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Peptide Lipidation

Lipidation of Cysteine or Cysteine-Containing Peptides Using the Thiol-Ene Reaction (CLipPA)

Sung-Hyun Yang,[a,b] Paul W. R. Harris,[a,b,c] Geoffrey M. Williams,[a,b,c] and Margaret A. Brimble*[a,b,c]

Abstract: The efficient synthesis of N^{α}-protected *S*-palmitoylated cysteine building blocks using thiol-ene coupling is described. These building blocks were incorporated into a resin-bound peptide under racemisation-suppressing conditions, and the degree of racemisation during the coupling process was assessed. The direct conjugation of vinyl palmitate with the sulfhydryl side-chain of a cysteine residue on a semiprotected peptide was also studied. The reaction gave both mono- and bispalmitoylated cysteine residues in varying proportions, depending on the reaction conditions adopted.

Introduction

The hydrothiolation of an unsaturated system, known as thiol-ene coupling, is a thermally or photochemically induced transformation that occurs by radical-promoted alkylation of a thiol with an unsaturated moiety, usually an alkene.[1] It has found particular utility in the fields of synthetic polymers,[2] synthetic chemistry, and bioconjugation,[3] owing to its atom economy, wide tolerance of functional groups, and straightforward reaction conditions. We have previously reported a successful application of the thiol-ene reaction to the efficient synthesis of self-adjuvating peptides by monopalmitoylation of fluorenylmethyloxycarbonyl (Fmoc)-cysteinyl or cysteinyl peptides with vinyl palmitate.[4] These monoacyl lipopeptide constructs, obtained in a single chemical step, have been shown to have immunogenic activity comparable to that of the corresponding, more synthetically demanding, less tractable, and more expensive Pam_3Cys motif. However, although the monoacyl lipopeptides were prepared in adequate quantities for biological evaluation, the overall yields and/or conversions in this initial study were unsatisfactory, thereby limiting their use as building blocks for more complex systems.

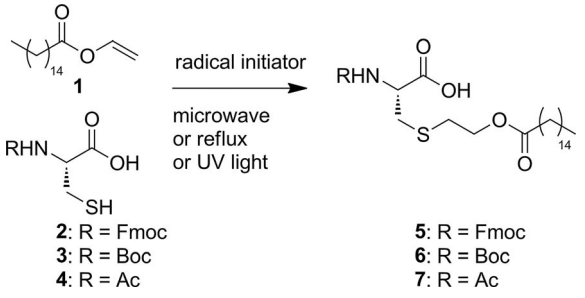
We now coin the term "CLipPA" — **C**ysteine **Lip**idation on a **P**eptide or **A**mino acid — to describe this reaction, and in this paper we report a detailed study of the CLipPA reaction between vinyl palmitate and N^{α}-protected cysteine derivatives, which gives the *S*-palmitoylated compounds in high yield and purity. These compounds can be used directly in solid-phase peptide synthesis to construct lipopeptides. We also report improved conditions for the highly selective mono *S*-palmitoylation of immunogenic peptide sequences to give lipopeptides in high yield and purity.

[a] *School of Chemical Sciences, The University of Auckland, 23 Symonds St., Auckland 1142, New Zealand*
E-mail: m.brimble@auckland.ac.nz
http://www.auckland.ac.nz/
[b] *School of Biological Sciences, The University of Auckland, 3A Symonds St, Auckland 1142, New Zealand*
[c] *Maurice Wilkins Centre for Molecular Biodiscovery, School of Biological Sciences, The University of Auckland, Auckland 1142, New Zealand*
Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/ejoc.201501375.

Results and Discussion

Lipidation of N^{α}-Protected Cysteines

We originally reported that vinyl palmitate (**1**) underwent the thiol-ene reaction with N^{α}-protected Fmoc-cysteine **2** after irradiation at 365 nm for 60 min with the initiator 2,2-dimethoxy-2-phenylacetophenone (DMPA) to give thioether **5** in moderate yield (44 %)[4a] (Scheme 1). Monitoring the reaction by HPLC however, revealed that **2** was completely consumed, which suggests that the reaction parameters and purification method could be improved to increase the yield of **5**.



Scheme 1. Synthesis of N^{α}-protected *S*-palmitoylated cysteines **5**–**7**.

In this work, we sought to modify the N^{α}-protecting group, the radical initiator, and the activation method in order to define optimal conditions to generate the *S*-palmitoylated N^{α}-protected cysteines in good yield. This synthetic goal is important as we envisaged that building blocks **5**, **6**, and **7**, prepared from appropriately protected cysteines **2**, **3**, or **4**, could be used directly in routine Fmoc or Boc (*tert*-butoxycarbonyl) solid-phase

peptide synthesis through acylation of any resin-bound amine. In order to ensure efficient coupling to the solid-phase resin, sufficient quantities of the amino acid building block are initially required, hence the need for a robust synthetic method to access these *S*-palmitoylated building blocks.

N^{α}-Protected Fmoc, *tert*-butyloxycarbonyl (Boc) or *N*-acetylated (Ac) cysteines **2**, **3**, and **4** were treated with an excess of vinyl palmitate in the presence of DMPA[5] or 2,2-azo-bis(2-methylpropionitrile)[6] (AIBN) in either dichloromethane or 1,2-dichloroethane. The reactions were carried out under thermal heating, microwave irradiation, or UV light, and the product(s) were purified directly by silica gel chromatography. Purification by reverse-phase HPLC resulted in poor recoveries, probably due to the hydrophobic nature of **5**, **6**, and **7**. The results are summarised in Table 1.

Table 1. Optimisation of the thiol-ene reaction using Fmoc-Cys **2**, Boc-Cys **3**, or Ac-Cys **4** with vinyl palmitate (**1**).

Entry		Initiator	Conditions[a]	Yield[c]
1	**2**	AIBN (1.0 equiv.)	μwave, 100 W, 70 °C, 80 min	**5** (41 %)
2	**2**	AIBN (0.5 equiv.)	reflux, 90 °C, 150 min[b]	**5** (55 %)
3	**2**	DMPA (0.2 equiv.)	hν (365 nm), 60 min	**5** (67 %)
4	**2**	DMPA (1 equiv.)	hν (365 nm), 60 min	**5** (82 %)
5	**3**	AIBN (0.5 equiv.)	reflux, 90 °C, 40 min[b]	**6** (54 %)[b]
6	**3**	AIBN (1 equiv.)	μwave, 100 W, 70 °C, 80 min	**6** (77 %)
7	**3**	DMPA (0.2 equiv.)	hν (365 nm), 60 min	**6** (78 %)
8	**3**	DMPA (1 equiv.)	hν (365 nm), 60 min	**6** (82 %)
9	**4**	DMPA (0.2 equiv.)	hν (365 nm), DTT, 60 min	**7** (74 %)
10	**4**	DMPA (0.2 equiv.)	hν (365 nm), 60 min	**7** (87 %)
11	**4**	DMPA (1. eq.)	hν (365 nm), 60 min	**7** (88 %)
12	**4**	AIBN (0.5 equiv.)	reflux, 90 °C, 40 min[b]	**7** (95 %)
13	**4**	AIBN (1 equiv.)	μwave, 100 W, 70 °C, 80 min	**7** (>99 %)

[a] CH_2Cl_2 as solvent. [b] Cl-$(CH_2)_2$-Cl as solvent. [c] Isolated yield after flash column chromatography on silica gel.

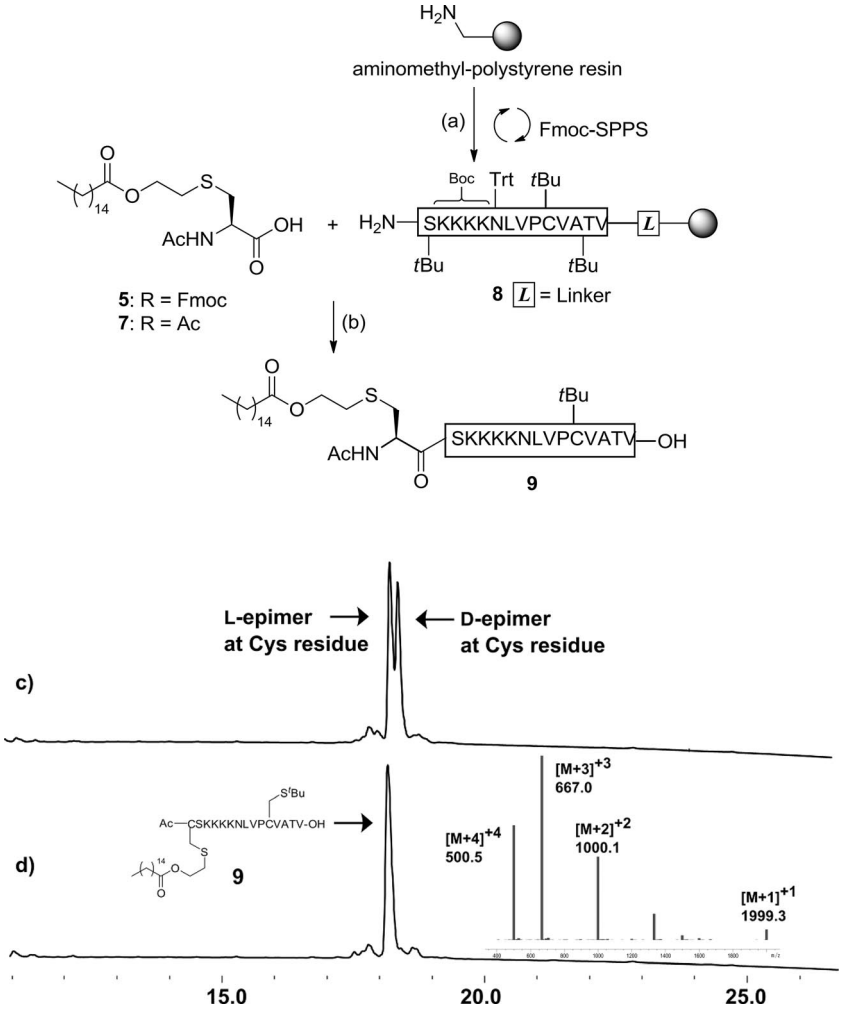
Fmoc-Cys-OH **2** underwent smooth conversion to *S*-palmitoylated product **5** (Table 1, entries 1–4), optimally using UV light and an excess of the radical initiator DMPA giving the desired product **5** in 82 % isolated yield (Table 1, entry 4). The use of conventional heating or microwave irradiation gave lower yields of 41 or 55 %, respectively (Table 1, entries 1 and 2), perhaps due to premature cleavage of the N^{α}-Fmoc protecting group. Boc-Cys-OH **3** was converted to *S*-palmitoylated product **6** under UV irradiation with an excess of DMPA in an isolated yield of 82 % after 1 h (Table 1, entry 8). Carrying out the reaction under thermal heating or microwave irradiation with AIBN as the radical initiator resulted in lower yields (Table 1, entries 5 and 6). This may be due to the instability of the *tert*-butyloxycarbonyl group, which may undergo de-*tert*-butylation and subsequent loss of CO_2 at high temperatures.

We next examined the thiol-ene chemistry using Ac-Cys-OH **4**, which bears an amide on the α-nitrogen (Table 1, entries 9–13). It has been shown that an *N*-acetyl group on the cysteine α-nitrogen plays an important role in modulating the immunogenic potency of an *S*-palmitoylated cysteinyl-serine (Cys-Ser) derivative.[7] We also reasoned that the acetamide moiety may be more stable to the reaction conditions than the Fmoc and Boc carbamate-based protecting groups. In contrast to the results obtained from N^{α}-protected cysteines **2** and **3**, Ac-Cys-OH **4** was quantitatively converted to *S*-palmitoylated product **7** under microwave irradiation (Table 1, entry 13) using AIBN as initiator, although other methods such as using UV light (Table 1, entries 10 and 11) or thermal heating in 1,2-dichloroethane (Table 1, entry 12) also gave excellent yields (87–95 %) of **7**. In all cases examined (Table 1), it was necessary to use the N^{α}-protected cysteine; attempting the thiol-ene reaction directly on native, unprotected cysteine using UV light and DMPA for 1 h gave none of the expected *S*-palmitoylated product.

Coupling of N^{α}-Protected *S*-Palmitoylated Cysteines 5 and 7 to Resin-Bound Peptide 8

With *S*-palmitoylated cysteine building blocks in hand, we went on to examine the solid-phase coupling of Fmoc-protected **5** or Ac-protected **7** to a model resin-bound peptide **8**. The peptide sequence SKKKK-NLVPMVATV-OH was chosen as a model peptide for this study. The sequence is derived from a truncated peptide epitope from the cytomegalovirus (CMV) ppUL83 protein, which is known to stimulate $CD8^+$ cytotoxic T-cells.[8] This peptide has previously been used in our group for the incorporation of a palmitoyl functionality using either copper(I)-catalysed azide–alkyne cycloaddition "CuAAC" chemistry[9] or a thiol-ene[4a] reaction. The SKKKK sequence was included as the four lysine residues greatly improve the solubility and tractability of the lipopeptides, and, when conjugated with palmitoylated cysteine, the motif has established itself as a potent TLR2 agonist.[10] Resin-bound peptide **8** was synthesised with the Met residue substituted for a Cys(*t*Bu) in order to demonstrate that a suitably protected sulfhydryl side-chain of a cysteine residue could be tolerated by the thiol-ene reaction conditions. Side-chain unmasking then gives a handle for further manipulation at this cysteine residue.

Peptidyl resin **8** was synthesised by elongation of the peptide chain using Fmoc-SPPS (solid-phase peptide synthesis), with the reagents HATU {1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate}, diisopropylethylamine (DIPEA), and piperidine in DMF (20 % v/v), at room temperature. Activation and coupling of cysteine residues without epimerisation is recognised to be problematic in SPPS, and is influenced by the choice of coupling reagent, base, solvent conditions, and the protecting group on the sulfhydryl side-chain.[11] Coupling of *S*-palmitoylated cysteine building block **5** or **7** was therefore achieved using PyBOP [(benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; 5 equiv.] and 2,4,6-trimethylpyridine (TMP; 5 equiv.) at room temperature, conditions known[12] to suppress racemisation (Scheme 2). The choice of N^{α}-protecting group for the amino acid may also affect the degree of racemisation that occurs during the formation of the activated ester.[12a] To examine this effect, resin-bound peptide **8** was either derivatised with **5**, and the Fmoc protecting group exchanged for an acetyl group before cleavage to give peptide **9**, or building block **7** was coupled to give **9** directly. Comparison of the HPLC traces revealed that a 1:1 ratio of epimers was formed when acetamide-protected building block **7** was used in the coupling reaction (Scheme 2, chromatogram c), whereas no detectable racemisa-



Scheme 2. Coupling of N^{α}-protected *S*-palmitoylated cysteine building blocks **5** or **7** with resin-bound peptide **8**. a) Iterative Fmoc-SPPS: i) deprotection of N^{α}-Fmoc group: piperidine/DMF (20 % v/v), room temp., 5 min × 2; ii) Coupling of Fmoc-AA-OH: Fmoc-AA-OH (5 equiv.), HATU (4.6 equiv.), DIPEA (6 equiv.), room temp., 40 min; b) i) **5** or **7**, PyBOP (5 equiv.), 2,4,6-trimethylpyridine (5 equiv.) room temp.; For building block **5**: ii) piperidine/DMF (20 % v/v) then Ac_2O/DMF (20 % v/v); iii) TFA/DODT/H_2O/TIPS (94:2.5:2.5:1 v/v, resin cleavage); c) HPLC chromatogram of crude **9** synthesised using building block **7**; d) HPLC chromatogram of crude **9** synthesised using building block **5**. Inset: observed ESI-MS spectrum of the main peak (calcd. mass: 1998.2; found 1999.3 $[M + H]^+$, 1000.1 $[M + 2H]^{2+}$, 667.0 $[M + 3H]^{3+}$, 500.5 $[M + 4H]^{4+}$). Chromatographic separations were carried out using a linear gradient of 5–95 % B over 30 min, ca. 3 % B per min. Buffer A: H_2O containing TFA (0.1 % v/v); Buffer B: acetonitrile containing TFA (0.1 % v/v).

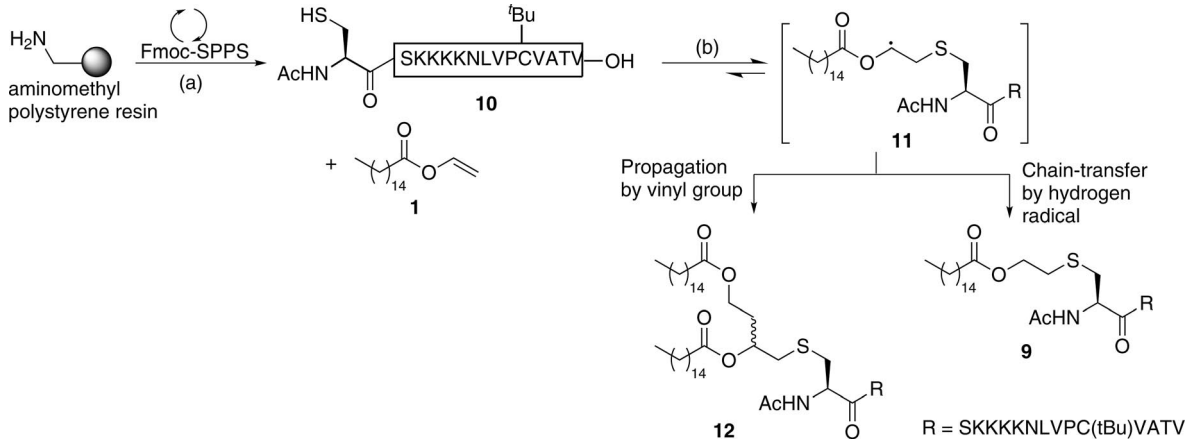
tion was observed when Fmoc-protected building block **5** was used (Scheme 2, chromatogram d). Thus, even though the couplings of **5** and **7** both proceeded to completion, Fmoc-protected building block **5** is clearly a more versatile reagent.

Direct Lipidation on a Semiprotected Peptide 10 Bearing a Free Cysteine Sulfhydryl Side-Chain

We next examined a more convergent approach for the synthesis of the desired product (i.e., **9**). This involved using the thiol-ene reaction to install the palmitoyl group onto the side-chain of a cysteine residue by direct conjugation of vinyl palmitate (**1**) with fully formed peptide **10** (Scheme 3). This strategy has been briefly reported by our group,[4] and we sought to further extend our understanding of this transformation. As a starting point, peptide **10**, bearing a free sulfhydryl side-chain, was synthesised using Fmoc-SPPS as described previously, and the *N*-terminus was acetylated. The peptide was liberated from the solid support by cleavage with cocktail A[13] (TFA/DODT/H_2O/TIPS, 94:2.5:2.5:1 v/v), and then purified by HPLC, ready for lipidation.

Optimisation of Reaction Solvent

Firstly, we examined the viability of the reaction between vinyl palmitate (**1**) and peptide **10** using the polar organic solvents *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and *N*-methyl-2-pyrrolidone (NMP) in an effort to fully solvate both the hydrophilic peptide and hydrophobic vinyl palmitate (**1**). The use of DMSO as solvent was discounted owing to the low solubility of vinyl palmitate (**1**), which led to low conversion of peptide **10** into the desired mono adduct (i.e., **9**) (data not shown). DMF proved a more suitable solvent in that all the reagents were soluble, and efficient conversion to product **9**



Scheme 3. CLipPA direct conjugation of vinyl palmitate (**1**) and semiprotected peptide **10**. a) Iterative Fmoc-SPPS: i) Deprotection of N^{α}-Fmoc group: piperidine/DMF (20 % v/v), room temp., 5 min × 2; ii) Coupling of Fmoc-AA-OH: Fmoc-AA-OH (5 equiv.), HCTU (4.6 equiv.), DIPEA (6 equiv.), room temp., 40 min; iii) piperidine/DMF (20 % v/v), room temp., 5 min × 2; then Ac_2O/DMF (20 % v/v) room temp., 10 min; iv) Cleavage of peptidyl resin: Cocktail A (TFA/DODT/H_2O/TIPS, 94:2.5:2.5:1 v/v), 2 h, room temp.; b) DMPA (0.5 equiv.), NMP, TFA (5 % v/v), additives (see Table 2), UV (365 nm), room temp. DODT = 2,2′-(ethylenedioxy)diethane dithiol; HCTU = 1-[bis(dimethylamino)methylene]-5-chlorobenzotriazolium 3-oxide hexafluorophosphate; TIPS = triisopropylsilane; TFA = trifluoroacetic acid.

was observed. However, the heat generated from mixing DMF and residual TFA (vide infra) induced the formation of a sulfoxide (+16 Da) by-product, thus complicating HPLC analysis and product recovery. We assume that this results from oxidation of the thioether moiety of the palmitoylated cysteine residue present in **9**. NMP was finally determined to be most suitable solvent for this transformation, and was therefore used exclusively for the subsequent optimisation studies.

Optimisation of Radical Quencher

Initially, we used our original reaction conditions[4a] to effect direct conjugation of vinyl palmitate (**1**) with semiprotected peptide **10**. The procedure entailed irradiating at 365 nm a solution of the radical initiator DMPA (0.5 equiv.) and the peptide in NMP as the solvent, with dithiothreitol (DTT; 3 equiv.) included as an exogenous thiol source. We had previously concluded that extraneous thiols were required to improve the product profile; they presumably decrease unwanted side-reactions such as the formation of telomers or mixed disulfides by facilitating the hydrogen abstraction process to quench the intermediate radical species (e.g., **11**) and give the desired product.[14] We have previously used various thiols such as reduced glutathione (GSH), 2,2′-(ethylenedioxy)diethanethiol (DODT), and (optimally) DTT. However, in this work, careful examination of the LC–MS profile of the reaction between peptide **10** and vinyl palmitate (**1**) revealed the formation of by-products with masses of 120 and 152 Da greater than product **9**, indicative of the addition of DTT and fragments thereof to carbon-centred radical **11**.[15]

We therefore substituted DTT with the hindered *tert*-butylthiol (*t*BuSH); this reagent should still act as an efficient chain-transfer agent, but its steric bulk should prevent the formation of *S*-alkylation by-products.[16] Gratifyingly, this proved to be the case, as no analogous by-products were detected by LC–MS. Thus, *tert*-butylthiol was preferentially used in subsequent experiments.

Addition of TFA and tert-Butylthiol

The first lipidation of semiprotected peptide **10** and vinyl palmitate (**1**) was carried out using DMPA as initiator, but excluding other additives (Table 2, entry 1). After irradiating the mixture for 30 min with UV light, a sample was analysed by LC–MS. This showed, in addition to DMPA degradation products (see Supporting Information Figure S2), a moderate conversion (58 %) of peptide **10** to the desired mono adduct (i.e., **9**; 84 % of product) and bis adduct **12** (16 % of product) (Figure 1). The formation of **12** arises from reaction of carbon-centred β-sulfanylalkyl radical intermediate **11** with a second equivalent of vinyl ester (Scheme 3); this process — telomerisation — occurs in competition with the expected quenching of **11** by a thiol leading to mono adduct **9** and propagation of the thiol-ene reaction.

An improved RP-HPLC profile was observed when trifluoroacetic acid (TFA; 5 % v/v) was added[14,17] (see Supporting Information Figure S4) to the reaction mixture; this had a minimal effect on the conversion rate. Decreasing the pH of the reaction mixture may suppress the formation of by-products by ensuring protonation of the electron rich side-chains of residues such as lysine, arginine, and histidine, which could otherwise participate in single-electron transfers and form radical species during the propagation step.

Next, the effect of adding of both *tert*-butylthiol and TFA to the reaction mixture was investigated. While addition of an exogenous thiol slightly favoured an increase of mono adduct **9** over bis adduct **12**, low conversion (<70 %) of peptide **10** was still problematic (Table 2, entry 2). Extending the reaction time to 60 min under identical reaction conditions did not alter the conversion of peptide **10** into products **9** and **12** (see Supporting Information Figure S3); in fact, the reaction appeared to stop within the initial 30 min of UV irradiation, and could not be driven to completion, even upon the addition of a second portion of both DMPA and vinyl palmitate (**1**). These pre-

Table 2. Optimisation of lipidation. Conjugation of peptide **10** and vinyl palmitate (**1**) in NMP[a] using DMPA[b] as radical initiator.

Entry	Vinyl palmitate (**1**) [equiv.][c]	*t*BuSH [equiv.][c]	TIPS [equiv.][c]	Conversion[f] [%]	Products[f]
1	7	0	0	58	**9** (84 %) **12** (16 %)
2	7	3	0	69	**9** (97 %) **12** (3 %)
3	70	3	0	84	**9** (65 %) **12** (35 %)
4	70	80	0	93	**9** (76 %) **12** (24 %)
5	70	80	40	94	**9** (88 %) **12** (12 %)
6	70	40	40	88	**9** (95 %) **12** (5 %)
7	70	80	80	94	**9** (95 %)[g] **12** (5 %)
8	70	0	80	78	**9** (67 %) **12** (33 %)
9	7	80	80	60	**9** (98 %) **12** (2 %)
10	20	80	80	81	**9** (> 99 %) **12** (<1 %)
11	35	80	80	92	**9** (97 %) **12** (3 %)
12	100	80	80	90	**9** (95 %) **12** (5 %)
13[d]	70	80	80	26	**9** (> 99 %) **12** (trace)
14[e]	70	80	80	91	**9** (96 %) **12** (4 %)

[a] 30 min reaction time, with 5 % TFA based on final reaction volume. [b] 0.5 equiv. relative to peptide **10**. [c] Molar equivalent relative to peptide **10**. [d] Dimethyl sulfoxide as solvent. [e] *N*,*N*′-Dimethylformamide as solvent. [f] Conversion of peptide **10**, mono adduct **9**, and bis adduct **12** is based on integration of the corresponding peaks on the RP-HPLC profile at 210 nm. The relative amounts of **9** and **12** are cited as percentages. [g] 72 % isolated yield after RP-HPLC purification.

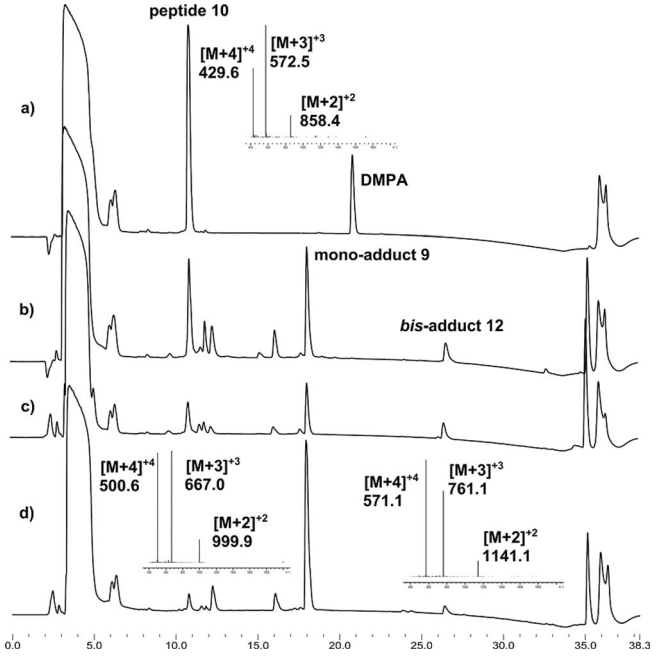


Figure 1. Lipidation reaction between vinyl palmitate (**1**) and peptide **10** using DMPA and UV light. a) HPLC chromatogram of Table 2, entry 1, $t = 0$ min; Inset: observed ESI-MS spectrum of the main peak for peptide **10** (calcd. mass: 1715.0; found 858.4 $[M + 2H]^{2+}$, 572.5 $[M + 3H]^{3+}$, 429.6 $[M + 4H]^{4+}$); b) HPLC chromatogram of Table 2, entry 1, $t = 30$ min; c) HPLC chromatogram of Table 2, entry 8, $t = 30$ min; d) HPLC chromatogram of Table 2, entry 7, $t = 30$ min; Inset: observed ESI-MS spectrum of the main peak for mono adduct **9** (calcd. mass: 1998.2; found 999.9 $[M + 2H]^{2+}$, 667.0 $[M + 3H]^{3+}$, 500.6 $[M + 4H]^{4+}$); for bis adduct **12** (calcd. mass: 2280.6; found 1141.1 $[M + 2H]^{2+}$, 761.1 $[M + 3H]^{3+}$, 571.1 $[M + 4H]^{4+}$); Chromatographic separations were carried out using a linear gradient of 5–95 % B over 30 min, ca. 3 % B per min. Buffer A: H_2O containing TFA (0.1 % v/v); Buffer B: acetonitrile containing TFA (0.1 % v/v).

liminary observations suggested that a rapid conversion of peptide **10** into product **9** is required in order to maximise conversion of the starting peptide. It is well known that the first step, in which carbon-centred radical intermediates such as **11** are formed, is reversible;[18] thus, the olefin and thiol are regenerated, and this may contribute to the low conversion observed in this case. We postulated that a higher conversion could be achieved by adding a larger excess of vinyl palmitate (**1**), as this would facilitate an increase in the rate of formation of carbon-centred radical intermediate **11**, and thence form mono adduct **9** via chain transfer with the hydrogen radical source. Indeed, upon addition of a large excess of vinyl palmitate (**1**; 70 equiv.), the overall conversion of peptide **10** increased markedly to 84 % (Table 2, entry 3, see Supporting Information Figure S6). However, this was accompanied by a corresponding increase in the proportion of bis adduct **12** to 35 %. It is interesting to note that despite the high concentration of vinyl palmitate, no higher-order propagations were observed.[14] Increasing the amount of *tert*-butylthiol (Table 2, entry 4, see Supporting Information Figure S7) enhanced the conversion of **10** further to 93 %, and diminished the proportion of **12** to 24 %. Presumably, this resulted from shifting the balance of competing pathways available to radical intermediate **11** — namely, reversibility, telomerisation and quenching — in favour of the radical-quenching route that leads to **9**.

Addition of a Coreductant

We next envisaged that the formation of bis adduct **12** could be suppressed by the introduction of a coreductant to the reaction mixture that facilitates more rapid hydrogen-radical abstraction from the coreductant to the carbon-centred radical intermediate **11**, thus giving the desired monopalmitoylated adduct **9**. Organosilanes are radical-based reducing agents whose hydrogen-donor ability can be fine-tuned by varying the substituents on the silicon atom.[19] Thus, judicious selection of the organosiliane reagent may induce the formation of the desired product (i.e., **9**) by fast hydrogen transfer to the carbon-centred radical intermediate **11**.

We chose readily available triisopropylsilane (TIPS) as the coreductant (40 equiv.) for the thiol-ene reaction (Table 2, entry 5, see Supporting Information Figure S7). Gratifyingly, using TIPS as a coreductant, the formation of bis adduct **12** was substantially diminished to 12 %, while peptide **10** was consumed with

Table 3. Conjugation of peptides **13** and **14** with vinyl palmitate (**1**) to form monopalmitoylated product under unoptimised[a] and optimised[b] conditions.

Entry	Peptide sequence	Unoptimised conditions [%][e]	Optimised conditions [%][e]
1	Peptide **13**[c] Ac-CSKKKKGARGPESRLLE-FYLAMPFATPMEAELARRSLAQDAPPL-OH	4	81
2	Peptide **14**[d] H_2N-CSKKKKVPGVLLKEFTV-SGNILTIRLTAADHR-OH	<1	46

[a] DMPA (0.5 equiv.), vinyl palmitate (7 equiv.) in NMP. [b] DMPA (0.5 equiv.), vinyl palmitate (70 equiv.), *tert*-butylthiol (80 equiv.), triisopropylsilane (80 equiv.), TFA (5 % v/v) in NMP. [c] Derived from NY-ESO-1 [79-116]. [d] Derived from NY-ESO-1 [118-143]. [e] Conversion of peptides **13** and **14** to corresponding monopalmitoylated product **S1** and **S2** is based on the integration of corresponding peaks on RP-HPLC profile at 230 nm.

>95 % conversion, and the desired product (i.e., **9**) was formed in 88 % yield. A slight improvement in the conversion of peptide **10** or the ratio of product **9** to **12** was observed when the amounts of *tert*-butylthiol and TIPS were varied slightly (Table 2, entries 6 and 7).

When TIPS was used as a sole reductant (without *t*BuSH) in the reaction, a large amount of bispalmitoylated by-product **12** was observed (Table 2, entry 8, see Supporting Information Figure S7). This experimental observation indicated that while *tert*-butylthiol or TIPS can be used as a sole hydrogen donor source for the radical chain process, a synergistic effect between these two reductants minimises the formation of **12**.

Final Comments

Lastly, having established the efficacy of the thiol and silane as a reducing pair, the relative quantity of vinyl palmitate (**1**) was progressively decreased to assess the effect of this on the efficiency of the reaction (Table 2, entries 9–12, see Supporting Information Figure S8). Little difference in conversion was observed for a 100- or 35-fold excess of **1**; however, a progressive decrease in the conversion of peptide **10**, from 81 to 60 %, was noted as the levels of **1** were reduced to a 20-fold and then to a 7-fold excess, respectively, with the optimal level clearly falling somewhere between a 20- and 30-fold excess. It was gratifying to note that for these experiments the formation of bispalmitoylated species **12** consistently remained at near-negligible levels.

With the optimised conditions for the thiol-ene reaction established, we finally reexamined the use of DMSO and DMF as solvents for the reaction (Table 2, entries 13 and 14, respectively, see Supporting Information Figures S9 and S10). Both solvents gave monopalmitoylated product **9** selectively, although only DMF (Table 2, entry 14) resulted in consumption of peptide **10** comparable to that observed when using NMP.

The conditions optimised for this peptide were then applied to other examples to assess the generality of the approach, particularly on longer peptides (See Supporting Information Figures S11–17). These results are summarised in Table 3.

In both cases, the palmitoylation process was considerably enhanced by using our optimised conditions, which demonstrates that complex substrates can be successfully monopalmitoylated. In the example of peptide **13**, the conversion (81 %) represents a considerable improvement over that previously reported[4b] for this peptide (41 %). It should be noted that in the case of peptide **13**, partial oxidation of the constituent methionine residues to the sulfoxides was observed, so the crude product mixture was reduced with tetrabutylammonium iodide in trifluoroacetic acid to simplify HPLC analysis.[20]

Conclusions

In conclusion, N^{α}-protected-cysteinyl building blocks **5**–**7**, functionalised with an *S*-palmitoyl fatty chain, were synthesised in high yield using a thiol-ene radical conjugation reaction. These pre-formed building blocks were directly incorporated into a peptide sequence using Fmoc-SPPS. Significant epimerisation was observed when coupling AcNH-Cys(palmitoyl)-OH **7**, but this did not take place with carbamate FmocNH-Cys(palmitoyl)-OH (**5**). Direct conjugation of the palmitoyl group onto the cysteine residue of unprotected peptide **10** using a thiol-ene reaction was also demonstrated. The key reagents for effective conjugation of peptide **10** with vinyl palmitate (**1**) were *tert*-butylthiol and triisopropylsilane, acting as dual hydrogen-donor components. The use of this dual reductant system effected high conversion of starting peptide **10**, giving mono adduct **9** as the major component, and minimising formation of bis adduct **12**. Our optimised conditions were applied to longer peptides **13** and **14**, which exclusively gave monopalmitoylated adducts in excellent to good yields that could not be accessed without the addition of *tert*-butylthiol and triisopropylsilane. We believe that the experimental conditions that have been carefully optimised in this study will be generally applicable as an efficient method for the site-specific lipidation of cysteine-containing polypeptides (CLipPA technology).

Experimental Section

General Remarks: All solvents and reagents were purchased from commercial sources, and were used without further purification. Fmoc-SPPS and other reactions were carried out under an air atmosphere without using anhydrous solvents. Aminomethyl-polystyrene (AM-PS) resin was synthesised "in house" as described.[21] Fmoc-amino acids were purchased from GL Biochem (Shanghai, China) with the following side-chain protection: Fmoc-Asn(Trt)-OH (Trt = trityl), Fmoc-Cys(Trt)-OH, Fmoc-Cys(*t*Bu)-OH (*t*Bu = *tert*-butyl), Fmoc-Lys(Boc)-OH (Boc = *tert*-butyloxycarbonyl), Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH.

Thin-layer chromatography (TLC) was carried out using Merck silica gel plates, and spots were visualised with UV light and/or developed using an ethanolic solution of vanillin or permanganate. Microwave irradiation was carried out with a CEM® Discover instrument. NMR spectra were recorded in $CDCl_3$ with a Bruker BRX300

spectrometer operating at 300 MHz for ^{1}H nuclei, and 75 MHz for ^{13}C nuclei. Chemical shifts are reported in parts per million (ppm) on the δ scale from tetramethylsilane (TMS), and were referenced to residual solvent peaks ($CDCl_3$: δ = 7.26 ppm for ^{1}H NMR, δ = 77.0 ppm for ^{13}C NMR). Coupling constants (*J*) are in Hertz (Hz). ^{1}H NMR spectroscopic data is reported as chemical shift in ppm, followed by multiplicity and relative integral. Multiplicities are reported as "s" (singlet), "br. s" (broad singlet), "d" (doublet), "dd" (doublet of doublets), "ddd" (doublet of doublets of doublets), "dt" (doublet of triplets), "t" (triplet), and "m" (multiplet). RP-HPLC was carried out with a Dionex UltiMate 3000 system equipped with a four-channel UV detector. Analytical RP-HPLC was carried out using a Phenomenex Gemini C-18 column (5 µm; 4.6 × 150 mm) at a flow rate of 1.0 mL/min. Semipreparative RP-HPLC was carried out using a Foxy Jr fraction collector with a Phenomenex Gemini C18 column (5 µm; 10.0 × 250 mm) at a flow rate of 5 mL/min, eluting using a one-step slow gradient protocol.[22] The solvent system used was A (0.1 % trifluoroacetic acid in H_2O) and B (0.1 % trifluoroacetic acid in acetonitrile). Peptide masses were confirmed by LC–MS using an Agilent 1120 Compact LC system with a Hewlett Packard Series 1100 MSD mass spectrometer using ESI in the positive mode. Optical rotations were recorded with a Rudolph Autopol® IV automatic polarimeter. Melting points were recorded with an X-4 melting-point apparatus with a microscope. Attenuated total reflection infrared (ATR-IR) spectroscopy was carried out with a Bruker Alpha spectrometer. High-resolution mass spectra (HRMS) were obtained using a spectrometer operating at a nominal accelerating voltage of 70 eV, or with a TOF-Q mass spectrometer.

General Procedure A: Synthesis of *S*-Palmitoylated Cysteine Building Blocks 5, 6, and 7: Vinyl palmitate (**1**; 1.5 equiv.) and 2,2-dimethoxy-2-phenylacetophenone (DMPA) were added to a solution of differentially protected cysteine **2**, **3**, or **4** in dichloromethane (100 mg/1.0 mL). The reaction mixture was irradiated at a wavelength of 365 nm using a UV lamp at room temperature. When TLC indicated that the reaction was complete, the mixture was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel to give the title compound.

(*R*)-2-({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)-3-{[2-(palmitoyloxy)ethyl]thio}propanoic Acid 5: Compound **2** (0.2 g, 0.58 mmol) was treated according to General Procedure A using DMPA (0.149 g, 0.58 mmol) and vinyl palmitate (0.25 g, 0.87 mmol) in dichloromethane (2 mL). The crude mixture was purified by flash column chromatography on silica gel ($MeOH/CH_2Cl_2$, 0.5:95) to give **5** (0.3 g, 82 %) as a white solid, m.p. 50.3–50.7 °C. R_f = 0.33 ($MeOH/CH_2Cl_2$, 0.5:95). $[\alpha]_D^{23}$ = –8.54 (*c* = 0.398, MeOH). IR (neat): $\tilde{\nu}$ = 3318.32, 2918.39, 2850.47, 1731.80, 1693.43, 1534.48, 1466.61, 1450.17 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.6 Hz, 2 H, Ar*H*), 7.60 (br. s, *J* = 6.80 Hz, 2 H, Ar*H*), 7.39 (t, *J* = 7.50 Hz, 2 H, Ar*H*), 7.31 (dt, *J* = 0.77, *J* = 7.50 Hz, 2 H, Ar*H*), 6.92–6.42 (br. s, 1 H, COO*H*), 5.72 (d, *J* = 7.66 Hz, 1 H, N*H*), 4.70–4.62 (m, 1 H, α-C*H*), 4.45–4.38 (m, 2 H, Fmoc-CH_2), 4.26–4.18 (m, 3 H, Fmoc-C*H* and SCH_2CH_2O), 3.14 (dd, *J* = 4.67, *J* = 13.66 Hz, 1 H, β-CH_{2a}), 3.06 (dd, *J* = 5.36, *J* = 13.84 Hz, 1 H, β-CH_{2b}), 2.78 (t, *J* = 6.40 Hz, 2 H, SCH_2CH_2O), 2.29 (t, *J* = 7.50 Hz, 2 H, $OCOCH_2CH_2$), 1.59 (m, 2 H), 1.28–1.21 (m, 24 H), 0.88 (t, *J* = 6.87 Hz, 3 H, CH_2CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.1 (quat., *C*=O), 173.9 (quat., *C*=O), 155.9 (quat., OCONH), 144.2 (CH, Ar*C*H), 143.69 (CH, Ar*C*H), 143.62 (CH, Ar*C*H), 141.3 (CH, Ar*C*H), 127.7 (CH, Ar*C*H), 127.0 (CH, Ar*C*H), 125.0 (CH, Ar*C*H), 119.9 (CH, Ar*C*H), 67.3 (CH_2, Fmoc-CH_2), 63.0 (CH_2, SCH_2CH_2O), 53.5 (CH, α-*C*H), 47.0 (CH, Fmoc-*C*H), 34.3 (CH_2, β-CH_2), 34.1 (CH_2, $OCOCH_2CH_2$), 31.9 (CH_2), 31.2 (CH_2, SCH_2CH_2O), 29.67 (CH_2), 29.64 (CH_2), 29.60 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 24.8 (CH_2), 22.6 (CH_2), 14.0 (CH_3, CH_2CH_3) ppm. HRMS (ESI): calcd. for $C_{36}H_{51}NO_6SNa$ $[M + Na]^+$ 648.3329; found 648.3328.

(*R*)-2-[(*tert*-Butyloxycarbonyl)amino]-3-{[2-(palmitoyloxy)ethyl]-thio}propanoic Acid (6): Compound **3** (0.2 g, 0.90 mmol) was treated according to General Procedure A using DMPA (0.23 g, 0.90 mmol) and vinyl palmitate (0.38 g, 1.35 mmol) in dichloromethane (2 mL). The crude mixture was purified by flash column chromatography on silica gel ($MeOH/CH_2Cl_2$, 0.5:95) to give **6** (0.37 g, 82 %) as a yellow oil. R_f = 0.33 ($MeOH/CH_2Cl_2$, 0.5:95). $[\alpha]_D^{23}$ = –7.0 (*c* = 0.742, MeOH). IR (neat): $\tilde{\nu}$ = 3370.72, 2915.81, 2850.44, 1735.03, 1687.58, 1519.59, 1471.76, 1415.78 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.71–8.84 (br. s, 1 H, COO*H*), 5.43–5.36 (m, 1 H, N*H*), 4.60–4.49 (m, 1 H, α-C*H*), 4.21 (t, *J* = 6.80 Hz, 2 H, SCH_2CH_2O), 3.14–2.93 (m, 2 H, β-CH_2), 2.78 (t, *J* = 6.73 Hz, 2 H, SCH_2CH_2O), 2.30 (t, *J* = 7.46 Hz, 2 H, $OCOCH_2CH_2$), 1.64–1.56 (m, 2 H), 1.44 [s, 9 H, $C(CH_3)_3$], 1.24 (s, 24 H), 0.56 (t, *J* = 6.82 Hz, 3 H, CH_2CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.7 (quat., *C*=O), 173.8 (quat., *C*=O), 155.4 (quat., OCONH), 80.5 [quat., $C(CH_3)_3$] 63.1 (CH_2, SCH_2CH_2O), 53.2 (CH, α-*C*H), 34.4 (CH_2, β-CH_2), 34.2 (CH_2, $OCOCH_2CH_2$), 31.9 (CH_2), 31.2 (CH_2, SCH_2CH_2O), 29.69 (CH_2), 29.66 (CH_2), 29.62 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 28.2 [CH_3, $C(CH_3)_3$], 28.1 (CH_2), 24.9 (CH_2), 22.6 (CH_2), 14.1 (CH_3, CH_2CH_3) ppm. HRMS (ESI): calcd. for $C_{26}H49NO_6SNa$ $[M + Na]^+$ 526.3173; found 526.3176.

(*R*)-2-Acetamido-3-{[2-(palmitoyloxy)ethyl]thio}propanoic Acid (7): Compound **4** (0.2 g, 1.22 mmol) was treated according to General Procedure A using DMPA (0.31 g, 1.22 mmol) and vinyl palmitate (0.52 g, 1.84 mmol) in dichloromethane (2 mL). The crude mixture was purified by flash column chromatography on silica gel ($MeOH/CH_2Cl_2$, 0.5:95) to give **7** (0.48 g, 88 %) as a colourless solid, m.p. 86.8–93.2 °C R_f = 0.08 ($MeOH/CH_2Cl_2$, 0.5:95). $[\alpha]_D^{23}$ = –5.7 (*c* = 0.374, MeOH). IR (neat): $\tilde{\nu}$ = 3328.93, 2916.77, 2850.09, 1736.25, 1703.47, 1617.83, 1545.29, 1467.03, 1419.40 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.21–7.70 (br. s, 1 H, COO*H*), 6.29 (d, *J* = 7.51 Hz, 1 H, N*H*), 4.79 (ddd, *J* = 5.06, *J* = 5.49, *J* = 6.94 Hz, 1 H, α-*CH*), 4.22 (ddd, *J* = 3.91, *J* = 6.90, *J* = 10.55 Hz, 2 H, SCH_2CH_2O), 3.12 (dd, *J* = 4.72, *J* = 14.08 Hz, 1 H, β-CH_{2a}), 3.05 (dd, *J* = 5.80, *J* = 13.93 Hz, 1 H, β-CH_{2b}), 2.78 (dt, *J* = 1.60, *J* = 6.55 Hz, 2 H, SCH_2CH_2O), 2.33 (t, *J* = 7.50 Hz, 2 H, $OCOCH_2CH_2$), 2.09 (s, 3 H, CH_3CO), 1.65–1.55 (m, 2 H), 1.27–1.22 (s, 24 H), 0.87 (t, *J* = 6.92 Hz, 3 H, CH_2CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.0 (quat., *C*=O), 172.7 (quat., *C*=O), 171.4 (quat., *C*=O), 62.9 (CH_2, SCH_2CH_2O), 52.1 (CH, α-*CH*), 34.2 (CH_2, $OCOCH_2CH_2$), 33.8 (CH_2, β-CH_2), 31.8 (CH_2), 31.2 (CH_2, SCH_2CH_2O), 29.6 (CH_2), 29.63 (CH_2), 29.60 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 24.8 (CH_2), 22.8 (CH_3, CH_3CO), 22.6 (CH_2), 14.0 (CH_3, CH_2CH_3) ppm. HRMS (ESI): calcd. for $C_{23}H_{43}NO_5SNa$ $[M + Na]^+$ 468.2754; found 468.2750.

General Procedure for Fmoc-SPPS: Aminomethyl-polystyrene resin (100 mg, 0.1 mmol, loading 1.0 mmol/g) was treated with Fmoc-Val-HMPP (HMPP = hydroxymethylphenoxyacetic acid) (105 mg, 0.2 mmol) and DIC (31 µL, 0.2 mmol) in a mixture of dichloromethane and DMF (1.9:0.1 v/v; 2 mL) for 1 h at room temperature. The completion of the coupling was monitored using the Kaiser test, and if the coupling was incomplete, the coupling procedure was repeated with freshly prepared reagent. Solid-phase peptide synthesis was performed using a Tribute peptide synthesiser (Protein technologies Inc.) using HATU/DIPEA for the coupling step for 40 min at room temperature, and a solution of piperidine in DMF (20 % v/v) for the Fmoc-deprotection step, repeated twice for 5 min at room temperature.

Resin-bound peptide 8: Target compound **8** (380 mg) was prepared using the General Procedure for Fmoc-SPPS.

Peptide 10: Elongation of the peptide chain was achieved through the General Procedure for Fmoc-SPPS. *N*-Terminal acetylation was completed using a solution of acetic anhydride in DMF (20 % v/v) and DIPEA (0.25 mL) for 15 min at room temperature. The resin-bound peptide was treated with TFA/TIPS/H_2O/DODT (94:1:2.5:2.5 v/v; 10 mL) for 2 h at room temperature. The TFA was evaporated by a flow of nitrogen, then the peptide was precipitated in cold diethyl ether, isolated by centrifugation, washed twice with cold diethyl ether, dissolved in acetonitrile/water (1:1, v/v, containing 0.1 % TFA v/v), and lyophilised to give the crude peptide. Purification by RP-HPLC using a semipreparative Gemini C-18 column (Phenomenex, 5 μm, 10.0 × 250 mm) gave peptide **10** (74 mg, 43 % based on a 0.1 mmol scale). MS: calcd. for $[M + 2H]^{2+}$ 858.5; found 858.6.

General Procedure B: Coupling of Building Blocks 5 or 7 With Resin-Bound Peptide 8: A solution of *S*-palmitoylated building block **5** (21 mg, 0.03 mmol) or **7** (15 mg, 0.03 mmol), PyBOP (17 mg, 0.03 mmol), and 2,4,6-trimethylpyridine (4.2 μL, 0.03 mmol) in DMF (1 mL) was added to resin-bound peptide **8** (30 mg, 8 μmol based on recovered peptidyl resin, 380 mg). The reaction mixture was left for 1 h at room temperature, and the completion of the coupling was monitored using the Kaiser test. If the coupling was incomplete, the coupling procedure was repeated with freshly prepared reagent. The resin-bound peptide was treated with TFA/TIPS/H_2O/DODT (94:1:2.5:2.5 v/v; 1 mL) for 1 h at room temperature. The TFA was then evaporated using a flow of nitrogen, the peptide was precipitated in cold diethyl ether, isolated by centrifugation, washed twice with cold diethyl ether, dissolved in acetonitrile/water (1:1, v/v, containing 0.1 % TFA v/v), and lyophilised to give the crude peptide.

General Procedure C: Direct Conjugation of Vinyl Palmitate (1) and Semiprotected Peptide 10: Stock solution 1: DMPA (6.5 mg, 25.3 μmol) in degassed *N*-methyl-2-pyrrolidone (0.5 mL); Stock solution 2: vinyl palmitate (**1**) in degassed *N*-methyl-2-pyrrolidone (required concentration). Peptide **10** (1.71 mg, 1.0 μmol) was dissolved in stock solution 1 (10 μL, 0.5 μmol), then *tert*-butylthiol, triisopropylsilane, trifluoroacetic acid (5 % v/v), and stock solution 2 were added. The reaction mixture was irradiated at wavelength of 365 nm using a UV lamp at room temperature, and samples were taken for LC–MS analysis at 30 min intervals. An analytical sample was prepared by quenching with Milli-Q water, and this was analysed using a Gemini C-18 column (Phenomenex, 5 μm, 4.6 × 150 mm).

Example of General Procedure C: Table 2, entry 7: Peptide **10** (5.13 mg, 3.0 μmol) was dissolved in stock solution 1 (30 μL, 1.51 μmol), then *tert*-butylthiol (27 μL, 0.24 mmol), triisopropylsilane (48.9 μL, 0.24 mmol), trifluoroacetic acid (5 % v/v; 20 μL), and stock solution 2 (59.1 mg, 0.21 mmol, 300 μL from 0.2 mg/μL) were added. Upon completion of the reaction, as monitored by RP-HPLC, the crude material was directly purified by RP-HPLC using a semipreparative diphenyl column (Vydac, 5 μm, 10.0 × 250 mm) to give peptide **9** (4.29 mg, 72 %). MS: calcd. for $[M + 2H]^{2+}$ 999.5; found 999.9.

Table 3, entry 1: Peptide **13** (1.23 mg, 0.25 μmol) was dissolved in degassed *N*-methyl-2-pyrrolidone (4.7 μL), stock solution 1 (2.5 μL, 0.13 μmol), and stock solution 2 (4.94 mg, 0.018 mmol, 10 μL from 0.495 mg/μL), and then *tert*-butylthiol (2.3 μL, 0.02 mmol), triisopropylsilane (4.1 μL, 0.02 mmol), and trifluoroacetic acid (5 % v/v; 1.4 μL) were added. The reaction mixture was irradiated at wavelength of 365 nm using a UV lamp at room temperature, and samples were taken for LC–MS analysis at 30 min intervals. An analytical sample was prepared by quenching with Milli-Q water, and this was analysed using an XTerra® MS C-18 column (Waters, 5 μm, 4.6 × 150 mm). The reaction mixture was lyophilised before methionine oxide reduction. Tetrabutylammonium iodide (9 mg, 0.025 mmol) was added to the crude peptide in TFA (28 μL) at 0 °C. After 1 min at the same temperature, the peptide was precipitated in cold diethyl ether, isolated by centrifugation, washed twice with cold diethyl ether, dissolved in acetonitrile/water (1:1, v/v, containing 0.1 % TFA v/v), and lyophilised to give the crude peptide. MS: calcd. for $[M + 3H]^{3+}$ 1734.2; found 1734.2.

Table 3, entry 2: Peptide **14** (0.88 mg, 0.25 μmol) was dissolved in degassed *N*-methyl-2-pyrrolidone (4.7 μL), stock solution 1 (2.5 μL, 0.13 μmol) and stock solution 2 (4.94 mg, 0.018 mmol, 10 μL from 0.495 mg/μL), and then *tert*-butylthiol (2.3 μL, 0.02 mmol), triisopropylsilane (4.1 μL, 0.02 mmol), and trifluoroacetic acid (5 % v/v; 1.4 μL) were added. The reaction mixture was irradiated at wavelength of 365 nm using a UV lamp at room temperature, and samples were taken for LC–MS analysis at 30 min intervals. An analytical sample was prepared by quenching with Milli-Q water, and this was analysed using an XTerra® MS C-18 column (Waters, 5 μm, 4.6 × 150 mm). MS: calcd. for $[M + 2H]^{2+}$ 1269.7; found 1269.4.

Keywords: Synthetic methods · Click chemistry · Peptides · Lipids · Thiols · Cysteine

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