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Sequence dependence of aspartimide formation during 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis

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SUMMARY

We have examined the sequence dependence of aspartimide formation during Fmoc-based solid-phase synthesis of the peptide Val-Lys-Asp-X-Tyr-Ile. The extent of aspartimide formation and subsequent conversion to the α - or β -piperidide was characterized and quantitated by analytical reversed-phase high-performance liquid chromatography and fast atom bombardment mass spectrometry. Aspartimide formation occurred for X = Arg(Pmc), Asn(Trt), Asp(OtBu), Cys(Acm), Gly, Ser, Thr and Thr(*t*Bu). No single approach was found that could inhibit this side reaction for all sequences. The most effective combinations, in general, for minimization of aspartimide formation were (i) *tert*-butyl side-chain protection of aspartate, piperidine for removal of the Fmoc group, and either 1-hydroxybenzotriazole or 2,4-dinitrophenol as an additive to the piperidine solution; or (ii) 1-adamantyl side-chain protection of aspartate and 1,8-diazabicyclo[5.4.0]undec-7-ene for removal of the Fmoc group.

INTRODUCTION

Successful solid-phase syntheses are dependent upon highly efficient coupling/deprotection cycles and minimization of deleterious side reactions. The cyclization of aspartate to form aspartimide has long been recognized as a substantial side reaction occurring during both synthesis and storage of peptides [1–3]. Aspartimide formation during peptide synthesis can be either acid or base catalyzed, with the kinetics of ring closure depending upon the nature and strength of the acid or base, the structure of the aspartate side-chain protecting group, and the aspartate carboxyl

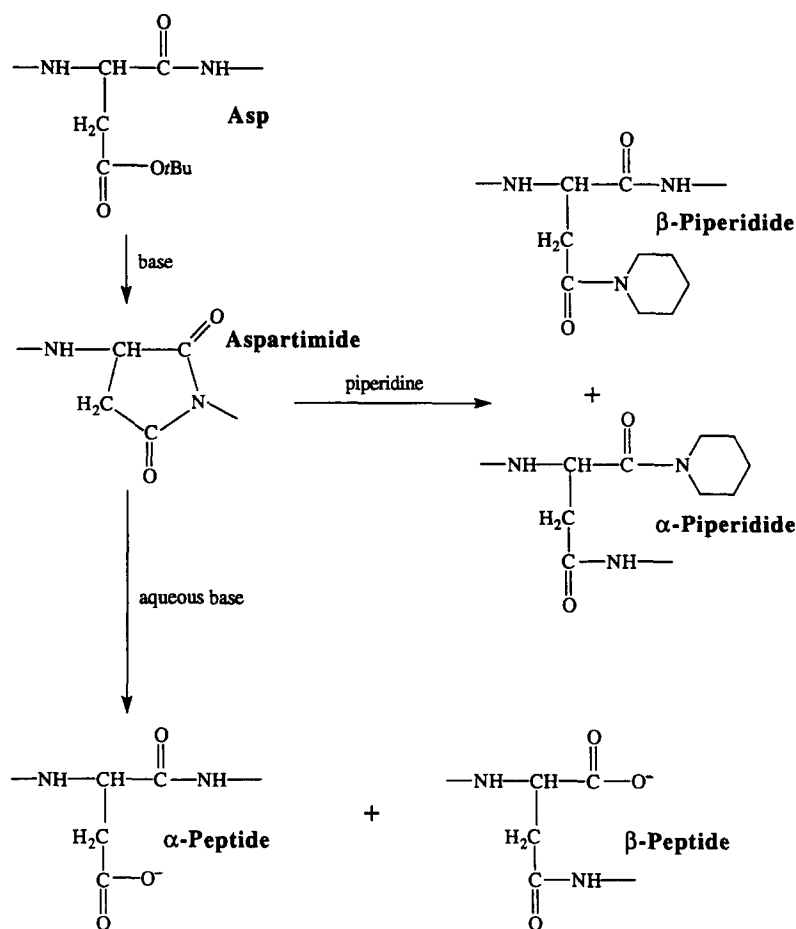
neighboring residue. Extensive studies have been published of aspartimide formation during *tert*-butoxycarbonyl (Boc)-based peptide synthesis which have focused on protecting group strategies [4,5], sequence dependence [6], and the nature and strength of the acid or tertiary base [5,7]. These studies found that aspartimide formation can be adequately suppressed by additives such as 1-hydroxybenzotriazole (HOBt) or 2,4-dinitrophenol (Dnp) during base neutralization [8] or by using 2-adamantyl (2-Ada) or cyclohexyl side-chain protection of aspartate instead of benzyl (Bzl) protection [4,5].

It had been assumed that for 9-fluorenyl-

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methoxycarbonyl (Fmoc)-based solid-phase synthesis, *tert*-butyl (*t*Bu) side-chain protection of aspartate inhibits aspartimide formation. However, Nicolás et al. [9] found substantial aspartimide formation (see Scheme 1) resulting from piperidine treatment of the resin-bound sequence Val-Lys(Boc)-Asp(*Ot*Bu)-Gly-Tyr(*t*Bu)-Ile. Subsequently, other sequences have been found to be susceptible to aspartimide formation during Fmoc solid-phase synthesis [10–13]. As shown in Scheme 1, aspartimide-containing peptides can be converted to the α - or β -piperidide by continued exposure to piperidine during solid-phase synthesis [11–14]. Alternatively, aqueous conditions could result in ring opening to either the α - or β -peptide. Ring opening to either the piperidide or

peptide is accompanied by aspartate racemization [15]. Although comprehensive studies have not been performed, aspartimide and piperidide formation have been shown to be dependent upon the peptide sequence, solvent polarity, and conformation of the peptide chain [11,13,16,17]. We have further examined the sequence dependence of aspartimide formation using the model peptide Val-Lys-Asp-X-Tyr-Ile, based on the initial studies of Nicolás et al. [9]. In addition, we have compared the effect of different bases [piperidine versus 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)], additives (HOBt and Dnp), and aspartate side-chain protecting groups [*t*Bu and 1-adamantyl (1-Ada)] [4,18] on aspartimide and subsequent piperidide formation.



Scheme 1. Formation and ring opening reactions of aspartimide.

METHODS

All standard peptide synthesis chemicals were analytical reagent grade or better and purchased from Applied Biosystems, Inc. (Foster City, CA) or Fisher (Pittsburgh, PA). DBU was obtained from Aldrich (Milwaukee, WI), trifluoroacetic acid (TFA) from Applied Biosystems, benzoic anhydride from Kodak, Fmoc-Ile-4-hydroxymethylphenoxy (HMP) resin (substitution level = 0.46 mmol/g) and benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) from Novabiochem (La Jolla, CA), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU) from Richelieu Biotechnologies (St.-Hyacinthe, Quebec), and all Fmoc-amino acids from Novabiochem or Millipore Corp. (Bedford, MA). All amino acids are of the L-configuration where applicable.

Incorporation of individual amino acids was by Fmoc solid-phase methodology on either an Applied Biosystems 431A peptide synthesizer or a Gilson automated multiple peptide synthesizer AMS 422. Cycles for the ABI 431A were as described [19], while cycles for the Gilson AMS 422 were modified from those described previously [20] by using double couplings with sixfold excesses of Fmoc-amino acids. All peptides were assembled on Fmoc-Ile-HMP resin, capped with a 10-fold excess of benzoic anhydride using standard coupling cycles, and cleaved and deprotected with H₂O–TFA (1:19) as described [21]. Peptides were purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on a Rainin AutoPrep system equipped with a Vydac C₁₈ column (15–20 µm particle size, 300 Å pore size, 250 × 22 mm). The elution gradient was 0–60% B in 60 min at a flow rate of 5.0 ml/min, where A was water containing 0.1% TFA and B was acetonitrile containing 0.1% TFA. Detection was at 229 nm. Analytical RP-HPLC was performed on a Hewlett-Packard 1090 Liquid Chromatograph equipped with a Dynamax C₁₈ column (5 µm particle size, 300 Å pore size, 250 × 4.6 mm). The elution gradient was 0–60% B in 45

min at a flow rate of 1.0 ml/min, where A was water containing 0.045% TFA and B was acetonitrile containing 0.035% TFA. Detection was at 230 nm. Elution peaks were integrated using the Hewlett-Packard ChemStation software set at peak width 0.100, threshold –2, and area reject <10% of the largest peak.

Edman degradation sequence analysis was performed on an Applied Biosystems 477A protein sequencer/120A analyzer and fast atom bombardment mass spectrometry (FABMS) on a VG 7070E-HF mass spectrometer as described previously [19,22]. Sequence analysis of selected peptide-resins indicated efficient assemblies. Mass spectral analysis of the benzoyl-Val-Lys-Asp-X-Tyr-Ile peptides (either purified or crude when no side products were seen by analytical RP-HPLC) gave (theoretical molecular weights are given in parentheses): X = Ala, [M+H]⁺ = 812.4 Da (812.8 Da); X = aminoisobutyric acid (Aib), [M+H]⁺ = 826.4 Da (826.9 Da); X = Arg (from Arg(Pmc)), [M+H]⁺ = 897.4 Da (897.9 Da); X = Asn (from Asn(Trt)), [M+H]⁺ = 855.4 Da (855.8 Da); X = Asp (from Asp(*O**t*Bu)), [M+H]⁺ = 856.5 Da (856.8 Da); X = Cys (from Cys(Trt)), [M+H]⁺ = 844.5 Da (844.9 Da); X = Cys(Acm), [M+H]⁺ = 915.4 Da (916.0 Da); X = Gln (from Gln(Trt)), [M+H]⁺ = 869.5 Da (869.9 Da); X = Glu (from Glu(*O**t*Bu)), [M+H]⁺ = 870.5 Da (870.9 Da); X = His (from His(Trt)), [M+H]⁺ = 878.5 Da (878.9 Da); X = Ile, [M+H]⁺ = 854.5 Da (854.9 Da); X = Leu, [M+H]⁺ = 854.5 Da (854.9 Da); X = Lys (from Lys(Boc)), [M+H]⁺ = 869.5 Da (869.9 Da); X = Met, [M+H]⁺ = 872.5 Da (873.0 Da); X = Phe, [M+H]⁺ = 888.5 Da (888.9 Da); X = Pro, [M+H]⁺ = 838.5 Da (838.9 Da); X = Ser, [M+H]⁺ = 828.5 Da (828.8 Da); X = Ser (from Ser(*t*Bu)), [M+H]⁺ = 828.5 Da (828.8 Da); X = Thr, [M+H]⁺ = 842.3 Da (842.9 Da); X = Thr (from Thr(*t*Bu)), [M+H]⁺ = 842.5 Da (842.9 Da); X = Trp, [M+H]⁺ = 927.4 Da (928.0 Da); X = Trp (from Trp(Boc)), [M+H]⁺ = 927.5 Da (928.0 Da); X = Tyr (from Tyr(*t*Bu)), [M+H]⁺ = 904.6 Da (904.9 Da); and X = Val, [M+H]⁺ = 840.5 Da (840.9 Da). FABMS analysis of benzoyl-Val-Lys-Asp-Gly-Tyr-Ile obtained us-

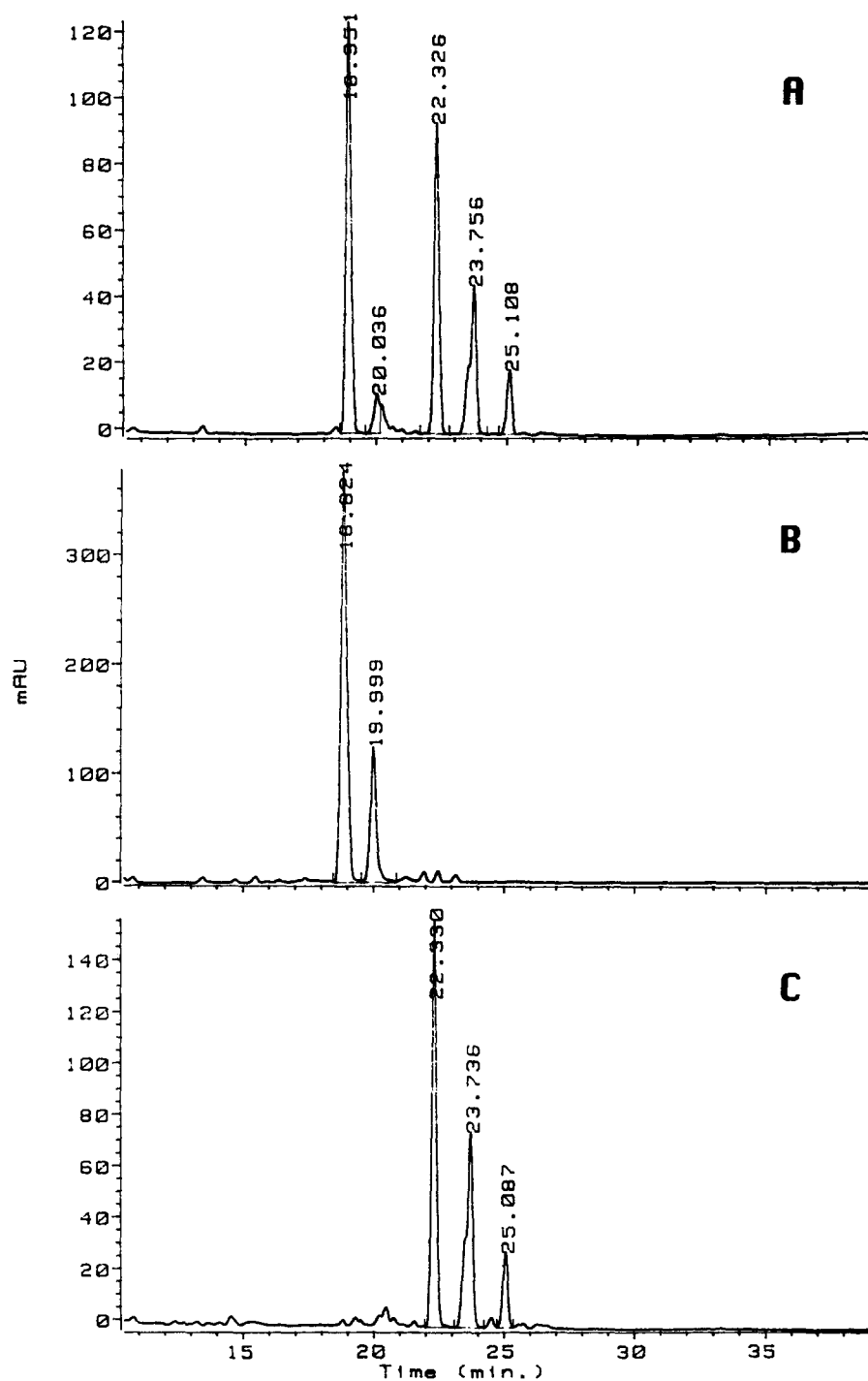


Fig. 1. RP-HPLC elution profiles of the cleavage products from (A) peptide-resin **1a** after 16 h piperidine treatment; (B) peptide-resin **1b** after 0 h piperidine treatment; and (C) peptide-resin **1b** after 16 h piperidine treatment. The peptides eluting at 18.9 min in (A) and 18.8 min in (B) are the desired products ($[M+H]^+ = 798.7$ and 798.3 Da). The peptide eluting at 20.0 min is the aspartimide-containing product ($[M+H]^+ = 780.3$ Da). The peptides eluting at 22.3, 23.7, and 25.1 min are L- or D-, α - or β -piperidine-containing products ($[M+H]^+ = 865.4$ Da).

ing Asp(*O*-1-Ada) gave $[M+H]^+ = 798.5$ Da (798.8 Da).

RESULTS

The conversion of side-chain protected aspartate to aspartimide can occur by repetitive base treatments throughout the synthesis of a peptide. We chose the sequence Val-Lys-Asp-Gly-Tyr-Ile to study aspartimide formation because of its susceptibility to base-catalyzed cyclization of the aspartate residue during synthesis [9]. Initially, to compare the extent of aspartimide formation, peptide-resins **1a** and **1b** were synthesized, where **1a** contained aspartate with *t*Bu side-chain (β -carboxyl) protection and **1b** contained aspartate with *t*Bu α -carboxyl protection and no side-chain

protection. During assembly of **1a** and **1b**, Fmoc removal for each amino acid utilized a 9 min treatment with piperidine and 0.1 M HOBT in NMP (1:4). Thus, the aspartate residue was exposed to the piperidine solution for 27 min. The peptide-resins were subsequently treated with piperidine-DMF (1:4) for 0, 2, 4, 6 and 16 h, after which they were cleaved and side-chain deprotected for 1 h, and the products were analyzed by RP-HPLC. After 0 h treatment, the cleavage product from **1a** contained only the desired material, eluting at 18.9 min ($[M+H]^+ = 798.7$ Da, theoretical 798.8 Da). A 16 h treatment of **1a** resulted in a product containing 39.3% of the desired material (elution time = 18.9 min), and several later eluting peptides (Fig. 1A). The peptide eluting at 20.0 min corresponded to an aspart-

TABLE I
SEQUENCE DEPENDENCE FOR PIPERIDINE-CATALYZED ASPARTIMIDE FORMATION OF BENZOYL-Val-Lys(Boc)-Asp(*Ot*Bu)-X-Tyr(*t*Bu)-Ile-HMP RESIN

X	Percentage desired product				
	t = 0 h	t = 2 h	t = 4 h	t = 6 h	t = 16 h
Ala	100	100	100	100	100
Aib	100	100	100	100	100
Arg(Pmc)	100	92.9	94.2	88.8	82.4
Asn(Trt)	100	100	100	100	77.2
Asp(<i>Ot</i> Bu)	100	100	100	96.1	88.5
Cys(Acm)	100	100	92.0	88.3	52.6
Cys(Trt)	100 ^a	100	100	100	100
Gln(Trt)	100	100	100	100	100
Glu(<i>Ot</i> Bu)	100	100	100	100	100
Gly	100	ND ^b	ND	ND	39.3
His(Trt)	100	100	100	100	100
Ile	100	100	100	100	100
Leu	100	100	100	100	100
Lys(Boc)	100	100	100	100	100
Met	100	100	100	100	100
Phe	100	100	100	100	100
Pro	100	100	100	100	100
Ser	100	82.9	62.7	53.9	32.7
Ser(<i>t</i> Bu)	100	100	100	100	100
Thr	81.0	62.5	44.4	36.1	12.5
Thr(<i>t</i> Bu)	100	100	100	100	67.1
Trp	100	100	100	100	100
Trp(Boc)	100	100	100	100	100
Tyr(<i>t</i> Bu)	100	100	100	100	100
Val	100	100	100	100	100

^a The product contained ~10% of a *t*-butylated peptide with the desired sequence ($[M+H]^+ = 900.6$ Da, theoretical 901.0 Da).

^b ND = not determined.

imide-containing product based on FABMS ($[M+H]^+ = 780.3$ Da, theoretical 780.8 Da). Peptides eluting at 22.3, 23.7 and 25.1 min corresponded to piperidide-containing products based on FABMS ($[M+H]^+ = 865.4$ Da, theoretical 865.8 Da). After 0 h treatment, the cleavage product from **1b** contained 75.3% of the desired material, eluting at 18.8 min ($[M+H]^+ = 798.3$ Da, theoretical 798.8 Da) and 24.7% of the aspartimide-containing peptide, eluting at 20.0 min (Fig. 1B). Thus, aspartimide formation occurred more rapidly for the β -Asp peptide compared to the α -Asp peptide. After a 16 h treatment only the piperidide-containing products of **1b** were seen, eluting at 22.3, 23.7 and 25.1 min (Fig. 1C).

1a benzoyl-Val-Lys(Boc)-Asp(*Or*Bu)-Gly-Tyr(*t*Bu)-Ile-HMP resin

1b benzoyl-Val-Lys(Boc)- β -Asp(α -*Or*Bu)-Gly-Tyr(*t*Bu)-Ile-HMP resin

To examine the sequence dependence of aspartimide formation, 25 different residues were substituted for glycine in the Val-Lys-Asp-Gly-Tyr-Ile sequence. During assembly of the 25 peptide-resins, the aspartate residue was exposed to piperidine and 0.1 M HOBt in DMF (1:4) for 1.85 h. Peptide-resins were subsequently treated with piperidine-DMF (1:4) for 0, 2, 4, 6 and 16 h, after which they were cleaved and side-chain deprotected for 1 h, and the products were analyzed by RP-HPLC (Table 1). The area of the desired

product peak was compared to those of later eluting product peaks corresponding to aspartimide- and piperidide-containing peptides (vide supra). With no additional piperidine treatment, cyclization of aspartate was seen for the sequence containing X = Thr. Additional piperidine treatment resulted in aspartimide formation for sequences containing X = Arg(Pmc) or Ser after 2 h, Cys(Acm) after 4 h, Asp(*Or*Bu) after 6 h, and Asn(Trt) or Thr(*t*Bu) after 16 h. The extent of aspartimide formation increased very slowly over time for X = Arg(Pmc), Asp(*Or*Bu), or Cys(Acm), but very rapidly for X = Ser or Thr. For all of these aspartimide-forming sequences, the use of DBU-piperidine-DMF (1:1:48) instead of piperidine-DMF (1:4) increased the rate of side reaction (not shown).

Inhibition of aspartimide formation was studied with HOBt or Dnp as additives to the piperidine solution. All sequences from Table 1 that gave aspartimide formation were exposed to a 16 h piperidine-DMF (1:4) treatment in the presence of 0.1 M potential inhibitor. In most cases, either HOBt or Dnp could inhibit, but not always eliminate, aspartate cyclization (Table 2). For the sequences containing X = Cys(Acm), Thr or Thr(*t*Bu), the use of Dnp as an additive significantly increased the yield of desired product from 53 to 76%, 12 to 55%, and 67 to 100%, respectively.

The synthesis of **1a** was repeated using Fmoc-Asp(*O*-1-Ada) to evaluate the influence of the aspartate side-chain protecting group on aspartimide formation. Treatment of benzoyl-Val-Lys-

TABLE 2
INHIBITION OF PIPERIDINE-CATALYZED ASPARTIMIDE FORMATION OF BENZOYL-Val-Lys(Boc)-Asp(*Or*Bu)-X-Tyr(*t*Bu)-Ile-HMP RESIN

X	Percentage desired product following 16 h piperidine treatment		
	No additive	HOBt	Dnp
Arg(Pmc)	82.4	85.2	95.2
Asn(Trt)	77.2	84.8	81.2
Asp(<i>Or</i> Bu)	88.5	92.5	100
Cys(Acm)	52.6	68.6	76.3
Ser	32.7	45.9	41.1
Thr	12.5	28.0	54.9
Thr(<i>t</i> Bu)	67.1	100	100

TABLE 3
ASPARTIMIDE FORMATION OF BENZOYL-Val-Lys(Boc)-Asp(*O*-1-Ada)-Gly-Tyr(*t*Bu)-Ile-HMP RESIN

Base	Additive	Time (h)	Percentage desired product
Piperidine	None	0	100
Piperidine	None	2	0
Piperidine	None	4	0
Piperidine	None	6	0
Piperidine	None	16	0
Piperidine	HOBt	16	69.3
Piperidine	Dnp	16	86.9
DBU	None	0	100
DBU	None	2	83.1
DBU	None	4	85.0
DBU	None	6	73.3
DBU	None	16	11.2
DBU	HOBt	16	11.6
DBU	Dnp	16	12.4

(Boc)-Asp(*O*-1-Ada)-Gly-Tyr(*t*Bu)-Ile-HMP resin (**1c**) with piperidine–DMF (1:4) resulted in higher levels of side products than when Asp(*O**t*Bu) was used for the same sequence. For example, a 16 h treatment of **1c** yielded none of the desired product (Table 3), whereas 39.3% of the desired product was obtained following identical treatment of **1a**. Both HOBt and Dnp were effective inhibitors of aspartimide formation of **1c** (Table 3). Treatment of **1c** with DBU was far less detrimental than piperidine. All time points beyond 0 h resulted in more desired product following DBU treatment than piperidine. Additives such as HOBt or Dnp had little effect on inhibition of DBU-catalyzed aspartimide formation (Table 3).

DISCUSSION AND CONCLUSIONS

The formation of aspartimide in aspartate-containing peptides has been documented for only a few sequences assembled by Fmoc chemistry [9–13,16,17,23]. A comprehensive study was clearly needed to determine if this side reaction is a significant problem during Fmoc solid-phase chemistry. We utilized the sequence Val-Lys-Asp-X-Tyr-Ile as a model for aspartimide formation. For X = Gly, there was no detectable conversion to aspartimide following peptide assembly, but 60.7% conversion after an additional 16 h expo-

sure to piperidine–DMF (1:4). Assuming a linear relationship, this corresponds to 0.63% conversion per 10 min treatment. Nicolás et al. [9] and Quibell et al. [23] found 0.46 and 0.5% conversion, respectively, per 10 min piperidine–DMF (1:4) treatment of resin-bound, side-chain protected Val-Lys-Asp-Gly-Tyr-Ile. Our results with respect to sequence dependence show that aspartimide formation occurs for X = Thr > Ser > Arg-(Pmc) = Cys(Acm) > Asp(*O**t*Bu) = Asn(Trt) = Thr(*t*Bu), where threonine and serine overwhelmingly gave the most rapid side-product formation. It is somewhat surprising that ring closure occurs readily in the presence of a bulky side chain [2] such as Arg(Pmc). A prior study of base-catalyzed aspartimide formation of Boc-Asp(OBzl)-X- β -naphthylamide indicated that conversion was most rapid for the residues X = Ser > Thr > Gly = Asn = Gln [6]. Thus, results of our study are consistent with this prior study. We did not examine X = Asn or Gln, as there does not appear to be much effect of Trt side-chain protection of a neighboring asparagine or glutamine on inhibition or enhancement of aspartimide formation [17]. Prior studies of aspartimide formation of Asp(*O**t*Bu)-X sequences during Fmoc chemistry found significant conversion when X = Gly, Ala, Asn, Asn(Trt), Gln, and Gln(Trt) [9–13,16,17]. While we did see aspartimide formation for X = Gly and

Asn(Trt), we did not for X = Ala and Gln(Trt). Other researchers have shown that not all Asp(*O**t*Bu)-Gly are susceptible to cyclization [24]. These results lend further support to the notion that aspartimide formation is both sequence and conformation dependent [11,17].

When DBU was used instead of piperidine, the rate of aspartimide formation increased for all sequences examined. This is consistent with prior results with an Asp(*O**t*Bu)-Asn(Trt) sequence [10]. Previously, DBU has been shown to remove the Fmoc group more rapidly than piperidine [24]. Fmoc group removal requires a base to abstract the acidic proton at the 9-position of the fluorene ring system, leading to β -elimination. If DBU more rapidly removes this proton than piperidine, then the increased rate of aspartimide formation may be the result of the rate of abstraction of the NH proton leading to cyclization.

A sufficient number of aspartimide-susceptible sequences have now been identified to warrant the development of general methods for inhibiting aspartate cyclization during Fmoc solid-phase synthesis. We have considered the (i) time of base treatment; (ii) use of additives during Fmoc removal; and (iii) protecting group strategy. Increased exposure to piperidine or DBU usually results in increased aspartimide and/or piperidine formation of susceptible sequences, as found previously [12,16]. Obviously, minimizing the length of base treatment of susceptible sequences will, in general, reduce aspartimide formation. Additives such as HOBt or Dnp were effective inhibitors when Asp(*O**t*Bu) or Asp(*O*-1-Ada) was used in combination with piperidine, similar to their effects on base-catalyzed aspartimide formation of Boc-Asp(OBzl)-Gly- β -naphthylamide [8] and Fmoc-Leu-Thr(*t*Bu)-Glu(*O**t*Bu)-Asp(*O**t*Bu)-Asn(Trt)-Val-Lys(Boc)-HMP resin [16,17]. The use of HOBt as an additive to piperidine solutions does not appear to have a detrimental effect on Fmoc removal [11,25]. However, neither HOBt nor Dnp was an effective inhibitor of aspartimide formation during DBU treatment of the Asp(*O*-1-Ada) sequence studied here.

The Ada group has been reported to suppress acid-catalyzed aspartimide formation better than the Bzl and cyclohexyl groups [4,18]. It is believed that the relative bulkiness of the Ada group is responsible for this inhibitory effect [4]. We found that the 1-Ada group was (i) more effective at suppressing DBU-catalyzed aspartimide formation than the piperidine-catalyzed side reaction; and (ii) less effective at suppressing piperidine-catalyzed aspartimide formation than *t*Bu group side-chain protection of aspartate. Based on Ada steric effects alone, result (i) is anticipated. However, steric effects do not explain result (ii). It is possible that Ada or *t*Bu provides minimal steric hindrance to piperidine abstraction of the NH proton.

Other approaches for potential inhibition of aspartimide formation can also be considered. Since the base-catalyzed reaction requires abstraction of the NH proton [2], there should be no cyclization when the carboxyl neighbor to aspartate is an *N*-alkyl amino acid. This concept is supported by our results, showing no aspartimide formation for the sequence containing an *N*-alkyl amino acid (X = Pro). In a similar fashion, a recent study showed minimization of aspartimide formation using the 2-hydroxy-4-methoxybenzyl (Hmb) *N*-protecting group prior to the incorporation of an aspartate residue [23]. An alternative method for inhibiting aspartimide formation would thus be solid-phase incorporation of an *N*-alkyl amino acid prior to Fmoc-Asp(*O**t*Bu). Based on our study, the most effective combinations for minimization of aspartimide formation were (i) *t*Bu side-chain protection of aspartate, piperidine for removal of the Fmoc group, and either HOBt or Dnp as an additive to the piperidine solution; or (ii) 1-Ada side-chain protection of aspartate and DBU for removal of the Fmoc group.

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