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ON THE USE OF S-t-BUTYLSULPHENYL GROUP FOR PROTECTION OF CYSTEINE IN SOLID-PHASE PEPTIDE SYNTHESIS USING FMOC-AMINO ACIDS.

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ABSTRACT

The utilization of the N^{α}-fluorenylmethoxycarbonyl derivative of S-t-butyl sulphenyl cysteine in solid-phase peptide synthesis for the preparation of several cysteine-containing peptides is described. When this derivative is located in the C-terminal position a side reaction prevents the formation of the desired product. Finally, a peptide containing a disulphide bond has been obtained in excellent yields by selective deprotection of the cysteines and disulfide bond formation on the solid support.

INTRODUCTION

The combined use of the base labile amino-protecting group 9-Fluorenylmethyloxycarbonyl (Fmoc) and t-butyl side chain protectors has found wide application on solid-phase peptide synthesis[1-4] . However, the adequate protection of side chains of some trifunctional amino acids like cysteine, arginine and histidine presents several problems[3]. Very recently, two reports on the use of several side-chain protecting groups for N^{α}-Fmoc-cysteine has been published[3,4] . In this paper we will describe our results using the t-butylsulphenyl group as a protecting group of cysteine[5] . This group can be deprotected under very mild conditions either after removal of the peptide from the resin and purification or when the peptide chain remains anchored to the support. In this case the oxidation can be achieved with the peptide on the resin and the final cleavage from the resin affords the desired peptide with the disulfide bond already formed. This shows the advantages of the solid-phase strategy for disulfide-bond formation due to the pseudo-dilution phenomena[6,7]

RESULTS

Sequences shown in table 1 were assembled on a p-alkoxybenzyl ester type resin . The first Fmoc-amino acids of the sequences were attached onto aminomethyl-polystyrene via performed handle derivatives as described[8] . The following groups were chosen for side chain protection :t-butyl (^{t}Bu) for glutamic and aspartic acids, serine, threonine and tyrosine, t-butyloxycarbonyl (Boc) for lysine, t-butylsulphenyl (^{t}BuS) for cysteine, 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr) for arginine and 9-fluorenylmethyloxycarbonyl (Fmoc) for histidine. Amino acids derivatives were incorporated using preformed

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symmetrical anhydrides (2 fold excess) or performed hydroxybenzotriazole esters (4 fold excess). This later method was employed for the incorporation of glutamine and asparagine to prevent nitrile formation and for the incorporation of glycine, histidine and arginine where problems during the symmetrical anhydride formation have been described[9,10] . Syntheses were controlled by the ninhydrine test[11] and by measuring the amount of N-(9-fluorenylmethyl) piperidine released during the deprotection steps[1].

Figure 1 : Sequence of the peptides synthesized

I CysAspAspValValAspAlaAspGluTyrLeuIleProGln
II ThrThrAlaCysHisGluPhePheGluHisGlu
III CysSerTrpLeuPheGlnAsnSerSerSerLysLeuProTyr
IV CysIleGlyProArgGlyGlyAspPheLeuTyrSerAspArgGluIle
V TyrLysProGlnAlaProGluLeuArgCys
VI TrpGlnProLysArgGluCys
VII CysAspGlyTyrProLysAspCysLysGly
└──────────────────┘

Trifluoroacetic acid treatment of the peptidyl-resins yielded the crude S-(t-butylsulphenyl)cysteinyl peptides which were purified by preparative HPLC (see figure 4). Special attention was made during the acid treatment in order to avoid thiols as a scavengers that could remove the t-butylsulphenyl group. In the tryptophan containing peptide (peptide III) the use of mercaptoethanol as a scavenger and antioxidant was avoided by using 2-methylindole and anisole The t-butylsulphenyl group was stable during the long acid treatment needed for the deprotection of the Mtr group of the arginine (TFA/thioanisole/phenol, 6hr, ref.10). Purified S-(t-butylsulphenyl) cysteinyl peptides were stored on this form and this protecting group was removed prior their use by treatment with mercaptoethanol. The structure of the purified peptides was verified by fast atom bombardement mass spectroscopy (FAB) (see table 1). Peptides I-IV presented the expected molecular ion and a characteristic peak at M-88, due to the loss of the t-butylsulphenyl group of the cysteine.

Peptides V and VI that have the t-butylsulphenyl cysteine at the C-terminal position did not present the correct molecular ion. Instead, we observed a peak 36 mass units lower than expected and the absence of the characteristic peak at M-88. A detailed study of the fragmentation pattern of the mass spectra of these peptides shown that the modification occurred on the C-terminal end. H-NMR of these peptides showed the presence of a clear peak due to t-butyl group but the products did not change by treatment with mercaptoethanol or DTT. On the other hand, the treatment of Fmoc-Cys(^{t}BuS)-resin and H-Cys(^{t}BuS)-resin with trifluoroacetic acid yielded the corresponding Fmoc-Cys(^{t}BuS)-OH and H-Cys(^{t}BuS)-OH indicating that, probably, an amide bond is needed in order to form the side product. Peptides V and VI were obtained without problems when the t-butyl group was used instead of t-butylsulphenyl for the protection of the C-terminal cysteine (data not shown). For these reasons we concluded that when t-butylsulphenylcysteine is at C-terminal a modification of this residue occurs, probably during the TFA treatment, that prevents the formation of the desired product.

The synthesis of peptide VII was done following the scheme on fig. 2. After solid-phase assembly of the peptide ,the Fmoc-decapeptidyl-resin was treated with mercaptoethanol in order to remove the t-butylsulphenyl protecting groups and the free thiol groups were oxidised with potassium

ferricyanide. After cleavage of the resin and removal of the rest of the protecting groups we were able to prove by HPLC that the protected lineal peptide was transformed into the desired cyclic peptide in practically quantitative yields (fig. 3). Peptide VII was isolated by semipreparative HPLC obtaining an overall yield (synthesis, cyclization, removal from the resin and deprotection) of 60 %. The purified peptide has been characterized by amino acid analysis, mass spectrometry and coelution in HPLC with a sample obtained previously by cyclization in solution.

Table 1. Mass spectra of the t-butylsulphenyl peptides I-IV, the peptide containinig a disulphide bond (VII) and the side products obtained during the preparation of peptides V and VI.

Peptide	M.S. m/e
(^{t}BuS)peptide I	1704 (M+Na), 1682 (M+H), 1593, 707, 620, 521, 422, 357, 244, 145.
(^{t}BuS)peptide II	1438 (M+H), 1350, 1422, 1394, 1337, 1309, 1264, 1236, 1220, 1172, 1127, 1043, 1028, 1000, 998, 896, 851, 749, 734, 704, 620, 481.
(^{t}BuS)peptide III	1790 (M+2Na), 1768 (M+Na), 1747 (M+H), 1659, 1330, 1267, 1245, 1049, 1006, 893, 720.
(^{t}BuS)peptide IV	1886 (M+H), 1798, 1642, 1524, 1486, 1441, 1427, 1371, 1326, 1284, 1271, 1239, 1214, 1199, 1183, 1157, 1142, 1121, 1076, 1042, 1027, 1008, 965, 921, 895, 880, 861, 818, 746, 731, 703, 689, 674, 646, 632, 619, 604, 587, 558, 532, 517, 443, 417, 402.
Peptide VII	1083 (M+H), 185.
Side product from the synthesis of peptide V	1019 (M+Na), 997 (M+H), 982, 953, 912, 868, 842, 796, 739, 713, 683, 666, 640, 585, 570, 556, 499, 483, 461, 442, 429, 391.
Side product from the synthesis of peptide VI	1278 (M+Na), 1256 (M+H), 1241, 1212, 1127, 1101, 1057, 1019, 993, 963, 945, 922, 894, 851, 834, 794, 766, 740, 725, 697, 667, 626, 596, 555, 537, 496, 468, 427, 383.

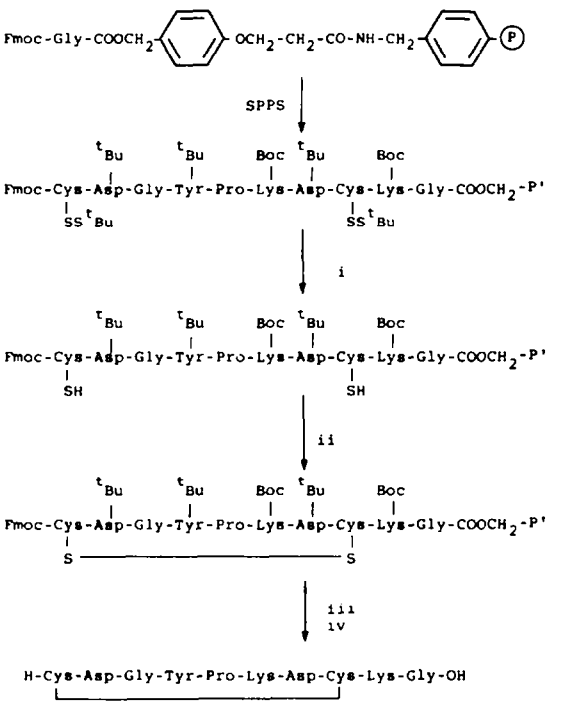
DISCUSSION

The t-butylsulphenyl group is resistant to acids like TFA and base[5,12], partially removed in strong acids like HF[13] and it is cleaved by mild reagents such as thiols[5] or phosphines[14]. The special characteristics of this group and the mildy acid conditions of side chain deprotection in Fmoc-t-butyl groups strategy has permitted us the purification, characterization and storage of (S-t-butylsulphenyl)cysteine containig peptides without the complication of free thiol groups.

However, when the t-butylsulphenyl group is on the C-terminal position, we have detected a side reaction that prevents the formation of the desired product. We have not been able to elucidate the structure of the side product formed. We think that a decrease on the molecular mass of 36 units may be due to the loss of two molecules of water. This is possible during the TFA treatment if one of the sulphurs of the S-t-butylsulphenyl group assists to the breakdown of the benzyl esther between peptide and resin (a similar reaction has been shown for homocysteine[15]) and the resulting intermediate undergoes to rearrengement with a loss of a molecule of water. Further investigations have to be done in order to characterize this new side reaction.

On the other hand, a peptide containing a disulphide bond has been obtained in high yield by selective removal of the t-butylsulphenyl group, disulphide formation on the solid support and, finally, cleavage and removal of protecting groups. As it has been described, the utilization of the pseudo-

Figure 2 : Synthesis of peptide VII



Boc: t-butyloxycarbonyl; ^{t}Bu: t-butyl; Fmoc: fluorenylmethyloxycarbonyl; (P): polystyrene-co-1%-divinylbenzene; SPPS: solid-phase peptide synthesis. i) 2-mercaptoethanol/DMF (1:1); ii) potassium ferricyanide in DMF/water; iii) 20% piperidine/DMF; iv) 70% trifluoroacetic acid in dichloromethane.

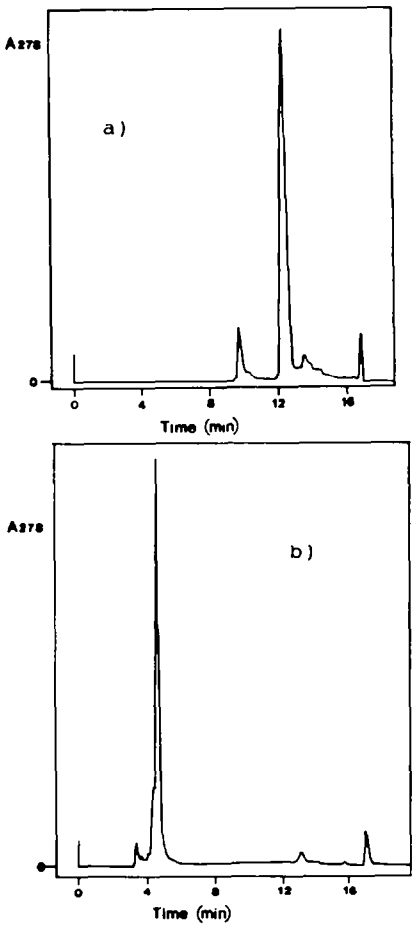


Figure 3: HPLC chromatogram a) of the crude di-t-butylsulphenyldecapeptide VII. Column Rainin Dynamax C-18, 25x1 cm. Flow rate: 3 mL/min. A:0.1% TFA in H_2O; B: 0.1% TFA in H_2O/CH_3CN (3:7). A 15 min linear gradient from 20 to 60%B.

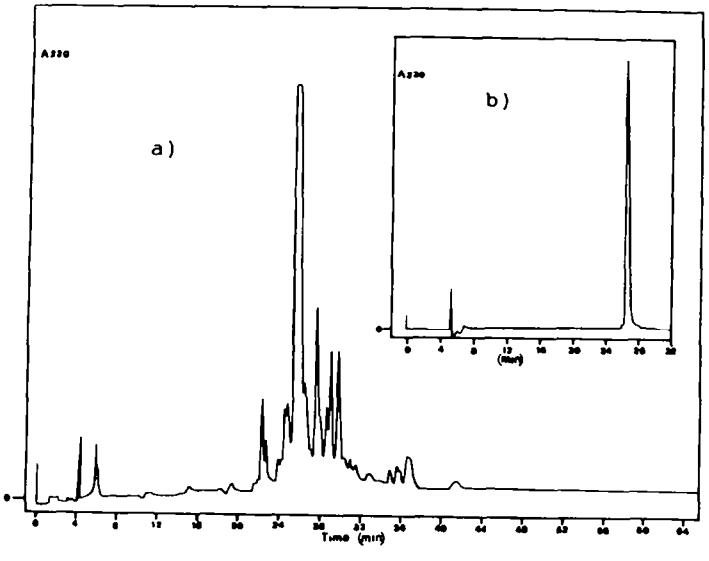


Figure 4 : a) Semi-preparative HPLC purification of peptide III. Same column and solvents than in fig.3. In this case a 50 min linear gradient from 10% to 100% B was used. b) analytical HPLC of the product corresponding to the main peak.

dilution properties of the peptides linked to polymers on the synthesis of disulphide bonds favors in grand extent intramolecular reactions in front of intermolecular reactions[6,7,16-18]. The method that here we describe is specially convenient because of the very mild conditions used during the selective deprotection of cysteine, cyclization and final deprotection. Furthermore, the major part of the reagents are commercially available. We think that this method will be useful for the rapid preparation of peptides containing disulphide bonds.

EXPERIMENTAL SECTION

Abbreviations not previously defined are as follows : ACN : acetonitrile; DCM: dichloromethane; DMF: N,N-dimethylformamide; TFA: trifluoroacetic acid. Protected amino acids were from Bachem Inc (Torrance,USA) except Fmoc-Arg(Mtr) and Fmoc-Cys(S^{t}Bu) that were from Bachem AG (Bubendorf, Switzerland). Aminomethylpolystyrene was prepared starting from poly(styrene-co-1%-divinylbenzene) 200-400 mesh (Bio-Rad, Richmond, USA) as previously described[19].

Peptide syntheses were performed on a semi-automatic peptide synthesizer designed for one of us (B.K.) and built by Systec Inc (Minnesota, USA). Fast-atom bombardment (FAB) mass spectra were recorded on a JOEL H X100 HF spectrometer and ^{1}H-NMR spectra on a Varian XL-200 spectrometer.

Solid-phase synthesis of peptides I-VII

Starting Fmoc-aminoacyl-p-alcoxybenzyl resins were prepared by synthesizing the corresponding 2,4,5-trichlorophenyl 4'-(N$^\alpha$-Fmoc-aminoacyloxymethyl)phenoxypropionate and coupling it onto aminomethylpolystyrene as recently described[8].

Syntheses were carried out on a 0.15 mmol scale (0.5 g of resin, 0.3 mmol/g) using the following procedure : 1) DMF 2 x 0.5 min ; 2) 20% piperidine /DMF 2 x 6 min; 3) DMF 4 x 1 min; 4) ACN 4 x 0.5 min; 5) nitrogen flow for 2 min; 6) Coupling reaction : 2 eq of symmetrical anhydride of Fmoc-amino acid or 4 eq of 1-hydroxybenzotriazole ester of the Fmoc-amino acid, shaked for 1-2 hrs; 7) DMF 4 x 1 min; 8) ACN 4 x 1 min. At this point, the completion of the coupling reactions was checked by the ninhydrin test[11]. If the ninhydrin test was positive, the coupling reaction was repeated and if the ninhydrin test was slightly positive, the free amino groups were acetylated with acetic anhydride/ ethyldiisopropylamine/ DMF (0.5/0.85/4 mL) for 30 min.

The collected filtrates from treatments 2) and 3) were combined, diluted with ethanol and used for the spectrophotometric determination of N-(9-fluorenylmethyl)piperidine at 301 nm (ε 7800).

Cleavage and purification of peptides I-VI

Peptides I, II and V were cleaved from the resin and deprotected by treatment with 70% TFA/DCM (15 mL) at room temperature for 4 hours. After filtration of the resin, the resin was washed with acetic acid (3 x 5 mL) and the combined filtrates were evaporated to dryness.

For the cleavage and deprotection of peptide IV (containing Arg(Mtr)), the resin was treated with TFA/phenol/thioanisole (95:2.5:2.5 v/v) at room temperature for 6 hours. After filtration of the resin, the combined filtrates were concentrated to a small volume and the crude peptide precipitated by addition of ether.

Peptide III (containing Trp plus Cys(S^{t}Bu)) was cleaved from the resin as follows[12] : Fmoc-peptidyl-resin (0.6 g, 0.2 mmol) was suspended to an ice-cold solution of 2-methylindole (0.1 g, 0.76 mmol) and anisole (1 mL) in TFA (9 mL). The resulting solution was kept under nitrogen at room temperature for 4 hrs. After filtration of the resin, precooled ether (30 mL) was added and the precipitate collected by centrifugation , washed with ether and dried.

Peptides were purified by preparative reversed-phase HPLC: Columns : Magnum 20 Partisil10 ODS-3 (Whatman) 50 x 2.2 cm and Dynamax C-18 (Rainin) 25 x 1 cm. Solvent A : 0.1 % TFA in water, solvent B : ACN / water (7:3) + 0.1 % TFA. Linear gradient from 10 %B to 80 %B. Flow rate : 9 mL/min (Magnum), 3 mL/min (Dynamax) (see for example figure 4).

Purified peptides were obtained in 20-40% overall yields. They were homogeneous by analytical HPLC and their structure was confirmed by FAB-mass spectroscopy (table 1) Prior to use, the remaining t-butylsulphenyl groups were removed by anovernight treatment with 2-mercaptoethanol followed by gel filtration chromatography (Sephadex G-10) or, simply, by precipitation of the peptide with ether. The removal of this group was monitored by analytical HPLC.

Preparation of H-Cys-Asp-Gly-Tyr-Pro-Lys-Asp-Cys-Lys-Gly-OH by cyclization on solid support.

The synthesis of the peptidyl-resin was carried out as described above. The overall yield (measured by the UV absorbance of the first and last deprotection washings at 301 nm) was found to be 90%. An aliquot of the Fmoc-

decapeptidyl-resin was deprotected with 20% piperidine/DMF and treated with 60% TFA in DCM at rom temperature for 2 hours. The HPLC chromatogram of the crude (Fig.3a) shows a major peak that corresponds to the desired di-t-butylsulphenyl derivative of the decapeptide ($M+ H^+$ = 1261, M-88 = 1173).

The remainder of the Fmoc-decapeptidyl-resin (aprox. 0.15 mmol) was treated with 5 mL of DMF/2-mercaptoethanol (1:1) for 5 hours at room temperature. The resin was washed with DMF, DCM, DMF, ethanol and DMF. The resin was suspended with 10 mL of DMF and 1 mL of 1M aqueous solution of potassium ferricyanide was added. The resulting suspension was agitated overnight. After this treatment, the resin was washed with DMF/water (1:1), water, DMF, DCM, and DMF, deprotected with 20% piperidine/DMF and treated with 60% TFA/DCM for 2 hours. The crude was purified by HPLC (Fig.3b). The purified decapeptide was isolated in a 60% with a correct mass spectra (M+H= 1083) and amino acid analysis (Asp 1.92; Pro 1.00, Gly 2.04, Tyr 0.92, Lys 2.11).

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