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Limiting racemization and aspartimide formation in microwave-enhanced Fmoc solid phase peptide synthesis

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Abstract: Microwave energy represents an efficient manner to accelerate both the deprotection and coupling reactions in 9-fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS). Typical SPPS side reactions including racemization and aspartimide formation can occur with microwave energy but can easily be controlled by routine use of optimized methods. Cysteine, histidine, and aspartic acid were susceptible to racemization during microwave SPPS of a model 20mer peptide containing all 20 natural amino acids. Lowering the microwave coupling temperature from 80 °C to 50 °C limited racemization of histidine and cysteine. Additionally, coupling of both histidine and cysteine can be performed conventionally while the rest of the peptide is synthesized using microwave without any deleterious effect, as racemization during the coupling reaction was limited to the activated ester state of the amino acids up to 80 °C. Use of the hindered amine, collidine, in the coupling reaction also minimized formation of D-cysteine. Aspartimide formation and subsequent racemization of aspartic acid was reduced by the addition of HOBt to the deprotection solution and/or use of piperazine in place of piperidine. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Fmoc solid phase peptide synthesis; microwave; racemization; aspartimide formation

INTRODUCTION

While use of microwave energy has become widespread for enhancing chemical reactions in organic synthesis, only recently has it been adopted in solid phase peptide synthesis (SPPS). This is primarily attributed to concern over side reactions and until recently a lack of availability of proper tools. There have been several publications using both domestic and single-mode microwave devices that report enhanced coupling reactions [1–10]. As several well-known side reactions have been noted in SPPS, there has been concern that microwave, while speeding up deprotection and coupling reaction rates, may also accelerate racemization, aspartimide formation, and other potential problems.

The application of microwave energy has proved to be a major enabling tool for many chemical applications requiring energy input. In the last 10 years, microwave synthesis has become widely accepted to increase reaction rates in organic synthesis up to 1000-fold. Unlike conventional heating, microwave energy directly activates any molecule with a dipole moment and allows for rapid heating at the molecular level.

In SPPS, reactions must proceed cleanly and efficiently in order to be successful. While certain peptide sequences are synthesized relatively easily, some sequences are much more difficult. During assembly of some peptides, sudden decreases in reaction rates resulting in incomplete couplings have been observed [11]. In some cases, repeated or prolonged reaction time showed no improvement in chain assembly. Optimal reaction conditions require a fully solvated peptide–polymer matrix that allows for efficient reagent penetration [12]. It has been noted that during the synthesis of difficult peptides the reaction matrix becomes partially inaccessible, typically 6–12 residues into chain assembly. This is thought to occur owing to formation of secondary structures that result in poor solvation of the peptide–polymer matrix. As a peptide is built stepwise on a resin bead, it can form aggregates with itself or neighboring chains via hydrogen bonding of the peptide backbone. Microwave energy represents a fast and efficient way to enhance both the deprotection and coupling reactions hindered by aggregation. In peptides, the *N*-terminal amine group and peptide backbone are polar, causing them to constantly try to align with the alternating electric field of the microwave. During peptide synthesis this can help break up the chain aggregation owing to intra and interchain association and allow for easier access to the solid phase reaction matrix (Figure 1).

The properties of proteins and peptides are critically dependent on the configuration of their chiral centers. The alteration of a single chiral center can have a drastic effect on biological activity. Therefore, racemization must be minimized during peptide synthesis. With the exception of glycine, all 20 standard amino acids contain a chiral center at the α-carbon atom. Additionally, isoleucine and threonine contain a chiral center in their side chains. In SPPS, racemization has been extensively documented during the coupling

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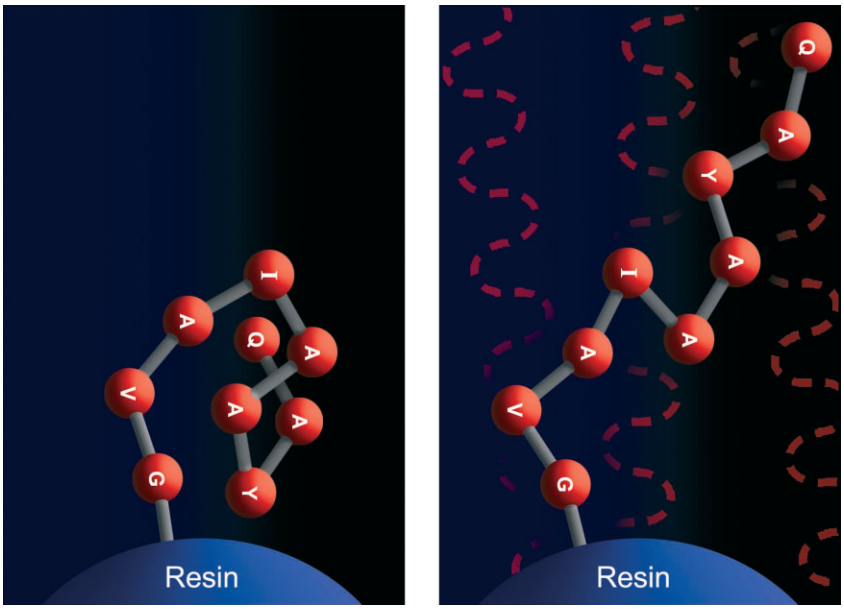

(a) (b)

Figure 1 Microwave energy disrupting aggregation through dipole rotation of the polar peptide backbone.

reaction [13–15]. The two main mechanisms of racemization are direct enolization and oxazolone formation [16].

Base-catalyzed enolization can occur during the coupling reaction. The conversion of the incoming amino acid to an activated ester increases the acidity of the α-carbon. This can tend to favor enolization, which leads to rearrangement about the α-carbon. The rate of racemization depends on the electron-withdrawing effect of the amino acid side chain, the temperature of the reaction, the reaction solvent, and the activator base. In SPPS, sterically hindered tertiary amines are used in an effort to minimize base-catalyzed removal of the α-carbon proton. (Figure 2).

Enantiomerization of the incoming amino acid residue is possible through formation of an oxazolone ring. This ring can then reopen to racemize about the α-carbon. Enantiomerization by oxazolone formation occurs more frequently in fragment condensation when connecting peptide fragments together. However,

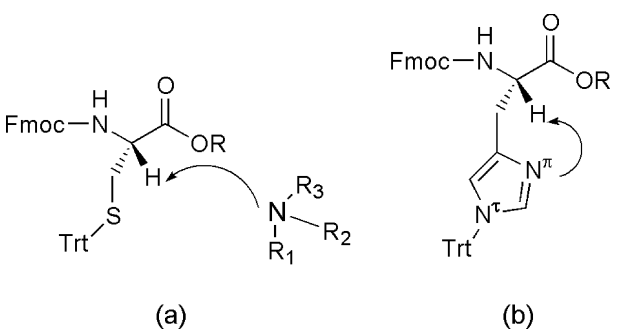

Figure 2 Potential mechanisms for racemization of activated Fmoc derivatives of (a) cysteine and (b) histidine.

certain *N*-urethane protected amino acids have been shown to be susceptible to this reaction, particularly histidine and cysteine [13].

Base-catalyzed aspartimide formation is a very common side reaction observed in 9-fluorenylmethyloxycarbonyl (Fmoc) SPPS [17,18]. This sequence-dependent side reaction occurs during the Fmoc deprotection reaction in peptides containing the 'Asp-AA' moiety, where AA = Gly, Asn, Ser, or Ala. The reaction involves the attack of the side chain ester of aspartic acid by the nitrogen atom attached to the α-carboxy group. Subsequent hydrolysis of the aspartimide ring gives rise to a mixture of α- and β-aspartyl peptides. In addition, nucleophilic attack of the imide ring by piperidine results in the formation of α- and β-piperidides. Aspartimide formation can be a serious problem in longer peptides with multiple Asp residues since each subsequent deprotection cycle after the 'Asp-AA' sequence further increases the formation of aspartimide. The bulkiness of the tert-butyl ester protecting group provides some defense against this unwanted side reaction. Only protection of the aspartyl amide bond with the 2-hydroxy-4-methoxybenzl (Hmb) group will eliminate aspartimide formation altogether [19]. Unfortunately, these derivatives are very expensive and not desirable for routine peptide synthesis.

Alternatively, incorporation of 0.1 M HOBt to the piperidine solution has shown to reduce aspartimide formation [20,21]. In addition, the use of piperazine in place of piperidine has demonstrated significantly lower levels of aspartimide formation [22]. As a precursor for the synthesis of phenylcyclidine (Angel Dust), piperidine is a controlled substance regulated by the Drug Enforcement Agency. Piperazine is a noncontrolled

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substance making it more accessible to laboratories than piperidine. Piperazine is also less odorous and toxic than piperidine. However, with a p*K*a of 9.8 compared to 11.1 for piperidine, piperazine is a slower deprotection reagent. For conventional synthesis of hydrophobic sequences, the use of piperazine may not be desirable.

The aim of the following study was to quantify and limit racemization and aspartimide formation in microwave-enhanced Fmoc SPPS. A 20mer peptide containing each of the natural 20 amino acids, with a selectively placed *C*-terminal Asp-Gly segment to encourage maximum aspartimide formation potential, served as the model peptide for the inquiry.

MATERIALS AND METHODS

Reagents

All Fmoc amino acids were obtained from Novabiochem (San Diego, CA) and contained the following side chain protecting groups: Asn(Trt), Asp(OtBu), Arg(Pbf), Cys(Trt), Gln(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), and Tyr(tBu). *N*-[(1*H*-Benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), *N*-hydroxybenzotriazole (HOBt), benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), and Rink amide MBHA resin were also obtained from Novabiochem. Diisopropylethylamine (DIEA), *N*-methylmorpholine (NMM), collidine (TMP), piperidine, piperazine, trifluoroacetic acid (TFA), thioanisole, 1,2-ethanedithiol (EDT), and phenol were obtained from Sigma Aldrich (St. Louis, MO). Dichloromethane (DCM), *N*,*N*-Dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), anhydrous ethyl ether, acetic acid, HPLC grade water, and HPLC grade acetonitrile were obtained from VWR (West Chester, PA).

CEM Liberty Automated Microwave Peptide Synthesizer

The Liberty system is a sequential peptide synthesizer capable of complete automated synthesis including cleavage of up to 12 different peptides. The system uses the single-mode microwave reactor, Discover, which has been widely used in the organic synthesis industry. The Liberty uses a standard 30 ml Teflon glass fritted reaction vessel for 0.025–1.0 mmol syntheses. The reaction vessel features a spray head for delivery of all reagents and a fiber-optic temperature probe for controlling the microwave power delivery. The system utilizes up to 25 stock solutions for amino acids and seven reagent ports that perform the following functions: main wash, secondary wash, deprotection, capping, activator, activator base, and cleavage. The system uses nitrogen pressure for transfer of all reagents and to provide an inert environment during synthesis. Nitrogen bubbling is used for mixing during deprotection, coupling, and cleavage reactions. The system uses metered sample loops for precise delivery of all amino acid, activator, activator base, and cleavage solutions. The Liberty is controlled by an external computer, which allows for complete control of each step in every cycle.

Peptide Synthesis: VYWTSPFMKLIHEQCNRADG-NH_2

A model peptide containing all 20 amino acids was synthesized under a variety of conditions using the CEM Liberty automated microwave peptide synthesizer on 0.152 g Rink amide MBHA resin (0.66 meq/g substitution). Deprotection was performed in two stages using a fresh reagent each time with one of the three solutions: (i) 20% piperidine in DMF; (ii) 20% piperidine with 0.1 M HOBt in DMF; or (iii) 5% piperazine with 0.1 M HOBt in DMF. An initial deprotection of 30 s at 50 W (5 min at 0 W for conventional synthesis) was followed by a 3-min deprotection at 50 W (15 min at 0 W for conventional synthesis) with a maximum temperature of 80 °C. Coupling reactions were performed in the presence of a 5-fold molar excess of 0.2 M Fmoc-protected amino acids dissolved in DMF with various types of activation: (i) HBTU : DIEA : AA (0.9 : 2 : 1); (ii) HBTU : HOBt : DIEA : AA (0.9 : 1 : 2 : 1); (iii) PyBOP : DIEA : AA (0.9 : 2 : 1); (iv) HBTU : NMM : AA (0.9 : 2 : 1); and (v) HBTU : TMP : AA (0.9 : 2 : 1), double coupling on valine. Coupling reactions were for 5 min at 40 W (30 min at 0 W for conventional synthesis) with a maximum temperature of 80 °C. In latter experiments, coupling conditions of cysteine and histidine were altered to 2 min at 0 W followed by 4 min at 40 W with a maximum temperature of 50 °C. Cleavage was performed using 10 ml of Reagent K (TFA/phenol/water/thioanisole/EDT; 82.5/5/5/5/2.5) for 180 min. Following cleavage, peptides were precipitated out and washed using ice-cold anhydrous ethyl ether.

Peptide Analysis

Prior to LC-MS analysis, all peptides were dissolved in 10% acetic acid solution and lyophilized to dryness. Analytical HPLC of peptide products was performed using a Waters Atlantis dC18 column (3 μm, 2.1 × 100 mm) at 214 nm. Separation was achieved by gradient elution of 5–60% solvent B (solvent A = 0.05% TFA in water; solvent B = 0.025% TFA in acetonitrile) over 60 min at a flow rate of 0.5 ml/min. Mass analysis was performed using an LCQ Advantage ion trap mass spectrometer with electrospray ionization (Thermo Electron, San Jose, CA). Racemization analysis of amino acids was performed by C.A.T. GmbH & Co. (Tuebingen, Germany) using a published GC-MS method that involves hydrolysis of the peptide in 6 N DCl/D_2O [23].

RESULTS AND DISCUSSION

The model 20mer peptide was first synthesized using conventional room temperature SPPS to determine baseline racemization levels for all amino acids (Table 1). While enantiomerization about a single chiral center can have a major impact on the biological activity of a peptide, it can be a very difficult task to quantify. Racemization was determined by GC-MS analysis of the peptide hydrolyzed into free amino acids in deuterated solvents. This method is more accurate and sensitive than quantification using C-18 or chiral HPLC columns. Conventional synthesis yields less than 1.5% D-enantiomer for all amino acids. However, a number of deletion products are present in the LC-MS, resulting in

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Table 1 Crude product purity measured by LC-MS and racemization of amino acids measured by GC-MS after hydrolysis of VYWTSPFMKLIHEQCNRADG-NH_2 with 6 N DCl/D_2O

Amino acid	Synthesis conditions							
	Conventional	Microwave						
	20% Piperidine in DMF	20% Piperidine in DMF (80 °C)	20% Piperidine with 0.1 M HOBt in DMF (80 °C)	20% Piperidine with 0.1 M HOBt in DMF (80 °C)	5% Piperazine with 0.1 M HOBt in DMF (80 °C)	5% Piperazine with 0.1 M HOBt in DMF (80 °C)	5% Piperazine with 0.1 M HOBt in DMF (80 °C)	5% Piperazine with 0.1 M HOBt in DMF (80 °C)
	HBTU/DIEA in DMF	HBTU/DIEA in DMF (80 °C)	HBTU/ HOBt/DIEA in DMF (80 °C)	PyBOP/DIEA in DMF (80 °C)	HBTU/DIEA in DMF (80 °C)	HBTU/TMP in DMF (80 °C)	HBTU/HOBt in DMF (80 °C) ([a]Cys, His)	HBTU/HOBt in DMF (80 °C) ([b]Cys, His)
D-Asp	1.19	9.60	3.50	3.83	1.18	0.91	1.62	Not determined
D-Ala	0.21	0.50	0.34	0.18	0.29	0.12	0.63	Not determined
D-Arg	0.18	0.17	0.21	0.18	0.15	<0.10	0.20	Not determined
D-Cys	1.09	<4.48	<7.00	<6.95	<3.96	<1.03	<3.16	<1.14
D-Glu	1.46	<1.14	<1.45	<1.04	<1.27	<1.26	<1.51	Not determined
D-His	0.65	9.40	8.40	12.30	10.91	8.49	1.59	0.67
D-Ile	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	Not determined
L-allo Ile	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	Not determined
D-allo Ile	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	Not determined
D-Leu	0.17	0.14	0.22	0.21	0.13	0.11	0.24	Not determined
D-Lys	0.10	0.11	0.15	0.16	<0.10	<0.10	0.14	Not determined
D-Met	0.48	0.37	0.81	0.44	0.27	0.39	0.49	Not determined
D-Phe	0.28	0.20	0.85	0.35	0.28	0.14	0.41	Not determined
D-Pro	<0.10	<0.10	<0.10	<0.10	<0.10	0.13	<0.10	Not determined
D-Ser	0.46	0.54	0.99	1.02	0.30	0.31	1.83	Not determined
D-Thr	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	Not determined
L-allo Thr	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	0.13	Not determined
D-allo Thr	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	0.12	Not determined
D-Trp	0.19	0.20	0.57	<0.60	0.71	0.17	0.80	Not determined
D-Tyr	0.42	0.35	0.72	0.86	0.45	0.64	0.88	Not determined
D-Val	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	Not determined
Crude product purity	68.4%	76.1%	88.9%	87.4%	87.6%	84.6%	86.7%	84.0%
Synthesis time (h)	23.3	9.2	9.2	9.2	9.2	9.2	9.2	10.1

[a] Microwave methods were modified for Cys and His couplings; 2 min at 0 W followed by 4 min at 40 W with a maximum temperature of 50 °C.
[b] The Cys and His coupling cycles were performed conventionally.

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only 68.4% crude product purity. An initial microwave-enhanced synthesis of the 20mer peptide with piperidine deprotection and HBTU/DIEA activation indicates that the sequence is susceptible to racemization at histidine (9.40% D-His), cysteine (4.48% D-Cys), and aspartic acid (9.60% D-Asp) residues. Racemization levels of the remaining amino acids were comparable to those obtained via conventional synthesis. Addition of HOBt to the HBTU coupling solution did not substantially reduce racemization levels. Synthesis of the 20mer peptide with PyBOP activation produced a similar degree of racemization as HBTU activation.

Histidine is susceptible to racemization through its own side chain. Typical side chain protection for histidine involves shielding the τ-nitrogen since it is more accessible [24]. However, the π-nitrogen is actually closer to the α-carbon proton and responsible for nucleophilic attack leading to α-carbon rearrangement via direct enolization. Derivatives of histidine, such as Fmoc-His(3-Bum)-OH or Fmoc-His(3-Bom)-OH, are available with side chain protection on the π-nitrogen that eliminate concern of histidine racemization [25]. However, these amino acid derivatives are expensive and not an attractive option for routine synthesis. A simpler and more cost-effective solution is lowering the His coupling temperature from 80 to 50 °C. When the coupling temperature was reduced, racemization of His was decreased from >8.00% D-His in previous microwave-enhanced syntheses to 1.59% D-His.

Cysteine racemization has been attributed to α-carbon proton abstraction by the tertiary amine in the coupling reaction. This has led to the use of hindered bases with significant bulkiness to limit their ability to reach the α-carbon proton. Substitution of DIEA with the more hindered base TMP led to substantial reduction of cysteine racemization, as shown in Table 1. However, use of TMP resulted in decreased coupling efficiency. A significant valine deletion was noted during the initial synthesis of the 20mer peptide, necessitating double coupling of valine on the sample sent for racemization analysis. Alteration in the activator base from DIEA to TMP has no measurable effect on histidine racemization. As with histidine, an easier solution for minimizing cysteine racemization is to reduce coupling temperature from 80 to 50 °C.

Table 2 Percentage of aspartimide and D-Asp measured by GC/MS after hydrolysis of VYWTSPFMKLIHEQCNRADG-NH_2 with 6N DCl/D_2O

Deprotection reagent	aspartimide (%)	D-Asp (%)
20% Piperidine in DMF	31.50	9.60
20% Piperidine with 0.1 M HOBt in DMF	9.10	3.83
5% Piperazine with 0.1 M HOBt in DMF	3.15	1.18

While coupling temperatures of up to 80 °C can be used during microwave SPPS to drive difficult reactions to completion, both histidine and cysteine require special attention. Reducing the coupling temperature to 50 °C had a positive effect for both amino acids. Additionally, once both histidine and cysteine are coupled, they do not show any further increase in racemization during the ensuing chain synthesis steps with microwave energy. Hence, racemization is limited to the activated ester state of the amino acid. This allows coupling of both amino acids to be performed conventionally at room temperature with no increased susceptibility to racemization from elevated temperatures of up to 80 °C during subsequent amino acid coupling and deprotection steps.

Aspartimide formation can create unwanted side-products and high levels of aspartic acid racemization, thereby reducing product purity. When Fmoc deprotection was performed using 20% piperidine in DMF, α and β-piperidides from base-catalyzed aspartimide ring opening were present in LC-MS analysis, resulting in a crude product purity of 76.1%. Fmoc deprotection with piperazine showed an overall reduction in aspartimide formation side-products. The α- and β-piperidides were not detected in syntheses with piperazine-based deprotection solutions, leading to greater product purities (roughly 85%). Microwave energy is able to accelerate Fmoc deprotection substantially with piperazine. Since no deletion products were evident in the LC-MS analysis of the model 20mer peptide, it is concluded that complete Fmoc removal can be accomplished with piperazine in 3 min. This allows for an efficient deprotection reaction with a very desirable reagent. Addition of 0.1 M HOBt reduced aspartimide formation with both piperidine- and piperazine-based deprotection solutions. Racemization of the aspartic acid that can occur through hydrolysis of the imide ring was found to be significantly less with piperazine in place of piperidine under microwave conditions at 80 °C and comparable to conventional synthesis with piperidine (Table 2).

CONCLUSIONS

Microwave energy has been used to enhance Fmoc SPPS as shown on the model 20mer peptide increasing in purity from 68.4% using conventional methodology to 84.0–86.7% under optimized microwave conditions. Since the successful synthesis of a peptide necessitates near 100% completion of two reactions per cycle, microwave energy represents an efficient way to drive both the deprotection and coupling reactions to completion. The result is higher product purities with reduced synthesis time. Common side reactions are observed during microwave SPPS, but can be easily overcome by routine use of optimized methods.

During the coupling reaction, racemization is a concern with histidine and cysteine both conventionally

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and with microwave SPPS. However, increases in racemization from excess microwave energy can be overcome by lowering the temperature to 50 °C for histidine and cysteine couplings or by substitution to a more hindered base such as TMP for cysteine couplings. Additionally, coupling of both histidine and cysteine can be performed conventionally while the rest of the peptide is synthesized using microwave without any increased effect on racemization. Racemization during the coupling reaction is limited to the activated ester state of the amino acid up to 80 °C. Available software and instrumentation allow such microwave methods to be performed automatically without user interface.

The Fmoc deprotection reaction is prone to aspartimide formation in peptides containing certain 'Asp-AA' sequences. While the addition of HOBt to the deprotection solution reduces aspartimide formation, the use of piperazine was shown to nearly eliminate aspartimide formation altogether. A key advantage of microwave is its ability to accelerate Fmoc deprotection with reagents that are more desirable to use and advantageous for prevention of aspartimide formation.

REFERENCES

1. Krchnak V, Vagner J. Color-monitored solid-phase multiple peptide synthesis under low-pressure continuous flow conditions. *Pept. Res.* 1990; **3**: 182–193.
2. Yu HM, Chen ST, Wang KT. Enhanced Coupling Efficiency in solid-phase peptide synthesis by microwave irradiation. *J. Org. Chem.* 1992; **57**: 4784–4875.
3. Rabinovich A, Rivier J. Accelerated protocols for solid-phase peptide synthesis at elevated temperature. *Am. Biotechnol. Lab.* 1994; **12**: 48–51.
4. Erdeyli M, Gogoll A. Rapid microwave-assisted solid phase peptide synthesis. *Synthesis* 2002; **11**: 1592–1596.
5. Murray J, Gellman S. Application of microwave irradiation to the synthesis of 14-helical β-peptides. *Org. Lett.* 2005; **7**: 1517–1520.
6. Olivos H, Alluri P, Reddy M, Salony D, Kodadek T. Microwave-assisted solid phase synthesis of peptoids. *Org. Lett.* 2002; **4**: 4057–4059.
7. Collins J, Collins M, Steorts R. Novel method for solid phase peptide synthesis using microwave energy. *Poster Presentation at the 18th American Peptide Symposium*, Boston, MA, 2003.
8. Matsushita T, Hinou H, Kurogochi M, Shimizu H, Nishimura S. Rapid microwave-assisted solid-phase glycopeptide synthesis. *Org. Lett.* 2005; **7**: 877–880.
9. Fara M, Diaz-Mochon J, Bradley M. Microwave-assisted coupling with DIC/HOBt for the synthesis of difficult peptoids and fluorescently labelled peptides- a gentle heat goes a long way. *Tetrahedron Lett.* 2006; **47**: 1011–1014.
10. Rybka A, Frank H. Microwave sssisted peptide synthesis A tool to replace classical SPPS?. *Poster Presentation at the 19th American Peptide Symposium*, San Diego, CA, 2005.
11. Atherton E, Sheppard R. In *Peptides, Structure and Function; Proceedings of the 9th American Peptide Symposium.*, Deber C, Hruby V, Kopple K (eds.) Pierce Chemical Co: Rockford, IL, 1985; 415.
12. Merrifield R, Littau V. In *Peptides*, Bricas E (ed.). North Holland: Amsterdam: 1968; 179.
13. Han Y, Albericio F, Barany G. Occurrence and minimization of cysteine racemization during stepwise solid-phase peptide synthesis. *J. Org. Chem.* 1997; **7**: 4307–4312.
14. Angell Y, Alsina J, Albericio F, Barany G. Practical protocols for step-wise solid-phase synthesis of cysteine-containing peptides. *J. Pept. Res.* 2002; **60**: 292–299.
15. Hogue Angeletti R, Bibbs L, Bonewald L, Fields G, Kelly J, McMurray J, Moore W, Weintraub S. Analysis of racemization during "standard" solid phase peptide synthesis: a multicenter study. *Techniques in Protein Chemistry VIII*. Academic Press: San Diego, CA, 1997; 875–890.
16. Kaiser T, Nicholson G, Kohlbau H, Voelter W. Racemization studies of Fmoc-Cys(Trt)-OH during stepwise Fmoc-solid phase peptide synthesis. *Tetrahedron Lett.* 1990; **37**: 1187–1190.
17. Nicolas E, Pedroso E, Giralt E. Formation of aspartimide peptides in Asp-Gly sequences. *Tetrahedron Lett.* 1989; **30**: 497–500.
18. Martinez J, Brodansky M. Side reactions in peptide synthesis. *Int. J. Pept. Protein Res.* 1978; **12**: 277–283.
19. Packman L. N-2-Hydroxy-4-methoxybenzyl (Hmb) backbone protection strategy prevents double aspartimide formation in a 'difficult' peptide sequence. *Tetrahedron Lett.* 1995; **36**: 7523–7526.
20. Dölling R, Beyermann M, Jaenel J, Kernchen F, Krause E, Franke P, Brudel M, Biernet M. Piperidine-mediated side product formation for Asp(OBu^t)-containing peptides. *J. Chem. Soc., Chem. Commun.* 1994; **7**: 853–854.
21. Lauer J, Fields C, Fields G. Sequence dependence of aspartimide formation during 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis. *Lett. Pept. Sci.* 1994; **1**: 197–205.
22. Wade J, Mathieu M, Macris M, Tregear G. Base induced side reactions in Fmoc solid-phase peptide synthesis: Minimization by use of piperazine as N-α-deprotection reagent. *Lett. Pept. Sci.* 2000; **7**: 107–112.
23. Kemp D. In *The Peptides: Analysis, Synthesis, Biology*, Gross E, Meienhoefer J (eds). Academic Press: New York, 1979; 315.
24. Harding S, Jones J, Sabirov A, Samukov V. im-Trityl protection of histidine. *J. Pept. Sci.* 1999; **5**: 368–373.
25. Mergler M, Dick F, Sax B, Weiler P, Vorherr T. Synthesis and application of Fmoc-His(3-Bum)-OH. *J. Pept. Sci.* 2001; **7**: 502–510.

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