Pergamon

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Tetrahedron Letters 44 (2003) 8673–8676

TETRAHEDRON LETTERS

Aspartyl methyl ester formation via aspartimide ring opening: a proposed modification of the protocols used in Boc- and Fmoc-based solid-phase peptide synthesis

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Received 16 July 2003; revised 11 September 2003; accepted 18 September 2003

Abstract—Aspartimide formation is still an unresolved problem in the solid-phase peptide synthesis of aspartic acid-containing peptides, following either Boc- or Fmoc-based synthetic strategies. α-Aspartyl peptides of high purity can be obtained, despite aspartimide formation, by incorporating an additional step in the Boc- and Fmoc-based solid-phase peptide synthesis protocols, consisting of treatment of the peptide-resin with methanol in the presence of 2% DIEA (v/v) for 15 min immediately after completion of the peptide chain elongation.

A well documented problem in the synthesis of aspartic acid-containing peptides and proteins is aspartimide formation.[1–8] This undesirable reaction has been proved to occur under both acidic and basic conditions, but in the latter case is faster.[3] The imide ring formed is susceptible to opening by nucleophilic attack on either of the carbonyl carbons, resulting in the two deprotected aspartate regioisomers, but with the isomer containing the β-amide bond being the main by-product. The rate of aspartimide formation is dependent on the β-carboxyl protecting group,[2,3] the acid[2] or base[9] used during the synthesis, and the peptide sequence.[3,10]

In Boc-based solid-phase peptide synthesis, acid-catalyzed aspartimide formation has been reported[2] to occur in the presence of both strong acids such as hydrogen fluoride (HF) and trifluoromethanesulfonic acid–trifluoroacetic acid (TFMSA–TFA), and milder acids such as TFA. Under these conditions, the extensively used (in Boc methodology) benzyl ester (Bzl) protecting group, is highly susceptible to aspartimide formation, while the cyclohexyl (cHex) group provides significantly lower rates. In Fmoc-based peptide chemistry, despite the absence of strongly acidic conditions and the use of the more hindered *tert*-butyl (Bu^t) protecting group, aspartimide formation also occurs via basic catalysis, usually during the prolonged treatment with piperidine (for Fmoc group deprotection) during the synthetic cycles.[5–8] In this case, imide ring-opening also gives α- and β-piperidides.

Various methods have been developed in order to minimize aspartimide formation, most of them being applicable to the Fmoc synthetic approach. The use of additives such as 1-hydroxybenzotriazole (HOBt) or 2,4-dinitrophenol (Dnp) with the base,[11] the protection of the peptide bond with the *N*-2-hydroxy-4-methoxybenzyl (Hmb) group,[8] and the use of side-chain protecting groups that give better protection against aspartimide formation have been reported.[12,13] However, acid- or base-catalysed ring closure seems to be a much more complicated problem since it is also dependent on the amino acid which follows the aspartic residue in the peptide sequence, as well as by the conformation of the peptide.[5] As well as Gly, Ser and Ala which favor aspartimide formation, His, Asn and Thr have also been reported to produce extensive imide formation.[2,3]

After the completion of the solid-phase synthesis of the peptide KKFDRGALHDENT (MW = 1530.5) in our laboratory following the Fmoc/Bu^t synthetic methodol-

Keywords: aspartimide; α-, β-aspartyl peptides; solid-phase protocols; peptide synthesis protocols; SPPS protocols; α-, β-aspartyl methyl esters.

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doi:10.1016/j.tetlet.2003.09.163

ogy,[†] the analysis of the crude products by LC–ESMS revealed a significant amount of three by-products (Fig. 1). The first by-product (~10%) suggested the formation of the aspartimide peptide (found $(M+2H^+-18)/2=757.44$, expected 757.25) and the second the opening of the imide ring and the subsequent α- and β-piperidides (~4.7%) (found $(M+2H^+67)/2=799.90$ expected = 799.75). We hypothesized that the third unexpected by-product (~5%) could be due to the formation of a methyl ester (found $(M+2H^+14)/2=$ 773.34, expected 773.25), apparently by the extensive use of methanol as a washing solvent during the synthesis.

Aiming to identify and elucidate the origin and the mechanism of formation of the third by-product ($(M+2H^+14)/2=773.34$), we decided to test the following: i) aspartimide ring opening by nucleophilic attack of methanol and, ii) methyl ester formation by transesterification of aspartic acid β-esters. Aspartimide ring opening by methanol was studied by ESMS, using the HPLC purified fraction consisting of two by-products, the aspartimide peptide and the methyl ester peptide, with the former being the main component (Figs. 1 and 2A). Samples of this fraction were dissolved in (1) acetonitrile (MeCN)/0.2% aq. NH_3 (45/55, v/v) as control sample, (2) methanol, (3) methanol containing *N*,*N*-diisopropylethylamine (DIEA) 2% (v/v), and (4) methanol containing piperidine 2% (v/v). The reaction was monitored by ESMS at times (a) after 15 min, (b) after 30 min and (c) after 24 h. Complete aspartimide hydrolysis was achieved within 30 min in MeCN/0.2% aq. NH_3 (45/55, v/v). In the presence of methanol, almost nothing changed within the first 15 min. However, after 30 min, although the aspartimide peptide was still the main component, the by-product had significantly increased $(M-2H^++14)/2=770.70$, expected 771.25). The composition of the sample changed dramatically after 24 h. The M+14 by-product became the main component, while the aspartimide peptide concentration was less than 10%. On the contrary, almost complete transformation to α-, and β-aspartyl methyl esters was observed after 15 min in the methanolic solution containing DIEA 2% (v/v) (Fig. 2B) and that containing piperidine 2% (v/v). In order to exclude the possibility that the peptide methyl esters obtained were formed during peptide synthesis, either by transesterification of the aspartic β-, or glutamic γ-carboxyl Bu^t esters, we studied Fmoc-Asp(OBu^t)-OH, and Fmoc-Glu(OBu^t)-OH in methanol, and methanol containing DIEA 2% (v/v). In both cases the Bu^t esters were stable even after 24 h. From the above findings, we concluded that the M+14 by-product is an aspartyl methyl ester of the peptide, which is mainly formed by the aspartimide ring-opening during washing with methanol.

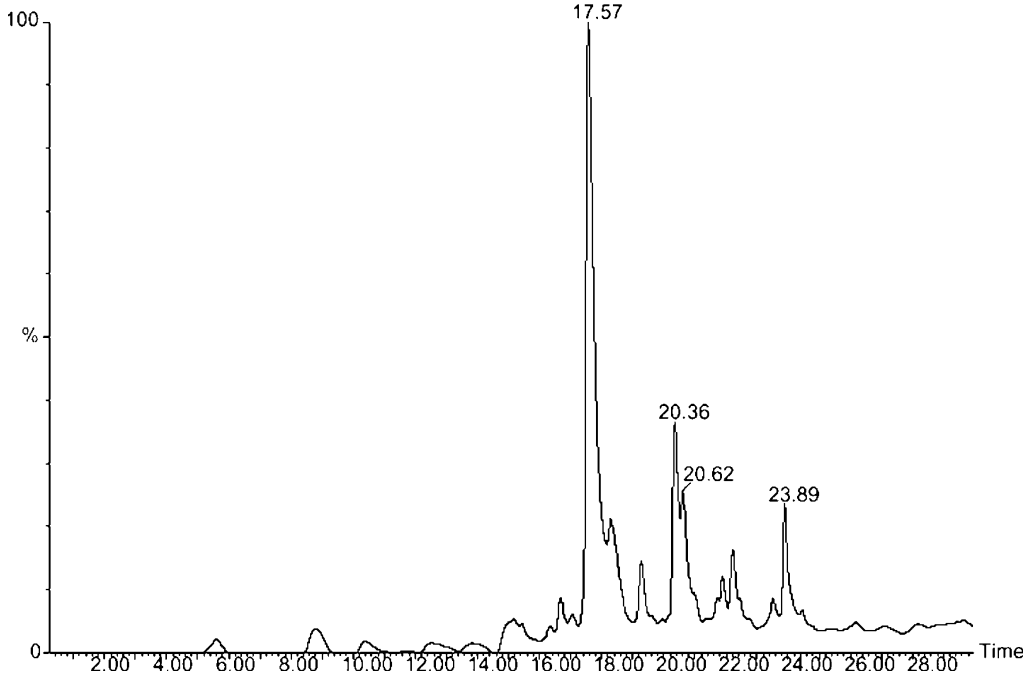

Figure 1. HPLC profile of the crude KKFDRGALHDENT peptide. HPLC elution conditions: (A) H_2O–0.1% TFA; (B) MeCN–0.1% TFA; linear gradient: 95% A to 70% A in 30 min. The major peak (t_R ~17.57 min) corresponds to the expected peptide, the peak at t_R ~20.36 min corresponds to the aspartimide (M–18), the peak at t_R ~20.62 min corresponds to the aspartyl methyl ester peptides (M+14), and the peak at t_R ~23.89 min corresponds to the aspartyl piperidide peptides (M+67).

[†] The synthesis was carried out using Fmoc-Thr(Bu^t)-Wang resin (substitution 0.63 mmol/g resin). A synthetic cycle consisted of the following steps: (i) deprotection of the Fmoc group: 20% piperidine/DMF (DMF, *N*,*N*-dimethylformamide), (ii) washings: DMF, MeOH, DMF, (iii) coupling: Fmoc-A.A.-OH/TBTU/HOBT/DIEA/mmol peptidyl-resin 3/3/3/9/1, 2 h (TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate), (iv) washings: DMF, MeOH, DMF. The peptide was cleaved from the solid support with a 95% TFA/2.5% H_2O/2.5% TIS solution (TIS, triisopropylsilane).

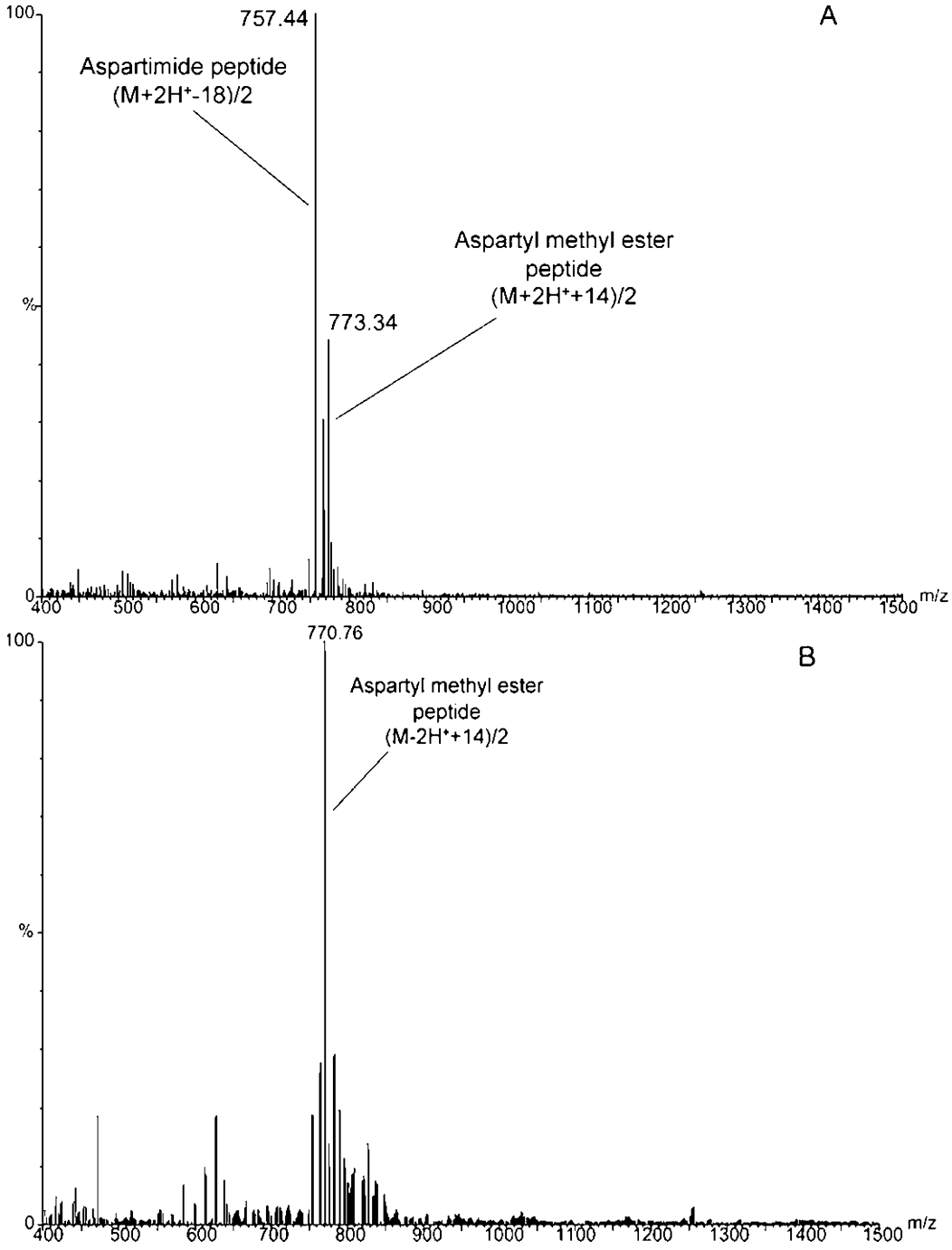

Figure 2. ESMS spectra of the isolated fraction containing the by-products aspartimide and aspartyl methyl ester peptides (see also Fig. 1) in MeCN/0.2% aq. NH_3 (45/55, v/v) immediately after preparation of the sample recorded in the positive mode (A), and in methanolic solution containing 2% DIEA, after 15 min recorded in the negative mode (B).

Taking advantage of these results and the fact that aspartyl α- and β-methyl esters in peptides can be more easily purified from the target peptide (Fig. 1) than the α- and β-peptides formed from aspartimide hydrolysis during either the cleavage step or the HPLC purification (in both cases acidic aqueous conditions are used), we decided to test the stability of the β- and γ-carboxyl protecting groups of Boc-Asp(OcHex)-OH, Boc-Asp(OBzl)-OH, Boc-Glu(OcHex)-OH and Boc-Glu-(OBzl)-OH in methanolic solution containing 2% DIEA. These amino acids are the only ones that could be affected by methanol in the presence of DIEA (transesterification). All the amino acid derivatives tested were stable in methanol containing 2% DIEA even after 1 h. Their stability under these conditions provides the possibility of introducing an additional and easily performed step in the Boc- or Fmoc-based peptide synthesis protocols. This step is proposed since aspartimide formation is still an unresolved problem in solid-phase peptide synthesis of aspartic acid-containing

peptides following either the Boc- or Fmoc-based synthetic strategies. Treatment of the peptide resin just before removal of the solid support will enhance the resolution of the target peptide and the by-products by HPLC.

In conclusion, our results suggest that aspartimide ring-opening by methanol in the presence of a small amount of base is a quantitative reaction which results in α- and β-aspartyl methyl ester peptides. The treatment of these peptides with methanol in the presence of 2% DIEA (v/v) for 15 min, immediately after completion of the peptide chain elongation, is proposed to be included as an additional step in both Boc- and Fmoc-based solid-phase peptide synthesis protocols. This step will facilitate peptide purification and prevent the formation of generally unresolved α- and β-aspartyl peptide mixtures.

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