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Comparative Studies of Nsc and Fmoc as N^{α}-protecting Groups for SPPS

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Abstract: 2-(4-Nitrophenyl)sulfonylethoxycarbonyl (Nsc) is an alternative base-labile N^{α}-protecting group to 9-fluorenylmethoxycarbonyl (Fmoc) for amino acids. The UV spectrum of the Nsc group exhibits moderate absorption at 380 nm which is excellent for real-time monitoring of the deprotection process. It also decreases the rearrangement of X-Asp, which can be a serious problem in SPPS. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Nsc-amino acids; peptide; coupling monitoring; rearrangement

INTRODUCTION

The concept of solid phase peptide synthesis (SPPS) enunciated by R. B. Merrifield has had a seminal influence on the synthesis of peptides/proteins, nucleic acids and, more recently, the development of libraries of compounds for biological screening. Although the earliest application of SPPS to peptide synthesis involved graded acid-liability of the N^{α}-amino protecting group (Boc) compared with the less acid-labile side chain protecting groups, a subsequent complementary protecting group strategy was developed. This involved the repetitive base cleavage of the N^{α}-Fmoc group in the presence of acid-labile side chain protecting groups. An important consequence of this approach was that the final cleavage of the product peptide from the polymeric solid phase could be achieved under mild acid conditions (TFA + scavengers), thus relatively unstable products were not exposed to strong acidic conditions at the end of the synthesis.

In solution phase synthesis it is routine practice to monitor each chemical reaction in real-time, however for SPPS this can prove difficult since only the reagents which are available in solution can be utilized for monitoring. Over many years it has been our practice to measure the UV absorption of the solution resulting from the deprotection of the N^{α}-Fmoc in one cycle and compare this with the succeeding N^{α}-Fmoc deprotections during the SPPS of peptides/proteins. The isosbestic point in the UV spectra of the piperidine adduct of dibenzofulvene, and the dibenzofulvene itself, is 302 nm. However, there are some problems associated with the use of the N^{α}-Fmoc protected amino acids in solid phase protein synthesis.

Firstly, the above monitoring does not include the crucial amide forming step, but rather describes the overall deprotection and coupling efficiency within a cycle. Although difficult to quantify, it would be valuable to assess the coupling reactions routinely and hence allow real-time intervention. In order to achieve this, the coupling reagent(s) must not ab-

Abbreviations: HOCt, ethyl 1-hydroxy-1H-1,2,3-triazole-4-carboxylate; Nsc, 2-(4-nitrophenyl)sulfonylethoxycarbonyl.

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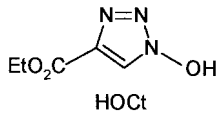
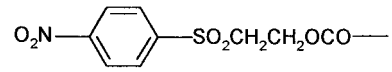


HOCt

Scheme 1



The Nsc protecting group

Scheme 2

sorb in the 300 nm UV region used for Fmoc monitoring. Hence, our newly designed HOCt reagent [1,2] has admirable solubility qualities and does not absorb above 250 nm in the UV (Scheme 1). There is a second consideration of the nature of the N^{α}-protecting group of peptides, during assembly, namely the slow deprotection of the N^{α}-Fmoc group which can occur over a period of time in DMF-based solvent systems. During slow coupling reactions undesired loss of N^{α}-Fmoc groups over time would cause a build up of by-products due to the presence of excess activated N^{α}-Fmoc protected amino acids used in each cycle of the SPPS.

Thus, it would be highly desirable to have N^{α}-protecting groups of amino acids which are designed to have good solubility properties and swift base-liability, but which also possess the following features:

(a) Exhibit a UV absorption of ca. 350–400 nm in the species produced on deprotection.
(b) Afford N^{α}-protected peptides which are stable for 24 h in DMF-based solvent systems.

Several years ago, we designed the base labile N^{α}-protecting group 2,2-bis(4′-nitrophenyl)ethan-1-oxycarbonyl urethanes (Bnpeoc) [3] as an alternative to Fmoc in solid phase peptide synthesis, however, this was not found to be optimal. More recently, a similar but much better N^{α}-protecting group 2-(4-nitrophenylsulfonyl)ethoxycarboxyl (Nsc) was designed and appeared to have the required features listed above (Scheme 2) [4–6]. These Nsc-amino acids are easily synthesized and commercially available crystalline compounds which are prerequisites for SPPS applications. In order to probe the above criteria, three peptides were synthesized accordingly:

1. **Substance P**: ArgProLysProGlnGlnPhePheGlyLeuMetNH_2
2. **Human big endothelin (1–38)**: CysSerCysSerSerLeuMetAspLysGluCysValTyrPheCysHisLeuAspIleIleTrpValAsnThrProGluHisValValProTyrGlyLeuGlySerProArgSerOH
3. **Partial bacteriophage MS2 coated peptide (8–25)**: ValLeuValAspAsnGlyGlyThrGlyAspValThrValAlaProSerAsnPheOH.

MATERIALS AND METHODS

All peptides described in this text were synthesized using an Applied Biosystem 430A Peptide Synthesiser (ABI) as described elsewhere [1,2] except that the Nsc group was used instead of Fmoc for the protection of the α-amino group and the Nsc deprotection was monitored at 380 nm instead of 302 nm. Wang resin was used for the synthesis of peptide acids and the tricyclic amide linker resin was used for the synthesis of peptide amide. The side chain protections are the same for most of the amino acids: O*t*Bu (Asp, Glu), *t*Bu (Ser, Tyr), Boc (Lys), Trt (His, Cys), Acm(Cys). However, Nsc-Arg-(Mts) was used instead of FmocArg(Pmc) since the Pmc analogue of Nsc-Arg was not available. The Nsc group was deprotected with 1% DBU/20% piperidine/DMF:dioxane (1:1) while the corresponding Fmoc group was deprotected with 20% piperidine in DMF:dioxane(1:1). Acetylation (capping) was carried out in a solution of 0.5 M acetic anhydride, 0.125 M DIEA (diisopropylethylamine) and 0.2% HOBt in DMF. The deprotection of Nsc and Fmoc was monitored at 380 and 302 nm, respectively, by a UV monitoring system fitted to the synthesizer. All the residues were incorporated through single couple cycles, using 1 mmol HOCt and 1 mmol DIC as coupling reagents.

The Cleavage, Purification and Analysis of Peptides

The peptides were cleaved from the resin with a mixture (10 ml) of TFA containing scavengers (TFA/EDT/H_2O/TIS = 10/1/1/0.2) except where Mts was used for side-chain protection of Arg. In this case, 0.2 ml of TMSOTf was added to the cleavage mixture instead of water and the cleavage time was extended from 2 to 4 h.

The crude peptides were precipitated with ether and washed with ether. The crude peptide was then purified by HPLC using C18 or C8 preparative columns as specified, eluting with a linear gradient of 0.1% TFA/H_2O with increasing concentration of 0.1% TFA/CH_3CN at 5 ml/min. The pure peptide was obtained by lyophilization and analysed by analytical HPLC, MALDI TOF/Electrospray mass spectroscopy and amino acid analysis.

Deprotection of Nsc

Substance P was synthesized three times. The coupling cycle was the same as described above but the deprotection files were varied as described below and the last deprotection file was found to be sufficient for Nsc deprotection.

1. 1% DBU/20% piperidine in DMF/dioxane (1:1), three deprotections of 6, 1.5 and 1.5 min, respectively, for each coupling cycle.
2. 1% DBU/20% piperidine in DMF/dioxane (1:1), three deprotections of 3, 1.5 and 1.5 min, respectively, for each coupling cycle.
3. 1% DBU/20% piperidine in DMF/dioxane (1:1), two deprotections of 2 and 1.5 min, respectively, for each coupling cycle.

Synthesis of Human Big Endothelin

Human big endothelin (human BigET) was synthesized under the same conditions described above and the deprotection cycles were 6, 1.5 and 1.5 min for Nsc amino acids and 4 and 2.5 min for Fmoc amino acids.

Synthesis of the Partial Bacteriophage MS2 Coat Protein (8–25)

This peptide was synthesized as described above and the deprotection cycles were 2.0 and 1.5 min.

Monitoring of the Nsc Deprotection

The Nsc group absorbs at 380 nm at a moderate intensity which allows the reaction mixture to be passed directly into the UV monitor without intervening dilution. This protocol increased the accuracy of the monitoring process.

RESULTS AND DISCUSSION

Nsc Deprotection Studies through the Synthesis of Substance P

It was found that deprotection of the Nsc group and subsequent monitoring of peptide synthesis on the Applied Biosystems 430A automated synthesizer could be conveniently measured at 380 nm. In addition, there was no need for the dilution stages which have to be incorporated in our protocol for the measurement of Fmoc deprotection at 302 nm since the deprotection solution could be monitored directly at 380 nm. This method was employed for monitoring the synthesis of all peptides using Nsc amino acids. Dioxan/DMF (1:1) was the solvent system used throughout the synthesis. The amount of piperidine and DBU required for efficient cleavage of the Nsc group had been investigated and published previously [4–6]. It was established that the optimal deprotection solution for fast deprotection was 1% DBU and 20% piperidine in the solvent mixture dioxan/DMF (1:1). The amount of time required for deprotection was also studied with respect to our existing protocols in which **each** deprotection stage in SPPS is repeated two or three times until no further UV absorbance is observed, i.e. complete removal of the protecting group. For the Nsc group three different time sequences were investigated for the deprotection, namely: 6.0, 1.5 and 1.5 min, 3.0, 1.5, 1.5 min, and 2.0 and 1.5 min. The latter was found to be satisfactory for deprotection of the Nsc group under the above reaction conditions. In the light of these results it is interesting to compare the properties of the Fmoc and Nsc groups outlined in Table 1.

Table 1 Comparison Between Fmoc and Nsc Protected Peptides

	Fmoc	Nsc
Cleavage rate ($t_{1/2}$)		
20% piperidine in DMF	10–15s	90–110 s
1% DBU/20% piperidine/DMF	—	12–15 s
Decomposition in DMF solution		
1 week	10%	<1%
3 weeks	40%	2%
Olefin-amine adduct formation	Fast, reversible	Very fast, irreversible
Polymerization during deprotection	Yes	No
Available UV monitoring range	302 nm	380 nm

The stability of the N^{α}-protecting group in DMF was found to be of particular importance when this group was to be left on for a long time, i.e. for fragment ligation. When trace of piperidine remains, the deprotection of Fmoc was much faster (data not shown). Nsc is obviously a better protecting group as far as stability is concerned.

The crude peptide obtained was >80% pure with a truncated peptide eluted 2 min earlier (Figure 1). It was purified on HPLC (Figure 2) and analysed by MALDI TOF MS (1349.1. expected 1348.6) and

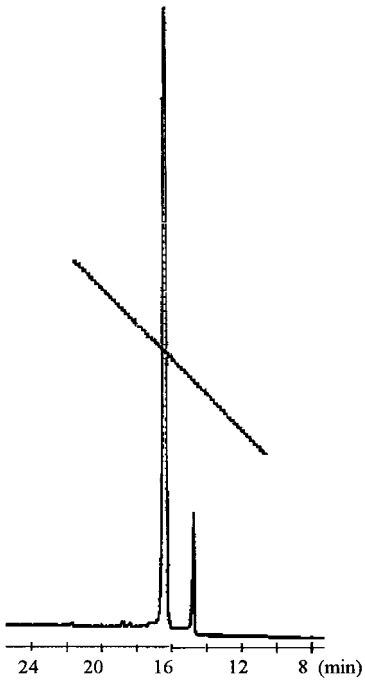


Figure 1 HPLC of substance P (crude) Hichrom C18, 214 nm. A: 0.1% TFA/H_2O; B: 0.1% TFA/CH_3CN; 1 ml/min, 10–90% B in 30 min.

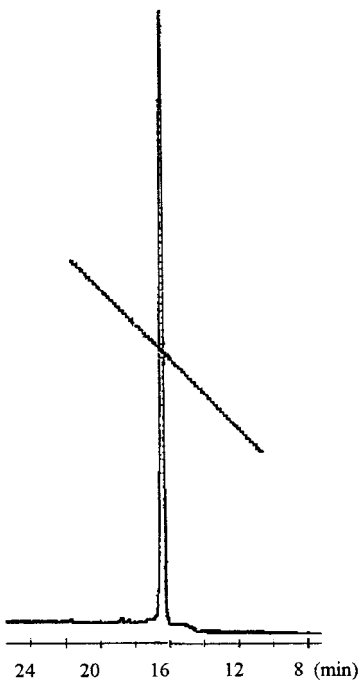


Figure 2 HPLC of substance P (pure), Hichrom C18, 214 nm. A: 0.1% TFA/H_2O; B: 0.1% TFA/CH_3CN; 1 ml/min, 10–90% B in 30 min.

amino acid analysis results are: Asx4 (3.81), Thr2 (1.80), Ser1 (0.94), Gly3 (2.86), Ala1 (1.07), Val4 (3.74), Leu 1 (1.04), Phe 1 (0.95), Pro 1 (1.02).

Coupling Efficiency Studies through the Synthesis of Human BigET

Human BigET was synthesized in order to investigate the coupling efficiency of this new N^{α}-protecting group. The coupling efficiency was monitored by the deprotection of Nsc (380 nm) and was compared with that of Fmoc (302 nm) (Figure 3). The result indicated that Nsc amino acids are comparable with the corresponding Fmoc analogues. The peptide was purified and folded (Figure 4) to give the correct mass (4282.8, expected 4282.9).

Rearrangement of Asp-X and the Synthesis of the Partial Bacteriophage MS2 Coated Protein (8–25)

A recurring problem in peptide synthesis is the tendency of Asp-X sequences to rearrange, via the imide intermediate, to the β-peptide structure [7,8]. This phenomenon is sequence dependent, particularly when X = Asn, Gly or Ser, and can be observed in the final acid cleavage and deprotection using Boc/Bzl methodology. In the case of the Fmoc/*t*Bu strategy the rearrangement can also occur during the repeated piperidine cleavage of the N^{α}-Fmoc groups to form β-piperidide [7,8]. It was therefore of great importance to test the Nsc N^{α}-protecting group under the deprotection conditions cited above. In this respect the partial sequence bacteriophage MS2 of coat protein (VLVDNGGTGDVTVAPSNF) has been shown to be extremely prone to rearrangement [7]. The content of the desired product was only 16.8% in the crude product and the ratio of desired product to piperidide product was 1:3 [7]. Investigations had been carried out to reduce this rearrangement by introducing a special protecting group for the β-carboxylic acid for Asp, but the results were not fully satisfactory [9]. This partial phage MS2 sequence was thus chosen as an example for SPPS to test the effects of the repeated base-deprotection of the Nsc group.

A synthesis was thus carried out using the corresponding Nsc amino acids. The HPLC trace of the crude peptide obtained is shown in Figure 5. A very shallow gradient was applied to permit optimal resolution. Each peak was collected and examined using electrospray mass spectrometry. The major peak was the desired product (1762.8, expected 1761.9) and the impurities are listed. Although both

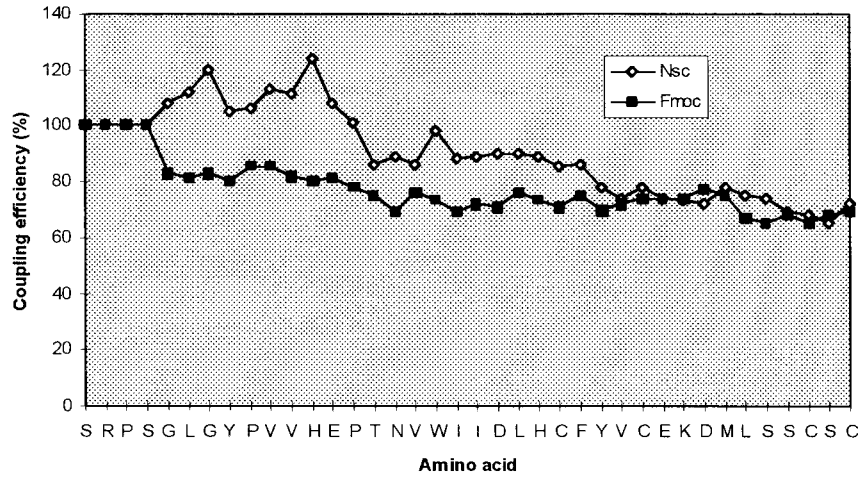


Figure 3 Deprotection profiles of Fmoc (302 nm) and Nsc (380 nm) in the synthesis of BigET(1–38) (human).

the cyclized aspartimide peptide and the β-piperidide peptide were observed, the desired product was the major product. The ratio of the desired product to the rearranged by-products is improved from 1:3 to 2:1 without recourse to the use of a special protecting group for the β-carboxylic acid of the key aspartic acid residue. This greatly improved the purification protocol and the quality of the final product (Figure 6). The amino acid analysis results

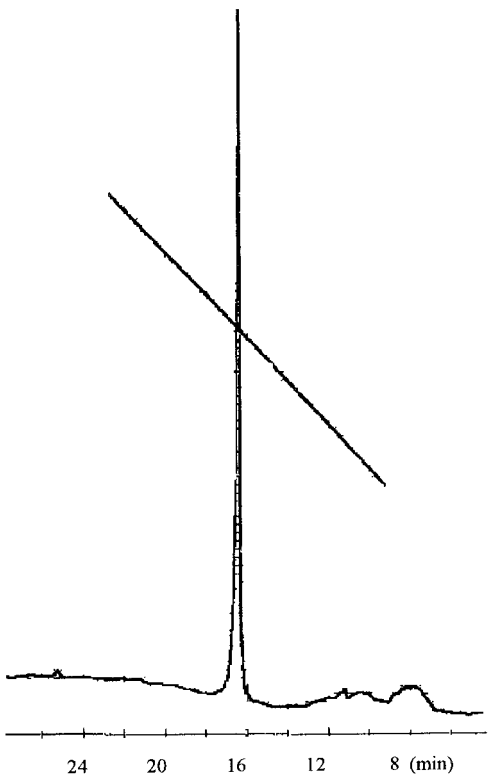


Figure 4 Human BigET(1–38) (pure, folded), Hichrom C18, 214 nm. A: 0.1% TFA/H_2O; B: 0.1% TFA/CH_3CN; 1 ml/min, 10–90% B in 30 min.

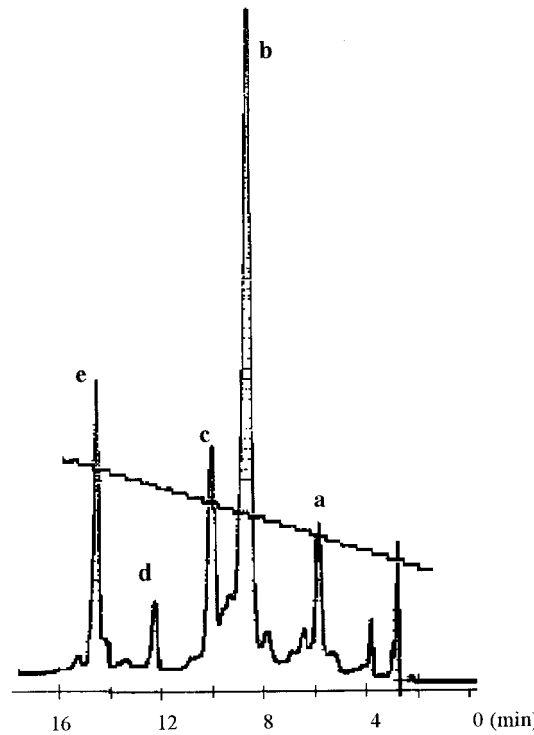


Figure 5 HPLC trace of crude bacteriophage MS2(8–25), synthesized using Nsc amino acids, Hichrom C18, 214 nm. A: 0.1% TFA/H_2O; B: 0.1% TFA/CH_3CN; 1 ml/min, 20–45% B in 30 min. (a) Ac-13-25(1265.2, expected 1264.3); (b) title peptide (1763.5, expected 1762.9); (c) cyclized aspartmide peptide (1746.7, expected 1744.9); (d) Ac-9-25 (1705.8, expected 1706); (e) β-piperidide peptide (1832.4, expected 1929.0).

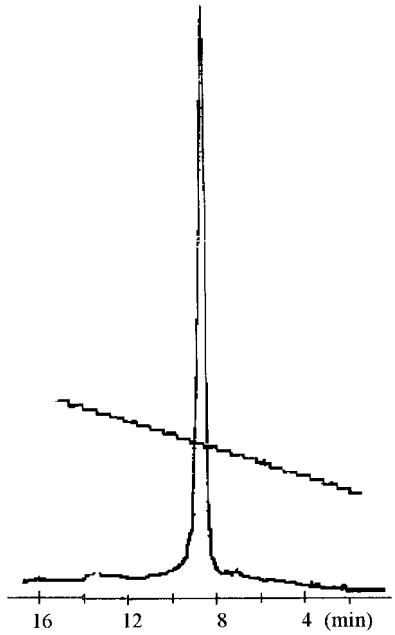


Figure 6 Bacteriophage MS2(8–25) (pure), Hichrom C18, 214 nm, 1 ml/min. A: 0.1% TFA/H_2O; B: 0.1% TFA/CH_3CN; 1 ml/min, 20–45% B in 30 min.

are (6 M HCl, 110°C, 24 h): Glx2 (2.15), Gly1 (1.03), Met1 (0.92), Leu1 (0.98) Phe2 (2.08), Lys1 (1.02), Arg1 (0.9), Pro 2 (1.79).

Acknowledgements

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