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α- and β- Aspartyl peptide ester formation *via* aspartimide ring opening

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Abstract: The undesirable reaction of aspartimide formation has been proved to occur under both acid and base conditions in solid-phase peptide synthesis and is dependent on the β-carboxyl protecting group, the acid or base used during the synthesis, as well as the peptide sequence. The hydrolysis of aspartimide-containing peptides, especially during HPLC purification, yields a mixture of α- and β-aspartyl peptides that can not be purified easily. A previous study demonstrated that treatment of aspartimide-containing peptides with methanol in the presence of 2% diisopropylethylamine in solution leads to α- and β-aspartyl peptide methyl esters. Taking advantage of these results and aiming at elucidating the optimal conditions for aspartimide ring opening, the effect of different types and concentrations of alcohols (primary and secondary) and bases (diisopropylethylamine, collidine, 4-pyrrolidinopyridine, 1-methyl-2-pyrrolidone, piperidine and KCN) was tested at various temperatures and reaction times. The best results were obtained with a combination of a primary alcohol and diisopropylethylamine, while aspartimide ring opening by secondary alcohols occurred only at high temperatures. The optimal conditions were also applied to solid-phase peptide synthesis. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: aspartimide; aspartimide chemistry; α- and β-aspartyl peptide esters

INTRODUCTION

The main side reaction in the synthesis of Asp-containing peptides is aspartimide formation (Asi: aspartimide; the abbreviation Asu was used in some publications to denote aspartimide [1,2] while the same abbreviation is proposed for α-aminosuberic acid [3]). In order to avoid future confusion we propose the use of Asi as abbreviation, derived from **As**parti**i**mide. Asi formation occurs under both acid and base conditions [1,4] (Figure 1). In Boc (*tert*-butyloxycarbonyl)-based solid-phase peptide synthesis (SPPS), acid-catalysed aspartimide formation has been reported to occur in the presence of both strong acids, such as HF and trifluoromethanesulfonic acid-trifluoroacetic acid (TFMSA-TFA), and milder acids, such as TFA [5]. In Fmoc (9-fluorenylmethoxycarbonyl)-based peptide chemistry aspartimide formation occurs via base catalysis, particularly with the prolonged use of piperidine or stronger bases [6,7] for the Fmoc group cleavage during the synthetic cycles. It has been established that this intramolecular cyclization occurs at higher rates under base conditions [4].

Several detailed studies have related the extent of Asi formation in Asp-containing peptides to: (i) the protocols used in peptide synthesis, i.e. the choice of Fmoc-deprotecting or coupling reagents [8,9], (ii) the type of Asp β-carboxyl protecting group [5,8], (iii) the amino acid which follows Asp in the sequence, -Asp-Xaa- (imide formation have been reported to occur when Xaa is Gly, Asn, Asp, Ser, Ala, Arg, Cys, His and Gln, while the extent of the reaction depends on whether the Boc/Bzl (benzyl) or the Fmoc/Bu^t (*tert*-butyl) synthetic approach is followed [7,10] and (iv) the peptide conformation [6,11].

Various methods have been proposed aimed at minimizing Asi formation. The use of HOBt (1-hydroxybenzotriazole) or 2,4-dinitrophenol (Dnp) with the base during Fmoc deprotection can partly, but not fully, suppress this side reaction [12]. In addition, these two reagents are not convenient when the peptide is attached to the resin via a base-labile linker. Another approach includes the protection of the amide group of the Xaa residue in the sequence -Asp-Xaa- with the Hmb (2-hydroxy-4-methoxybenzyl) group [13]. However, this method is not compatible with the Boc-based chemistry. In addition, difficulties have been reported in the Fmoc-based chemistry, including incomplete couplings due to the bulkiness of Hmb, as well as incomplete backbone deprotection in cases of amino acids other than glycine [10]. In general, electron donating and/or sterically hindered β-carboxyl protecting groups, such as cyclohexyl ester in the Boc-based chemistry [5] and *tert*-butyl ester in the Fmoc/Bu^t approach [8], can also minimize Asi formation. In some cases, and especially in the synthesis of long peptides, considerable amounts of Asi-containing peptides and related by-products have been obtained.

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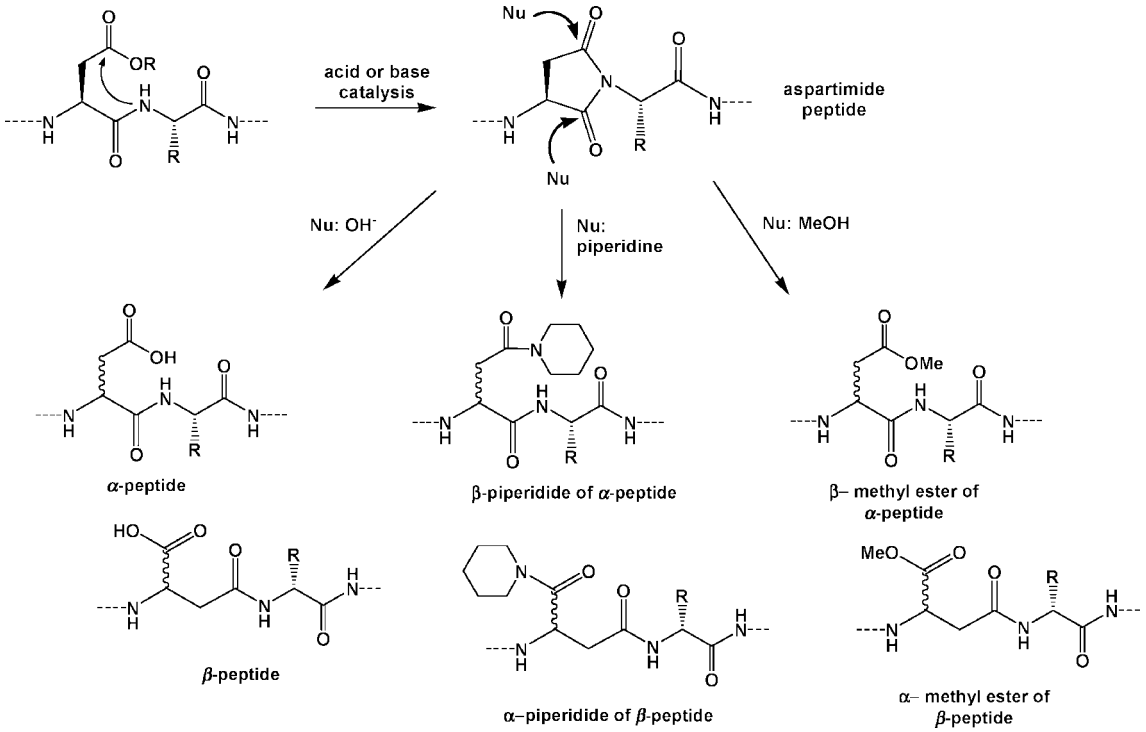

Figure 1 Mechanism of Asi formation and the products from its reaction with nucleophiles.

Once the imide ring is formed, the susceptibility of the two carbonyl carbons to nucleophiles such as H_2O, piperidine, etc., leads to opening of the ring (Figure 1). The hydrolysis of the succinimide moiety produces a mixture of α- and β-aspartyl peptides with the latter being the main product [14]. Enantiomerization of Asp can also take place by treatment of Asi peptides with strong bases [15]. The main problem caused by this reaction, apart from the reduced yield of the target α-peptide, is the difficult separation and purification of the two products (α- and β-aspartyl peptides). We have recently reported [16] a method of Asi ring opening, consisting of treatment of the Asi-containing peptide with a solution of 2% DIEA in methanol (v/v) yielding α- and β-aspartyl peptide methyl esters. A great advantage of this method is an easier purification of the two products, which does not afford the usually unresolved mixture of α- and β-aspartyl peptides resulting from the hydrolysis of the imide ring during purification.

Despite the fact that the cyclization of an Asp residue to Asi is generally an undesirable side reaction in peptide synthesis, the chemical and structural properties of the 5-membered ring can be significantly useful. 2,5-Dioxopiperazines, which are often used as templates for the synthesis of peptidomimetics [17], can be produced by the intramolecular attack of a dipeptide *N*-terminus on the aminosuccinyl carbonyl group [18]. However, the main advantage of the Asi-containing peptides is that the geometry of the succinimide residue is identical to that predicted for the second residue of a type II′ β-turn [19]. Therefore, the imide ring can be used for inducing a type II′ β-turn in the backbone conformation of a peptide [20,21]. Due to this conformational behavior, Asi-containing peptides have found application in structure-activity studies [14,22]. Various methods that improve the imide ring formation have been explored, e.g. treatment of the peptide-resin with tertiary amines (10% triethylamine in CH_2Cl_2 for 15 h) before removal of the solid support with TFMSA, or the Fmoc deprotection with 50% piperidine in DMF (v/v) for 20 min [2].

The aim of this study was to elucidate the effect of different types of alcohols and bases at various concentrations, temperatures and time of reactions, on the succinimide ring opening by esterification. The imide ring opening in all of the Asi-containing peptides was explored by varying the following conditions: (i) the type of alcohols used for esterification (i.e. primary and secondary alcohols), (ii) the type and concentration of the base, (iii) the temperature and (iv) the time of reaction. Four Asi-containing model peptides Ac-Asi-Val-Arg-OH (Ac, acetyl) (**1**), Ac-Asi-Ala-Lys-OH (**2**), Ac-Asi-Gly-Ala-OH (**3**), and Ac-Asi-Arg-NH_2 (**4**) were synthesized and used in this study.

MATERIALS AND METHODS

HBTU, HOBt, Fmoc amino acids, Rink AM resin and Wang resin were purchased from Neosystem Laboratoire, (Strasbourg, France). Solvents were purchased from Labscan, (Dublin, Ireland), while TFA and DIEA were Merck-Schuchardt

(Darmstadt, Germany) products. All reagents and solvents were used without further purification.

Peptide Synthesis

The synthesis of the Asi-containing model peptides Ac-Asi-Val-Arg-OH (**1**), Ac-Asi-Ala-Lys-OH (**2**) and Ac-Asi-Gly-Ala-OH (**3**) was carried out manually by a stepwise solid-phase procedure on Wang resin. The peptide Ac-Asi-Arg-NH_2 (**4**) was synthesized on Rink amide resin. The Fmoc group was used for N^{α}-protection while the amino acid side chain functional groups were protected as: Boc for Lys, Pbf (2,2,5,7,8-pentamethyl-chromane-6-sulfonyl) for Arg and Bzl for Asp. All coupling reactions were performed using a molar ratio of amino acid/HBTU/HOBt/DIEA/resin (3 : 3 : 3 : 6 : 1). The Fmoc cleavage protocol consisted of two treatments (5 and 10 min) with 20% piperidine in DMF. Acetylation was performed at the *N*-terminal amino group of compounds **1**–**4** with $(Ac)_2O$ in pyridine in an $(Ac)_2O$/peptide-resin 30 : 1 molar ratio. The peptides **1**–**4** were cleaved from the resin using the TFA/TIS (triisopropylsilane)/H_2O (95 : 2.5 : 2.5, v/v) method at room temperature for 3 h.

The crude peptides were purified by high performance liquid chromatography (HPLC) (semi-preparative reversed phase C_{18} column) using a gradient elution with the following solvents: A, H_2O/0.1% TFA and B, CH_3CN/0.1% TFA. The purity of the Asi-containing peptides was evaluated by analytical HPLC and the correct molecular mass was confirmed by electro spray ionization–mass spectroscopy (ES-MS) (Table 1).

Reaction Monitoring

The transformations of the Asi-containing peptides to the corresponding α- and β-aspartyl peptide esters were monitored by recording comparative ES-MS spectra under the same instrumental conditions (cone voltage: 50 V; source temperature: 60 °C, flow rate: 5 µl/min) on a Platform II quadropole mass spectrometer (Figures 2A–D). The ES-MS spectra were recorded in the positive ion mode, except that of model **3** for which the negative ion mode was used due to the lack of positive charges. The ES-MS type of monitoring of the reaction was also imposed by the fact that the Asi-containing peptides and the resulting α- and β-aspartyl peptide esters in most of the cases gave almost overlapping peaks in the analytical HPLC spectra.

RESULTS AND DISCUSSION

Aspartimide Formation

The -OBzl group was chosen for the protection of the Asp side chain in order to improve the yields of the Asi-containing peptides needed for this study. The nature of the amino acid Xaa that follows the Asp in the sequence Asp-Xaa and the type of the Asp β-carboxyl group protection, as already known, play an important role in Asi formation. Following the standard Fmoc synthetic approach (i.e. piperidine as Fmoc deprotecting reagent) and having the Asp β-carboxyl group protected with the -OBzl group, the synthesis of the tripeptide Ac-Asp-Gly-Ala-OH resulted in a quantitative Asi formation (100%) (Table 1). However, this was not the case when Xaa was Val (0%), Ala (18%) and Arg (9%) (Table 1). On the other hand, further treatment of the peptide-resin with 20% piperidine in DMF (v/v) for 30 min after the acetylation proved to be more efficient in imide ring formation. As shown in Table 1, the most drastic change was in the case of the -Asp-Arg-containing peptide which provided 83% of Asi peptide **4** while the –Asp-Val- and –Asp-Ala- containing peptides were also affected (73% of **1** and 51% of **2**, respectively). Therefore, Asi formation can be achieved in high yields, even in cases of a sterically hindered amino acid like Val following Asp in the sequence, by (i) protecting the Asp β-carboxyl group with the benzyloxy group and (ii) prolonged treatment of the peptide-resin with piperidine. The latter parameter might not be necessary in the synthesis of long peptide chains due to the repetitive deprotection steps with piperidine (however, this case was not tested).

Aspartimide Ring Opening in Solution

As mentioned above, the imide ring can be transformed to α- and β-aspartyl methyl esters (Figure 1) by treatment with a methanol solution of 2% DIEA (v/v) [15]. This method of Asi ring opening can be significantly important in order to avoid the generally difficult separation of a mixture consisting of α- and β-aspartyl peptides, which are formed by hydrolysis of the imide ring during the HPLC purification

Table 1 Yields of the Asi-containing Peptides Depending on the Piperidine Treatment and ESI-MS Data

Peptide	% Asi- containing peptide[a]	% Asi- containing peptide[b]	Expected MW	Found MW	Retention time (min)
Ac-Asi-Val-Arg-OH (**1**)	0	51	412.45	412.39	12.7
Ac-Asi-Ala-Lys-OH (**2**)	18	73	356.38	356.41	5.2
Ac-Asi-Gly-Ala-OH (**3**)	100	—	285.27	285.12	9.3
Ac-Asi-Arg-NH_2 (**4**)	9	83	312.34	312.23	8.4

[a] After treatment with piperidine for 15 min.
[b] After two treatments (1 × 15 min and 1 × 30 min) with piperidine, before and after acetylation of the peptide resin, respectively.

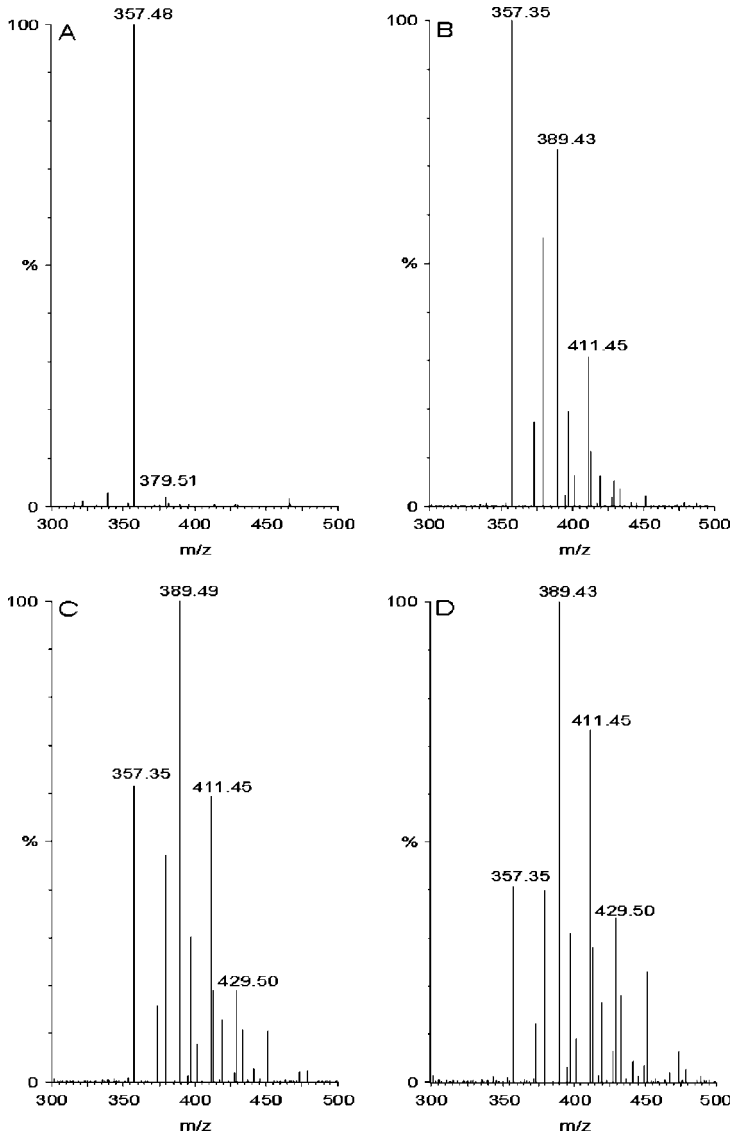

Figure 2 Monitoring of the Asi ring opening with ES-MS spectra recorded under the same instrumental conditions (cone voltage: 50 V; temperature: 60 °C, flow rate: 5 µl/min). The Ac-Asi-Ala-Lys-OH model in MeOH after 3 days (A), in DCM/MeOH (1 : 2, v/v) in the presence of 0.1% DIEA (v/v) after 15 min (B), after 30 min (C), and after 45 min (D). The peak assignments are the following: Ac-Asi-Ala-Lys-OH ($M + H^+ = 357.48$, $M + Na^+ = 379.51$), α- and β-aspartyl peptide methyl esters of Ac-Asp-Ala-Lys-OH ($M + H^+ = 389.43$, $M + Na^+ = 411.45$, $M + K^+ = 429.50$).

procedures. Therefore, various conditions for optimizing the esterification reaction were examined by using the purified Asi-containing peptides as models.

The first factor examined was the effect of various types of alcohols in the presence of 2% DIEA (Table 2). The transformation of the Ac-Asi-Arg-NH_2 peptide to the corresponding α- and β-methyl ester peptides does not occur in the absence of the base (2% DIEA) even after 3 days at room temperature. Apparently, the presence of the base is of great significance for the imide ring opening. In the presence of 2% DIEA in methanol (v/v), 67% of Ac-Asi-Arg-NH_2 was transformed into the corresponding α- and β-aspartyl methyl ester peptides in 30 min. The rate of this transformation was even higher in the case of Ac-Asi-Ala-Lys-OH, which, under the same conditions, provided 77% α- and β-aspartyl methyl ester peptides in 15 min. The esterification of Ac-Asi-Arg-NH_2 was less efficient with 2% DIEA in *n*-butanol (v/v) (55% in 30 min), while the secondary alcohols isopropanol and cyclohexanol proved to be less reactive. Therefore, it is concluded that the presence of a base as catalyst is necessary for the imide ring opening with alcohols, while primary alcohols and in particular methanol, are the most appropriate nucleophiles for the esterification.

The strongest effect of the temperature on imide ring opening by alcohols in the presence of DIEA was observed in the case of secondary alcohols (Table 3). The yields of α- and β-aspartyl ester peptides produced by methanol or 1-butanol did not change by increasing

Table 2 Influence of the Type of Alcohol on Asi Ring Opening in Solution at Room Temperature

Peptide	Alcohol	Base	Time (min)	% Asi- containing peptide[a]	% α-, β- Aspartyl peptide ester[a]
Ac-Asi-Arg-NH_2	Methanol	0% DIEA	3 days	100	0
Ac-Asi-Arg-NH_2	Methanol	2% DIEA	30	33	67
Ac-Asi-Ala-Lys-OH	Methanol	2% DIEA	15	23	77
Ac-Asi-Arg-NH_2	*n*-Butanol	2% DIEA	30	45	55
Ac-Asi-Arg-NH_2	Isopropanol	2% DIEA	30	65	35
Ac-Asi-Arg-NH_2	Cyclohexanol	2% DIEA	30	96	4

[a] The percentages represent the relative peak intensities estimated from the related ES-MS spectra. See also text.

Table 3 Influence of the Temperature on Asi Ring Opening by Various Alcohols in the Presence of 2% DIEA

Peptide	Alcohol	Base	Time (min)	Temp. (°C)	% Asi- containing peptide[a]	% α-, β- Aspartyl peptide ester[a]
Ac-Asi-Arg-NH_2	Methanol	0% DIEA	3 days	25	100	0
Ac-Asi-Arg-NH_2	Methanol	2% DIEA	30	25	33	67
Ac-Asi-Arg-NH_2	Methanol	2% DIEA	30	70	35	65
Ac-Asi-Arg-NH_2	*n*-Butanol	2% DIEA	30	25	45	55
Ac-Asi-Arg-NH_2	*n*-Butanol	2% DIEA	30	70	47	53
Ac-Asi-Arg-NH_2	Isopropanol	2% DIEA	60	25	63	37
Ac-Asi-Arg-NH_2	Isopropanol	2% DIEA	60	70	49	51
Ac-Asi-Arg-NH_2	Cyclohexanol	2% DIEA	24 h	25	75	25
Ac-Asi-Arg-NH_2	Cyclohexanol	2% DIEA	24 h	70	59	41

[a] The percentages represent the relative peak intensities estimated from the related ES-MS spectra. See also text.

Table 4 Influence of the Nature of the Base on Asi Ring Opening

Peptide	Alcohol	Base	Time (min)	Temp. (°C)	% Asi-containing peptide[a]	% α-, β- Aspartyl peptide ester[a]
Ac-Asi-Arg-NH_2	Methanol	0% DIEA	3 days	25	100	0
Ac-Asi-Arg-NH_2	Methanol	2% DIEA 2%	15	25	35	65
Ac-Asi-Arg-NH_2	Methanol	4-Pyrrolidino-pyridine	15	25	28	72
Ac-Asi-Arg-NH_2	Methanol	0.1% KCN	15	25	33	67
Ac-Asi-Arg-NH_2	Cyclohexanol	2% DIEA	24 h	70	59	41
Ac-Asi-Arg-NH_2	Cyclohexanol	2% Collidine 2%	24 h	70	89	11
Ac-Asi-Arg-NH_2	Cyclohexanol	1-Methyl-2-pyrrolidone 2%	24 h	70	80	20
Ac-Asi-Arg-NH_2	Cyclohexanol	4-Pyrrolidino-pyridine	24 h	70	65	35

[a] The percentages represent the relative peak intensities estimated from the related ES-MS spectra. See also text.

the temperature up to 70 °C. On the contrary, in the case of isopropanol and cyclohexanol, the increase of temperature improved the yield of esterification compared with those observed at room temperature for the same alcohols. However, these yields were not better, even at longer times of reaction, than those obtained for methanol and *n*-butanol at room temperature.

The type and the strength of the base used for the imide ring opening of the Asi-containing peptides were proved to be very important. Treatment of the Ac-Asi-Arg-NH_2 peptide with solutions of collidine, 1-methyl-2-pyrrolidone and 4-pyrrolidino pyridine [2% base in methanol or cyclohexanol (v/v)] provided significantly lower yields, even at higher temperatures (70 °C) and times of reaction (24 h), compared with those obtained with 2% DIEA (Table 4). An exception occurred with the MeOH solutions of either 0.1% KCN or 2% 4-pyrrolidinopyridine, with the yields of the esterification products (67% and 72%, respectively) being comparable

to that obtained in the case of 2% DIEA in MeOH (65%).

Taking into consideration the fact that MeOH proved to be the most appropriate alcohol for the esterification of the Asi-containing peptides, the effect of varying the DIEA concentration was tested. By decreasing the concentration of DIEA to 0.1% in methanol (v/v), almost the same results were obtained as in the case of using 2% DIEA (Table 5). Longer times were needed to achieve comparable results when mixtures of DCM/MeOH were used instead of pure MeOH (Table 5 and Figure 2). Therefore, it is concluded that even lower concentrations of DIEA can afford a quantitative esterification of the Asi-containing peptides in MeOH solution.

Aspartimide Ring Opening in Solid Phase

The estimation of the optimal conditions for the Asi ring opening by esterification to the corresponding α- and β-aspartyl peptide methyl esters in solution prompted us to check the applicability of the above experiments in solid phase as well. Thus, after completion of the synthesis of the Ac-Asp-Ala-Lys-OH sequence, treatment with 20% piperidine in DMF for 30 min and removal of the solid support with 95% aqueous TFA resulted in the Ac-Asi-Ala-Lys-OH peptide as the main product in a 73% yield (Table 1). However, when the peptide-resin was treated before the removal of the solid support with 2% DIEA in methanol for 60 min, only 29% of Ac-Asi-Ala-Lys-OH and 53% of α- and β-aspartyl peptide methyl esters were obtained (Table 6). The use of a lower concentration of DIEA (0.1%), in contrast to the results obtained in solution, provided a significantly higher yield of the Asi-containing peptide (51%) and a lower yield of α- and β-aspartyl peptide methyl esters (22%). Therefore, it is concluded that a concentration of 2% (v/v) DIEA is required for a sufficient imide ring opening in the solid phase. Taking into account the fact that DCM swells the resin more efficiently than MeOH it was thought that the use of 2% DIEA in a mixture of DCM/MeOH might provide even better yields of α- and β-aspartyl peptide methyl esters in the solid phase. However, this was not the case. Indeed, the treatment of the Ac-Asp-Ala-Lys-resin (the treatment with piperidine was performed as discussed above) with a mixture of 2% DIEA in DCM/MeOH (1 : 2, v/v) for 60 min provided 45% of Asi-containing peptide and a 41% of α- and β-aspartyl peptide methyl esters. After treatment with the same mixture for 4 h, the results were slightly improved (36% and 51%, respectively) (Table 6). Surprisingly, treatment of the Ac-Asi-Ala-Lys-OH peptide in solution with the mixture of DCM/MeOH (1 : 2, v/v) in the presence of 0.5% DIEA provided 72%

Table 5 Influence of the Base Concentration on Asi Ring Opening at Room Temperature

Peptide	Solvent	Base	Time (min)	% Asi- containing peptide[a]	% α-, β- Aspartyl peptide ester[a]
Ac-Asi-Ala-Lys-OH	MeOH	0% DIEA	3 days	100	0
Ac-Asi-Ala-Lys-OH	MeOH	0.1%DIEA	15	23	77
Ac-Asi-Ala-Lys-OH	MeOH	2% DIEA	15	23	77
Ac-Asi-Ala-Lys-OH	DCM/MeOH (1 : 1, v/v)	0.1%DIEA	15	83	17
Ac-Asi-Ala-Lys-OH	DCM/MeOH (1 : 1 v/v)	0.1%DIEA	45	54	46
Ac-Asi-Ala-Lys-OH	DCM/MeOH (1 : 2, v/v)	0.1%DIEA	15	60	40
Ac-Asi-Ala-Lys-OH	DCM/MeOH (1 : 2, v/v)	0.1% DIEA	45	31	69
Ac-Asi-Ala-Lys-OH	DCM/MeOH (1 : 2, v/v)	0.5% DIEA	15	52	48
Ac-Asi-Ala-Lys-OH	DCM/MeOH (1 : 2, v/v)	0.5% DIEA	45	27	72

[a] The percentages represent the relative peak intensities estimated from the related ES-MS spectra. See also text.

Table 6 Extent of Asi Ring Opening in Various DCM/MeOH Ratios and Contents of DIEA in Solid-Phase Reactions

Peptide	Solvent	Base	Time (min)	% Asi- containing peptide[a]	% α-, β- Aspartyl peptide ester[a]	% By-products[a]
Ac-Asi-Ala-Lys-OH	—	—	—	73	0	27
Ac-Asi-Ala-Lys-OH	MeOH	0.1% DIEA	60	51	22	27
Ac-Asi-Ala-Lys-OH	MeOH	2% DIEA	60	29	53	18
Ac-Asi-Ala-Lys-OH	DCM/MeOH (1 : 2, v/v)	2% DIEA	60	45	41	14
Ac-Asi-Ala-Lys-OH	DCM/MeOH (1 : 2, v/v)	2% DIEA	240	36	51	13

[a] The percentages represent the relative peak intensities estimated from the related ES-MS spectra. See also text.

of α- and β-aspartyl peptide methyl esters after 45 min (Table 5). Finally, the effect of the MeOH solution of 2% DIEA on the *tert*-butyl and cyclohexyl Asp β-carboxyl protecting groups was tested. No significant influence was observed.

CONCLUSION

Our results suggest that quantitative Asi formation in the Fmoc-based solid-phase synthesis of Asp-containing peptides can be achieved by using the benzyl group as Asp β-carboxyl protection and prolonged treatment of the peptide-resin with piperidine (even in cases of sterically hindered amino acids following Asp in the peptide sequence).

The Asi transformation to α- and β-aspartyl peptide methyl esters, which are easily separated from the α-aspartyl peptide, can be obtained in high yield with methanol in the presence of a catalytic amount of base at room temperature. The best conditions in solution (0.1% DIEA in methanol for 15 min at room temperature) can be applied before the step of the purification procedure. In solid-phase peptide synthesis, treatment of the peptide-resin after the completion of the sequence with 2% DIEA in methanol for 1 h at room temperature seems to be the most effective of the conditions tested in this study. The latter procedure can be used both in Fmoc- and Boc-based SPPS methodologies, since the *tert*-butyl and cyclohexyl Asp β-carboxyl protecting groups are not affected by a methanol solution of 2% DIEA.

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