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ORIGINAL ARTICLE

Lipidated peptides via post-synthetic thioalkylation promoted by molecular sieves

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Abstract A thioalkylation procedure, which uses molecular sieves to promote the reaction, was exploited to provide peptides with useful functional groups (lipidic moieties), naturally occurring on proteins as post-translational modifications. The procedure was further implemented to synthesize tailor-made lipidated peptides, interesting tools to investigate biological processes involving their Ras parent proteins. Moreover, the one-pot preparation of multi-alkylated peptides confirms the versatility and flexibility of the employed methodology.

Keywords Peptide thioalkylation · Molecular sieves · Lipidation · Farnesylation · Multi derivatization

Introduction

Post-translational modifications occurring on cysteines are essential for the biological function of many proteins, such as those belonging to the Ras family, that play a crucial role in cancer (Bourne et al. 1990; Boguski and McCormick 1993; Vetter and Wittinghofer 2001). In fact, misregulated Ras signaling is involved in the establishment of around 30 % of human cancers.

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The study of Ras signal transduction is an important field, as many useful drugs to combat diseases, such as cancer, may develop from full understanding of this important pathway (Brunsveld et al. 2006b). In particular, the three isoforms H, N-, and K-Ras are post-translationally lipidated on a cysteine residue, at their C-termini, via S-isoprenylation (farnesyl or geranylgeranyl moieties) and S-palmitoylation. These modifications, by possibly anchoring the protein to the membranes, may likely influence the biological function (Hancock et al. 1990; Hall 1994).

Thus far, the synthesis of lipidated protein segments has been performed by employing two different strategies; the first in which a prelipidated amino acid derivative is introduced into a peptide sequence during its synthesis (Brunsveld et al. 2006a, b; Lee and Vince 1978; Brown et al. 1991; Perrey and Uckun 2001) and the second based on lipidation performed on a peptide provided with a free thiol group (Berezovski et al. 2002; Gomashchi et al. 1995; Okeley and Gelb 2004). Several protocols that used lipidated cysteine building blocks have been already described, such as the Vince et al. method which allows preparation of alkylated cysteines reducing the cystine with sodium in liquid ammonia and adding the appropriate alkyl halide, as subsequent step (Lee and Vince 1978). Poulter et al. synthesized a farnesylated cysteine and a cysteine methyl ester by employing farnesyl chloride in an ammonia/methanol solution (Brown et al. 1991). Uckun et al. performed the S-alkylation of an *N*-acetyl cysteine with different alkyl bromides and used sodium ethoxide as base (Perrey and Uckun 2001). More recently, Waldmann and co-workers published an elegant thiol-ene reaction to alkylate a cysteine derivative; this strategy implemented as substrates 1-hexadecene and a cysteine derivative in presence of a radical initiator (AIBN) (Triola et al. 2008). As concerning with the S-lipidation method selectively performed on peptide

sequences, it is worth mentioning the valuable protocol by Naider and co-workers which succeeded in the farnesylation of peptides using acidic conditions in presence of $Zn(OAc)_2$ and an organic aqueous cocktail as solvent (Naider and Becker 1997; Xue et al. 1992). Further examples of this kind of strategy are represented by the Poulter's protocol, which allows S-alkylation in presence of an organic base (DIPEA) (Berezovski et al. 2002), and Gelb's procedure, which consents to prenylate a peptide under inorganic basic conditions (such as a solution of 0.5 M $KHCO_3$) (Gomashchi et al. 1995). Moreover, the Waldman procedure (Triola et al. 2008) has been also employed to lipidate fully synthesized peptides useful as self-adjuvanting antigenic sequences (Wright et al. 2013).

Herein, we describe a mild and chemoselective procedure for introducing lipid functionalities, like farnesyl and hexadecyl groups, on peptide sequences useful for investigating the biological processes of the related proteins. The method uses molecular sieves to promote the reaction, and can be further employed to perform multi-synthetic modifications on the same molecule.

Materials and methods

Chemicals and equipment

Fmoc-protected amino acids, Rink Amide MBHA resin, *N*-hydroxybenzotriazole (HOBT), benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium (PyBOP) were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland), piperidine and diisopropylethylamine (DIPEA) were purchased from Fluka (Milwaukee, WI), all remaining solvents were purchased from Aldrich (St Louis, MI) or Fluka (Milwaukee, WI) and were used without further purification, unless otherwise stated. Molecular sieves type 4 Å (beads, diameter 1.6 mm) were purchased from Aldrich and activated by heating at 280 °C for 4 h under vacuum.

LC/MS analysis

Analytical RP-HPLC runs were carried out using a C18 column, 4.6 × 250 mm with a flow rate of 1.0 mL min^{-1}. Preparative RP-HPLC was carried out using a C18 column, 22 × 250 mm with a flow rate of 20 mL min^{-1}. For all the RP-HPLC procedures the system solvent used was: H_2O 0.1 % TFA (A) and CH_3CN 0.1 % TFA (B), with a linear gradient starting from 30 to 90 % B in 30 min and detection at 210 and 280 nm. LC–ES–MS data were obtained using a Finnigan Surveyor MSQ single quadrupole electrospray ionization mass spectrometer coupled with a Finnigan Surveyor HPLC or LCQ DECA XP MAX (ThermoFinnigan).

NMR analysis

Proton NMR spectra were recorded on a 400-MHz spectrometer equipped with a z-gradient 5-mm triple-resonance probe or a 600-MHz instrument provided with a cold probe. Experiments were acquired at 298 K; samples consisted of compounds (~1 mg) dissolved in 600 µL of DMSO-d6 (99.9 % D).

1D proton spectra were recorded with 16–64 scans and a relaxation delay of 1 s. 2D [^{1}H, ^{1}H] TOCSY (Total Correlation Spectroscopy) (Griesinger et al. 1988) and 2D [^{1}H, ^{1}H] ROESY (Rotating frame Overhauser Enhancement Spectroscopy) (Bax and Davis 1985) experiments were recorded with mixing times of 70 and 200 ms, respectively. A 2D [^{1}H, ^{1}H] DQCOSY (Double Quantum Filter Correlation Spectroscopy) (Piantini et al. 1982) experiment was also acquired to confirm chemical shift assignments. 2D experiments for resonance assignments were usually acquired with 16–64 scans, 128–256 FIDs in t1, 1,024 or 2,048 data points in t2. Chemical shifts were referenced to residual DMSO at 2.55 ppm. Spectra were processed with the software vnmrj_1.1D. 2D experiments were analyzed with the program NEASY (Bartels et al. 1995) as implemented in the Cara (Computed Aided Resonance Assignment) software package (http://www.nmr.ch).

General procedure for peptide synthesis

Peptide synthesis was carried out manually by solid-phase method using the standard Fmoc-protecting group strategy. Appropriate Fmoc-amino acid derivatives were employed and a Rink Amide MBHA resin (0.7 mmol g^{-1} substitution; 50 µmol scale) was used as solid support, as it releases peptides amidated at C-terminus upon acid treatment. All Fmoc-amino acids were activated by in situ PyBop/HOBt//DIPEA activation procedure.

Amino acid coupling steps were monitored by Kaiser test after 60-min coupling cycles. Fmoc-deprotection was performed with 20 % piperidine in DMF for 5 + 10 min. Peptide N-terminus was acetylated by treatment with a mixture of acetic anhydride (4.7 %) and Pyridine (4 %) in DMF for 10 min. The cleavage from the solid support and the simultaneous deprotection of all side chains were performed by suspending the fully protected compound resins in TFA/H_2O/TIS (97: 2: 1) for 3 h. The peptides were isolated by precipitation in cold diethyl ether and centrifuged to form a pellet.

AcGlyTrpCys(Far)HisValAlaNH_2 (1a)

The peptide synthesis was performed as previously described (synthesis scale 0.050 mmol). Subsequently, 10 mg of **1** crude product (0.014 mmol) were placed into a

vial and dissolved in DMF (3 mL) under argon atmosphere. Then, the obtained solution was transferred, using a syringe, in a 10-mL round-bottom flask covered by a rubber top and containing 4 Å molecular sieves (3–3.5 g), previously activated at 280 °C for 4 h under vacuum (10^{-4} mBar). After a few minutes, *trans, trans*-farnesyl bromide (4.6 μL, 0.017 mmol) was added by a syringe and the obtained solution was stirred under argon atmosphere at room temperature for 3 h. Then, the mixture was centrifuged to eliminate the sieves and the supernatant was concentrated under vacuum. RP-HPLC and mass spectrometry analysis were performed on the obtained crude product and, after purification, **1a** was recovered in 54 % yield (5.4 mg), as estimated after lyophilization. HPLC: t_R = 17.60 min; ES–MS: calculated $[M + H]^+$, 918.6, found m/z 917.4. ^{1}H NMR (600 MHz, DMSO) δ 8.13, 3.79–3.61, 1.84 (Gly H_N, Hα-Hα′, acetyl CH_3); 8.08, 4.62, 3.19–3.01, 7.19, 10.86, 7.03, 7.62, 7.09, 7.37 (Trp H_N, Hα, Hβ-Hβ′, Hδ1, Hε1, Hζ3, Hε3, Hη2, Hζ2); 8.31, 4.46, 2.82–2.70 (Cys H_N, Hα, Hβ-Hβ′), 5.23, 5.13, 5.11, 3.20, 2.08, 2.03, 1.97, 1.67, 1.66, 1.63 (farnesyl); 8.31, 4.72, 3.13–2.99, 7.36, 8.94 (His H_N, Hα, Hβ-Hβ′, Hδ2, Hε1); 7.86, 4.22, 2.08, 0.92 (Val H_N, Hα, Hβ, Hγ); 8.15, 4.26, 1.27, 7.28–7.03 (Ala H_N, Hα, Hβ, $CONH_2$).

AcGlyTrpCys(HD)HisValAlaNH$_2$ (1b)

Peptide **1b** was synthesized, characterized and purified under the experimental conditions described above. The hexadecyl bromide was employed as alkylating agent (5.2 μL, 0.017 mmol). The final RP-HPLC purification provided compound **1b** in 40 % yield (4.0 mg). HPLC: t_R = 21.85 - min; ES–MS: calculated $[M + H]^+$, 937.7, found m/z 937.4. ^{1}H NMR (600 MHz, DMSO) δ 8.13, 3.79–3.61, 1.86 (Gly H_N, Hα-Hα′, acetyl CH_3); 8.07, 4.58, 3.18–2.97, 7.17, 10.86, 7.03, 7.62, 7.09, 7.35 (Trp H_N, Hα, Hβ-Hβ′, Hδ1, Hε1, Hζ3, Hε3, Hη2, Hζ2); 8.27, 4.40, 2.82–2.74 (Cys H_N, Hα, Hβ-Hβ′), 2.55, 1.51, 1.36, 1.29, 0.90 (HD); 8.38, 4.73, 3.12–3.04, 7.42, 8.93 (His H_N, Hα, Hβ-Hβ′, Hδ2, Hε1); 7.82, 4.21, 2.07, 0.91 (Val H_N, Hα, Hβ, Hγ); 8.14, 4.26, 1.26, 7.28–7.00 (Ala H_N, Hα, Hβ, $CONH_2$).

AcGlyAspArgCys(Far)TrpCys(Far)HisValAlaNH$_2$ (2a)

Peptide **2a** was synthesized, characterized and purified under the experimental conditions described above. The farnesyl bromide was employed in ratio of 1 equivalent compared to the sulfhydryl groups to be alkylated (5.5 μL, 0.022 mmol). The final RP-HPLC purification provided compound **2a** in 45 % yield (4.5 mg). HPLC: t_R = 20.18 min; ES–MS: calculated $[M + H]^+$, 1,494.9, found m/z 1,495.7. ^{1}H NMR (600 MHz, DMSO) δ 8.27, 3.74, 1.90 (Gly H_N, Hα-Hα′, acetyl CH_3); 8.35, 4.60, 2.72–2.56 (Asp H_N, Hα, Hβ-Hβ′); 7.96, 4.30, 1.73–1.55, 1.56, 3.08, 7.42 (Arg H_N, Hα, Hβ-Hβ′, Hγ, Hδ, $_N$Hε); 7.99, 4.46, 2.79–2.58 (Cys H_N, Hα, Hβ-Hβ′), 5.21, 5.12, 5.09, 3.19, 2.05, 2.02, 1.98, 1.67, 1.59 (farnesyl); 8.20, 4.58, 2.97–3.18, 7.16, 10.80, 6.98, 7.58, 7.08, 7.35 (Trp H_N, Hα, Hβ-Hβ′, Hδ1, Hε1, Hζ3, Hε3, Hη2, Hζ2); 8.16, 4.46, 2.76–2.67 (Cys H_N, Hα, Hβ-Hβ′), 5.21, 5.12, 5.09, 3.19, 2.05, 2.02, 1.98, 1.67, 1.59 (farnesyl); 8.40, 4.69, 3.03 (His H_N, Hα, Hβ-Hβ′); 7.86, 4.19, 2.06, 0.87 (Val H_N, Hα, Hβ, Hγ); 8.16, 4.26, 1.23, 7.27–7.02 (Ala H_N, Hα, Hβ, $CONH_2$).

AcGlyCys(Acm)MetGlyLeuProCys(Far)NH$_2$ (3a)

Peptide **3a** was synthesized, characterized and purified under the experimental conditions described above. The farnesyl bromide was employed as alkylating agent (3.7 μL, 0.016 mmol). The final RP-HPLC purification provided compound **3a** in 40 % yield (4.0 mg). HPLC: t_R = 17.37 min; ES–MS: calculated $[M + H]^+$, 996.5, found m/z 996.1

AcGlyCys(HD)MetGlyLeuProSerNH$_2$ (4b)

Peptide **4b** was synthesized, characterized and purified under the experimental conditions described above. The hexadecyl bromide was employed as alkylating agent (5.2 μL, 0.017 mmol). The final RP-HPLC purification provided compound **4b** in 48 % yield (4.8 mg). HPLC: t_R = 21.54 min; ES–MS: calculated $[M + H]^+$, 929.7, found m/z 929.2

AcLys(Ns)GlyCys(Bn)ValAlaNH$_2$ (5c)

Peptide **5c** was synthesized, characterized and purified under the experimental conditions described above. Concerning the employed Fmoc-Lys(Ns)-OH derivative, it was synthesized as previously reported (De Luca et al. 2005). The alkylating agent was benzyl bromide (1.6 μL, 0.013 mmol). The final RP-HPLC purification provided compound **5c** in 58 % yield (6.0 mg). HPLC: t_R = 17.67 min; ES–MS: calculated $[M + H]^+$, 793.3, found m/z 792.9. ^{1}H NMR (600 MHz, DMSO) δ 8.06, 4.17, 1.59–1.47, 1.28, 1.44, 2.89, 8.10 (Lys H_N, Hα, Hβ-Hβ′, Hγ, Hδ, Hε, Hζ), 8.04, 7.97, 7.89 (Ns), 1.87 (acetyl CH_3); 8.24, 3.77 (Gly H_N, Hα-Hα′); 8.09, 4.63, 2.79–2.62 (Cys H_N, Hα, Hβ-Hβ′), 3.80, 7.27, 7.36 (Bn); 8.08, 4.19, 2.05, 0.89 (Val H_N, Hα, Hβ, Hγ); 7.90, 4.23, 1.20, 7.26–7.04 (Ala H_N, Hα, Hβ, $CONH_2$).

AcLys(Far)GlyCys(Bn)ValAlaNH$_2$ (7)

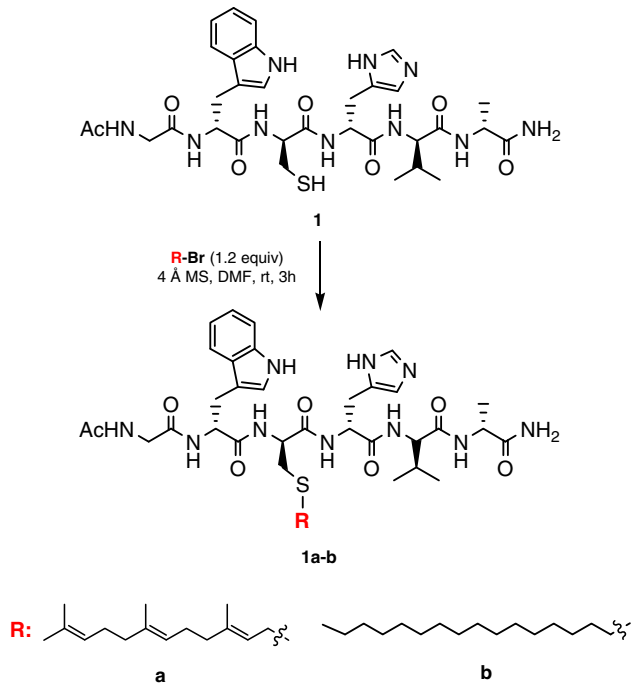
Peptide **5c** was synthesized, characterized under the experimental conditions described above (peptide synthesis

scale: 0.1 mmol). Concerning the first alkylation, it was performed on 20 mg of compound **5** (0.028 mmol dissolved in 3 mL of DMF) using benzyl bromide as alkylation agent (3.1 μL, 0.025 mmol). The obtained mixture was stirred at room temperature for 1 h. After having removed the molecular sieves by centrifugation, the supernatant was added, under argon atmosphere, to another round-bottom flask containing fresh activated molecular sieves and *trans*, *trans*-farnesyl bromide (8.3 μL, 0.032 mmol) was added as second alkylating agent to provide compound **6**. The obtained mixture was stirred at room temperature overnight. Subsequently, the nosyl group was removed by adding to the supernatant DBU (10 eq) and 2-mercapto-ethanol (10 eq). The solution was stirred at room temperature for 20 min and the crude product was isolated by precipitation in cold diethyl ether and purified by RP-HPLC to give compound **7** in 20 % yield (4.0 mg), after lyophilization. HPLC (**6**) : $t_R = 21.44$ min; ES–MS (**6**): calculated $[M + H]^+$, 997.5, found m/z 997.6. HPLC (**7**): $t_R = 20.21$ min; ES–MS (**7**): calculated $[M + H]^+$, 812.5, found m/z 812.0. ^{1}H NMR (**7**) (600 MHz, DMSO) δ 8.11, 4.24, 1.71–1.55, 1.35, 1.55, 2.84 (Lys H_N, Hα, Hβ-Hβ′, Hγ, Hδ, Hε), 5.24, 5.14, 5.12, 3.57, 2.12, 2.08, 1.99, 1.72, 1.69, 1.62 (farnesyl), 1.89 (acetyl CH_3); 8.24, 3.79 (Gly H_N, Hα-Hα′); 8.11, 4.65, 2.81–2.65 (Cys H_N, Hα, Hβ-Hβ′), 3.80, 7.27, 7.36 (Bn); 8.08, 4.21, 2.06, 0.90 (Val H_N, Hα, Hβ, Hγ); 7.90, 4.24, 1.22, 7.27–7.05 (Ala H_N, Hα, Hβ, $CONH_2$).

Results and discussion

Using the standard Fmoc-protocol the generic peptide sequence **1,** acetylated at the N-terminus and amidated at the carboxylic extremity, was synthesized to be used as substrate for the subsequent S-farnesylation (Scheme 1, compound **1a**). This reaction was performed in DMF under an Ar atmosphere, and in presence of activated 4 Å molecular sieves that, as previously described (Calce et al. 2013), act as mild base to promote the farnesylation by employing 1.2 equivalent of farnesyl bromide. The obtained mixture was stirred at room temperature for 3 h (Scheme 1). Afterward, the molecular sieves were removed by centrifugation and the obtained crude product was purified by reverse-phase chromatography. As shown in Table 1, the final product was characterized by high yield, as estimated by HPLC integration of the alkylated peptide compared to starting peptide and other multi-alkylated byproducts, if any.

Since the S-farnesylation is performed in solution on a fully deprotected peptide sequence, our method avoids subjecting the farnesyl moiety to acid treatment. In fact, the strong acidic conditions, requested for the peptide cleavage



Scheme 1 S-lipidation of peptide sequences

Table 1 Efficiency of S-alkylation reaction

Entry	Peptide	Yield (%)	Time (h)
1a	AcGlyTrpCys(**Far**)HisValAlaNH$_2$	90	3
1b	AcGlyTrpCys(**HD**)HisValAlaNH$_2$	70	3
2a	AcGlyAspArgCys(**Far**)TrpCys(**Far**)HisValAlaNH$_2$	85	3
3a	AcGlyCys(Acm)MetGlyLeuProCys(**Far**)NH$_2$	70	3
4b	AcGlyCys(**HD**)MetGlyLeuProSerNH$_2$	75	3
5c	AcLys(Ns)GlyCys(**Bn**)ValAlaNH$_2$	90	1
6	AcLys(Ns, **Far**)GlyCys(**Bn**)ValAlaNH$_2$	50	o.n.

in traditional synthetic routes, may affect the stereochemical integrity of the trans-double bonds of the farnesyl group (Naider and Becker 1997; Mullen et al. 2011).

In Fig. 1 NMR characterization of peptide **1a** is reported. Insertion of a farnesyl chain can be appreciated in the 1D [^{1}H] spectrum, where strong signals around 5 ppm, due to the CH protons of the farnesyl group involved in double bond formations, can be noticed (Fig. 1a). All spin systems are easily observable in the 2D TOCSY (Griesinger et al. 1988) spectrum (Fig. 1b) in which correlations arising from the farnesyl tail appear as well (Fig. 1c). The SCH_2 methylene protons, inserted by farnesylation, present a characteristic chemical shift at 3.20 ppm (Fig. 1a, c); ROE contacts (Bax and Davis 1985) between a few protons belonging to the farnesyl tail and those of cysteine (such as Hββ') indicate that farnesylation is selectively achieved on this amino acid.

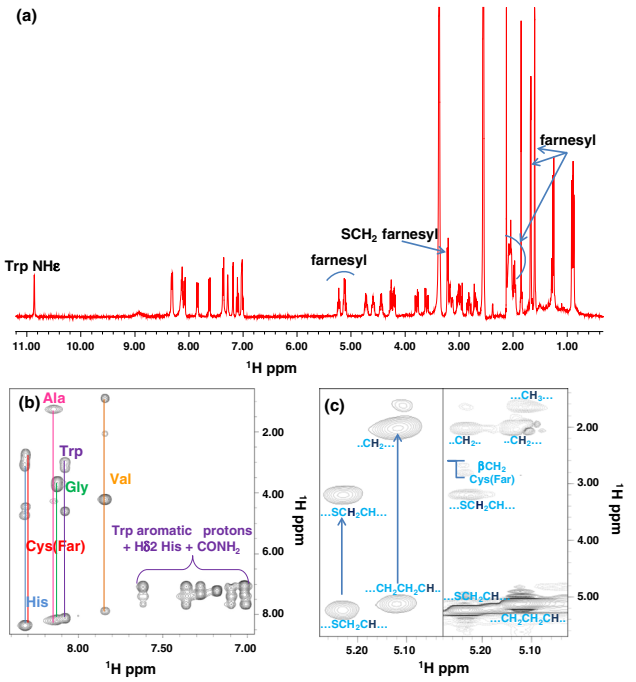


Fig. 1 **a** 1D [^{1}H] NMR experiment of AcGlyTrpCys(Far)HisValAlaNH$_2$ peptide. **b** 2D [^{1}H, ^{1}H] TOCSY spectrum with reported assignments for different amino acids. **c** Comparison between 2D [^{1}H, ^{1}H] TOCSY (*left panel*) and ROESY (*right panel*) spectra. Correlations, in the aliphatic protons region, arising from the farnesyl tail are shown in the reported expansion

Since S-palmitoylation represents another important post-translational modification of Ras superfamily (Bourne et al. 1990; Boguski and McCormick 1993; Vetter and Wittinghofer 2001; Hancock et al. 1990; Hall 1994), we also synthesized the peptide **1b**, using the hexadecyl bromide as alkylating agent (Scheme 1). Indeed, the hexadecyl moiety is a non-hydrolyzable by enzymes mimic of the palmitoyl arm. The synthesis of lipidated natural peptide sequences which incorporate S-hexadecyl modifications instead of the naturally occurring *S*-palmitoylated cysteine is well documented (Bader et al. 2000; Kuhn et al. 2001; Lumbierres et al. 2005; Weise et al. 2009).

Compound **1b** was obtained in quite high yield (Table 1) after 3 h. A conventional protocol, which uses an organic base (TEA) to promote S-alkylation, with such a low electrophilic group, such as a long alkyl moiety, can generate the lipidated peptide in only modest yields, after a long chromatographic purification (Triola et al. 2008). A different synthetic route, which employs a solution of ammonia in methanol as base, fails to provide good yields with alkylating agents consisting of long aliphatic chains, such as the hexadecyl group (Triola et al. 2008). On the other hand, the usage of a strong base to efficiently hexadecylate a cysteine is known to induce racemization of the amino acid chiral center (Triola et al. 2008). Thus, with respect to all these cited procedures, our method, which is based on mild reaction conditions, turns to be particularly efficient to link a lipidic tail to a peptide.

In a subsequent step, a relevant result was obtained with the synthesis of compound **2a**. It contains two farnesyl groups inserted on cysteines separated by one amino acid residue and other sensitive functional groups on amino acids such as Asp, Arg, Trp and His (Table 1). In fact, while it has been reported that the synthesis of doubly isoprenylated peptides, characterized by -CC and -CXC sequences, is difficult to perform in good yields (Naider and Becker 1997), our methodology allowed the synthesis of **2a** in a high yield using 1 equivalent of bromide, with respect to the sulfhydryl group, and the standard reported reaction time (3 h).

As an example of useful application of our synthetic strategy, we prepared in high yields several tailor-made lipidated peptides (**3a** and **4b**), representing the characteristic functional parts of their Ras parent proteins (Fig. 2). Design and synthesis of such peptides have been previously described, as well as their implementation in a variety of biophysical and biochemical studies aiming at elucidating biological aspects of the Ras protein family (Kuhn et al. 2001).

Moreover, to explore a synthetic route to introduce different substituents on the same molecule, we investigated the reactivity of a cysteine in presence of the nosyl-protected lysine (De Luca et al. 2005; Monfregola and De Luca 2011; Monfregola et al. 2012) and prepared compound **5c** (Scheme 2). After one hour, the most abundant product was the mono-S-alkylated while the di-alkylated peptide was present in a quite low percentage (<5 %).

Encouraged by these results, multiple post-synthetic modifications on the same peptide sequence were performed (Scheme 2). For instance, compound **6** was obtained by playing with the different reactivity exhibited, under the employed alkylation conditions, by the *N*-ε-

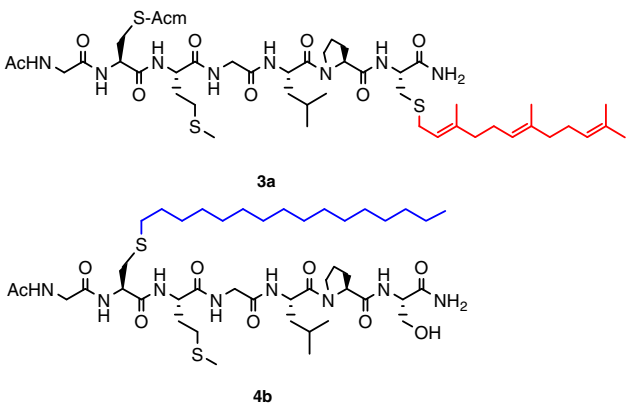
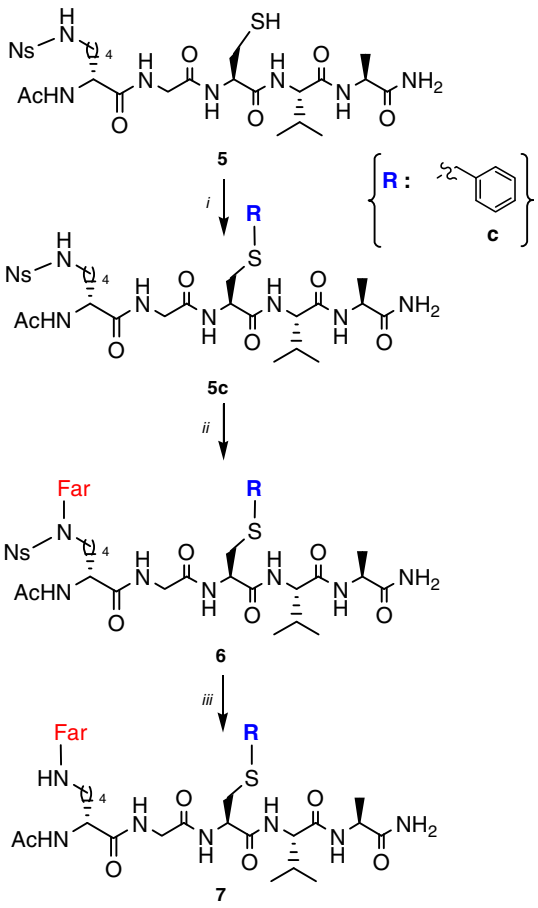


Fig. 2 Lipidated natural peptide sequences



Scheme 2 Multi-alkylation on cysteine and lysine residues. Reagents and conditions (*i*) BnBr (0.9 equiv), DMF, 4 Å MS, rt, 1 h; (*ii*) FarBr (1 equiv), DMF, 4 Å MS, rt, 12 h; (*iii*) DBU (10 equiv), βME (10 equiv), DMF, rt, 20 min

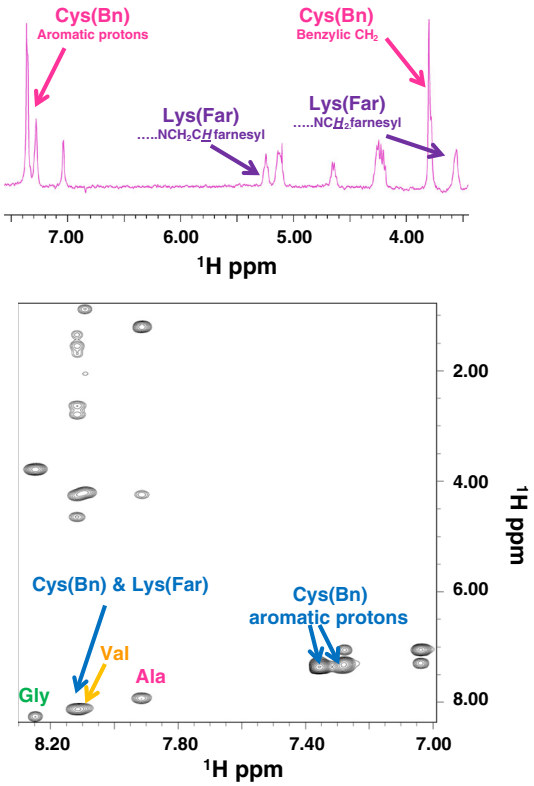


Fig. 3 NMR characterization of the AcLys(Far)GlyCys(Bn)ValAlaNH$_2$ peptide. 1D [^{1}H] and 2D [^{1}H, ^{1}H] TOCSY experiments are shown in the *upper* and *lower panels*, respectively. In detail, a spectral region containing a few distinctive signals belonging to Cys(Bn) and Lys(Far) is reported in the upper section. In the lower inset, TOCSY correlations between $\mathbf{H}_N$ and aliphatic protons together with those arising from the benzyl ring and the protecting CON$\mathbf{H}_2$ C-terminal group are observable

nosyl-protected lysine with respect to the cysteine side chain. Specifically, two alkylation reactions in consecutive steps were performed to obtain **6** (Scheme 2). The cysteine sulfhydryl group was firstly alkylated with a yield of around 85 %, by treating the peptide with benzyl bromide in presence of activated molecular sieves. In a subsequent step, after having replaced the molecular sieves, a second alkylating agent (farnesyl bromide) was added to the reaction mixture and was stirred for 15 h (Monfregola et al. 2012). The di-alkylated product was obtained in yields close to 70–80 %. After removal of the *N*-ε-nosyl-protecting group (Monfregola et al. 2012), the peptide AcLys(Far)GlyCys(Bn)ValAlaNH$_2$ was analyzed by NMR spectroscopy (Fig. 3).

The presence of a Cys(Bn) is well represented by typical NMR signals (Fig. 3). In particular, the SCH$_2$ benzyl protons originate a peak at 3.80 ppm that is partially overlapped with those arising from the Hα Gly protons (Fig. 3, upper panel); moreover, benzylic aromatic protons give signals centered at 7.36 and 7.27 ppm (Fig. 3).

Lysine farnesylation is indicated by the appearance of characteristic sets of peaks belonging to the farnesyl tail (Fig. 3, upper panel); it is worth noting that, moving farnesylation from the cysteine to the lysine, an expected change in the chemical shift of the newly generated *X*-CH$_2$ methylene protons ($X = S$ or N), from 3.20 to 3.60 ppm, is observed. All other poly-alkylation products were widely characterized by NMR spectroscopy that further proved the obtainment of the desired compounds (Supplemental Data).

Conclusion

In summary, we reported on the synthesis of peptides reproducing natural sequences from the protein N-Ras C-terminus, provided with different lipidic moieties attached on a cysteine residue.

The versatility of the method was further proved by introducing two different substituents in a peptide sequence. The relevant aspect of this procedure consists in

inserting the two modifications in one-pot synthesis. This result can be considered completely innovative, since, to best of our knowledge, it is not described in the literature any synthetic protocol, which introduces two different substituents, on the same peptide sequence, without requiring purifications after each reaction step.

Taking advantage of the flexibility of the described strategy, in the close future we are planning to synthesize several natural peptides characterized by the presence of either different combinations of lipid patterns, to study the role of Ras lipid anchor in signal transduction pathways, and/or other functionalities, to investigate biophysical and biochemical properties of complex biological systems.

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Conflict of interest The authors declare that they have no conflict of interest.

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