A method for S- and O-palmitoylation of peptides: synthesis of pulmonary surfactant protein-C models

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A method for O- and S-palmitoylation of non-protected peptides has been developed. The peptides are treated with excess of palmitoyl chloride in 100 % trifluoroacetic acid for 10 min at room temperature. The acidic conditions prevent acylation of amino groups, which is only significant after prolonged treatment (hours to days). The tripeptides Gly-Cys-Phe and Gly-Ser-Phe were converted into the respective S- and O-palmitoylated compounds, and the hydrophobic pulmonary surfactant protein-C model peptides, LRIPCCPVNLKRLLVVV [SP-C(1–17)] and FGIPSSPVLKRLLILLLLLLLILLLILGALLMGL [SP-C(Leu)] were converted into their respective S,S- and O,O-dipalmitoylated peptides. The reactions were virtually quantitative, and the palmitoylated peptides were isolated in about 75–80 % yield after reversed-phase HPLC purification. CD spectroscopy showed that S,S-dipalmitoylation of SP-C(1–17) affects the peptide secondary structure (substantial increase in the α-helix content) in dodecylphosphocholine micelles.

Key words: lipopeptide, peptide structure, protein acylation.

INTRODUCTION

Fatty acylation occurs co- or post-translationally in several different protein families which cover a wide range of functional properties [1]. The acyl chains are linked either via thioesters or via amide bonds. The thioesters are most commonly palmitoyl (C_{16}) groups linked to cysteine residues. The proteins carrying ester-linked fatty acyl chains are, as a rule, associated with lipid membranes. A significant number of these proteins are, however, also hydrophobic in the absence of the acyl moieties. The fatty acyl esters are generally considered to be enzymically linked [1,2] but non-enzymic [3,4] addition has also been suggested.

Fatty acyl amides have been found either in the form of myristoyl (C_{14}) groups linked to α-amino groups or, in a few cases, as palmitoyl groups bound to lysine ϵ-amino groups. Myristoyl groups are enzymically added co-translationally to proteins that carry a defined N-terminal signal sequence. N^{ϵ}-Palmitoyl-lysine has so far only been detected in adenylate cyclase from *Bordetella pertussis*, where it is formed by a specific enzyme [5] and as a probably non-enzymic modification of a fraction of the pulmonary-surfactant-associated polypeptide C (SP-C) [6].

Several different functional roles of the fatty acyl moieties are possible, including modulating effects on protein–lipid interactions, protein–protein interactions and protein conformation [7]. The labile nature of thioester-linked acyl chains suggests that they can undergo acylation/deacylation cycles [8] and hence influence the interactions of the protein to which they are attached in a reversible manner. SP-C is a 4.2 kDa lipopeptide that contains two palmitoyl groups linked to Cys^5 and Cys^6 [9]. Evidence is now accumulating that the presence of the fatty acyl moieties is required for the lipopeptide to be functionally fully active [10–12]. From a structural point of view it has been reported that the palmitoyl groups both increase [12–14] and decrease [10,15] the helical content of the peptide. This discrepancy may be caused by the fact that different procedures have been employed in order to deacylate native SP-C, and that the peptide can undergo a conversion from helical to β-sheet conformation [14]. A method for stoichiometric formation of thio- and oxy-esters would facilitate clear-cut analysis of the acyl-chain effects on structure and function of SP-C and other acylated peptides. Use of an S-palmitoylated cysteine building block in solid-phase peptide synthesis appears problematic, owing to the instability of the thioester, although one method makes it possible to add an N-protected S-palmitoyl-cysteine at the N-terminus of a peptide [16]. Other methods for S-acylation of peptides include formation of thioesters of acyl carrier protein using *N*-acylimidazoles [17] and less-than-quantitative palmitoylation of detergent-solubilized SP-C with palmitoyl-CoA [18].

EXPERIMENTAL

Trifluoroacetic acid (TFA), di-isopropylethylamine (DIPEA, Fluka) and palmitoyl chloride (Aldrich), dimethylformamide (DMF) and dichloromethane (DCM) were distilled under atmospheric pressure. Hydroxybenzotriazole (HOBt), di-isopropylcarbodi-imide (DIPCDI, Aldrich) and dicyclohexylcarbodi-imide (DCC; Lancaster Synthesis Ltd., Easgate, Lancs., U.K.) were of commercial quality. Amino acids and building blocks for the tripeptide syntheses were from Bachem, and the chloromethylated resin (Bio-Beads Sx1) used in the Gly-Cys-Phe synthesis was from Bio-Rad. The palmitoylation reactions were monitored by reversed-phase (RP-) HPLC (Vydak C_{18} column, 4.6 mm diameter × 250 mm long; The Separations Group, Hesperia, CA, U.S.A.) employing a flow rate of 0.7 ml/min and the same eluents and gradient as for the corresponding HPLC purifications of compounds **3**, **6** and **7** (see below). CD

Abbreviations used: Boc, butoxycarbonyl; Boc-Cys(mBzl)OH, *N*-t-butylcarbamoyl-*S*-(4-methylbenzyl)cysteine; DCM, dichloromethane; DMF, dimethylformamide; DIPCDI, di-isopropylcarbodi-imide; DIPEA, di-isopropylethylamine; ESI, electrospray ionization; HOBt, hydroxybenzotriazole; MALDI-TOF, matrix-assisted laser-desorption time-of-flight; RP-, reversed phase; r.t., retention time; SP, surfactant protein; TFA, trifluoroacetic acid.

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spectroscopy and matrix-assisted laser-desorption time-of-flight (MALDI-TOF) and electrospray ionization (ESI) MS were performed as described previously [6,14,19]. ^{1}H- and ^{13}C-NMR analyses were performed with a JEOL GSX-270 spectrometer.

Polypeptides 4 and 5

The polypeptides SP-C(1–17) (**4**) [20] and SP-C(Leu) (**5**) [21] were synthesized by t-butyloxycarbonyl chemistry and purified by RP-HPLC {**4** as in [20] and **5** [retention time (r.t.) 33 min, 81 % propan-2-ol] as for **6**}.

S-Palmitoylcysteine hydrochloride (1)

Palmitoyl chloride (0.34 g, 1.2 mmol, 1.2 equiv.) was added to L-cysteine (100 mg, 0.83 mmol) in distilled trifluoroacetic acid (TFA) (4 ml), and the mixture was stirred at room temperature. After 5 min the product precipitated as a white powder, which was filtered and washed with chloroform.Yield 115 mg (38 %). Elemental analysis confirmed the identity of the product. Calculated (%): C, 57.62; H, 9.67; N, 3.54; S, 8.10; found (%): C, 57.60; H, 10.13; N, 3.59; S, 8.28. ^{13}C NMR (DMSO, 30 °C, δ in p.p.m.): 13.84 (CH$_3$CH$_2$), 27.84–28.95 (13 × CH$_2$), 43.22 (CH$_2$CO), 31.21 (βC, Cys), 51.53 (αC, Cys), 168.89 (CO, Cys), 197.23 (SCO).

Gly-Ser-Phe (2a)

DCC (128.06 mg, 0.8 mmol) was added to HOBt (108 mg, 0.8 mmol) dissolved in DCM (5 ml), and the mixture was stirred at room temperature. Butoxycarbonyl (Boc)-Gly-OH (140 mg, 0.8 mmol) was added, and, after stirring for 1 h, the precipitated urea was filtered off and the solvent was evaporated. The residue was dissolved in DMF (5 ml) and Ser-Phe-OH (100 mg, 0.39 mmol) and DIPEA (200 μl, 1.2 mmol) were added. After 2 h the reaction was complete (TLC; butan-2-ol/acetic acid/water, 4:2:2, by vol.; $R_F = 0.9$). After solvent evaporation the product was precipitated in warm chloroform (10 ml), filtered off and dried *in vacuo* to give 130 mg of material (82 %). The protected tripeptide (28 mg, 0.068 mmol) was dissolved in 50 % (v/v) TFA in DCM (0.68 ml) and stirred at room temperature for 20 min. The solvent was evaporated, whereafter the residue was three times redissolved in DCM and evaporated. The product was dissolved in water (5ml) and passed through an amberlite resin [IR-45 (OH)]. The product was obtained as white powder after evaporation. Purification was performed by RP-HPLC (Vydak C_{18} column, 22 mm × 250 mm) using a linear gradient of 0–60 % acetonitrile in water over 60 min (flow rate 10 ml/min), both solutions containing 0.1 % TFA; r.t. 21min, 22 % acetonitrile. Yield 20 mg, 0.064 mmol, (95 %). ^{1}H NMR (^{2}H$_2$O): d 7.39 (5H Phe); 4.64 and 4.45 (2H αCH Ser and Phe); 3.84 (2H, CH_2 Gly); 3.80 (2H, βCH_2 Ser); 3.12–3.05 (2 × dd, 2H, βCH_2 Phe). ^{13}C NMR (^{2}H$_2$O): d 178.9, 172.9 and 168.8 (3 × C = O); 138.9–128.7 (aromatic Phe); 62.3 (βCH$_2$ Ser); 56.8 and 56.4 (2 × αCH Ser and Phe); 41.3 (CH$_2$ Gly); 38.2 (βCH$_2$ Phe).

Gly-Cys-Phe (2b)

The peptide **2b** was synthesized manually using solid-phase technique with Boc-protected amino acids [22,23]. The first amino acid was covalently linked to a chloromethylated polystyrene resin [1.25 mequiv./g) according to the KF method [24] in DMF, i.e. chloromethylated resin Bio-Beads S-X1 (2 g) was combined with Boc-Phe (796 mg, 3 mequiv.) and KF (443 mg, 10 mequiv.) in distilled DMF (15 ml). The mixture was stirred for 3 days at 50 °C. The resin was loaded on an apparatus for manual solid-phase peptide synthesis. The solvent was removed and the resin was washed with DCM (2 × 15 ml), DMF (2 × 15 ml), water (2 × 15 ml), ethanol (2 × 15 ml), DMF (15 ml) and DCM (15 ml). *N*-t-butylcarbamoyl-*S*-(4-methylbenzyl)cysteine [Boc-Cys(mBzl)OH] and Boc-glycine-OH were coupled to the growing peptide as active esters (using HOBt and DIPCDI) and DCM/DMF (3:1, v/v) as the coupling solvent [25–27]. The coupling reactions were monitored by the ninhydrin test [28]. DIPCDI (378 mg, 464 μl, 3 mmol), HOBt (610 mg, 4.5 mmol) and Boc-Cys(mBzl)OH (976 mg, 3 mmol) were dissolved in a mixture of distilled DCM (15 ml) and dry DMF (5 ml) in a 50 ml round-bottomed flask. The mixture was stirred for 30 min. In the meantime the resin in the reaction vessel was washed with, 10 % DIPEA in DCM (2 × 15 ml), DCM (2 × 15 ml) and DMF (2 × 15 ml). The mixture with the activated Boc-Cys(mBzl)OH was then added to the resin. After 2 h the solvent was removed and the resin was washed with DMF (2 × 20 ml) and DCM (2 × 20 ml). After a ninhydrin test, the resin was further washed with DCM (4 × 20 ml). The coupling of Boc-Gly-OH to the growing peptide was done using the same procedure. For deprotection of the Boc groups, 50 % TFA in DCM (15 ml) was used. The solvent was removed after 2 min and another portion of 50 % TFA in DCM was added and left on the support for 30 min. The resin was subsequently washed with DCM (2 × 20 ml), DMF (2 × 20 ml), ethanol (2 × 20 ml), DMF (20 ml) and DCM (20 ml). The peptide was deprotected and cleaved from the resin using HF [29]. The resin was washed with DCM (5 × 15 ml) and dried under nitrogen followed by desiccation under vacuum. The peptide resin was placed in a Teflon reaction vessel, and anisole (3 ml) was added. The vessel was cooled to −70 °C in a solid CO_2/ethanol mixture for 10 min. The reaction vessel was evacuated for 5 min, whereupon HF (27 ml) was condensed on to the peptide resin. The reaction mixture was stirred for 30 min at 0 °C. The HF was evaporated, the residue dissolved in 40 % (v/v) acetic acid (40 ml), and the mixture was washed with diethyl ether (3 × 30 ml) and ethyl acetate (30 ml). The aqueous layers were combined and the solvent was evaporated under reduced pressure. The peptide was desalted on a Sephadex G-10 column in aqueous ammonium hydrogen carbonate. Purification was performed by RP-HPLC as for **2a** (r.t. 28 min, 28 % acetonitrile). ^{1}H NMR (^{2}H$_2$O): d 7.17 (5H Phe); 4.51 (m, 2H, αCH Phe and Cys); 3.65 (s, 2H, CH_2 Gly); 3.09 (dd, 1H, J 13.9 and 5.5 Hz βCH Phe); 2.87 (m, 2H, βCH_2 Cys): 2.66 (dd, 1H, J 14.3 and 8.8 Hz βCH Phe). ^{13}C NMR (^{2}H$_2$O/[^{2}H]acetic acid): d 178.4, 172.9 and 169.1 (C = O Phe, Cys and Gly); 138.7–129.2 (aromatic, Phe); 57.7 and 56.4 (αCH Phe and Cys); 42.9 (CH$_2$ Gly); 38.9 (βCH$_2$ Phe); 28.0 (βCH$_2$ Cys).

O-Palmitoyl-Gly-Ser-Phe (3a)

Gly-Ser-Phe-OH (2.3 mg, 7.44 μmol) was dissolved in distilled TFA (744 μl) and palmitoyl chloride (20 mg, 74.4 μmol, 10 equiv.) was added. The reaction was quenched with 80 % ethanol (6.69 ml) after 10 min. The product was purified by RP-HPLC (Vydak C_{18} column, 22 mm × 250 mm) using a linear gradient of propan-2-ol in 60 % methanol over 40 min (flow rate 7 ml/min), both solutions containing 0.1 % TFA (r.t. 23 min, 58 % propan-2-ol). Isolated yield 3 mg, 5.48 μmol (74 %). MS (fast atom bombardment, $M+1$); m/z, found: 547.3616, calculated: 547.3621. ^{1}H NMR ([^{2}H]chloroform/[^{2}H]methanol/tetrahydrofuran): d 7.13 (5H Phe); 4.66 and 4.62 (2H, 2 × αCH Ser and Phe); 4.28 (2H βCH_2 Ser); 3.80 (2H, CH_2 Gly); 3.13–3.01 (2H, βCH_2 Phe); 2.22 (2H, CH_2C═O Palmitoyl glycine; Palm);

1.48–1.18 (26H, 13 × CH_2 Palm); 0.85 (3H, CH_3 Palm). ^{13}C NMR ([^{2}H]chloroform/[^{2}H]methanol/TFA): d 174.7, 173.9, 169.8 and 166.6 (4 × *C* = O); 139.1–112.9 (aromatic Phe); 63.5 (β*C*H$_2$ Ser); 54.3 and 53.9 (2 × αCH Ser and Phe); 41.2 (*C*H$_2$ Phe); 37.3 (β*C*H$_2$ Phe); 34.1–23.1 (*C*H$_2$ Palm); 14.2 (*C*H$_3$ Palm).

S-Palmitoyl-Gly-Cys-Phe (3b)

Gly-Cys-Phe-OH (3.2 mg, 9.5 μmol) was dissolved in distilled TFA (1 ml) and palmitoyl chloride (58 μl, 190 μmol) was added. After 10 min the reaction was quenched with aq. 80 % ethanol (9 ml). The product was purified by RP-HPLC (as for **6**) (r.t. 17 min, 45 % propan-2-ol). Isolated yield 4.3 mg, 7.7 μmol (78 %). MS (ESI, $M+1$); m/z, found: 564.2, calculated: 564.35. ^{1}H NMR ([^{2}H]methanol/TFA) d 7.25 (5H, Phe); 4.68 and 4.61 (2 × dd, 2H, αC*H* Phe and Cys); 3.65 (s, 2H, CH_2 Gly); 3.3–3.0 (m, 4H, βCH_2 Phe and Cys); 2.57 (t, 2H, CH_2C = O Palm); 1.64 (m, 2H, CH_2CH$_2$C = O Palm); 1.25 (24H, 12CH_2 Palm), 0.93 (3H, CH_3 Palm). ^{13}C NMR ([^{2}H]methanol/TFA): d 200.9 [*C* = O(S)]); 174.4, 171.6 and 167.4 (3 × *C* = O); 138.2–114.6 (aromatic Phe); 55.4 and 54.4 (2 × α*C*H Phe and Cys); 44.9 (*C*H$_2$ Gly); 41.7 (βCH$_2$ Phe); 38.4 (*C*H$_2$C = O Palm); 33.1–26.7 (*C*H$_2$ Palm and β*C*H$_2$ Cys); 14.2 (*C*H$_3$ Palm).

S,S-Dipalmitoyl-SP-C(1–17) (6)

LRIPCCPVNLKRLLVVV (**4**) (3 mg, 1.55 μmol) was dissolved in distilled TFA (150 μl) and palmitoyl chloride (9.40 μl, 31 μmol, 20 equiv.) was added. After 10 min, the reaction was quenched with 80 % ethanol (2.85 ml). Purification was performed using RP-HPLC (Vydak C$_{18}$column, 22 mm × 250 mm) and a linear gradient of propan-2-ol in 75 % ethanol over 40 min (flow rate 7 ml/min) (both solutions containing 0.1 % TFA), r.t. 22 min, 56 % propan-2-ol. Isolated yield was 78 % as determined by quantitative amino acid analysis. MALDI-TOF MS (positive ion); m/z: 2414 (calculated 2412). The ^{1}H-NMR chemical shifts (recorded as for **4** in [20]) of the β hydrogen atoms of the cysteine residues also confirmed the presence of the *S*-palmitoyl groups, i.e., $\delta = 3.35$ (β-CH_2-Cys5), 3.12 and 3.40 (β-CH_2-Cys6), whereas $\delta = 2.88$, 2.72 and 2.99 in the non-palmitoylated peptide.

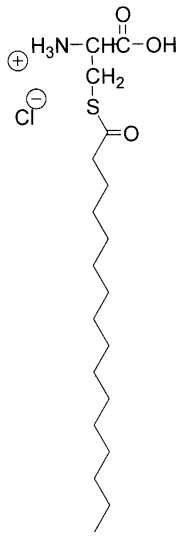
O,O-Dipalmitoyl-SP-C(Leu) (7)

FGGIPSSPVLKRLLILLLLLLLILLLILGALLMGL (**5**) (3.85 mg, 1.05 μmol) was dissolved in distilled TFA (100 μl) and palmitoyl chloride (6.2 μl, 21 μmol, 20 equiv.) was added. After 10 min, the reaction was quenched with 80 % ethanol (1.88 ml). Purification was performed using RP-HPLC as for **6** (r.t. 40 min, 100 % propan-2-ol). Isolated yield was 75 % as determined by quantitative amino acid analysis. MALDI-TOF MS (positive ion): m/z: 4145 (calculated 4143).

RESULTS AND DISCUSSION

Neither of the published methods for S-palmitoylation of peptides seemed ideal for our goals. The method involving use of *N*-acylimidazoles has the advantage of giving selectively more S-acylation than O- or N-acylation. However, the method requires strict control of conditions in order to avoid N-acylation, and the necessity to use aqueous buffers poses solubility problems when working with hydrophobic peptides. Since SP-C and peptide models thereof are sparingly soluble in water, we decided to try non-aqueous conditions instead. Furthermore, these models usually contain either serine or cysteine, but not both, which limits the need for selective palmitoylation of hydroxy or thiol groups in the presence of nitrogen nucleophiles. This would be most easily achieved by using acidic conditions.

The idea of simply dissolving the peptide in 100 % TFA and then adding commercially available palmitoyl chloride was first tested on cysteine (**1**). This palmitoylated amino acid can be difficult to isolate, owing to the possibility of intramolecular transacylation. However, a substantial amount of the product precipitated out of the solution as the hydrochloride and was verified to be the S-palmitoyl derivative, **1**. A number of peptides containing either serine or cysteine residues were then synthesized to investigate this method of palmitoylation further.



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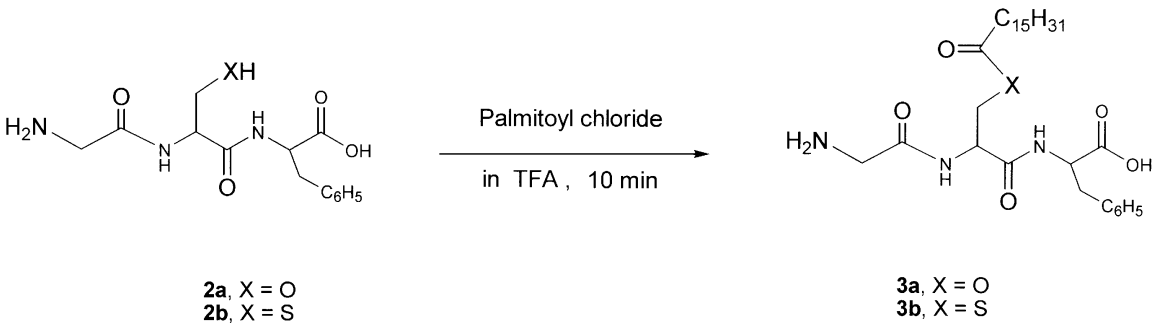
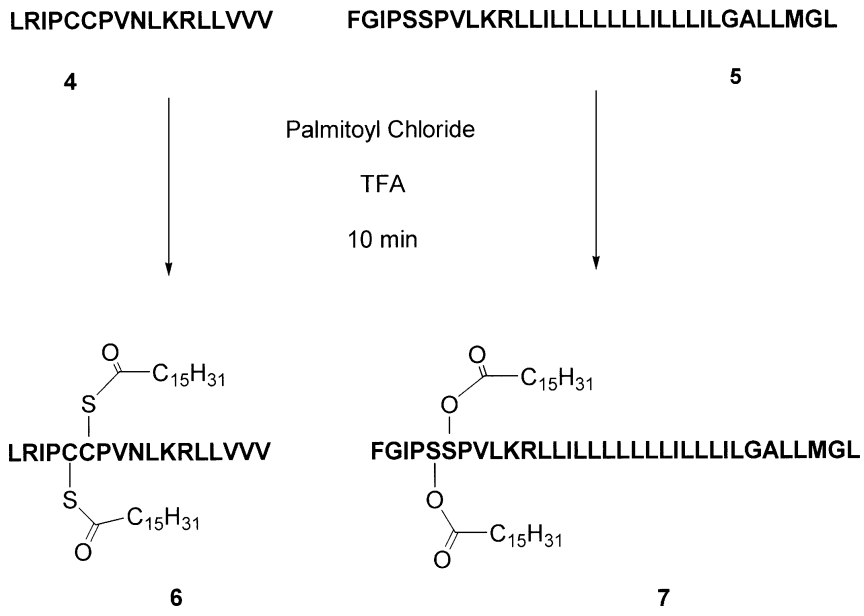
A serine-containing tripeptide Gly-Ser-Phe (**2a**) was synthesized in solution by coupling of the unprotected Ser-Phe dipeptide with *N*-t-butylcarbamoylglycine (Boc-Gly) using DCC and HOBt for the condensation reaction and subsequently deprotecting the N-terminus. A cysteine-containing tripeptide Gly-Cys-Phe (**2b**) was synthesized manually using the solid-phase technique and Boc-protected amino acids (4-methylbenzyl for S-protection of the cysteine building block). After purification by HPLC these tripeptides were treated with 10 equiv. of palmitoyl chloride (0.1 M) in TFA (Scheme 1). The reactions were monitored by analysis of quenched (with 80 % aq. ethanol) aliquots by RP-HPLC. The reactions proceeded quickly and seemed almost quantitative after the first aliquot was withdrawn (1 min). No substantial change was observed and, after 10 min reaction time, only one product peak could be seen in the chromatograms. After quenching the reactions with 80 % aq. ethanol, the products were isolated by RP-HPLC, giving 74 and 78 % isolated yields. Analysis of the products by NMR and MS unambiguously verified that the products are the O- and S-palmitoylated tripeptides (**3a** and **3b**).

Two models of SP-C were then synthesized using a standard solid-phase-synthesis protocol. One peptide [SP-C(1–17), **4**] corresponds to the N-terminal 17 amino acid residues of native porcine SP-C (i.e. LRIPCCPVNLKRLLVVV) and has previously been studied by NMR spectroscopy and found to be helical at positions 11–17, but otherwise to occupy a flexible disordered conformation. The other polypeptide, SP-C(Leu) (**5**), is a full-length analogue of human SP-C, where the valine residues in the hydrophobic part of the molecule are replaced by leucine residues and the two cysteine residues are replaced by serine residues (i.e. FGIPSSPVLKRLLILLLLLLLILLLILG-ALLMGL). These model peptides were dissolved in TFA and



Scheme 1 Synthesis of S- and O-palmitoylated tripeptides



Scheme 2 Synthesis of S,S- and O,O-palmitoylated SP-C analogues

treated with excess palmitoyl chloride (0.2 M, 10 equiv. per cysteine/serine residue) for 10 min (Scheme 2). This treatment gave conversion of the respective peptide into a product that in both cases was eluted later during RP-HPLC analysis, with only small amounts of other compounds being detected. The products could be isolated by RP-HPLC in 75–80 % overall yield, and MS analysis identified the products as the dipalmitoylated peptides **6** and **7**.

Hydrolysis of the dipalmitoylated peptides **6** and **7** in 0.1 M NaOH was complete after 1 h at room temperature, which confirmed that the palmitoyl groups were attached to the cysteine or serine residues respectively and not via amide bonds (Figure 1). However, upon prolonged treatment (several hours to days) of the peptides with palmitoyl chloride, additional reactions occured which were observed as later-eluted fractions during RP-HPLC analysis. These are probably peptides that are tri- or tetra-palmitoylated, formed by slower subsequent reactions with amino groups. The molecular masses of these compounds as determined by MS were also consistent with this conclusion.

In order to investigate the influence of the TFA concentration on the palmitoylation of hydrophobic peptides, a number of reactions with peptide **4** under similar conditions as above but with various amounts of TFA (15–60 %) in chloroform (chosen for solubility reasons) were performed. It is clear that the rate of reaction decreases with increasing amount of chloroform, e.g., with 25 % TFA only about 30 % of the peptide was palmitoylated after 10 min and, most importantly, some (≈ 3 %) tripalmitoylated peptide, due to N-acylation, was formed even after this short reaction time. With 60 % TFA about 80 % of dipalmitoyl peptide was formed in 10 min (the remaining 20 % equally distributed between the non-palmitoylated and the monopalmitoylated peptide). As mentioned above, 100 % TFA as solvent gives virtually complete conversion into the dipalmitoylpeptide. TFA as the sole solvent seems to be the safest choice, since it gives the fastest conversion with least risk of concomitant N-palmitoylation. With a considerably smaller amount of TFA this is clearly an unavoidable side-reaction. It should also be mentioned that, when doubling the concentration of palmitoyl chloride, the formation of tripalmitoylated peptide could be detected at high concentrations of TFA, suggesting that concentrations of palmitoyl chloride higher than about 0.2 M should be avoided. If for solubility reasons one needs to mix in an organic solvent, this should be done with caution. Our results indicate that it is advantageous to maximize the amount of TFA, and it is probably not advisable to use much less than 50 %, at least not when the co-solvent is relatively non-polar. With more

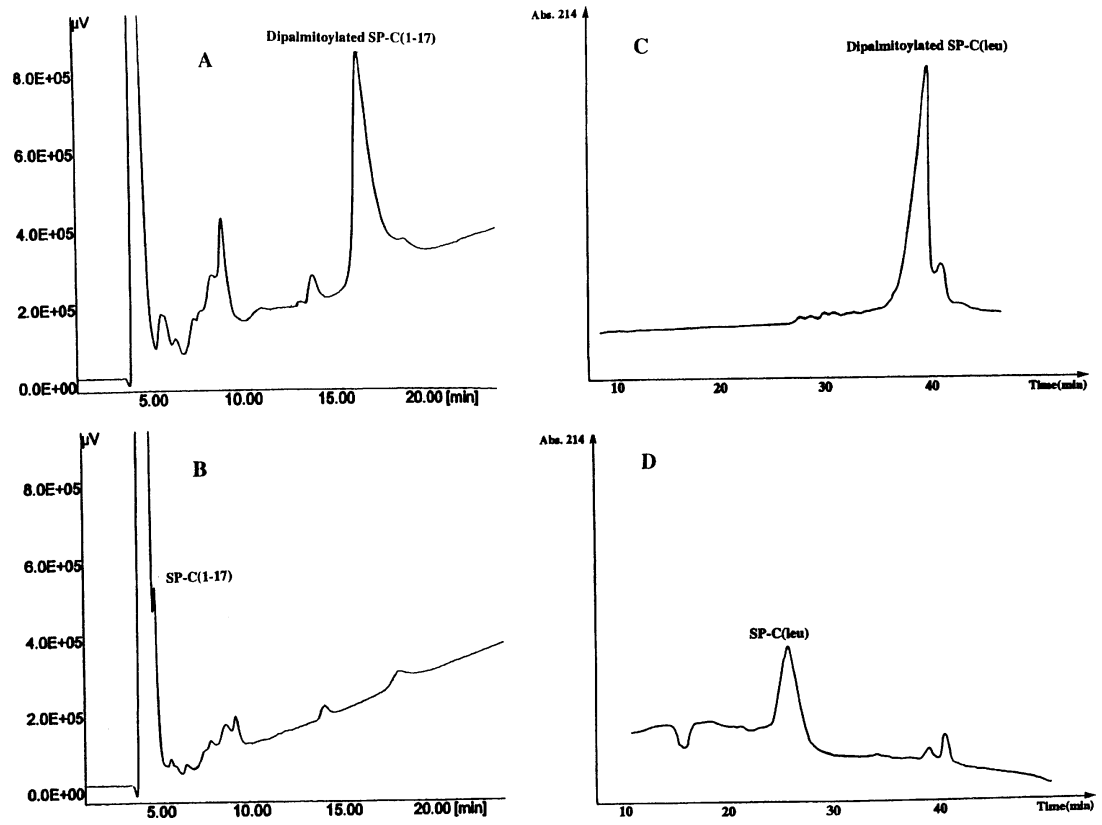


Figure 1 RP-HPLC analysis of the dipalmitoylated peptides 6 and 7 and their hydrolysis products after 1 h treatment with NaOH

Dipalmitoylated SP-C(1–17) (**A**) and dipalmitoylated SP-C(Leu) (**C**) and the products obtained by hydrolysis (**B**) and (**D**) respectively were analysed as described in the Experimental section. '2.0E+05' (etc.) on the ordinate means 2×10^5 (etc.). In (**C**) and (**D**), 'Abs. 214' is absorbance at 214 nm and the ordinate scale is in arbitrary units.

polar aprotic solvents one can, if necessary, lower the TFA concentration further, since the acidity of TFA and the basicity of amino groups will be higher (and N-protonation thus more efficient), and the palmitoylation reaction faster.

When 100% ethanol was used for quenching of the reactions, we could observe the formation of some additional later-eluted products that had a mass corresponding to an additional ethyl group. Presumably, these products are ethyl esters formed at the C-terminus by acid-catalysed esterification and/or with palmitoyl chloride as condensing agent. The amount of these by-products was reduced to a virtually non-detectable level when instead 80% aq. ethanol was used for quenching. Attempts to palmitoylate synthetic depalmitoyl-SP-C, i.e. containing a polyvalyl segment instead of the polyleucyl part of SP-C(Leu), seemed to proceed smoothly, but the product was difficult to analyse, since the solubility behaviour was such that neither good RP-HPLC nor MS analysis could be achieved. It is difficult to see any reason why the palmitoylation would not also proceed smoothly with this peptide, and our guess is that we have as yet been unable to find conditions under which the palmitoylated peptide can adopt the native α-helical conformation and that it instead seems to aggregate. This is in agreement with the observation that native helical SP-C unfolds into an extended conformation in 100% acids [21].

The dipalmitoylated peptides **6** and **7** were also analysed by CD spectroscopy, and the CD spectrum of **7** was virtually identical with that of **5**, which gives a spectrum typical for an α-helical conformation. The conformation of **4** is known from both

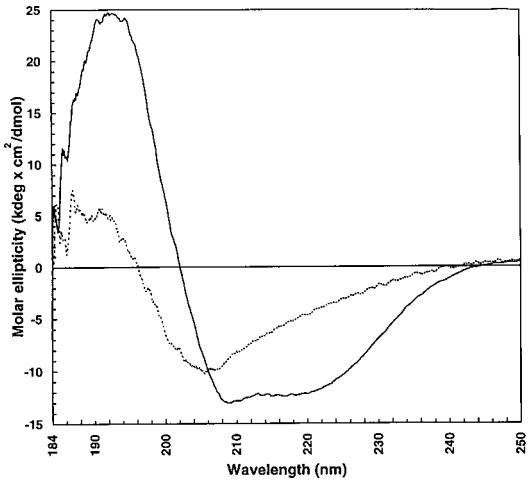


Figure 2 CD spectra of SP-C (1–17) (broken line) and S,S-dipalmitoylSP-C(1–17) (continuous line) in 10 mM dodecylphosphocholine/50 mM sodium phosphate buffer, pH 6.0 at 22 °C

NMR and CD measurements to form a helix between positions 11 and 17, but to be otherwise disordered [20]. However, the CD spectrum shows that the S,S-dipalmitoylated SP-C(1–17) (**6**) is largely in an α-helical conformation (Figure 2). This is an

intriguing finding and suggests that the palmitoyl groups enhance the tendency of the peptide to adopt this conformation in dodecylphosphocholine micelles. Whether this reflects the function of the palmitoyl group in the native SP-C remains to be explored.

We now clearly have an efficient method by which we can palmitoylate various peptide models in order to investigate the role of palmitoyl groups. The method is limited to peptides that contain only hydroxy or thiol groups to be acylated, since, under the conditions used, it would be difficult to get selective acylation of either group in presence of the other. On the other hand an advantage of the method is that it can be executed without risk of substantial amide formation, since the time window from complete O- or S-acylation to reaction with amino groups is quite substantial. An excess of reagent can thus be used, which makes the reaction more reproducible, owing to a virtually constant concentration of reagent, and the reaction becomes less sensitive to moisture and should only require a minimum of experimental experience to execute. A reaction time of 0.5–1 min seems sufficient for a complete reaction, but to account for most cases we would recommend a standard time of 10 min, which also gives a good margin to the considerably slower further amino acylations that could occur. The method should also be generally applicable to O- and S-acylation of peptides with acyl groups other than palmitoyl, as long as these do not contain too acid-sensitive functionalities.

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