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Acid-Mediated Prevention of Aspartimide Formation in Solid Phase Peptide Synthesis

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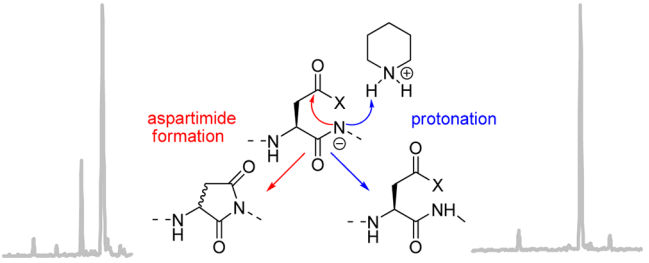
ABSTRACT



Aspartimide formation is one of the major obstacles that impedes the solid phase synthesis of large peptides and proteins. Until now, no cost-effective strategy to suppress this side reaction has been developed. Here it is demonstrated that addition of small amounts of organic acids to the standard Fmoc cleavage agent piperidine efficiently prevents formation of aspartimide side products. This effect is shown to be virtually independent of the acid strength.

Advances in molecular biology have paved the way for patient-specific therapies. Many of these novel drugs are proteins, which unfortunately show unfavorable pharmacokinetic behavior as a consequence of their high molecular weight. The *downsizing* of such proteins to their effective chemophore, such as an antibody to its epitope recognition site,[1] would provide an ideal strategy for closing the gap between drugs of a molecular weight below 500 Da, corresponding to the traditional understanding of druglikeness, and today's proteinaceous drug candidates. Furthermore, this strategy would be applicable for chemical synthesis techniques, thereby avoiding complications that arise with drug approval of recombinant proteins.

Solid phase peptide synthesis (SPPS), originally developed by Merrifield,[2] is a key technology for drug synthesis within the *downsizing* development strategy. The synthesis of large peptides by SPPS can be achieved by chemical ligation; however, side reactions can hamper the overall synthesis leading to decreased yields and/or quality. Aspartimide is a major pitfall in Fmoc-based SPPS.[3] Its formation, which can either be acid or base catalyzed, occurs while the piperidine-catalyzed Fmoc cleavage of peptides containing aspartic acid.[4] The propensity of aspartimide formation mainly depends on the aspartate carboxyl neighboring residue.[5] Aspartimide is, therefore, the result of an attack of an amidate species at the carbonyl carbon of the OtBu protected side chain carboxylate of aspartic acid (Scheme 1).

Many approaches have been developed to suppress the formation of aspartimides. Mergler et al. achieved significant improvements by using sterically demanding Asp side chain protection groups such as 3-methylpent-3-yl (OMpe)[6] and β-2,3,4-trimethyl-pent-3-yl.[7] Replacement

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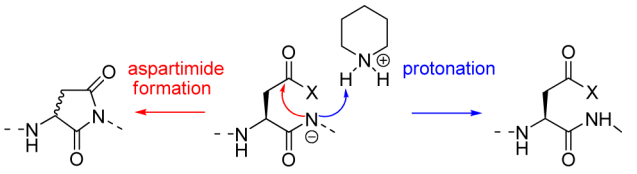
Scheme 1. Proposed Mechanism of Aspartimide Formation and Its Piperidinium Ion Mediated Suppression



of piperidine (pK_a = 11.12) with the milder base piperazine (pK_a = 9.73) can also reduce aspartimide formation, however, at the cost of the reaction rate.[8] This can be overcome by microwave heating;[9] however this must be carefully controlled to avoid other side reactions.[10] Complete prevention of aspartimide formation can be achieved using *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) as a backbone protecting group.[11] However, Hmb protected amino acids show low coupling efficiencies and so must be used as dipeptide building blocks. Furthermore, Hmb-protected building blocks are difficult to synthesize and only the dipeptide containing glycine (Fmoc-Asp(tBu)-(Hmb)Gly) is commercially available. The addition of hydroxybenzotriazole (HOBt) to the piperidine deprotection agent has been shown to slightly reduce the formation of aspartimide in Fmoc-based SPPS. However, commercial HOBt hydrate contains ~12% water, and its explosive potential together with the restricted availibility and light sensitivity would cause problems for its routine application in SPPS. Interestingly, 2,4-dinitrophenol[12] and ethyl 2-cyano-2-(hydroxyimino)-acetate (Oxyma)[13] have also been described as additives for reducing aspartimide formation. This effect might be attributed to their acidic character. Consequently, suppression of aspartimide formation by adding small amounts of organic acids to the deprotection agent piperidine was studied.

PreS9-33-y, a 26-mer peptide derived from the HBV surface antigen (NPLGFFPDHQLDPAFRANTANPDWDy-NH_2), was used to analyze aspartimide formation. This peptide contains three sites that are prone to aspartimide formation (Asp-D-Tyr, Asp-Trp, and Asp-His). The fourth Asp containing motif (Asp-Pro) is hindered due to the steric nature of proline. The motif Asp-Trp is not susceptible to aspartimide formation. As Asp-X motifs containing a D-amino acid are highly prone to aspartimide formation, this is the most probable site of formation. The close proximity of this motif to the resin may be an explanation for the absence of piperidide formation due to steric hindrance preventing the attack of piperidine.[14]

To determine the influence of acids on the formation of aspartimide in the deprotection step, PreS9-33-y was synthesized either with or without 5% formic acid (v/v in piperidine). The synthesis under standard conditions (no formic acid) led to a massive occurrence of aspartimide (Figure 1).

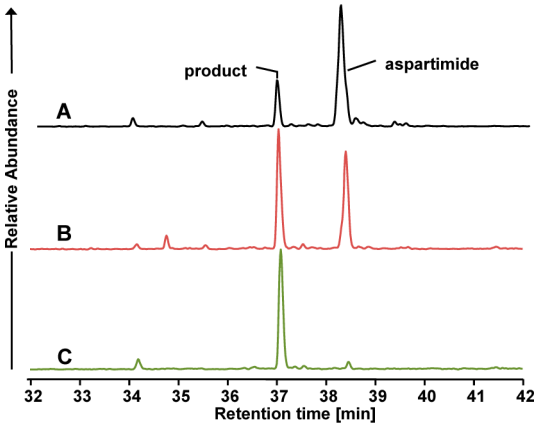


Figure 1. Analysis of aspartimide formation during solid phase synthesis. Reversed-phase HPLC of crude PreS9-33-y synthesized under (A) standard conditions, (B) using Fmoc-Asp-(OMpe)-OH and (C) with 5% formic acid.

In a second synthesis, the peptide was synthesized using Fmoc-Asp(OMpe)-OH. Under these conditions HPLC analysis revealed a decrease in aspartimide formation, although high levels of aspartimide remained (50% as compared to 81%). Finally, addition of 5% formic acid to piperidine significantly reduced aspartimide formation (13% aspartimide). Thus, addition of formic acid was superior to the usage of the sterical hindered side chain protecting group OMpe.

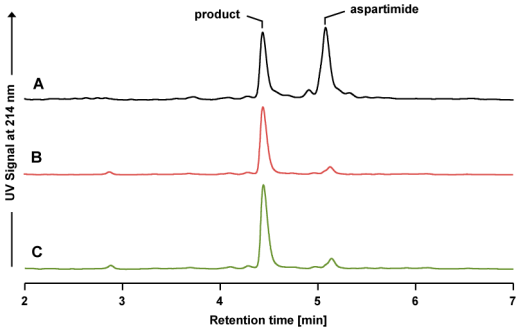


Figure 2. Analysis of aspartimide formation by piperidine treatment of resin bound PreS9-33-y. RP-HPLC analyses of a sample incubated with 20% piperidine (A), 20% piperidine with 0.1 equiv formic acid (B), and the untreated control (C).

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To simulate deprotection conditions of prolonged synthesis, the resin bound peptide, synthesized in the presence of formic acid, was incubated with piperidine (20% v/v in DMF) alone or with piperidine containing 0.1 equiv of formic acid at 50 °C for 60 min. Under these conditions, treatment with piperidine alone caused the formation of 59% aspartimide (Figure 2). Addition of 0.1 equiv of formic acid, on the other hand, completely prevented any further aspartimide formation (13% aspartimide).

A series of 18 different acids covering the range of pK_a values of common organic acids (0.1 equiv each) were added to 20% piperidine in DMF to establish whether prevention of aspartimide formation depends on the acid used. Analytic HPLC analysis did not show a correlation between the pK_a and the efficiency of aspartimide suppression (Table 1). With apparent independence from their pK_a values, all acids were shown to suppress aspartimide formation compared to the untreated sample. Citric acid and oxalic acid formed precipitates in the cleavage solution. Attempts to use inorganic acids such as HCl and sulfuric acid failed due to the low solubility of their piperidinium salts in piperidine. Interestingly, the amino sulfonic acid taurine gave results similar to those obtained with the common organic acids. The concentration dependency was determined using different equivalents of formic acid, HOBt, 4-nitrophenol, and hexafluoroisopropanol to identify optimal concentions for solid phase protein synthesis (Table 2). While low concentrations of formic acid, hydroxybenzotriazole (HOBt), and 4-nitrophenol dramatically suppressed aspartimide formation, HFIP did not show a significant effect, indicating that effects other than the pK_a (values determined from aqueous media) are responsible for this phenomenon. A strong base, 1,8-diazabicyclo[5.4.0]undec-7-en (DBU), was tested to simulate the even harsher Fmoc cleavage conditions (2% DBU and 20% piperidine in DMF) used to deprotect cleavage-resistant Fmoc groups.[15] This led to 67% aspartimide (compared to 17% with formic acid). Using DBU, the piperidide, which was not observed in the syntheses with pure piperidine, was formed in ~1.6% yield as determined by integration of the respective LC/MS signals. Using 0.1 equiv of formic acid, only 0.4% of the piperidine adduct could be detected. Furthermore, a second aspartimide was formed after treatment with DBU (26%), which was not visible in the sample treated with additional formic acid (0.6%). Thus, small amounts of acid prevented aspartimide formation even under very harsh deprotection conditions. Small amounts of formic acid (5% v/v in

Table 1. Piperidine-Catalyzed Aspartimide Formation of Resin Bound PreS9-33-y under the Influence of Different Organic Acids

		relative yields [%]	
acid	pK_{a1}, pK_{a2}	product[a]	aspartimide[a]
TFMSA[b]	−13	85.1	8.1
TFA	0.30	86.8	13.2
trichloroacetic acid	0.77	85.3	14.7
PTSA	0.7	88.9	11.1
dichloroacetic acid	1.25	90.1	9.9
taurine	1.5, 8.74	84.1	15.9
chloroacetic acid	2.87	88.4	11.6
formic acid	3.75	87.3	12.7
2,4-dinitrophenol	4.09	87.3	12.7
ascorbic acid	4.17, 11.6	88.2	11.8
benzoic acid	4.2	88.2	11.8
HOBt	4.6	88.9	11.1
acetic acid	4.75	87.9	12.2
4-nitrophenol	7.2	87.2	12.8
HFIP	9.6	67.9	32.1
phenol	10.0	88.1	11.9
benzenesulfonamide	10.1	88.2	11.8
20% piperidine		41.3	58.7
untreated		88.2	11.8

[a] Determined by RP-HPLC (UV detection at 214 nm). [b] Trifluoromethanesulfonic acid (TFMSA) caused the formation of side products besides aspartimide.

Table 2. Concentration Dependent Suppression of Piperidine Mediated Aspartimide Formation of Resin Bound PreS9-33-y by Different Acids

		relative yields [%]	
acid	mol equiv relative to piperidine	product[a]	aspartimide[a]
formic acid	1	93.6	6.4
	0.5	91.2	8.8
	0.2	90.6	9.4
	0.1	91.6	8.4
	0.05	87.8	12.2
	0.01	87.8	12.2
HOBt	1	90.6	9.4
	0.5	90.1	9.9
	0.2	88.8	11.2
	0.1	86.2	13.8
	0.05	85.6	14.4
	0.01	80.8	19.2
4-nitrophenol	1	92.7	7.3
	0.5	92.3	7.7
	0.2	90.3	9.7
	0.1	88.7	11.3
	0.05	87.1	12.9
	0.01	82.2	17.8
HFIP	1	92.5	7.5
	0.5	88.6	11.4
	0.2	74	26
	0.1	64.4	35.6
	0.05	54.3	45.7
	0.01	60	40
no acid	0	41	59

[a] Determined by RP-HPLC (UV detection at 214 nm).

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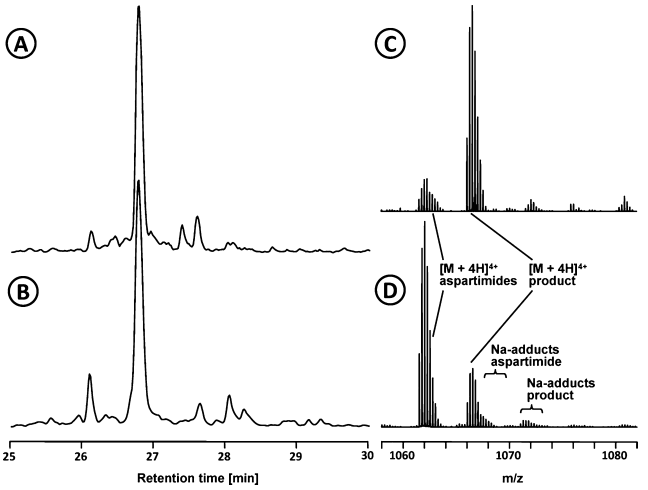

Figure 3. Product region of the UPLC-MS analysis of the crude reaction product of the SPPS of parathyroid (46−84) amide. Total ion current obtained with (A) and without the addition of acid (B). As the aspartimides eltue near the product, the positive ion mode ESI-MS mass spectrum obtained at the front of the product peak shows strong aspartimide signals. While the reaction with acid (C) leads to a significant aspartimide reduction in favor of the product, the aspartimides dominate after reaction without acid (D).

piperidine) almost completely prevented aspartimide formation in the PreS9-33-y peptide. Under normal conditions, this peptide was prone to significant amounts of aspartimide. Simulation of prolonged incubation under harsh conditions confirmed those results. Furthermore, it was shown that this was not formic acid specific, but a general effect of acids during piperidine driven deprotection.

With the exception of TFMSA and HFIP, all acids (0.1 equiv) reduced the level of aspartimide as comparred to the untreated control. It can therefore be concluded that this is due to a decrease of the amount of nucleophiles of adjacent amides by protonation of the amidate form. Even low amounts of acid (e.g., 0.05 equiv) prevented aspartimide formation.

The peptide parathyroid hormone (PTH) is known to be difficult to synthesize by SPPS.[16] To test the efficiency of aspartimide suppression strategy described above, a comperative synthesis of a PTH fragment (46−84 = AGSQ-RPRKKEDNVLVESHEKSLGEADKADVNVLTKA-KSQ-NH_2) was performed using standard conditions or addition of 5% formic acid to the piperidine. Mass spectrometry showed the formation of aspartimide at all three possible sites in the peptide synthesized under standard conditions (Figure 3). As expected, the addition of 5% formic acid reduced aspartimide formation by ~90%, as determined by integration of the respective LC/MS signals.

Today, extensive synthesis strategies are required to avoid aspartic acid in drug developmental candidates. The described method therefore provides an efficient means of preventing aspartimide formation and therefore presents an interesting alternative to the usage of backbone protected dipeptides, the *gold standard* for prevention of aspartimide side products. In contrast to the commercialy available dipeptides that are limited to specific motifs, this method can be used for all Asp containing peptides.

In conclusion, a straightforward, efficient, and cost-effective method for the prevention of aspartimide during deprotection in Fmoc-based solid phase peptide synthesis is described. This study clearly showed that the addition of organic acids to the piperidine cleavage solution does not have any limitations. Since aspartic acid is found in most oligopeptides, the authors recommend that, in general, 5% (v/v) formic acid (fresh baches to avoid eventual side reactions) should be added to piperidine based Fmoc cleavage mixtures.

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Supporting Information Available. Materials and methods; reversed-phase HPLC and HPLC-MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.