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A New Protecting Group For Aspartic Acid That Minimizes Piperidine-Catalyzed Aspartimide Formation In Fmoc Solid Phase Peptide Synthesis

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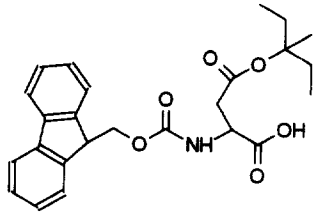
Abstract: The β-3-methylpent-3-yl ester of Fmoc aspartic acid, Fmoc-Asp(OMpe)-OH, is presented as a new protecting group for aspartic acid which provides good protection against base-catalyzed aspartimide formation in Fmoc solid phase peptide synthesis. Copyright © 1996 Elsevier Science Ltd

Acid- or base-catalyzed aspartimide formation has long been recognized as an important side reaction in Boc solid phase peptide synthesis[1]. For Fmoc-based synthesis this side reaction has not been considered a problem, because the *tert*-butyl protecting group gives good protection against base-catalyzed aspartimide formation and super strong acids like HF are not used. However, base-catalyzed aspartimide formation has recently been reported in Fmoc chemistry when piperidine is used for deprotection of the Fmoc group[2-5]. During piperidine treatment the imide ring may be opened by piperidine by nucleophilic attack on either of the carbonyl carbons resulting in α- or β-piperidides[6] and since the imide is sensitive to racemization a mixture of D- and L-aspartyl peptides are formed[7]. These reactions give rise to a heterogenous product which apart from reducing the yield of the desired peptide may lead to difficulties in purifying the peptide.

Base-catalyzed aspartimide formation is dependent on the peptide sequence[8], the base[9] and the β-carboxyl protecting group[10]. A systematic study by Lauer et al[3] showed that significant amounts of imide was formed upon prolonged piperidine treatment of a model peptide where different amino acids were inserted in the n+1 position, in particular for glycine or unprotected serine or threonine. In this study more aspartimide was formed upon piperidine treatment when aspartic acid was protected with the β-1-adamantyl group[11,12] (1-Ada) than when aspartic acid was protected with the β-*tert*-butyl (*t*Bu) group. Dölling et al[2] showed that in the synthesis of analogues of CRH where L-amino acids in the positions n+2 and n+3 were substituted with D-amino acids the rate of aspartimide formation was significantly increased. This shows that the sequence and the conformation of the peptide chain is of great importance for the rate of this side reaction. It is therefore evident that in the synthesis of long peptides, or complex peptides with unnatural amino acids, aspartimide and piperidide formation will become a serious problem in Fmoc chemistry.

Different approaches have been used to minimize base-catalyzed aspartimide formation in Fmoc peptide synthesis. The side reaction is reduced by adding HOBt or Dnp with the base[13]. Protection of the peptide bond by the N-(2-hydroxy-4-methoxybenzyl) (Hmb) group[14] prevents attack of the amide nitrogen on the β-carboxyl group, thereby eliminating aspartimide formation, but coupling to Hmb-protected amino acids is very slow and precludes the use of standard protocols. A different approach is to use a protecting group that gives better protection against aspartimide formation than the *t*Bu and 1-Ada groups. We have previously designed and synthesized a new protecting group for aspartic acid, the β-2,4-dimethyl-3-pentyl ester, which prevents base-catalyzed aspartimide formation in Boc solid phase peptide synthesis[15]. In this paper we have

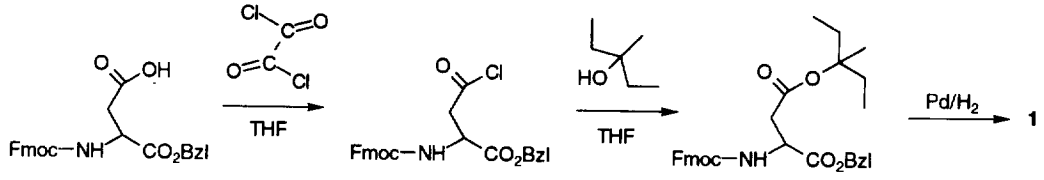
used a similar approach for Fmoc-based synthesis and suggest the use of Fmoc-aspartic acid with the side chain protected as a β-3-methylpent-3-yl (Mpe) ester (see figure 1).



1

Fig. 1. Fmoc-Asp(OMpe)-OH

Fmoc-Asp(OMpe)-OH[16] **1** was synthesized according to the following scheme:



2 **3** **4**

The key step in the synthesis was the esterfication of the β-carboxyl group by the sterically hindered 3-methyl-3-pentanol. The use of dicyclohexylcarbodimide and DMAP resulted in low yields. On the other hand, if the acid chloride **3**, synthesized by treating **2** with oxalyl chloride, is dissolved in a small volume of THF, diluted with a 20-fold volume excess of 3-methyl-3-pentanol and heated under reduced pressure to 50°C for 1 hour in a rotavapor, **3** is obtained as the major product[17]. Catalytical hydrogenation of **3** gives **1** in 20-25% overall yield.

To compare the effects on piperidine-catalyzed aspartimide formation we synthesized the peptide Val-Lys(Boc)-Asp(OX)-Gly-Tyr(*t*Bu)-Ile (where X= *t*Bu, 1-Ada and Mpe, respectively), which has previously been shown to be a good model system to study piperidine-catalyzed aspartimide formation[3]. The peptide was synthesized on a TFA-labile resin[18] and all couplings were carried out using 2 eq Fmoc amino acid, 2 eq TBTU, 2 eq HOBt and 4 eq DIEA in DMF. Deprotection of the Fmoc group was carried out using 20% piperidine/DMF for 10 min. The protected, resin-bound peptide was subsequently treated with 20% piperidine/DMF for 20 h. The peptides were cleaved from the resin with 95% TFA/H_2O for 30 min and were analyzed by analytical RP-HPLC using a C_{18} column. The peptides were eluted by 0% B in 2 min, 0-15% B in 2 min and 15-60% B in 21 min at a flow rate of 700 µl/min. The solvents used were A: 0.1% TFA/H_2O and B: 0.1% TFA/acetonitrile. As a reference peptides that had not been treated with piperidine were cleaved and analyzed in the same manner.

The HPLC elution profiles of the piperidine-treated peptides are shown in figure 2. Analyses of the peaks with plasma desorption mass spectrometry showed that the first peptide, eluting at 10.9 min, was the correct product with a molecular weight of 692. The second peptide, eluting at 11.3 min, had a mass difference of -18 compared to the correct peptide, which corresponds to the imide. The peptides eluting at 13.0, 13.4, 13.7 and 14.2 min, respectively, all had mass differences of +67, which corresponds to peptides

where the imide has been opened by piperidine to give piperidides. The four separated peaks probably correspond to α-and β-, D- and L-piperidides. The peak with a retention time of 12.1 min represents a by-product not related to aspartimide formation which was also present in the HPLC elution profiles of the peptides that had not been treated with piperidine.

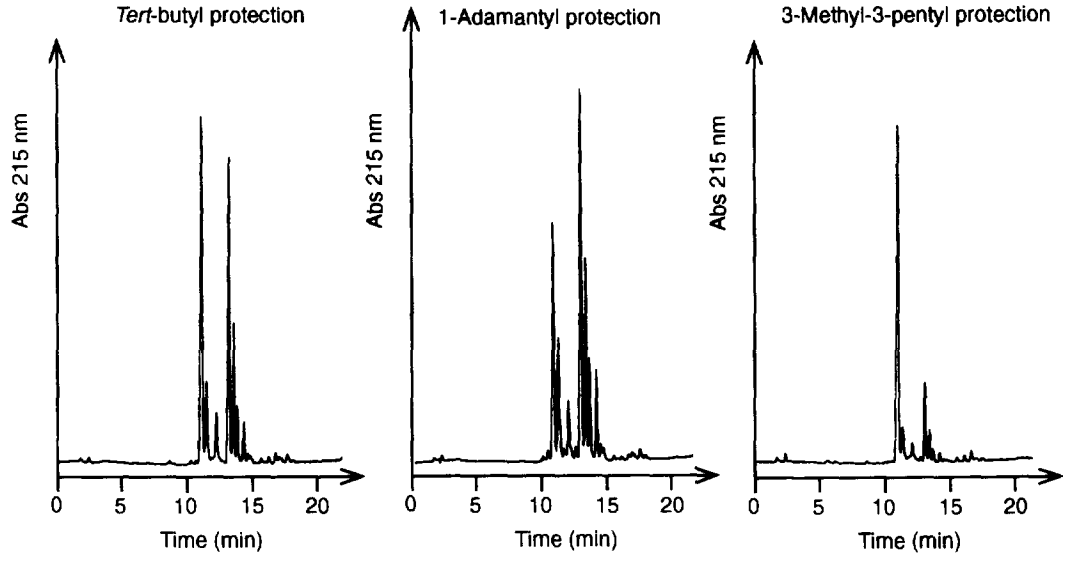


Fig. 2. HPLC elution profiles of the peptide Val-Lys-Asp-Gly-Tyr-Ile-NH_2 treated with 20 % piperidine/DMF for 20 h.

As can be seen in figure 2 and table 1 the new Mpe protecting group gives significantly better protection against base-catalyzed aspartimide formation than the *t*Bu and 1-Ada protecting groups. The difference in structure between the new protecting group and the commonly used *tert*-butyl protecting group is rather small, but the added methyl groups have a substantial effect on the rate of aspartimide formation. The flexibility and sterical hindrance of the alkyl chains is increased, thereby interfering with the attack of the amide bond nitrogen on the β-carboxyl group and/or the abstraction of the NH proton at the amide bond. The β-1-adamantyl ester increases the rate of aspartimide formation, probably because this protecting group is more rigid and provides less sterical hindrance.

Table 1. Aspartimide and piperidide peptides formed by treatment of the model peptide Val-Lys-Asp-Gly-Tyr-Ile-NH_2 with 20% piperidine/DMF for 20 h*

Asp protecting group	Imide-related by-products
Tert-butyl	58%
1-Adamantyl	81%
3-Methyl-3-pentyl	25%

* Total treatment: 20h + 3x10 min to deprotect the Fmoc group in the synthesis of the peptides

In conclusion this study shows that the use of the new Mpe protecting group for aspartic acid drastically decreases piperidine-catalyzed aspartimide formation in Fmoc synthesis. In combination with the previously mentioned methods to reduce aspartimide formation by the addition of HOBt or Dnp this protecting group should in many cases give sufficient protection for the synthesis of long peptides containing sensitive aspartyl sequences with Fmoc chemistry. The protecting group could probably be further improved by increasing the length of the alkyl chains, thereby increasing the sterical hindrance, and this concept is under current investigation in our laboratory.

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16. Fmoc-Asp(OMpe)-OH: amorphous solid, $[\alpha]_D^{20}$ +31 (*c* 1, $CHCl_3$).

    ^1H NMR (500 MHz; $CDCl_3$; Me_4Si) δ 7.77 (d, *J* 7.5, 2H, Fmoc H4 and H5), 7.62 and 7.61 (d, *J* 7.5, 2H, Fmoc H1 and H8), 7.41 (t, *J* 7.5, 2H, Fmoc H3 and H6), 7.32 (t, *J* 7.5, 2H, Fmoc H2 and H7), 5.90 (d, *J* 8.6, 1H, NH), 4.68 (m, *J* 8.6 and 2x4.5, 1H, Hα), 4.44 and 4.37 (m, *J* 7.3 and 10.6, 2H, Fmoc CH_2), 4.25 (t, *J* 7.3, 1H, Fmoc H9), 3.06 and 2.85 (m, *J* 4.5 and 17.4; 4.6 and 17.4, 2H, Hβ), 1.91 and 1.77 (m, 2H, $C(C\underline{H}_2CH_3)_2CH_3$), 1.88 and 1.75 (m, 2H, $C(C\underline{H}_2CH_3)_2CH_3$), 1.39 (s, 3H, $C(CH_2CH_3)_2C\underline{H}_3$) and 0.86 (t, 6H, *J* 7.4 , $CCH_2C\underline{H}_3)_2CH_3$).

    ^{13}C NMR (126 MHz; $CDCl_3$; Me_4Si) δ 175.9 (1C, COOH), 170.1 (1C, $CH_2\underline{C}OOC$), 156.1 (1C, Fmoc CO); 143.8 and 143.6 (2C, Fmoc C8a and C9a), 141.2 (2C, Fmoc C4a and C4b), 127.7 (2C, Fmoc C3 and C6), 127.0 (2C, Fmoc C2 and C7), 125.1 (2C, Fmoc C1 and C8), 119.9 (2C, Fmoc C4 and C5), 87.7 (1C, $\underline{C}(CH_2CH_3)_2CH_3$), 67.4 (1C, Fmoc CH_2), 50.3 (1C, Cα), 47.0 (1C, Fmoc C9), 37.4 (1C, Cβ), 30.3 (2C, $C(\underline{C}H_2CH_3)_2CH_3$), 22.7 (1C, $C(CH_2CH_3)_2\underline{C}H_3$) and 7.9 (2C, $C(CH_2\underline{C}H_3)_2CH_3$).
17. Fmoc-Asp(OMpe)-OBzl: m. p. 85°C, $[\alpha]_D^{20}$ +17 (*c* 1, $CHCl_3$).

    ^1H NMR (500 MHz; $CDCl_3$; Me_4Si) δ 7.78 (d, *J* 7.5, 2H, Fmoc H4 and H5), 7.62 and 7.61 (d, *J* 7.5, 2H, Fmoc H1 and H8), 7.42 (t, *J* 7.5, 2H, Fmoc H3 and H6), 7.37 (m, 2H, Bzl *o*-H), 7.36 (m, 2H, Bzl *m*-H), 7.34 (m, 1H, Bzl *p*-H), 7.32 (t, *J* 7.5, 2H, Fmoc H2 and H7), 5.90 (d, *J* 8.6, 1H, NH), 5.23 and 5.22 (m, *J* 12.3, 2H, Bzl-CH_2), 4.68 (m, *J* 8.6, 4.5 and 3.6 1H, Hα), 4.44 and 4.37 (m, *J* 7.3 and 10.6, 2H, Fmoc CH_2), 4.25 (t, *J* 7.3, 1H, Fmoc H9), 3.04 (m, *J* 3.6 and 17.2, 1H, Hβ), 2.88 (m, *J* 4.5 and 17.2, 1H, Hβ), 1.87 and 1.74 (m, 2H, $C(C\underline{H}_2CH_3)_2CH_3$), 1.87 and 1.72 (m, 2H, $C(C\underline{H}_2CH_3)_2CH_3$), 1.36 (s, 3H, $C(CH_2CH_3)_2C\underline{H}_3$) and 0.84 (t, 6H, *J* 7.4 , $CCH_2C\underline{H}_3)_2CH_3$).

    ^{13}C NMR (126 MHz; $CDCl_3$; Me_4Si) δ 170.8 (1C, $\underline{C}OOBzl$), 169.8 (1C, $CH_2\underline{C}OOC$), 156.0 (1C, Fmoc CO), 143.9 and 143.7 (2C, Fmoc C8a and C9a), 141.2 (2C, Fmoc C4a and C4b), 135.2 (1C, Bzl s-C), 128.5 (1C, Bzl *p*-C), 128.4 (2C, Bzl *m*-C), 128.2 (2C, Bzl *o*-C), 127.6 (2C, Fmoc C3 and C6), 127.0 (2C, Fmoc C2 and C7), 125.1 (1C, Fmoc C1 and C8), 87.2 (1C, $\underline{C}(CH_2CH_3)_2CH_3$), 67.4 (1C, Bzl CH_2), 67.2 (1C, Fmoc CH_2), 50.5 (1C, Cα), 47.0 (1C, Fmoc C9), 37.5 (1C, Cβ), 30.3 and 30.2 (2C, $C(\underline{C}H_2CH_3)_2CH_3$), 22.7 (1C, $C(CH_2CH_3)_2\underline{C}H_3$) and 7.9 (2C, $C(CH_2\underline{C}H_3)_2CH_3$).
18. p-[(R,S)-α-[1-(9H-Fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid anchored to 4-methylbenzhydrylamine resin.

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