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Monitoring and quantification on solid support of a by-product formation during peptide synthesis by Tof-SIMS

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Abstract

To evaluate the amount of pyroglutamic acid formation during the synthesis of glutamic acid containing peptides, resin beads were substituted by known amounts of Fmoc-Glu(O'Bu)-Phe-Ala-NH_2 and the corresponding side-product Pyr-Phe-Ala-NH_2 and directly analyzed by time of flight-secondary ion mass spectrometry. Detection and quantification of pyroglutamic acid was achieved without release of the peptide chains in solution.

In solid phase peptide synthesis, any side-reaction which might occur at any stage of the synthesis depending on the nature of the amino acid involved[1] is commonly evidenced after release of the growing chain from the solid support at the final step and subsequent analysis by conventional spectroscopic methods such as NMR and mass spectrometry (fast atom bombardment,[2] electrospray ionization[3] and matrix assisted laser desorption ionization[4]).

However, an analytical method allowing analysis of peptides still anchored to the insoluble polymer would provide a step by step monitoring of the whole synthetic process where quantitative yields are expected. Such control would also be highly suited in combinatorial chemistry for the identification of peptide libraries generated by the mix and split method since each resin bead is functionalized by a unique sequence.[5] In addition to solid state NMR[6] (cross polarization/magic angle spinning) and IR,[7] mass spectrometry was applied to the in situ monitoring of supported synthesis. Compounds anchored via a photolabile linker were simultaneously cleaved and ionized upon MALDI irradiation.[8] As such a method was restricted to syntheses involving photolabile handles, we investigated the time of flight-secondary ion mass spectrometry[9] (Tof-SIMS) for the direct control of peptide solid phase synthesis on various supports including polystyrene,[10] polyamide[11] and plastic pins.[12]

In SIMS, the solid sample is directly bombarded by a beam of primary ions releasing secondary ions which are characteristic of its surface. Thus, the most accessible N-terminal residue exhibited abundant

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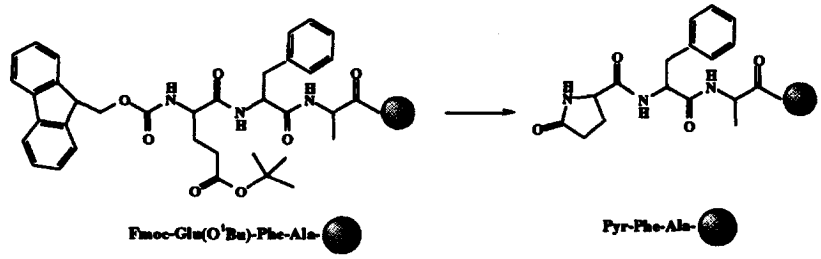
ions enabling deprotection and coupling reactions to be followed.[10–12] Besides, amino acids which were linked to the polymer by either covalent or ionic bonds were differentiated according to the nature of the ions produced upon the SIMS bombardment.[13] Finally, our method was validated by the identification of beads in mixtures issued from mix and split syntheses by imaging Tof-SIMS studies.[14] To complement our studies, we then investigated the detection and quantification of side-products in solid phase peptide synthesis. The cyclization of glutamic acid (Glu) into pyroglutamic acid (Pyr) (Scheme 1) was chosen as a model.



Scheme 1.

The tripeptide Fmoc-Glu(O^{t}Bu)-Phe-Ala-NH_2 and the corresponding chain Pyr-Phe-Ala-NH_2 were simultaneously built on polystyrene beads according to standard protocols.[15] Various batches were prepared where the percentage of the pyroglutamic acid containing sequence was set up at 0, 30, 70 and 100%. Aliquots of resin were then sampled and submitted to HF treatment in order to check by conventional HPLC methods the respective amounts of each chain prior to Tof-SIMS experiments[16] as shown in Table 1.

Table 1
Studied samples

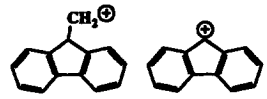
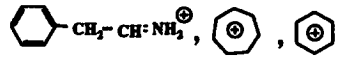
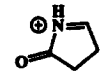
Fmoc-Glu(O^{t}Bu)-Phe-Ala / Pyr-Phe-Ala	Theoretical percentage of Pyr-Phe-Ala	Measured percentage of Pyr-Phe-Ala*	Measured ratio I(84) / I(120)	Measured ratio I(84) / I(115)
Batch A	0	0	0.33	0.30
Batch B	30	38	1.00	0.72
Batch C	70	75	1.00	1.14
Batch D	100	100	1.62	1.85

* Relative values (ratios between the Pyr-Phe-Ala-NH_2 and Fmoc-Glu(O^{t}Bu)-Phe-Ala-NH_2 peaks by HPLC at 214 nm on C_{18} column (4.6 x 50 mm), 1 ml/min, 0 to 100% CH_3CN + 0.1% TFA in 15 min)

According to our previous studies,[10–12] the amino acids were detected as immonium ions in the positive mode and the protecting groups exhibited characteristic ions as listed in Table 2. Indeed, the phenylalanine, glutamic acid and pyroglutamic acid residues were identified by the ions at *m/z* 120, 102 and 84 Th, respectively. The intensity of the first ion (*m/z* 120) should remain constant whereas the two other (*m/z* 102 and 84) should vary according to the percentage of the Pyr-Phe-Ala-NH_2 tripeptide. Thus, the ion at *m/z* 120 Th was chosen as a probe to quantify the pyroglutamic acid by-product. The Tof-SIMS positive spectra of few resin beads from batches **A** to **D** are displayed in Fig. 1a–d, the ion at *m/z* 115 Th being attributed to polystyrene.

The fact that the ion at *m/z* 84 Th was detected when the pyroglutamic acid was not present (Fig. 1a) was explained by fragmentation of the glutamic acid immonium ion (*m/z* 102) losing a molecule of H_2O. The intensity of the ion at 84 Th was increased from batches **A** to **D** whereas the ion at *m/z* 102

Table 2
Characteristic ions detected in the positive mode

Structure	Ion	m/z
Fmoc		179 / 165
^{t}Bu	$C_4H_9^+$	57
Phe		120 / 91 / 77
Pyr		84

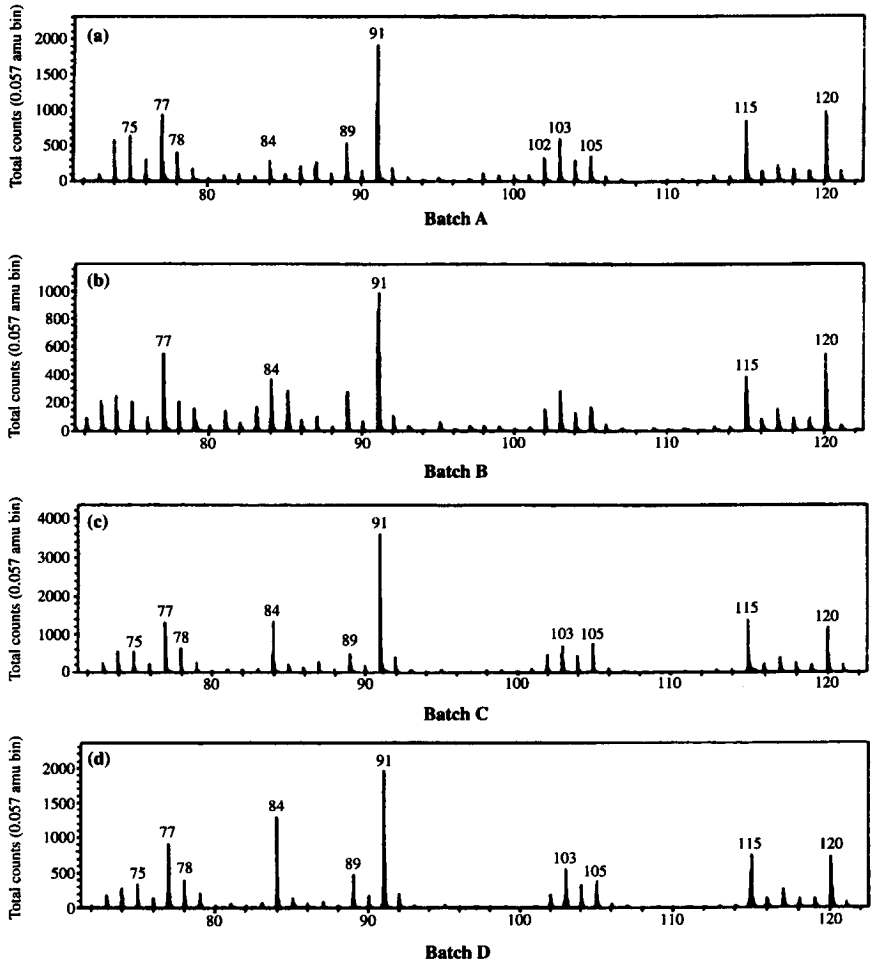
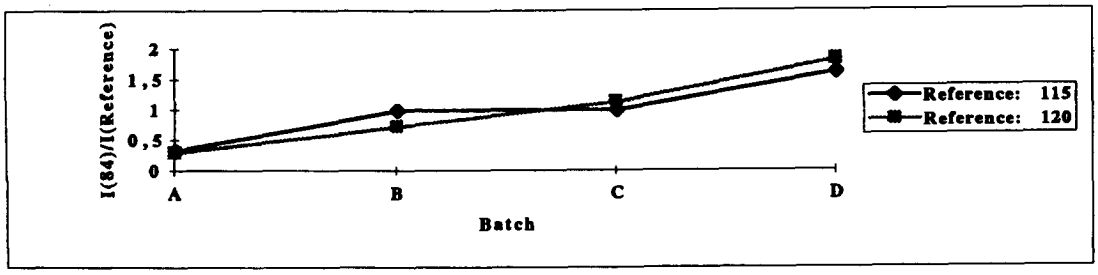


Figure 1.

simultaneously disappeared as expected (Fig. 1a–d). More precisely, the measured ratios between the abundance of the immonium ions of pyroglutamic acid (*m/z* 84 Th) and phenylalanine (*m/z* 120 Th) or polystyrene (*m/z* 115) are gathered in Table 1. The two curves shown in Scheme 2 are parallel, meaning that the presence of a phenylalanine residue was not compulsory for quantitative analysis. Besides, the ions related to the Fmoc protection (*m/z* 165 and 179 Th in the positive mode) and to the ^{t}Bu moiety (*m/z* 57) diminished with an increasing amount of Pyr-Phe-Ala-NH_2 (data not shown).



Scheme 2.

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15. The tripeptides were synthesized on 1 g of MBHA resin (Novabiochem, Meudon, France) loaded at 0.8 mmol/g. For each coupling, 2 equivalents of the Fmoc-protected amino acid dissolved in Analar grade dimethylformamide (DMF) were activated by 2.2 equivalents of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and 2.2 equivalents of diisopropylethylamine (DIEA) before shaking with the resin for 2 hours. After washings with DMF, CH_3OH, CH_2Cl_2 and DMF, the Fmoc protection was removed by two treatments (3 min and 15 min) with a piperidine solution in DMF (20%, v/v). Fmoc-Ala-OH, Fmoc-Phe-OH and a mixture of Fmoc-Glu(O'Bu)-OH with Pyr-OH (quantity adjusted according to the percentage of desired by-product) were successively coupled.
16. The resin beads were deposited on an adhesive foil and the mass spectra were recorded on a Tof-SIMS spectrometer designed by the Charles Evans company according to a procedure already described.[10]