Synthesis of Palmitoyl-thioester T-cell Epitopes of Myelin Proteolipid Protein (PLP). Comparison of Two Thiol Protecting Groups (StBu and Mmt) for On-resin Acylation

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Abstract: In order to test the effect of thiopalmitoylation on the encephalitogenic properties of two proteolipid protein (PLP) T-cell epitopes, we have studied the on-resin S-palmitoylation of peptides, synthesized using the Fmoc/tBu strategy. The use of two Cys protecting groups was investigated: the *tert*-butyl-sulfenyl (StBu) and the methoxytrityl (Mmt). Our studies show that the ease of deprotection of the thiol protected with StBu was sequence dependent. The deprotection of Cys(StBu) was difficult in the case of the two peptides PLP(104–117) and PLP(139–151). Neither of the two Cys(StBu) (Cys^{108} and Cys^{140}, respectively) could be deprotected with tributylphosphine. β-mercaptoethanol was only efficient for the deprotection of Cys(StBu)^{140} at 85°C and at 135°C for Cys^{108}. The two palmitoylated peptides could be obtained in good yield starting from Cys protected with Mmt. Our conclusion is that the Mmt group is the more versatile protecting group of the thiol for use in the on-resin synthesis of thiopalmitoylated peptides. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: proteolipid protein; T-cell epitopes; on-resin thioacylation; thioester; palmitoylation; Cys(StBu); Cys(Mmt); lipopeptide

INTRODUCTION

Experimental allergic encephalomyelitis (EAE) is a T-cell mediated disease that is used as an animal model for human multiple sclerosis. It can be induced by the injection of central nervous system (CNS) myelin proteins myelin basic protein (MBP), PLP, myelin oligodendrocyte glycoprotein (MOG) in complete Freund's adjuvant. The SJL mouse is widely used in EAE studies because of its enhanced susceptibility following immunization with either MBP, PLP, or their peptides [1].

It has been shown that there are two immunodominant encephalitogenic determinants of proteolipid protein (PLP) for SJL mice, PLP(139–151) [2] and PLP(178–191) [3] as well as one cryptic

encephalitogenic determinant, PLP(104–117) [4]. PLP, the major protein of CNS myelin, is a tetraspan membrane protein that contains covalently bound fatty acids, predominantly palmitic acid. According to Weimbs and Stoffel [5], PLP is palmitoylated at six Cys residues via thioester linkage. Two of these Cys at residues 108 and 140 are contained within the two encephalitogenic determinants PLP(104–117) and PLP(139–145), respectively. It is therefore important to study the influence of palmitoylation on the encephalitogenic activity of PLP(104–117) and PLP(139–151) in SJL mice.

For this purpose, we have investigated the selective acylation of PLP(104–117) and PLP(139–151) thiols (Cys^{108} and Cys^{140}, respectively) on the resin bound peptide after Fmoc assembly, in order to develop a general and reliable method. The use of two Cys protecting groups was investigated, namely the *tert*-butylsulfenyl (StBu) [6] and the

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methoxytrityl (Mmt) [7]. We indeed succeeded in the synthesis of both thiopalmitoylated peptides, but our study shows that the reactivity of the thiol protecting group is sequence dependent. Mmt was shown to be the more versatile thiol protecting group for on-resin thioacylation.

MATERIALS AND METHODS

Materials

Fmoc-L-amino acids were purchased from Novabiochem (Meudon, France) or Neosystem (Strasbourg, France). Preloaded Wang resins were obtained from Novabiochem and BOP from Neosystem. Tri-n-butylphosphine (nBu$_3$P) was purchased from Lancaster (Strasbourg, France) and palmitoylchloride (Palm-Cl) from Fluka (St Quentin Fallavier, France). Dimethylformamide (DMF) was purchased from SDS (Peypin, France). All other chemicals were of the purest grade available.

Peptide Synthesis

Peptides (0.1 or 0.2 mmol) were synthesized on a table top peptide synthesizer, ACT 90, on a Wang resin using the Fmoc/tBu strategy and BOP as coupling reagent. Typically, successive single couplings were performed with 4 equivalents of Fmoc amino acid and were monitored with the Kaiser colour test. Ile[107] of PLP(104–117) required 8 equivalents for complete coupling.

Fmoc amino acid side-chain protecting groups were Asp(OtBu), Cys(Trt), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), Tyr(tBu), Cys(Mmt), Cys(StBu). Boc-His(Boc) and Boc-Lys(Boc) were coupled as N-terminal amino acids. Boc-Lys(Boc)-OH and Boc-His(Boc)-OH were obtained by conversion of their DCHA salt.

Peptides were cleaved from the resin with the 'low odour mixture' (87.5% TFA, 5% phenol, 5% H$_2$O, 2.5% TIS), precipitated in ether (ethyl ether or *tert*-butyl methyl ether) and lyophilized after solubilization in 10% acetic acid.

Deprotection of the *S*-(StBu) Cysteinyl Residue

PLP(139–151) peptide resin (50 mg) was introduced into a round bottom flask equipped with a condenser. A freshly prepared mixture of β-mercaptoethanol/DMF (1/1, v/v) (5 ml) was added. The mixture was heated overnight at 85°C. After filtration, the peptide resin was washed with DMF.

Deprotection of the *S*-(Mmt) Cysteinyl Residue

The peptide resin (30 mg) was treated with a solution of 2% TFA in dichloromethane (DCM) for 10 min in a glass reaction vessel equipped with a sintered glass filter using nitrogen for mixing. After filtration the peptide resin was washed twice with DCM. The deprotection and washing steps were repeated five times. The peptide resin was finally washed with DCM and DMF.

Palmitoylation of Free SH Group on the Solid Support

In a glass reaction vessel equipped with a sintered glass filter, the free thiol peptide resin (30 mg) was suspended in DMF (8 ml) containing palmitoyl chloride (20 equivalents). DMAP (0.1 equivalent) dissolved in distilled pyridine (1.5 ml) was then added. After reaction overnight at room temperature, the peptide resin was filtered off, washed with DMF, DCM and then dried in a vacuum.

Peptide Characterization

Peptides were analysed by reversed phase high performance liquid chromatography (RP-HPLC) on an analytical Delta-pak C18 column (300 Å pore size, 5 μm particle size) or an analytical Aquapore-OD 300 column, using a Waters HPLC system. Solvent A was H$_2$O/0.1% TFA and solvent B was CH$_3$CN/H$_2$O (80/20)/0.09% TFA. Elution was conducted at a flow rate of 1 ml/min and detection was performed at 214 nm.

PLP(104–117)Cys(Pam) and PLP(139–151)Cys(Pam) were analysed using a stepwise gradient from 10 to 100% B over 40 min (condition A). PLP(139–151)Cys(Pam) was also analysed using a stepwise gradient from 20 to 100% B over 20 min (condition B).

Peptides were characterized by measurement of their mass by electrospray ionization mass spectrometry (ESMS) in the positive mode on a Bio-Q apparatus.

RESULTS

The syntheses of PLP(104–117) (KTTI**C**GK-GLSATVT) and PLP(139–151) (H**C**LGKWLGHPDKF) were performed on a semi-automated peptide synthesizer on preloaded Wang resins using the Fmoc/tBu strategy. Cys[108] and Cys[140] were coupled either as Fmoc-Cys(StBu)-OH or Fmoc-Cys(Mmt)-OH. Boc-Lys(Boc)-OH and Boc-His(Boc)-OH were

coupled as *N*-terminal amino acids in order to protect the *N*-terminus during palmitoylation. Single couplings with 4 equivalents of amino acid were used except for Ile[107] which needed 8 equivalents for complete coupling.

PLP(104–117)

Deprotection of Cys(StBu)[108] and thiopalmitoylation.
Two reducing reagents were tested for deprotection. The resin-bound peptide was first treated with tributylphosphine (20 equivalents/DMF) during 24 h at room temperature. No deprotection was detected according to HPLC and ESMS analysis.

We then used β-mercaptoethanol in DMF (1/1, v/v) as a reducing reagent and studied the thiol deprotection under various conditions of time (16 or 24 h) and temperature (85–135°C). As shown in Table 1, the best result was obtained after treatment of the peptide-resin with β-mercaptoethanol during 24 h at 135°C (77% deprotection in experiment 4).

The free thiol residue was then directly acylated on the resin with palmitoyl chloride but no palmitoylated peptide could be detected. We tried to improve the acylation step by changing the solvents (DCM or N-methyl pyrrolidone (NMP)) or by using preactivated palmitic acid but without success. These results suggested that the resin had somehow been degraded by the temperature rendering the thiol group no longer accessible to the acylating reagents.

We then tried to deprotect the Cys(StBu) with β-mercaptoethanol in a mixture of NMP/H$_2$O (9/1, v/v) as described by Beekman *et al.* [8] at lower temperature (85°C) and for various times. The deprotection was directly followed by palmitoylation and the products were analysed by HPLC after cleavage of the peptide from the resin. Table 2 shows that

Table 1 Deprotection of Cys(StBu)[108] with β-Mercaptoethanol/DMF (1/1, v/v) at Various Times and Temperatures

Experiment	Conditions of deprotection β-mercaptoethanol/ DMF	Results (% Cys(SH))
1	RT, 26 h	0
2	85°C, 16 h	21
3	120°C, 16 h	73
4	135°C, 24 h	77

Percentage of deprotection was determined by HPLC after cleavage of the peptide from the resin.

Table 2 Deprotection of Cys(StBu)[108] with β-Mercaptoethanol in NMP/H$_2$O (9/1, v/v) at Different Times and Temperatures

Experiment	Conditions of deprotection β-mercaptoethanol, NMP/H$_2$O	Results after acylation
1	85°C, 2 × 24 h	27% Cys(SH) 17% Cys(Pam)
2	85°C, 4 × 24 h	65% Cys(SH) 4% Cys(Pam)
3	95°C, 48 h	68% Cys(SH) 0% Cys(Pam)

Reduction was directly followed by palmitoylation and the percentage of the different peptides was determined by HPLC after cleavage from the resin.

although the level of deprotection was greater than 50% in experiments 2 and 3, the acylation was very poor and decreased with temperature.
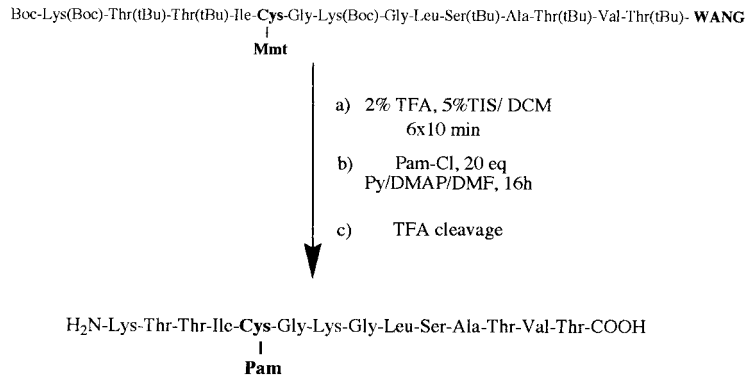
Deprotection of Cys(Mmt)[108] and palmitoylation. The
Mmt group is sensitive to dilute TFA. We have studied the on-resin deprotection of Cys(Mmt)[108] with two different concentrations of TFA (1% and 2%) in DCM for different times. The deprotection was directly followed by acylation with palmitoyl chloride. After cleavage from the resin, the crude peptide was analysed by HPLC. Eighty-six percent of thiopalmitoylated PLP(104–117) was obtained after deprotection of the Cys(Mmt)[108] with 2% TFA/DCM, 6 × 10 min.

Scheme 1 summarizes the procedure used for the synthesis of PLP(104–117)Cys(Pam) and the elution profile of the crude acylated peptide is shown in Figure 1. PLP(104–117)Cys(Pam) could be easily purified by HPLC and the mass measured by ESMS (1618.1) confirmed its identity.

PLP(139–151)

Deprotection of Cys(StBu)[140] and palmitoylation. As
in the case of Cys(StBu)[108], tributylphosphine (in DMF or NMP/H$_2$O, 9/1) was inefficient for the deprotection of Cys(StBu)[140].

We then tested β-mercaptoethanol at two different temperatures for Cys deprotection. One-hundred percent of the free thiol was obtained after treatment of the resin-bound peptide with β-mercaptoethanol in DMF (1/1, v/v) at 85°C overnight, while at 55°C no deprotection was observed. Thiopalmitoylated

Boc-Lys(Boc)-Thr(tBu)-Thr(tBu)-Ile-**Cys**-Gly-Lys(Boc)-Gly-Leu-Ser(tBu)-Ala-Thr(tBu)-Val-Thr(tBu)- **WANG**

|
Mmt

a)   2% TFA, 5%TIS/ DCM
        6x10 min

b)   Pam-Cl, 20 eq
           Py/DMAP/DMF, 16h

c)   TFA cleavage

H$_2$N-Lys-Thr-Thr-Ile-**Cys**-Gly-Lys-Gly-Leu-Ser-Ala-Thr-Val-Thr-COOH

|
Pam

Scheme 1   Solid-phase synthesis of PLP(104–117)Cys(Pam).
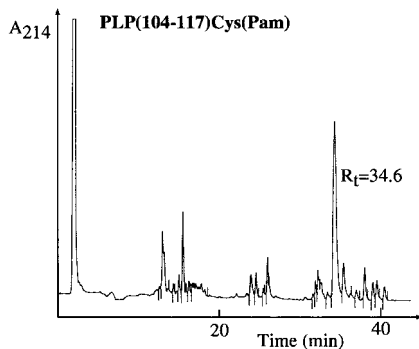
Figure 1   RP-HPLC trace (condition A) of crude thioacylated PLP(104–117) synthesized according to Scheme 1.

PLP(139–151) was finally synthesized as described in Scheme 2 in high yield as shown by the elution profile of the crude peptide (Figure 2). The mass of the purified palmitoylated peptide (1776.1) was in agreement with the calculated mass (1776.2).
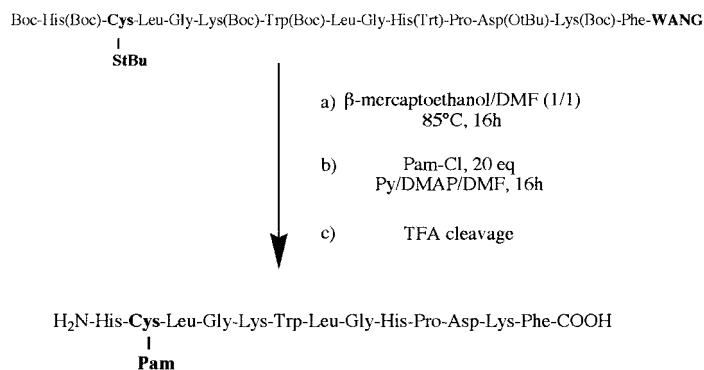
Deprotection of Cys(Mmt)[140] and palmitoylation.

PLP(139–151)-bound resin protected on the Cys[140] with Mmt was treated under the same conditions as

PLP(104–117) Cys(Mmt)[108] (2% TFA/DCM, 6 × 10 min). After direct acylation and cleavage from the resin, the thiopalmitoylated PLP(139–151) was obtained in very good yield according to the HPLC profile (Figure 3). No trace of non-palmitoylated peptide could be detected on HPLC and ESMS, showing that the deprotection and acylation steps were almost quantitative.

DISCUSSION

In the present study an efficient and general procedure has been developed for the synthesis of thiopalmitoylated peptides. This approach employs the on-resin palmitoylation of a Cys thiol group selectively protected during assembly by the Mmt group.

In this paper we have explored the use of two thiol protecting groups: StBu and Mmt. Our studies showed that the deprotection of Cys(StBu) was difficult and that tributylphosphine was inefficient as reducing agent. β-Mercaptoethanol was used with success only in the case of Cys(StBu)[140] but at

Boc-His(Boc)-**Cys**-Leu-Gly-Lys(Boc)-Trp(Boc)-Leu-Gly-His(Trt)-Pro-Asp(OtBu)-Lys(Boc)-Phe-**WANG**

|
StBu

a) β-mercaptoethanol/DMF (1/1)
        85°C, 16h

b)   Pam-Cl, 20 eq
           Py/DMAP/DMF, 16h

c)   TFA cleavage

H$_2$N-His-**Cys**-Leu-Gly-Lys-Trp-Leu-Gly-His-Pro-Asp-Lys-Phe-COOH

|
Pam

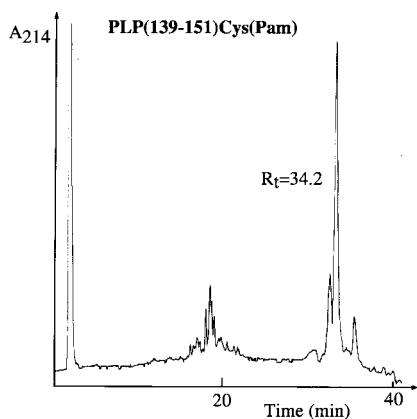Scheme 2   Solid-phase synthesis of PLP(139–151)Cys(Pam).

Figure 2   RP-HPLC trace (condition A) of crude thioacylated PLP(139–151) synthesized according to Scheme 2.

high temperature. These results indicate that the reactivity of Cys(StBu) seems to depend on the peptide sequence. In particular, the poor reactivity of Cys(StBu)[108] can be explained by steric hindrance: indeed, coupling of Ile[107] to Cys[108] needed 8 equivalents of amino acid for complete coupling. Furthermore, in preliminary studies on a shorter peptide, PLP(104–111), deprotection of Cys(StBu)[108] was successful using tributylphosphine in DMF (data not shown).

Beekman *et al.* [8] successfully performed the on-resin synthesis of thiopalmitoylated peptides using tributylphosphine (in NMP/H$_2$O, 9/1) for deprotection of a Cys(StBu) at the *N*-terminus. In the synthesis of the pulmonary surfactant SPC, Mayer-Fligge *et al.* [9] were able to deprotect the two Cys(StBu) to be palmitoylated with β-mercaptoethanol at room temperature. They did not mention the use of tributylphosphine. These examples and our results do indeed show that the on-resin deprotection of Cys(StBu) is sequence dependent.

In our hands, the use of Mmt for the selective protection of Cys thiol groups seems to be more versatile and can be removed under mild conditions (diluted TFA, RT) and allowed the on-resin thiopalmitoylation of PLP(104–117) and PLP(139–151) in nearly quantitative yield. The two palmitoylated T-cell epitopes were tested for their immunological properties. Preliminary results show that thioacylation significantly increases their encephalitogenic and immunogenic properties (Denis B, Trifilieff E, Greer. Palmitoylation of PLP encephalitogenic peptides via a thioester linkage enhances immunogenicity and encephalitogenicity, manuscript in preparation).

As thioacylated peptides have proved to be important in several other biological activities such as
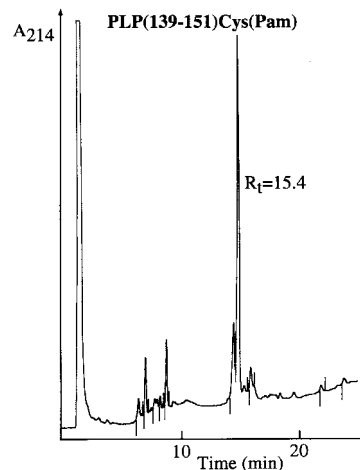

Figure 3   RP-HPLC trace (condition B) of crude PLP(139–151)Cys(Pam), obtained starting from Cys[140] protected with Mmt, according to the conditions described in Scheme 1.

enhancement of peptide immunogenicity and their use as synthetic vaccines [8] and pulmonary surfactants [9] or as tools to elucidate mechanisms of biological transduction [10], it is important to have a general and reliable method for the synthesis of such lipopeptides. Our study shows that the use of Mmt as the Cys thiol protecting group, offers the best hope for on-resin synthesis of any thioacylated peptide.

Acknowledgements

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