cys-(Far)CO_2Me under microwave irradiation. Lipopeptides were isolated in 8 to 20 mg amounts after purification by preparative HPLC. For detailed descriptions and HPLC conditions see the Supporting Information.

Ac-Gly-Cys(S*t*Bu)-Met-Gly-Leu-ProCys(Far)-CO_2Me (8): 18 % yield; $[\alpha]_D^{20} = -51.42$ (c = 0.14, $CHCl_3$); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.88 (t, 6H, J = 6.83 Hz, 6H, Leu-CH_3), 1.28 (s, 9H, *t*Bu), 1.44 (m, 2H, Leu-Hβ), 1.53 (s, 6H, J = 1.61 Hz, Far-CH_3), 1.64 (d, 6H, J = 4.11 Hz, Far-CH_3), 1.66 (m, 1H, Leu-Hγ), 1.85 (s, 3H, Ac), 1.92 (m, 4H, Far-CH_2, Pro-Hγa, Met-Hβa), 2.01 (m, 8H, 3 Far-CH_2, Pro-Hγb, Met-Hβb), 2.02 (s, 3H, Met-CH_3), 2.64–2.72 (m, 1H, Cys(S*t*Bu)-Hβa), 2.75 (m, 0.5H, Cys(Far)-Hβa), 2.81–2.76 (m, 1H, Cys(S*t*Bu)-Hβb), 2.86 (dd, 1H, J = 13.89, 5.38 Hz, Cys(Far)-Hβa), 2.94 (m, 1H, Cys(Far)-Hβb), 3.17 (tdd, 2H, J = 19.76, 12.99, 8.08 Hz, Cys(Far)-CH_2), 3.50 (m, 1H, Pro-Hδa), 3.62, 3.65 (s, 3H, OCH_3), 3.71 (m, 5H, Pro-Hδa, Gly-Hα), 4.23–4.32 (m, 1H, Met-Hα), 4.39 (m, 2H, Pro-Hα, Cys(S*t*Bu)-Hα), 4.50 (m, 2H, Cys-(Far)-Hα), 4.57 (m 1H, Leu-Hα), 5.07 (q, 2H, J = 5.36 Hz, Far), 5.15 (m, 1H, Far), 7.97 (m, 2H, Leu-NH, Gly-NH), 8.09 (d, 1H, J = 7.84 Hz, Met-NH), 8.16 (t, 1H, J = 5.66 Hz, Gly-NH), 8.21 (d,1H, J = 7.84 Hz, Cys-(S*t*Bu)-NH), 8.25 ppm (d, 1H, J = 7.84 Hz, 1H, Cys(Far)-NH); LC-MS (ESI): m/z: calcd for $C_{48}H_{84}N_8O_9S_4$: 1028.51 $[M+H]^+$; found: 1027.84 $[M+H]^+$, 1044.18 $[M+NH_4]^+$; t_R = 10.57 min; ES MS: m/z: calcd for $C_{48}H_{84}N_8O_9S_4$: 1028.50514; found 1028.50645 $[M+H]^+$, 1050.48756 $[M+Na]^+$.

Ac-Gly-Cys(Hd)-Met-Gly-Leu-ProCys(Far)-CO_2Me (9): 16 % yield; $[\alpha]_D^{20} = -34.64$ (c = 0.28, $CHCl_3$).^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.85 (m, 9H, Leu-CH_3, $HDCH_3$), 1.21 (m, 24H, CH_2HD), 1.45 (m, 3H, CH_2HD, Leu-Hγ), 1.54 (s, 6H, Far-CH_3), 1.61 (d, 6H, J = 1.34 Hz, Far-CH_3), 1.67 (m, 2H, Leu-Hβ), 1.80–2.06 (m, 14H, Far-CH_2, HD-CH_2, Pro-Hγ, Met-Hβ), 1.83 (s, 3H, Ac), 2.02 (s, 3H, Met-CH_3), 2.53–2.35 (m, 4H, Pro-Hβ, Met- Hγ), 2.69–2.61 (m, 2H, Cys(Far)-Hβa, Cys(HD)-Hβa), 2.84–2.74 (m, 2H, Cys(Far)-Hβb, Cys(HD)-Hβb), 3.14 (ddd, J = 25.39, 12.93. 7.63 Hz, 2H, Cys(Far)-CH_2), 3.50 (m, 1H, Pro-Hδa), 3.60 (s, 3H, OCH_3), 3.71–3.58 (m, 3H, Pro-Hδb, Gly-Hα), 4.25 (dt, J = 8.68, 4.80 Cys-(Far)-Hα), 4.43–4.34 (m, 3H, Met-Hα, Pro-Hα, Cys(HD) Hα), 4.56 (dt, 1H, J = 8.64, 5.87 Hz, Leu-Hα), 5.05 (dd, 2H, J = 12.54, 5.98 Hz, Far), 5.14 (t, 1H, J = 7.57 Hz, Far), 7.93–8.21 ppm (m, 6H, Gly-NH, Leu-NH, Cys-(Far)-NH, Cys(HD)-NH, Met-NH); LC-MS: m/z: calcd for $C_{60}H_{105}N_7O_9S_3$: 1164.72; found: 1164.33 $[M+H]^+$, 1181.04 $[M+NH_3]^+$; t_R = 11.98 min; ES MS: m/z: calcd for $C_{60}H_{105}N_7O_9S_3$: 1164.72087 $[M+H]^+$; found: 1164.72168 $[M+H]^+$, 1186.70339 $[M+Na]^+$.

Ac-Gly-Cys(Trt)-Met-Gly-Leu-ProCys(Far)-CO_2Me (10): 12% yield; $[\alpha]_D^{20} = -1.61$($c = 0.31$, $CHCl_3$); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta =$ 0.83 (m, 6H, Leu-CH_3), 1.29–1.48 (m, 3H, Leu-Hβ, Leu-Hγ), 1.52 (s, 6H, Far-CH_3), 1.60 (s, 6H, Far-CH_3), 1.67 (m, 2H, Leu-Hβ), 1.84–2.06 (m, 12H, Far-CH_2, Pro-Hγ, Met-Hβ), 1.82 (s, 3H, Ac), 1.97 (s, 3H, Met-CH_3), 2.36–2.41 (m, 2H, Pro-Hα), 2.63–2.69 (m, 1H, Cys(Far)-Hβa), 2.75–2.79 (m, 2H, Cys(Far)-Hβb, Cys(Trt)-Hβa), 2.79–2.92 (m, 1H, Cys(Trt)-Hβb), 3.15 (ddd, $J =$19.53, 12.10, 5.72 Hz, 2H, Cys(Far)-CH_2), 3.49 (m, 1H, Pro-Hδa), 3.60 (s, 3H, OCH_3), 3.63 (m, 5H, Pro-Hδb), 3.68 (t, 2H, $J =$ 6.03 Hz, Gly-Hα, 4.17–4.22 (m, 1H, Cys(Far)-Hα), 4.30 (m, 1H, Met-Hα), 4.37 (m, 2H, Pro-Hα, Cys(Trt)-Hα), 4.55 (dd, 1H, $J =$13.88, 8.68 Hz, Leu-Hα), 5.05 (d, 2H, $J =$5.25 Hz, Far), 5.15 (dd, 1H, $J =$15.67, 8.01 Hz, Far). 7.19–7.71 (m, 15H, Ar), 7.90–7.95 (m, 2H, Gly-NH, Leu-NH), 8.01 (m, 1H, Cys-Far-NH), 8.11 (t, 1H, $J =$5.47 Hz, Gly-NH), 8.17 (d, 1H, $J =$8.02 Hz, Met-NH), 8.20 ppm (d, 1H, $J =$7.63 Hz, Cys(Trt)-NH); LC-MS: m/z: calcd for $C_{63}H_{87}N_7O_9S_3$: 1181.57 $[M+H]^+$; found 1182.06 $[M+H]^+$, 1204.41 $[M+Na]^+$; t_R = 11.13 min; ES MS: m/z: calcd for $C_{63}H_{87}N_7O_9S_3$: 1182.58104 $[M+H]^+$, found: 1182.58104 $[M+H]^+$.

Ac-Gly-Cys(Pal)-Met-Gly-Leu-ProCys(Far)-CO_2Me (11): ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta =$0.85 (m, 9H, Leu-CH_3, PalCH_3), 1.19 (m, 24H, CH_2Pal), 1.35 (m, 3H, CH_2HD, Leu-Hγ), 1.52 (s, 6H, Far-CH_3), 1.60 (s, 6H, Far-CH_3), 1.67 (m, 2H, Leu-Hβ), 1.82–2.01 (m, 14H, Far-CH_2, Pal-CH_2, Pro-Hγ, Met-Hβ), 1.89 (s, 3H, Ac), 1.93 (s, 3H, Met-CH_3), 2.40–2.59 (m, 4H, Pro-Hβ, Met- Hγ), 2.63–2.70 (m, 2H, Cys(Far)-Hβa, Cys(Pal)-Hβa), 2.86–2.91 (m, 2H, Cys(Far)-Hβ), 2.95–3.20 (m, 4H, Cys-(Far)-CH_2, Cys(Pal)-Hβ), 3.51 (m, 1H, Pro-Hδa), 3.65 (s, 3H, OCH_3), 3.57–3.69 (m, 1H, Pro-Hδb), 3.75 (t, 2H, $J =$3.84 Hz, Gly-Hα), 4.45–4.68 (m, 5H, Cys(Far)-Hα, Met-Hα, Pro-Hα, Cys(Pal) Hα, Leu-Hα), 5.02 (m, 2H, Far), 5.13 (m, 1H, Far), 7.28–7.32 (m, 2H, Gly-NH, Leu-NH), 7.40–7.42 (m, 1H, Cys-(Far)-NH), 7.51 (m, 2H, Gly-NH, Cys(Pal)-NH), 7.70 ppm (m, 1H, Met-NH); LC-MS: m/z: calcd for $C_{60}H_{103}N_7O_{10}S_3$: 1178.70 $[M+H]^+$;found 1178.19 $[M+H]^+$, 1194.34 $[M+NH_3]^+$; t_R = 11.48 min; ES MS: m/z: calcd for $C_{60}H_{103}N_7O_{10}S_3$: 1178.700013 $[M+H]^+$; found 1178.70107 $[M+H]^+$.

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