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FORMATION OF ASPARTIMIDE PEPTIDES IN Asp-Gly SEQUENCES

Ernesto Nicolás, Enrique Pedroso, and Ernest Giralt*

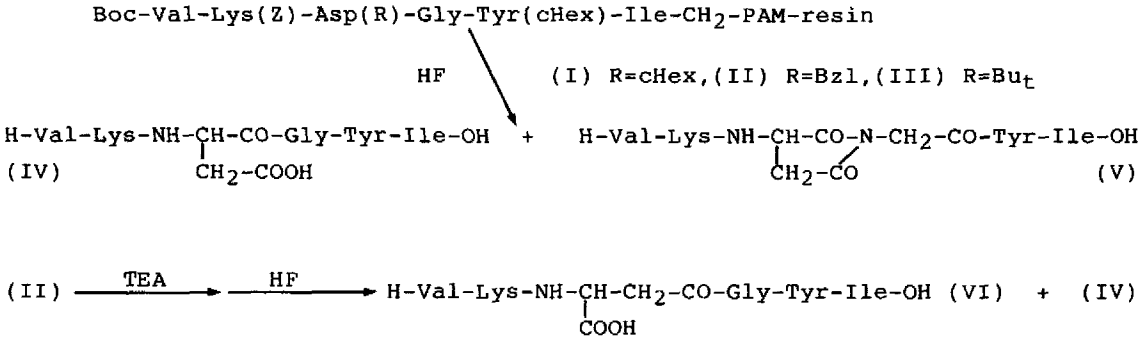
Universitat de Barcelona; Departament de Química Orgànica
Facultat de Química; Martí i Franquès 1, 08028. Barcelona (Spain)

Summary: *The behaviour of protected H-Val-Lys-Asp-Gly-Tyr-Ile-OH towards imide formation was evaluated. The use of a carboxylic acid temporary protecting group is not a safe strategy for avoiding aspartimide formation during HF treatment. Piperidine showed a marked tendency to form aspartimides during Fmoc deprotection.*

Formation of aspartimide peptides is perhaps one of the most characteristic examples of sequence dependent undesirable processes in solid phase peptide synthesis. Although these by-products (formed in the main, during acidic cleavage of the peptide from the resin) can be hydrolysed to obtain the desired peptide, the concomitant formation of significant amounts of the β-aspartyl peptide renders the separation and characterisation of the target peptide difficult (1). This side reaction is especially critical in sequences Asp-X where X is glycine, serine or threonine (2-10), and depends on the protecting group used for the side chain of aspartic acid; in this regard, the use of the benzyl group has given rise to the poorest results.

It has been reported that a good strategy for avoiding aspartimide formation is the use of a temporary protecting group which allows the acidolysis step of the peptide-resin bond (generally by treatment with hydrogen fluoride) to be carried out with the carboxyl group already deprotected (11,12). Recently we have used this approach in the synthesis of H-Val-Lys-Asp-Gly-OH corresponding to the 1-4 fragment of toxin II of the scorpion Androctonus australis Hector (13). However, the appearance in the literature of new acid labile protecting groups for aspartic acid (14-17) (e. g. the cyclohexyl group), the increasing application of strategies based on Fmoc aminoacids, and the reports of alternative methods for the cleavage of peptide-resin bonds have prompted us to carry out a complete study of this topic. For this purpose, we have evaluated the formation of aspartimide under different conditions in the synthesis of H-Val-Lys-Asp-Gly-Tyr-Ile-OH (1-6 fragment pertaining to the toxin above mentioned). We have chosen for this study the cyclohexyl (I), benzyl (II) and t-butyl (III) groups (the last as a temporary protecting group), perhaps the most representative series of aspartic acid side chain protecting groups.

The synthesis of the three peptide-resins was performed on a PAM resin (18), using as protecting groups the cyclohexyl group for tyrosine and benzy-



loxycarbonyl group for lysine (scheme). When the aspartic acid side chain was protected as a benzyl- or cyclohexyl-esters the conventional Boc strategy was used, whereas the fourth and fifth aminoacids were incorporated as their Fmoc derivatives when t-butyl was the protecting group (2.5 excess in all cases). The deprotection of the Boc group was carried out with a 30% solution of TFA in DCM over 30 min. (the same for the t-butyl ester); when Fmoc was the protecting group a 20% solution of piperidine in DMF, over 10 min, was used; all couplings were ninhydrin negative in two hours.

A sample of peptide-imide V was prepared by treating II with 10% of TEA in DCM for 24 hours at room temperature (scheme). After HF cleavage IV and VI were separated by ion-exchange chromatography (CMC, ammonium acetate buffer; pH 5.5, 5 mS), yielding 50% of each (82% yield from peptide-resin). These were characterised by aminoacid analysis, ^{1}H NMR and IR spectroscopy and mass spectrometry (FAB). After a careful adjustment of the experimental conditions, reversed phase HPLC allowed the separation and quantification of artificial mixtures of compounds IV, V and VI (19). This procedure has been used for the characterisation of the mixtures obtained after acidic treatment of peptide-resins described below.

Treatment of peptide-resins I, II and III with anhydrous HF under standard conditions (see table I, first row) yielded peptide IV with a constant amount of aspartimide V (5-6 %). The non-dependence of the extension of the secondary reaction upon the nature of the acid labile protecting group suggests that the 5-6 % of V comes from the cyclisation of the free carboxylic acid of compound IV during the HF treatment. To confirm this, a purified sample of IV was treated with anhydrous HF and analysed by HPLC. A similar amount (5.9 %) of V was detected.

These results show that, for our model peptide, the use of a temporary protecting group, as t-butyl, for the aspartic acid side chain in order to minimise aspartimide formation is not an efficient approach. Formation of aspartimide takes place whether or not the aspartic acid is protected and apparently takes place on the unprotected peptide.

As mentioned above we have extended our study to evaluate the influence of the conditions used for the peptide-resin bond acidolysis on the amount of aspartimide formation. As shown in table I we have compared the behaviour of III in the so-called "high HF" conditions (20) (using either p-cresol or anisole as scavengers), "low-high HF" and TFMSA. The worst result (12 % of aspartimide compared to 5-6 % using high HF) arose from the use of low-high HF and may be due to the long-time exposure of the peptide to the acid. Also, long treatment with a strong acid such as TFMSA gave an intermediate result (8.6 %).

Finally, we have tried to evaluate the extent of the side reaction during the growth of the peptide on the resin, especially with regard to the influence of DIEA or piperidine as basic catalysts of this process. From our experimental results (table I) we can conclude that DIEA has practically no effect on the formation of the by-product. In contrast, the more basic and more nucleophilic piperidine strongly promotes aspartimide formation, especially for II with a ca. 100 % conversion after 10 min. For I, the loss of the cyclohexyl group is appreciably lower but significant, involving 2.5 % of imide formation per deprotection step. As might be expected, t-butyl protection is much more resistant to nucleophilic attack probably due to the poor leaving group. Nevertheless, the extent of the side reaction (0.3 % per deprotection cycle) can not be considered negligible in a long synthesis.

	(I)	(II)	(III)
HF/10% p-cresol, 1 h, 0°C	6.0	5.5	5.3(5.2(a))
HF/10% anisole, 1 h, 0°C	n.d.(c)	n.d.	5.9
HF/DMS/p-cresol (6.5:2.5:1), 2 h, 0°C followed by HF/10% p-cresol, 1 h, 0°C	n.d.	n.d.	12.0
TFMSA/TFA/DMS/p-cresol(10:50:30:10),4 h, 0°C	n.d.	n.d.	8.6
5% DIEA in DCM, 10 h, r.t.	6.5	6.4	6.4
20% Piperidine in DMF, 4 h, r.t.(b)	67.5	100.0	11.0

(a) Obtained after HF treatment of pure peptide. (b) The results shown are the sum of the quantities of aspartimide and piperidides formed, as identified by aminoacid analysis and enzymic hydrolysis after HPLC collection. (c) not determined.

Table I. Percentage amounts of V found in the synthesis of VI using different protecting groups, and different acidic and basic conditions

One must take into account that aspartimide formation, like other secondary reactions, is probably highly sequence dependent but, nevertheless, these results raise some concerns as to the suitability of the widely used "Fmoc" peptide synthesis strategy for the step-wise synthesis of long peptide chains with sequences like Asp-Gly, prone to aspartimide formation, near the C-terminal end of the chain.

Abreviations: Boc, t-butoxycarbonyl; cHex, cyclohexyl, CMC, carboxymethylcelullose; DCM, dichloromethane; DIEA, diisopropylethylamine; DMS, dimethyl sulphide; FAB, fast atom bombardment; Fmoc, fluorenylmethoxycarbonyl; HF, hydrogen fluoride; HPLC, high performance liquid chromatography; tBu, t-butyl; TEA, triethylamine; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulphonic acid; Z, benzyloxycarbonyl.

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(1) This side reaction may often go unnoticed unless care is taken in the purification and characterisation conditions of the by-products. It must be remembered that both α and β peptides give aspartic acid by acid hydrolysis.

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