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A Side-reaction in the SPPS of Trp-containing Peptides

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Abstract: Syntheses of several Trp-containing peptides on a Wang solid support afforded significant amounts of a side-product. ^{1}H-NMR and MS data showed that an unexpected alkylation by the linker has occurred on the indole nucleus. This was observed whatever the scavenger used, and whatever the position of the Trp residue in the sequence, unless it was in the *C*-terminal position. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: side-reaction; Trp; alkylation; Wang resin; TFA cleavage

INTRODUCTION

In the course of a program dealing with Trp-containing peptides syntheses on solid support, we have been faced with an intriguing side-reaction. The acidic final TFA cleavage of the Delta Sleep Inducing Peptide (DSIP), a Trp-containing peptide synthesized on Wang resin using the classical Fmoc strategy, led to significant amounts of a side-product along with the expected peptide (Figure 1).

Surprisingly, all the syntheses of several Trp-containing sequences we carried out afforded a side-product. The repeatable and systematic presence of significant amounts of side-product prompted us to investigate the corresponding side-reaction in more detail.

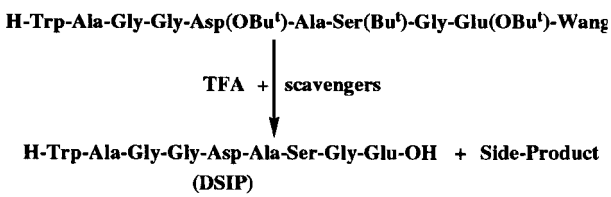

Figure 1 TFA cleavage of DSIP synthesis on a Wang resin.

MATERIALS AND METHODS

Reagents

Amino acid derivatives were purchased from Senn Chemicals (Gentilly, France), preloaded resins from Novabiochem (Meu Don, France) and Advanced ChemTech (Louisville, KY, USA), anisole from Prolabo (Cedex, France), ethanedithiol and triisopropylsilane from Aldrich (Milwaukee, WI, USA). TFA of synthesis quality was purchased from Merck (Nogent sur Marne, France), and distilled. Dichloromethane was pre-dried overnight over $CaCl_2$, distilled from K_2CO_3 and stored away from bright light in a brown bottle. Triethylamine was distilled from KOH/ninhydrin and stored away from bright light. Water was obtained from a Milli-Q plus system (Millipore, Cedex, France), and acetonitrile from Merck.

Abbreviations: SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; Mtr, methoxytrityl; Fmoc, 9-fluorenylmethoxycarbonyl; BOP, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole.

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Peptide Synthesis

DSIP and the heptapeptide studied were synthesized with standard Fmoc α-protection [1] first introduced by Carpino and Han [2,3] on a 433A peptide synthesizer employing HBTU/HOBt-activation. Shorter sequences were synthesized using a home-made rotating reaction vessel with preloaded resins; Fmoc amino acids were coupled with the BOP reagent. Reactions were monitored with the colour Kaiser test [4]. Deprotection steps were carried out with piperidine (20%) in DMF.

Cleavage and Deprotection

Typical procedure: The dry resin was placed into a rotating reaction vessel and TFA containing appropriate scavengers (25 ml/g resin) was added. After 2 h stirring, the resin was filtered, followed by a TFA washing. The filtrates were combined and concentrated under reduced pressure. Isolation of the peptide was achieved by precipitation and centrifugation in diethyl ether (three times). In the case of water-soluble peptides, after removing of the TFA, the residue was dissolved in water and the aqueous layer washed with diethyl ether (three times) and then freeze-dried.

For the Boc-indole protected peptides, the time of TFA treatment was doubled.

Chromatography

Thin layer chromatography (TLC) was performed on Merck precoated silica gel $60F_{254}$ plates and spots were visualized by ultraviolet light or by staining with phosphomolybdic acid. Flash chromatography was performed using Merck silica gel 60 (230–400 mesh). The HPLC analyses were carried out on a Waters Millennium apparatus with: a photodiode array detector 996, length 214 nm; using as column a reversed phase Nucleosil C18, 5µ, 250 × 10 mm; the flow: 1 ml/min, conducted using a 30 min gradient of 10–100% aqueous acetonitrile containing 0.1% TFA; solvent A: H_2O, TFA 0.1%; solvent B: CH_3CN, TFA 0.1%. The preparative apparatus was a Water PrepLC 4000 system with a reversed phase nucleosil Delta Pak C_{18}, 15µ, 100 Å, 40 × 100 mm and a Guard Pak C_{18},15µ, 100 Å, 40 × 10 mm in a PrepLC 40 mm Chamber Assembly. The tunable absorbance detector was a Waters 486 operated at 214 nm. Crude and purified peptides were chromatographed in the presence of 0.1% TFA and elution was conducted using aqueous acetonitrile appropriated gradient containing 0.1% TFA at a flow rate of 50 ml/min.

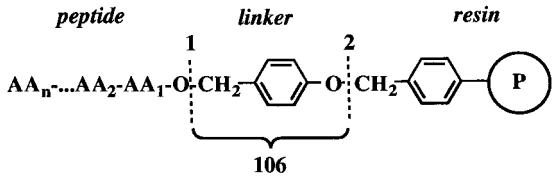


Figure 2 Linker structure of Wang resin.

Mass Spectroscopy and NMR

Positive ion fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMS SX102 (Jeol Ltd, Tokyo, Japan). This instrument is of BE design. Xenon was used in the FAB experiments. The energy of the neutral atom beam was 3 keV (emission current: 20 mA). Calibration was accomplished using Ultramark 1621 (Heraeus, Karlsruhe, Germany) as a reference. The FAB mass spectra were measured at a resolution of 1000. CAD spectra were taken using xenon as the collision gas at a pressure to reduce the beam of the parent ions by 30%. The collision cell is located in the region before the magnetic sector. The collision energy was 6 keV. The resulting fragment ions were analysed by a B/E linked scan method. The data were acquired and processed with an HP Apollo series 400 using the JEOL complement software. Glycerol was used as the matrix. Solutions of the studied peptides in methanol (1 µl containing *c.* 1–10 µg) were dissolved directly in a drop of glycerol on the target. Glycerol was obtained from a commercial supplier (Aldrich).

ESI mass spectra were recorded on a Platform II quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray ion source. The mass spectrometer was calibrated in the positive ion mode using a mixture of NaI and CsI. Data were acquired in the scan mode from m/z 100 to m/z 1000 in 4 s. Fifteen scans were summed to produce the final spectrum. Samples were dissolved in the mixture $H_2O/CH_3CN/HCOOH$ (49:49:2, v/v) and infused into the ESI source at a flow rate of 10 µl/min. Voltages were set at +3.5 kV for the capillary and adjusted for the sampling cone so that fragment ions were the most abundant. The source was heated at 60°C.

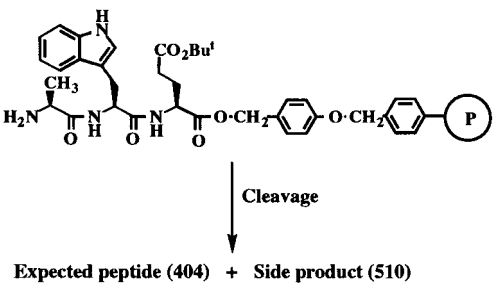
RESULTS AND DISCUSSION

By using the LC/MS technique, we showed that the side-product exhibited an increase in molecular weight of 106 compared to the desired peptide. The

increase in molecular weight correlates with the linker structure in the Wang resin (Figure 2).

While the normal cleavage position 1 leads to the expected peptide, cleavage at position 2 would afford an ester derivative having a +106 molecular weight. Linked scan-MS and ^{1}H-NMR analyses allowed us to exclude this structure for the side-product. Indeed, linked scan-MS experiments on the side-product obtained from Ala-Trp-Glu-resin cleavage having a molecular weight of 510 revealed fragments incompatible with the supposed ester since the Trp residue seemed to bear the +106 weight increment (Figure 3).

^{1}H-NMR of the side-product displayed modified signals for the indole protons compared to the ^{1}H-NMR of an unmodified Trp-side chain (Figure 4) indicating that the 2-position was involved in the alkylation reaction. In addition, a higher integration



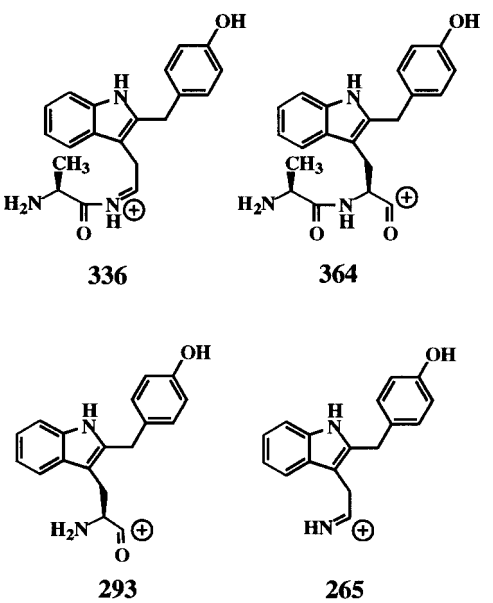


Figure 3 Fragmentation obtained from linked scan-MS experiments on the side-product obtained from Ala-Trp-Glu-resin cleavage having a molecular weight of 510.

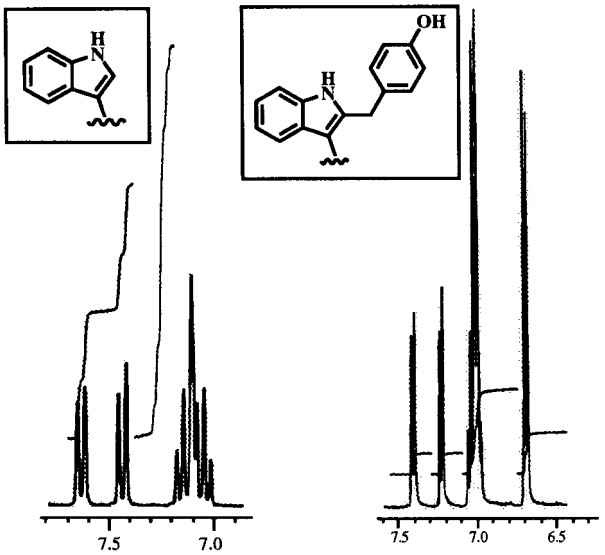


Figure 4 Comparison of aromatic region of the NMR of the side-product and Trp side-chain.

of the overall aromatic protons (increased from five to eight protons) was in agreement with the proposed structure.

Both MS and NMR data are consistent with the 2-substitution of the Trp indole ring of tryptophan, well known when carbocations (usually resulting from Boc or Bu^t group cleavage) are present in the mixture [5] or during the cleavage of Mtr group from arginine [6]. In the case of our syntheses, the side-product presumably arises by cleavage at both position 1 and position 2 (Figure 1), followed by alkylation of the indole nucleus.

This side-product SP1 was detectable in significant amounts in all syntheses of endogenic analogues of the DSIP that were carried out. In some cases, a second side-product SP2 was also present although to a lesser extent (Figure 5).

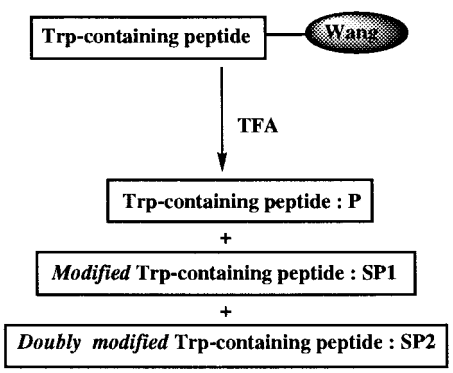


Figure 5 General TFA cleavage of the Trp-containing peptide synthesized on Wang resin.

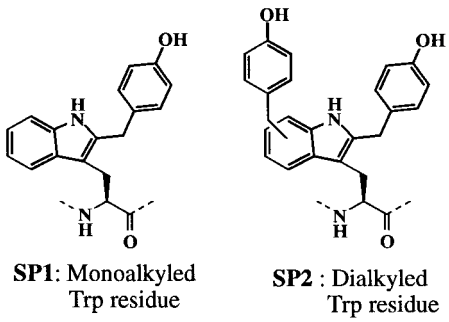


Figure 6 Structures of side-products.

The increase in weight of SP2 was determined to be twice 106, attributed to a double alkylation of the indole nucleus (Figure 6).

We have analysed the different factors responsible for this side-reaction. We first questioned the batch of the Wang resin that was used. Changing the batches as well as the suppliers (Novabiochem, Advanced ChemTech) did not affected the formation of the side-products. Improving the quality (by distillation) of the TFA used had no influence on the formation of the side-product. Dilution of the TFA with dichloromethane (TFA/CH_2Cl_2/anisole, 40:50:10) did not change the results. We also examined the effect of the position of the Trp residue in the peptide chain as well as the scavengers that were used (Table 1).

The side-product was obtained in significant amount when Trp was within the peptide chain (entries 2, 5, 12) or at the *N*-terminus (entries 1, 3, 4). Little or no side-reaction was obtained when Trp was linked to the resin as the *C*-terminal residue of the peptide (entries 6, 7, 9, 14, 15). This is in sharp contrast with the side-reaction reported by Atherton *et al.* [7] which occurs when Trp is in the *C*-terminal position. These authors described a tethering of the peptide to the resin support via the side chain of a *C*-terminal Trp residue through an intramolecular process as depicted in Figure 7.

The *N*-terminal protection is not involved in the side-reaction since unprotected dipeptide (entry 3) and the same Fmoc-protected dipeptide (entry 4) produced 25 and 29% of side-products, respectively. When Trp was directly linked to the Wang resin, either having a free amino group (entry 6) or Fmoc-protected (entry 7), no side-reaction was observed. Boc-protection of the indole nitrogen [8] totally prevented the side-reaction (entries 8 and 9).

The nature of the amino acid linked to the resin had no influence on the formation of the side-product. Switching from Glu (entry 3) to Ala (entry 10) only slightly decreased the rate of formation of the monoalkylated Trp-containing peptide SP1 and traces of dialkylated peptide SP2 were also detected.

The role of the scavenger also appeared to be essential. Replacing anisole (conditions I) (entry 10)

Table 1 Percentage of the Side-Products SP1 and SP2 Depending on Trp Position in Starting Peptides and TFA Cleavage Conditions (Scavengers)

Entry	Substrate	Cleavage	*P* (%)	SP1 (%)	SP2 (%)
1	H-**Trp**-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-Wang	I	75	25	
2	H-Ala-Ser-Gly-**Trp**-Ala-Gly-Glu-Wang	I	86	14	
3	H-**Trp**-Glu(O*t*Bu)-Wang	I	75	25	
4	Fmoc-**Trp**-Glu(O*t*Bu)-Wang	I	69	29	2
5	H-Ala-**Trp**-Glu(O*t*Bu)-Wang	I	75	21	4
6	H-**Trp**-Wang	I	98	2	
7	Fmoc-**Trp**-Wang	I	98	2	
8	H-**Trp**(Boc)-Glu(O*t*Bu)-Wang	I	100		
9	H-**Trp**(Boc)-Wang	I	98	2	
10	H-**Trp**-Ala-Wang	I	79	19	2
11	H-**Trp**-Ala-Wang	II	24	51	25
12	H-Ala-**Trp**-Glu(O*t*Bu)-Wang	III	56	38	6
13	Fmoc-Ala-Wang	IV	100	[a]	
14	Fmoc-Ala-**Trp**-Wang	V	96	4[a]	
15	Fmoc-Ala-**Trp**-Wang	VI	100		

I: TFA/anisole, 80:20 vol; II: TFA/ethanedithiol/triisopropylsilane, 80:10:10 vol; III: TFA; IV: TFA/Fmoc-Trp-OH (10 equivalents); V: TFA/anisole/Fmoc-Trp-OH; VI: TFA/anisole/indole.
P, product; SP1, side-product with an increased weight of 106; SP2, side-product with an increased weight of 2×106.
[a] No Fmoc-Trp-OH + 106.

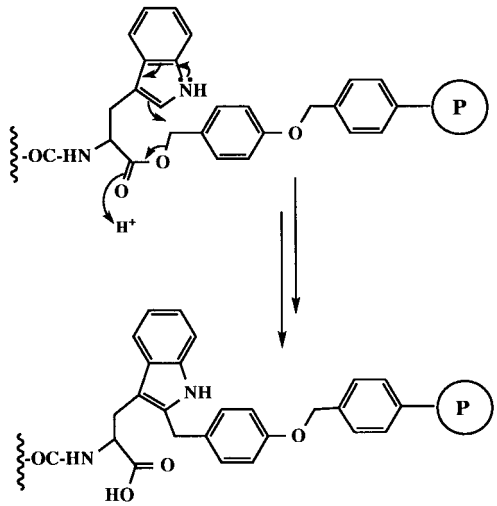


Figure 7 Intramolecular tethering of the peptide to the resin support via the side chain of a *C*-terminal Trp residue.

by ethanedithiol/triisopropylsilane (conditions II) (entry 11) significantly increased the rate of formation of both mono- and dialkylated peptides. Removal of the peptide from the resin without any scavenger (conditions III) (entry 12) increased the formation of the monoalkylated compound SP1 from 26% (entry 5) to 38% and the dialkylated compound SP2 from 4 to 6%.

We have investigated whether the Trp residue of Fmoc-Trp-OH in solution could be subjected to alkylation. No alkylation of Fmoc-Trp-OH could be detected when it was used as a scavenger (entry 13). Fmoc-Trp-OH (conditions V) or indole (conditions VI) were also added to the cleavage medium to establish whether they could catalyse the side-reaction. In these cases, 4% of the alkylated peptide was detected with Fmoc-Trp-OH (entry 14) and none with indole (entry 15).

It is worth noting here that replacing Trp with 7-azatryptophan [9,10] in the peptide H-Trp-Ala-OH led to totally different results. The TFA cleavage afforded a pure peptide with no side-product, due to the lower reactivity of the 2-position in the 7-aza case.

CONCLUSION

We have pointed out a side-reaction occurring during syntheses of several Trp-containing peptides on a Wang solid support. ^{1}H-NMR and MS allowed us to establish that an unanticipated alkylation by the linker occurred on the indole nucleus, whatever the scavenger used, and whatever the position of the Trp residue in the sequence, except in the *C*-terminal position.

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