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Characterisation of TNF-α-related peptides by high-performance liquid chromatography–mass spectrometry and high-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Ionspray triple quadrupole mass spectrometry and high-performance liquid chromatography were used to investigate the products from the solid phase synthesis of (H)–Leu–Thr–Glu–Asn–(OH), a TNF-α active-site probe. The target sequence was assembled using *tert.*-butoxycarbonyl (Boc) chemistry in stepwise fashion from the C-terminal on an Boc–Asn–OCH_2–Pam–copoly(styrene–divinylbenzene) resin [Pam = 4-(carboxamidomethyl)benzyl ester]. The crude product was deprotected and cleaved from the resin by HF–*p*-cresol treatment for 1 h at 0°C. HPLC analysis at 214 nm indicated two late-eluting major products and an early-eluting product. Preparative HPLC demonstrated that the early-eluting product contained *ca.* 80% of the expected recovered sample mass. Each component was then directly analysed by mass spectrometry and tandem mass spectrometry. The early eluting peak was confirmed as the desired LTEN sequence. Synthesis of the same sequence using 9-fluorenyl methoxycarbonyl (Fmoc) chemistry gave an identical product and confirmed the above analysis. The most significant by-product was derived from arylation of the glutamyl group by the quencher *p*-cresol. The likely origins of the by-products are discussed.

INTRODUCTION

The role of synthetic peptides is central to many areas of biomedical research [1]. Their synthesis by the solid phase method (SPPS) has become the most common and widely accepted technique since its introduction by Merrifield in 1963 [2]. Recently, we have explored the possibility of using small peptides to probe the putative binding site of TNF-α; the synthesis and characterisation of the tetrapeptide (H)–Leu–Thr–Glu–Asn–(OH) was part of that program and is the subject of this report.

Despite optimisation in many laboratories the success of the SPPS is not guaranteed. Indeed, by-products of the chemical synthesis of peptides are typically detected by HPLC analysis and are often tentatively assigned on past experience with little or no evidence as to their structure. The development of modern, faster and more efficient SPPS protocols demands high-resolution techniques for the structural determination of minor by-products.

Mass spectrometry and in particular ionspray mass spectrometry [3] has been recently demonstrated to meet many of the above demands. This technique requires only picomole quantities of sample and thus allows the direct investigation of synthetic peptide mixtures. Two types of data are readily accessible from ionspray mass spectrometry. The ability of peptides to form multiply charged ions of the type $[M + nH]^{n+}$ has lead to accurate mass determination (within 0.01%)

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over an extended mass range (up to M_r 250 000). In combination with a triple quadrupole, the singly or multiply charged ions can be induced to fragment and give significant covalent structural information [4,5]. In many cases complete sequence information can be obtained by MS–MS analysis of the singly and multiply charged molecular ions of peptides with molecular masses up to 2500.

Here we illustrate the use in peptide synthesis of ionspray mass spectrometry when combined with HPLC in the structural characterisation of the polypeptide LTEN and associated by-products in the chemical synthesis. The major by-product is due to a previously identified side reaction in synthetic peptide chemistry.

EXPERIMENTAL

Chemicals and reagents

All *tert.*-butoxycarbonyl (Boc)- and 9-fluorenyl methoxycarbonyl (Fmoc)-L-amino acids and reagents used during chain assembly were peptide synthesis grade supplied by Auspep (Parkville, Victoria, Australia). Acetonitrile (HPLC grade) was purchased from Millipore Waters (Milford, MA, USA). Deionized water was used throughout and prepared by a Milli-Q water purification system (Millipore Waters). Screw-cap glass peptide synthesis reaction vessels (20 ml) with a sintered glass filter frit were obtained from Embel Scientific Glassware (Queensland, Australia). An all-Kel-F apparatus (Toho; from Peptide Institute, Osaka) was used for HF cleavage. Argon, helium and nitrogen (all ultrapure grade) were from CIG, Australia.

Peptide synthesis

Chain assembly

(H)–Leu–Thr–Glu–Asn–(OH) was synthesised on a Boc–Asn–OCH_2–Pam–copoly-(styrene–divinylbenzene) resin [Pam = 4-(carboxamidomethyl)benzyl ester] [0.79 mequiv./g] or on a Fmoc–Asn(Trt)–OCH_2–Wang resin [0.37 mequiv./g] by manual stepwise solid phase peptide synthesis according to our *in situ* neutralisation protocols [6,7]. The following side chain protecting groups were used: Boc synthesis: synthesis 1: Thr, benzyl; Glu, benzyl; synthesis 2: Thr, benzyl; Glu, cyclohexyl (chxl). Fmoc synthesis: Thr, *tert.*-butyl; Glu, *tert.*-butyl; Asn, trityl.

All operations were performed manually in glass reaction vessels with PTFE-lined screw caps. The peptide resin was agitated by gentle inversion (Milligen SPPS shaker) during the N^{α}-deprotection and coupling steps.

Deprotection/cleavage

Boc. Boc-synthesised peptides were first treated with 2×1 washes of trifluoroacetic acid (TFA) to remove the N^{α}-Boc group, washed with dimethylformamide (DMF), dichloromethane (DCM), and air dried. Side chain protecting groups were removed and the peptide simultaneously cleaved from the resin by treatment with HF–*p*-cresol (9:1). HF cleavage was performed at various temperatures: 0°C/1 h; −10°C/90 min and −15°C/2 h. A low–high HF cleavage was also performed as described by Tam [8]. The HF was removed under reduced pressure at 0°C, the crude peptide product was precipitated and washed with ice-cold diethyl ether then dissolved in 50% aqueous acetic acid and lyophilised to give crude product.

Fmoc. LTEN synthesised using Fmoc chemistry cycles was first treated with 50% piperidine–DMF (2×1 min) and the peptide-resin washed with DMF, DCM, and air-dried. The peptide was then cleaved with TFA–triethylsilane (TES) (95:5) for 90 min at room temperature. TFA was removed under vacuum, the resulting oil was dissolved in water and the TES was removed by extraction with diethyl ether. The aqueous peptide solution was then lyophilised to give crude LTEN.

High-performance liquid chromatography

Analytical HPLC was performed on a Waters 600E solvent-delivery system equipped with a 484 absorbance detector and 745B data module. Peptides were analysed on a Waters reversed-phase analytical C_{18} column (Delta pak, 150 × 3.9 mm I.D.). Separations were carried out using a linear gradient of 5–55% B over 50 min at a

flow-rate of 1 ml/min. Solvent A was 0.1% aqueous TFA; solvent B was acetonitrile–water–TFA (90:10:0.08). The eluent was monitored at 214 nm. For preparative work peptide samples were dissolved in acetonitrile–water–TFA (35:65:0.1) and injected onto the column.

Mass spectrometry

Mass spectra were acquired on a PE-SCIEX API III triple quadrupole mass spectrometer equipped with an ionspray atmospheric pressure ionization source. Samples, collected from HPLC runs were infused directly into the ionization chamber of the mass spectrometer. Sample droplets were ionized at a positive potential of 5.6 kV into the gas phase and entered the analyser through an interface plate and subsequently through an orifice of 100–120 μm. Mass spectra were obtained over the m/z range of 200 to 600 u in 3 s with a scan step of 0.2 u.

For tandem mass spectrometry the molecular ions were bombarded in quadrupole-2 with argon; collision cell thickness was $350 \cdot 10^{12}$ atoms/cm^2 and a collision energy of 10–20 V. Quadrupole-3 was then scanned from 50 m/z to just above the parent ion molecular mass in 3 s with a step size of 0.2 u. Mass spectra were typically acquired in 5–15 min on an Apple Macintosh IIfx computer and were processed using the software package MacSpec (Sciex, Toronto, Canada). Interpretation of the spectra was aided by MacBiospec (Sciex).

RESULTS

Peptide synthesis

The tetrapeptide LTEN was synthesised in high yield using Boc chemistry. Stepwise SPPS gave near quantitative yields in the assembly of the protected peptide with an average coupling efficiency of greater than 99.9% as determined by quantitative ninhydrin analysis [9]. Similar results were obtained when the sequence was assembled with either benzyl or cyclohexyl sidechain protection for glutamic acid. Crude peptide mixtures were obtained initially using standard HF cleavage protocols (0°C, *p*-cresol).

In a separate synthesis the same sequence was assembled using Fmoc chemistry with an average coupling efficiency of 99.4%.

Analysis of crude LTEN by HPLC

The crude synthetic products derived from two separate Boc chemistry syntheses were examined by HPLC analysis. The two chromatograms (Fig. 1) were essentially identical and contained four products that were peptidic (labelled A, B, C and D). While the late-eluting peak D appeared to be the major product, preparative chromatography established that the early-eluting peak A contained 80% of the expected mass recovery. HF cleavage performed at several different conditions gave identical chromatograms of the crude products.

In a separate Fmoc chemistry synthesis of LTEN, a single product was obtained which co-eluted with peak A above (data not shown).

Mass spectrometric analysis

Aliquots of the crude peptide products were subjected to ionspray mass spectrometric analysis. Mass spectra of the crude product from the two different Boc syntheses were identical, showing two major ions at m/z 476 and m/z 566 and a smaller ion at m/z 532 (Fig. 2). Preparative HPLC followed by direct infusion of the collected peaks into the mass spectrometer al-

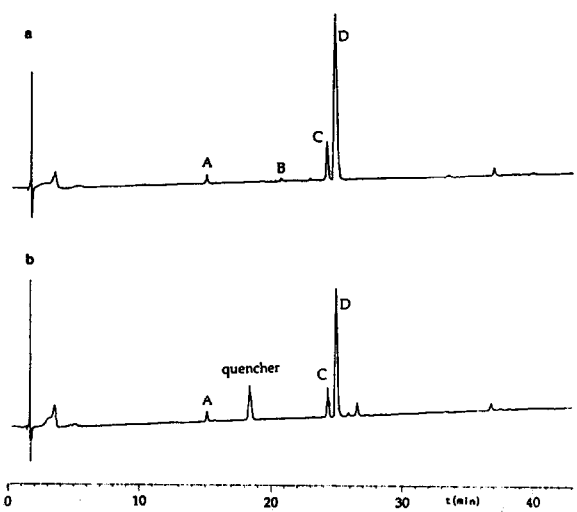

Fig. 1. Chromatograms for Boc-synthesised LTEN. (a) With benzyl protected glutamic acid; (b) with cyclohexyl protected glutamic acid.

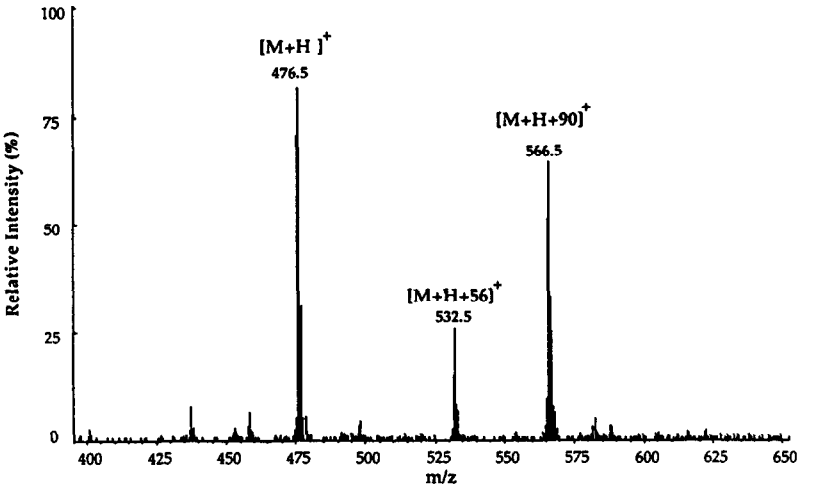

Fig. 2. Mass spectrum of crude LTEN synthesised by Boc chemistry.

TABLE I

MOLECULAR MASS ASSIGNMENTS FOR COMPONENTS IN HPLC FRACTIONS A–D (FIG. 1)

Peak	Molecular mass
A	476 $[M+H]^+$
B	532 $[M+57]^+$
C	566 $[M+91]^+$
D	566 $[M+91]^+$

lowed masses to be assigned to the individual components of the HPLC analysis (Table I).

In order to confirm that fraction A was indeed the target peptide LTEN the molecular ion at m/z 476 was analysed by MS–MS (Fig. 3). A series of fragments arising from the collision-induced dissociation (CID) of the parent ion were observed. Both the b and y series were present in abundance. By comparison with the expected fragmentation pattern of the tetrapep-

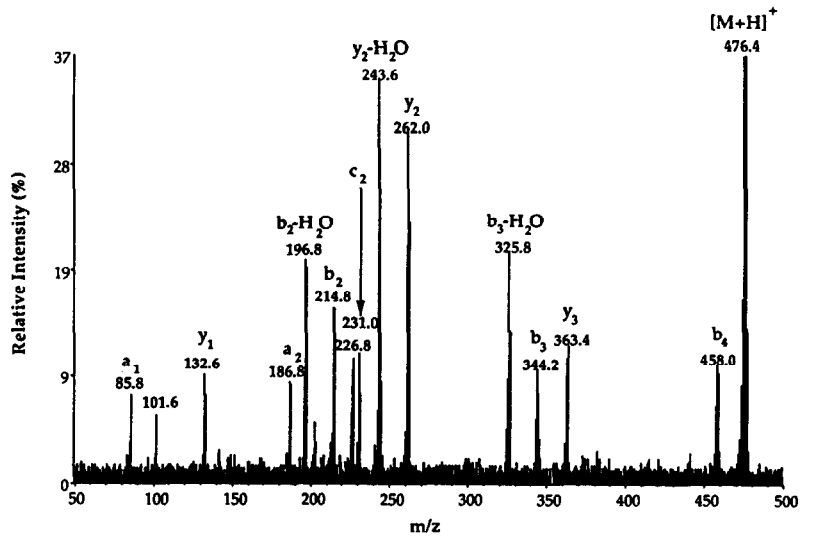

Fig. 3. MS–MS analysis of purified LTEN.

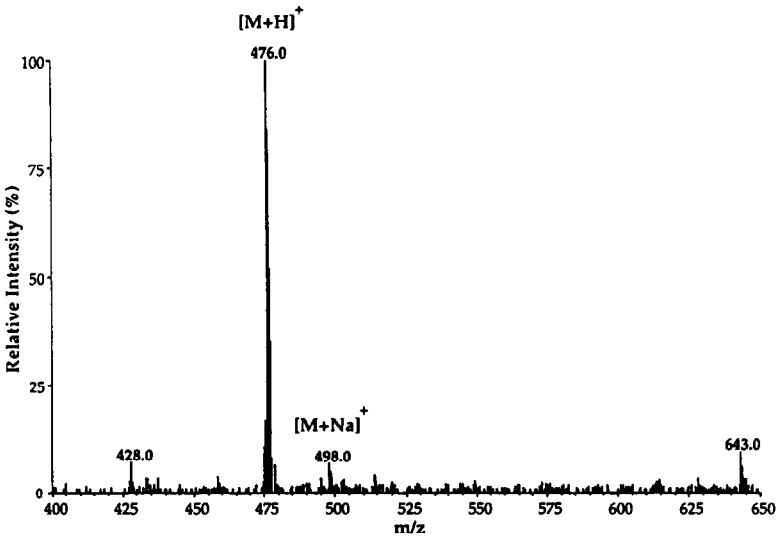

Fig. 4. Mass spectrum of crude LTEN synthesised by Fmoc chemistry.

tide LTEN (derived from MacBiospec) the sequence was readily confirmed. Further, the product obtained from the Fmoc synthesis of LTEN displayed the expected $[M + H]^+$ ion at m/z 476 (Fig. 4).

Structural characterisation of by-products derived from Boc synthesis

Three by-products were isolated by preparative HPLC and directly analysed by MS and MS–MS. The trace by-product from fraction B gave a molecular ion at m/z 532 (Table I); the extra mass of 57 u indicated the presence of a *tert.* butyl adduct. Further work will be required to determine its precise location.

Two other by-product peaks had molecular masses of m/z 566 (fractions C and D); the extra mass of 91 u indicated the presence of either a benzyl- or *p*-cresol-derived adducts of LTEN. MS–MS analysis located the "extra" mass on the threonine on fraction C (data not shown) and on the glutamate on fraction D.

DISCUSSION

Despite the continuing efforts of many research groups world-wide to develop rapid and highly effective synthetic protocols, SPPS has not become routine. Even in so-called simple sequences a variety of chemically derived artefacts may be observed. The present study illustrates this point and is concerned with the investigation of the products derived from the solid phase peptide synthesis of the tetrapeptide, (H)–Leu–Thr–Glu–Asn–(OH). This was achieved through a combination of HPLC and HPLC–MS analysis of the crude synthetic products.

Characterisation of the target sequence

HPLC analysis of the several products from Boc and Fmoc syntheses allowed the tentative assignment of the target sequence. This was the major product from the Fmoc synthesis and co-eluted at 15.0 min with the apparent minor product, peak A, observed in both Boc syntheses (Fig. 1). Mass spectrometric analysis of peak A supported the assignment with a molecular ion observed at m/z 476; this was also observed as the major ion from the Fmoc synthesis (Fig. 4). To fully confirm the sequence the molecular ion was analysed by MS–MS where all expected fragments were observed and assigned as shown (Fig. 3).

As expected from the above analyses, preparative HPLC demonstrated that peak A contained the bulk of the product mixture and it appeared likely that the "apparent major products" (peaks

C and D) contained strongly UV-absorbing chromophores.

Characterisation of by-products

Crude LTEN (synthesis 1) was subjected to preparative HPLC in order to identify and characterise by-products detected by analytical HPLC and to relate those by-products to the components present in the MS of the crude product. The three by-products detected by analytical HPLC (Fig. 1) in peaks B, C and D were collected and directly analysed by MS and MS–MS except for peak B (an apparent *tert.* butyl carbocation adduct) which was not investigated further.

Residual protecting groups. The second most abundant by-product (peak C, Fig. 1) had a molecular ion at m/z 566 compared to m/z 476 of the target LTEN. This mass difference of 90 u is consistent with a residual benzyl group on either the threonine or glutamyl residue or a *p*-cresol adduct from the glutamyl acylium ion, a known side reaction under HF cleavage conditions [10,11]. MS–MS analysis of the molecular ion located the residual mass on the threonine residue.

Quencher derived by-product. The most abundant by-product (peak D, Fig. 1) also had a molecular ion at m/z 566. MS–MS analysis of the molecular ion located the residual mass of 90 u on the glutamyl residue (Fig. 5). As peak D was common to both Boc syntheses which used different protecting groups for glutamic acid (benzyl in synthesis 1; cyclohexyl in synthesis 2) the possibility of a residual benzyl group on Glu was readily discounted. The most likely explanation was that a reaction had occurred between the sidechain of the glutamyl residue (as the acylium ion) with the *p*-cresol used as scavenger in the HF deprotection. This reaction has been well described [10,11] and characterized by classical techniques but has neither been reported nor characterised by MS analysis. The transformation is outlined in Fig. 6.

As the acylium ion generation is known to be sensitive to temperature and the nature of the sidechain protecting group [11,12] we repeated the HF cleavage for both Boc syntheses at lower temperatures (−15°C→ −20°C). In contrast to previous observations no significant change in the proportion of the intact tetrapeptide to *p*-cresylated peptide was observed. Additionally, we performed a low–high cleavage on the all-benzyl protected resin peptide (synthesis 1). In principle this would allow the removal of the

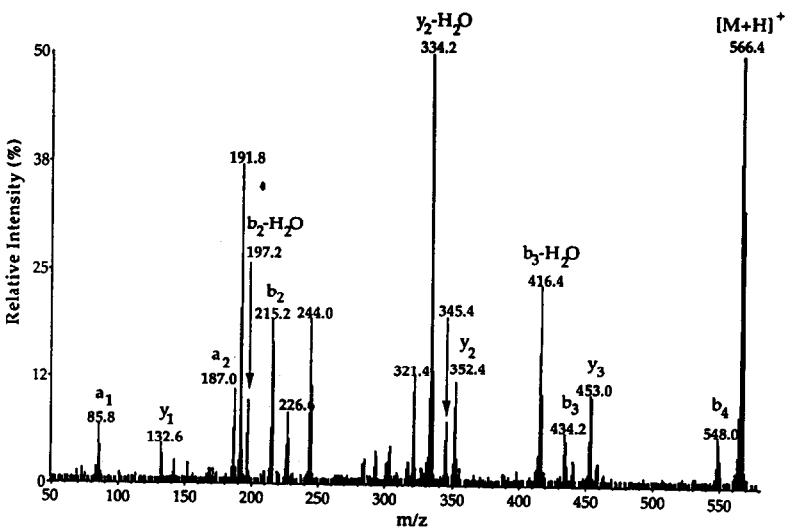

Fig. 5. MS–MS spectrum of Boc-synthesised LTEN by-product (peak D, Fig. 1).

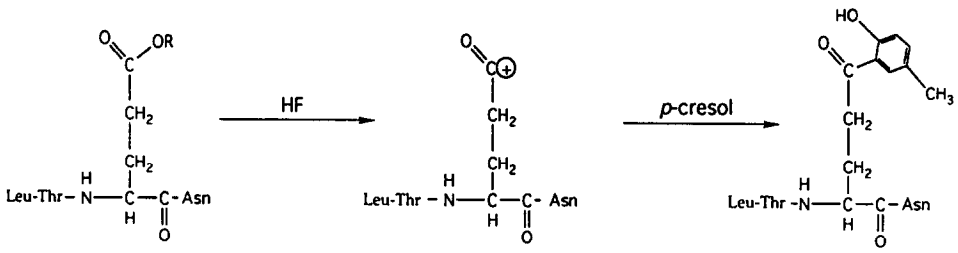

Fig. 6. Suggested mechanism of attack on the glutamyl residue by *p*-cresol.

benzyl ester by an S_N2 mechanism and avoid the acylium ion formation under stronger acid conditions (presumably by an $A_{AC}1$ mechanism). This was indeed the case and a dramatic improvement was observed both HPLC and MS analysis (data not shown). Peak D (the acylium adduct) now became a minor contributor to the observed impurities (*ca.* 1–3%).

CONCLUSIONS

The results obtained in the analysis of the tetrapeptide, (H)–Leu–Thr–Glu–Asn–(OH) obtained from several synthetic approaches show that ionspray MS and MS–MS can be routinely used in methodology development. Thus, synthetic products can be quickly analysed, by-products detected and the nature and the site of peptide modification identified.

In our Boc syntheses of LTEN it was clear that of the by-products observed, by far the most significant (10–15%) was that due to acylium ion generation. This is a direct result of strong acid cleavage. Its formation was markedly reduced by manipulation of the final cleavage conditions which augurs well for such treatments in other complex synthetic peptide product mixtures.

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