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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(*Ot*Bu), 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*-butyloxycarbonyl (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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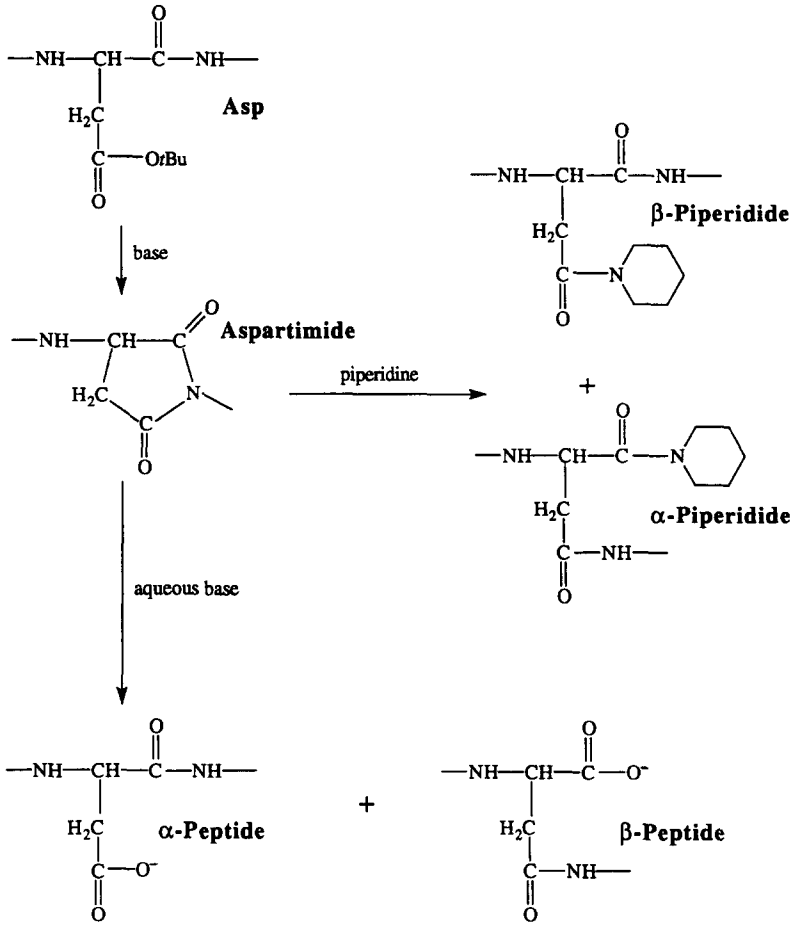
methoxycarbonyl (Fmoc)-based solid-phase synthesis, *tert*-butyl (*t*Bu) side-chain protection of aspartate inhibits aspartimide formation. However, Nicolás et al. [9] found substantial aspartimide formation (see Scheme 1) resulting from piperidine treatment of the resin-bound sequence Val-Lys(Boc)-Asp(*Ot*Bu)-Gly-Tyr(*t*Bu)-Ile. 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 H_2O–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 C_{18} 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 C_{18} 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(*Ot*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(*Ot*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-

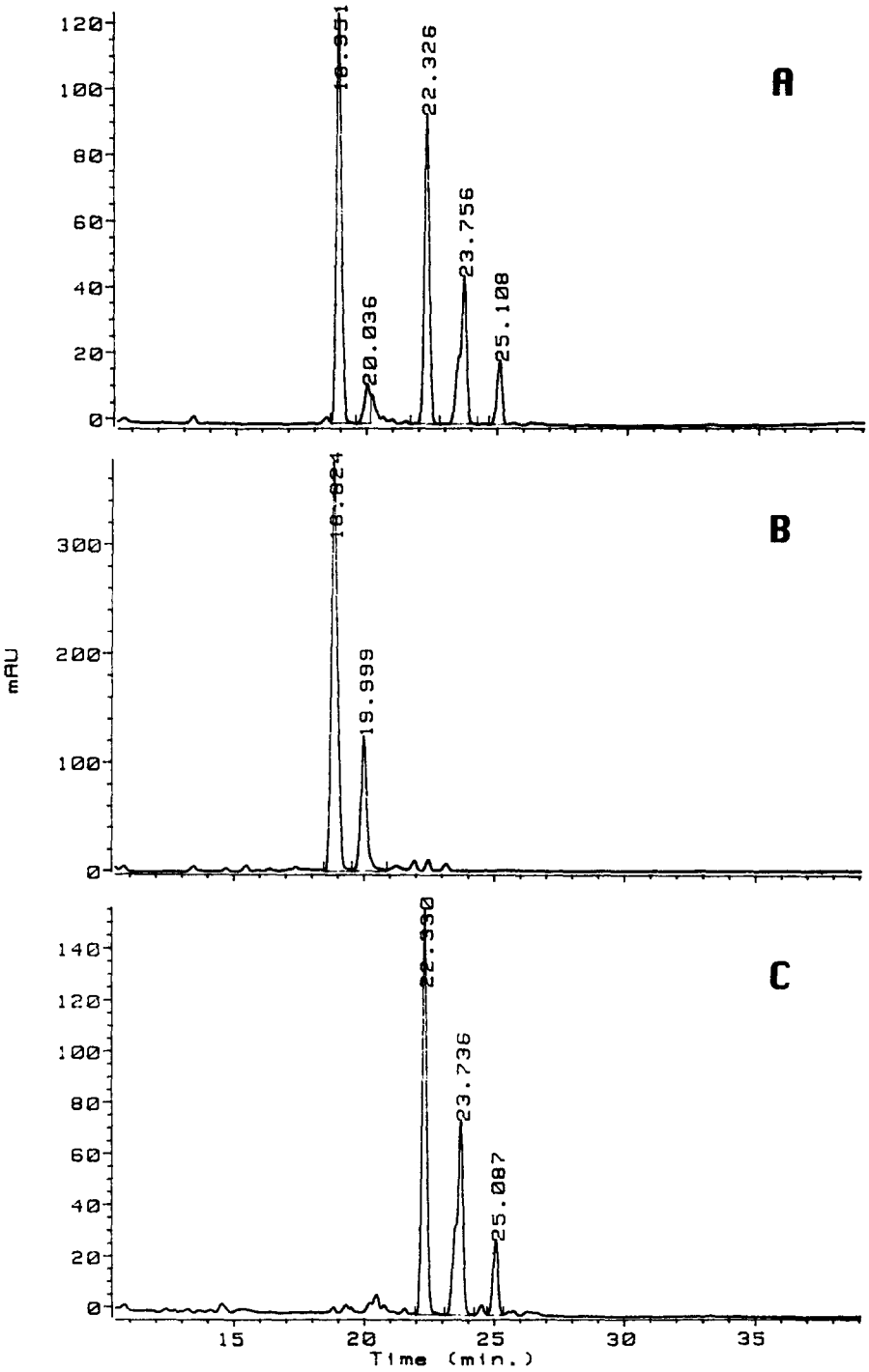


Fig. 1. RP-HPLC elution profiles of the cleavage products from (A) peptide-resin **1a** after 16 h piperidine treatment; (B) peptide-resin **1b** after 0 h piperidine treatment; and (C) peptide-resin **1b** after 16 h piperidine treatment. The peptides eluting at 18.9 min in (A) and 18.8 min in (B) are 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-piperidide-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 1
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(*Ot*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(*Ot*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(*t*Bu)	100	100	100	100	100
Thr	81.0	62.5	44.4	36.1	12.5
Thr(*t*Bu)	100	100	100	100	67.1
Trp	100	100	100	100	100
Trp(Boc)	100	100	100	100	100
Tyr(*t*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.

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