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How to Tackle Aspartimide Formation - A Systematic Comparison of Different Methods

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Introduction

Aspartimide formation is still a serious challenge in peptide synthesis. This side reaction is strongly sequence dependent and preferably occurs at Asp-Aaa motifs (Aaa = Gly, Asp, Asn, Gln or Arg). In a first step, the cyclic aspartimide is formed, which can re-open in a second reaction leading to (epimerized) α- and β-Asp peptides and corresponding piperidides. Thus, all in all ten different products can be formed. Over the last decades, several approaches to solve this problem have been developed.

In this work, we systematically compared the combination of various strategies on different model peptides. The steric effect of Asp side chain protecting groups was investigated (OtBu, OEpe, OBno) [1]. The influence of various Fmoc cleaving reagents was studied, including acidic additives [2]. Furthermore, these results were compared to the application of dimethoxybenzyl (Dmb) as backbone protection and cyanosulfurylide (CSY) [3] as side chain protection. Finally, our identified optimal conditions were tested in the synthesis of other peptide sequences prone to aspartimide formation.

Results and Discussion

Three short aspartimide-prone model peptides: VKDGYI-OH, VKDDYI-OH and VKDRYI-OH were synthesized to investigate the sequence dependency of aspartimide formation. The standard protection Asp(OtBu) was applied. In order to minimize aspartimide formation during synthesis, the very mild Fmoc cleaving reagent 50% morpholine/0.1 M formic acid (FA) was used.

Comparing different strategies, repeated Fmoc cleaving steps were simulated by applying various cleavage cocktails to the resin-bound peptide for 18 h. Incubation with pure DMF served as negative control. The released peptides were analyzed by HPLC and the formation of aspartimide/piperidides was quantified.

We observed a strong sequence dependency already with the standard cleaving reagent 30% piperidine - with DG being the most sensitive motif. Aspartimide/piperidide formation was less pronounced for the DR and DD motif.

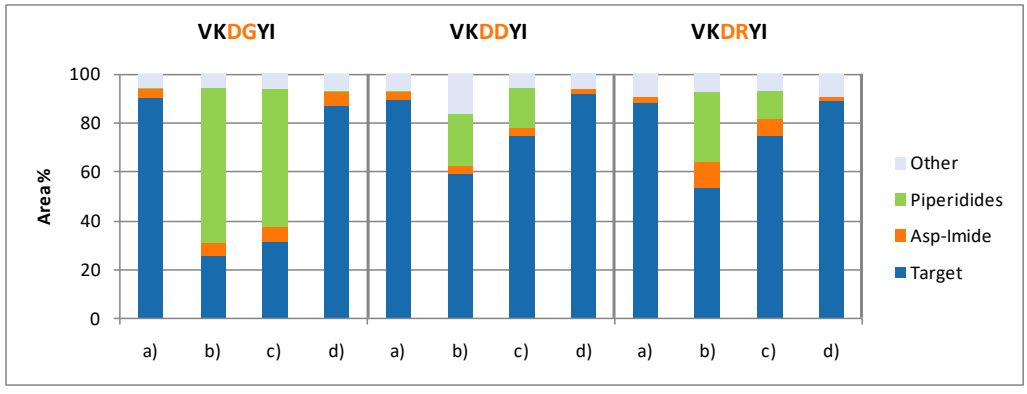

Fig. 1. Product composition after incubation of peptide resin with respective reagent for 18 h. a) DMF only, b) 30% piperidine, c) 30% piperidine/0.1 M FA, d) 50% morpholine.

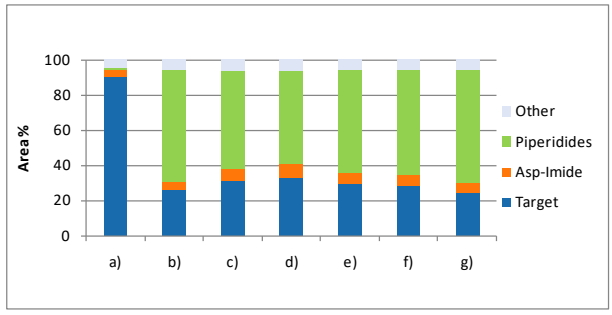

Fig. 2. Effect of acidic additives. a) DMF only, b) 30% piperidine, c) 30% piperidine/0.1 M FA, d) 30% piperidine/0.5 M FA, e) 30% piperidine/0.1 M NH_4OAc, f) 30% piperidine/0.1 M HOBt, g) 30% piperidine/0.1 M trifluoroethanol.

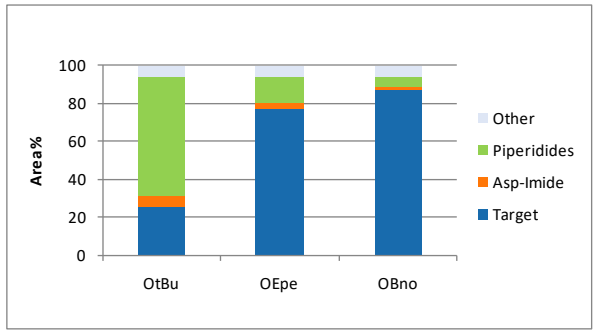

Fig. 3. Effect of different side chain protection groups.

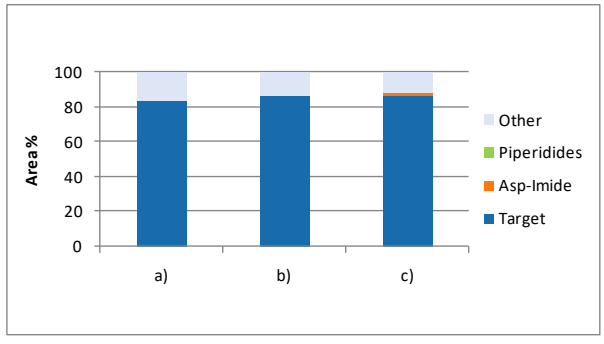

Fig. 4. Effect of Dmb backbone protection. a) DMF only, b) 30% piperidine, c) 2% DBU/2% piperidine.

In a next step we investigated the basicity of the cleavage reagent. The three model peptides were treated either with 30% piperidine, 30% piperidine/0.1 M formic acid or 50% morpholine (Figure 1). When using piperidine, a considerate amount of aspartimide/piperidides was observed, while morpholine showed almost no aspartimide formation. This effect correlates with the lower basicity of morpholine (pK_a piperidine = 11.2; pK_a morpholine = 8.4). Although morpholine reduces the aspartimide formation tremendously, it is often not sufficient for a complete Fmoc cleavage and stronger bases are necessary.

We also studied the influence of acidic additives. Addition of 0.1 M FA to piperidine significantly reduced the occurrence of byproducts for the DR and DD sequences. The effect was less pronounced for the most sensitive DG sequence. Various other acidic additives show similar results. Addition of formic acid, ammonium acetate, HOBt, or trifluoroethanol reduced the formation of aspartimide/piperidides in case of the most sensitive DG sequence only slightly (Figure 2).

Therefore, the steric effect of different Asp side chain protecting groups (OtBu, OEpe, OBno) was investigated (Figure 3). The bulkiness of the Asp protective groups has a large influence on aspartimide formation [1]. With increasing steric demand of the protecting group, aspartimide formation is diminished. OBno suppresses aspartimide/piperidide formation to a great extent even at DG sequences (Figure 3).

Dmb backbone protection offers a different strategy that completely prevents aspartimide formation (Figure 4). Even the very strong combination of DBU/piperidine produced almost no apartimide. Introducing Fmoc-Asp(OtBu)-(Dmb)Gly-OH however, needs sometimes more elaborated conditions due to the low coupling efficiency of such dipeptide building blocks at difficult positions. The formation of other byproducts during the synthesis lowered the yield of the target peptide additionally, e.g. we observed that tryptophane residues act as scavenger for Dmb upon TFA cleavage (data not shown).

Table 1. Analysis of naturally occurring peptide sequences.

Sequence[a]	*Asp-PG*	*Cleavage conditions*	*Crude/ area %*	*Isolated yield/%*
ASYKVTLKTPDGDNVITVPD [5]	OtBu (3x)	30% piperidine	66	29
	OtBu (3x)	30% piperidine/0.1 M FA	71	33
	OBno (3x)	30% piperidine	76	42
	CSY (3x)	30% piperidine	73	37
NPLGFFPDHQLDPAFRANTANPDWDy [2]	OtBu (3x)	30% piperidine	53	23
	OtBu (3x)	30% piperidine/0.1 M FA	58	24
	OBno (3x)	30% piperidine	63	27
	CSY (3x)	30% piperidine	64	14

[a]*All peptides were synthesized as C-terminal amides*

The Asp(CSY) protection recently introduced by Bode *et al.* shows complete suppression of aspartimide formation. However, other undefined side products were observed, even when applying morpholine (Figure 5). In contrast to other protecting schemes, the stable C-C-bond of the sulfur ylide allows the use of DBU as Fmoc cleaving reagent. Only in case of the DD motif, a significant drop of target yield was detected. We attribute this to the (expected) formation of aspartimide at DY, since regular Asp(OtBu) was used in this position. The cleavage of the CSY group in a second step went smoothly with approximately two equivalents of *N*-chlorosuccinimide (NCS). A larger excess of NCS led to multiple chlorinated products at the tyrosine residue.

Finally, our identified optimal conditions were tested in the synthesis of longer, naturally occurring peptide sequences prone to aspartimide formation. Synthesis was performed with different Asp protecting groups and Fmoc cleaving reagents (Table 1). In these cases, no additional incubation of the resin bound peptides with Fmoc cleaving reagents was performed. Application of CSY requires an additional cleavage step with NCS after final deprotection. All peptides were purified by preparative HPLC and isolated yields were determined. Using OBno or CSY protection resulted in increased crude yields for both sequences compared to standard OtBu protection. Addition of FA to the Fmoc cleaving cocktail improved the yields to a certain degree. Overall yields improved in the order OtBu, OtBu/FA, OBno similar to the model peptides. CSY showed comparable yields to OBno only for the first peptide.

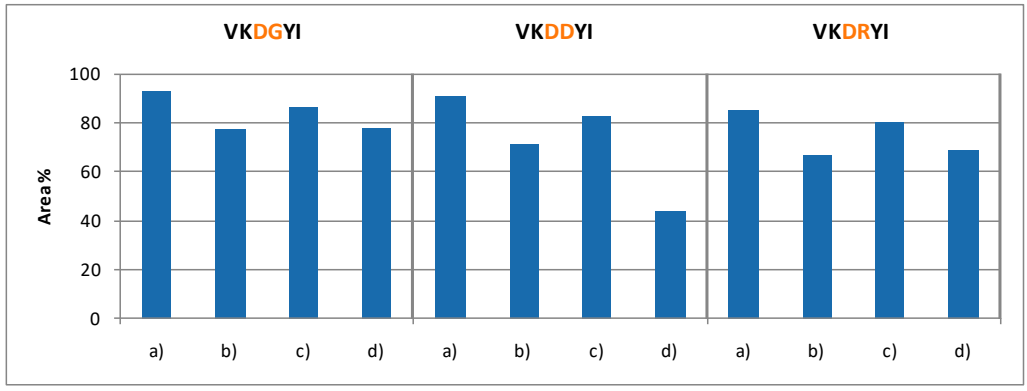

Fig. 5. Amount of target sequence after incubation of Asp(CSY)-peptide resins with respective reagent for 18 h. a) DMF only, b) 30% piperidine, c) 50% morpholine, d) 2% DBU.

In the second sequence we observed a byproduct with a Trp oxidation (confirmed by LC-MS/MS analysis), which was very difficult to separate by HPLC resulting in lower yield. Chlorination of Tyr was avoided by careful titration of NCS and monitoring by LC-MS. We could not prevent oxidation of Trp by this approach. Other literature known side reactions with NCS [4] can be circumvented by simple alterations: oxidation of Cys by disulfide protective groups (e.g. StBu or SIT) and oxidation of Met by substitution by norleucine.

Although aspartimide formation has been studied for decades, a universal low-cost method to circumvent this side reaction is still lacking. A simple and inexpensive approach is the standard Asp(OtBu) protection and addition of an acidic additive (e.g. FA) to the Fmoc cleavage cocktail. For the majority of peptides, these conditions reduce aspartimide formation to an acceptable level. In difficult cases, the use of special residues like Asp(OBno), Asp(CSY) or Dmb backbone protection should be considered. These more cost intensive special derivatives guarantee almost complete suppression of aspartimide-related side products.

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References

1. Behrendt, R., et al. *J. Pept. Sci.* **22**, 92-97 (2016), https://doi.org/10.1002/psc.2844
2. Michels, T., et al. *Org. Lett.* **14**, 5218-5221 (2012), https://doi.org/10.1021/ol3007925
3. Mergler, M., et al. *J. Pept. Sci.* **9,** 518-526 (2003), https://doi.org/10.1002/psc.473
4. Neumann, K., et al. *Nat. Commun.* **11**, 982 (2020), https://doi.org/10.1038/s41467-020-14755-6
5. Packman, L.C. *Tetrahedron Lett.* **36**, 7523-7526 (1995), https://doi.org/10.1016/0040-4039(95)01522-1