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The application of an aryl hydrazine linker prevents ß-elimination side products in the SPPS of *C*-terminal cysteine peptides

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Solid-phase synthesis allows for the preparation of some complex cysteine-containing peptides with both a high yield and purity. However, side reactions during chain elongation such as modification of amino acid residues have been found in *C*-terminal cysteine peptides. We identified 3-(1-piperidinyl)-alanine peptides, corroborated the mechanism of the side reaction, and introduced an efficient approach for the Fmoc-based synthesis of *C*-terminal cysteine peptides using an aryl hydrazine linker.

Supporting information may be found in the online version of this article

Keywords: aryl hydrazine linker; cysteine peptides; microwave; SPPS

Introduction

Cysteine is a key amino acid due to its role in the formation of disulfide bridges in many bioactive peptides and it appears at the *C*-terminal in some important peptide chains such as somatostatin, conotoxins, and lyp-1, etc. [1,2]. Fmoc-based SPPS has been proved both efficient and versatile, providing stepwise and fragment assembly procedures, analytical and monitoring techniques, and solutions to some of the previously limiting chemical problems [3,4]. However, when *C*-terminal cysteine peptides are synthesized on hydroxymethyl-based resins, a 3-(1-piperidinyl)-alanine residue can be formed at the *C*-terminus [5]. This side reaction might be explained by a base-catalyzed β-elimination of the protected thiol of the side chain to give a dehydroalanine intermediate, which is trapped by a nucleophilic addition of the deprotection base piperidine (Scheme 1).

We prepared three linear peptides (Figure 1) and the model tetrapeptide of H-Phe-Phe-Gly-Cys-OH (Figure 3a) under microwave irradiation using standard Fmoc-based SPPS on Wang resin to estimate the extend of the formation of cysteine-derived side products.

Then, we adapted a more efficient synthesis of *C*-terminal cysteine peptides, using the *N*-Fmoc-4-hydrazino-benzoic acid (Fmoc-HBA) building block [6] as an oxidation-labile linker for anchoring to the Wang resin. Peptides could be assembled directly on the solid support via the use of aryl hydrazine linker under standard Fmoc protocols. Aryl hydrazine strategy allows the efficient synthesis of *C*-terminal cysteine peptides, even under microwave conditions.

Materials and Methods

General

All reactions were carried out under an inert atmosphere with dry solvents under anhydrous conditions, unless otherwise stated. Fmoc amino acids and derivatives were obtained from GL Biochem Ltd (China). Fmoc Rink amide MBHA resin and Wang resin were purchased from C-Strong Co., Ltd (China). Microwave irradiation procedures were performed in a Liberty® focused single mode microwave synthesis system (CEM, North Carolina, USA), which produced continuous irradiation at 2450 MHz. Temperatures were monitored with an optical fiber. Reaction mixture was bubbled with N_2 during the irradiation. The HPLC analysis was performed on a Shimadzu 2010C HPLC system. For purification, Shimadzu RP-HPLC system was used. The ESI-MS spectra of the peptides were obtained with an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technology, Palo Alto, CA, USA). The ^{1}H NMR spectra and ^{13}C NMR spectra were recorded at 303 K with a Bruker AV-500 spectrometer and Bruker AV-300 spectrometer, respectively.

General Procedure for Preparation of Peptides under Microwave Irradiation

Wang resin (0.1 mmol) or Fmoc Rink amide MBHA resin (0.1 mmol) was placed in a peptide synthesis vessel, swollen in DCM. For Fmoc Rink amide MBHA resin, it should be deprotected with 20% piperidine in 5 ml DMF for 4 min under microwave irradiation (microwave power: 10 W) firstly. After washing three times with DMF, a mixed solution of 0.3 mmol Fmoc-AA-OH, 0.3 mmol HBTU, 0.3 mmol HOBt, and 0.6 mmol DIPEA dissolved in 4 ml DMF was added to the vessel. Then the mixture was bubbled with N_2 for 10 min under microwave irradiation (microwave power: 10 W) and washed three times with DMF. The temperature of

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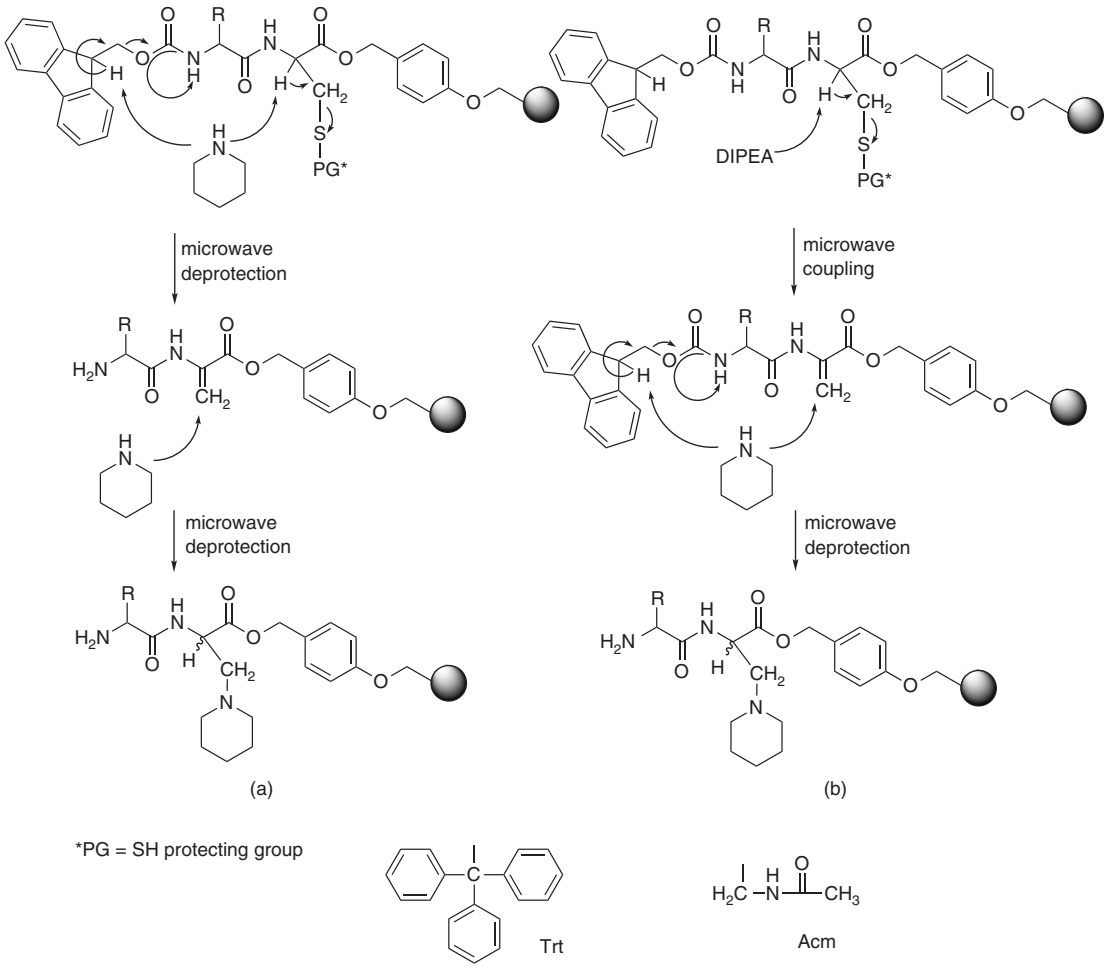

Scheme 1. Assumption of 3-(1-piperidinyl)-alanine residue formation.

H-Cys-Gly-Asn-Lys-Arg-Thr-Arg-Gly-Cys-OH

Lyp-1

H-Cys-Lys-Gly-Gly-Arg-Ala-Lys-Asp-Cys-OH

M-08

H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH

Somatostatin

Figure 1. Sequences of peptides used in these studies.

reaction mixtures under microwave irradiation was kept below 50 °C. Qualitative ninhydrin test was applied to examine whether there were free amino groups. The procedures of deprotection and coupling were repeated with relevant Fmoc-protected amino acids to give peptide resin. Then resin was washed successively with DMF three times.

Procedure for Preparation of Peptides under Normal Condition

Wang resin (0.1 mmol) swollen in DMF was transferred to a reaction tube. After washing three times with DMF, a mixed solution of 0.3 mmol Fmoc-AA-OH, 0.3 mmol HBTU, 0.3 mmol HOBt, and 0.6 mmol DIPEA dissolved in 4 ml DMF was added to the reaction tube and the temperature of reaction was kept at room temperature for 1.5 h. The completion of each coupling step was confirmed by the qualitative ninhydrin test. The procedures of deprotection and coupling were repeated with relevant Fmoc-protected amino acids to assembly the peptide sequence on the resin. Then resin was washed successively with DMF three times.

Procedure for Peptides Cleavage from the Resin

Final peptide was cleaved from the resin with 7 ml Reagent K (TFA/thioanisole/water/phenol/EDT, 82.5 : 5 : 5 : 5 : 2.5 v/v) for 1.5 h at room temperature. Peptide was precipitated by addition of 50 ml cold ethyl ether; after centrifugation, the ether was removed, the peptide pellet was resuspended in cold ethyl ether, and this process was repeated three times.

Procedure for Peptide Synthesis using Aryl Hydrazine Linker

Wang resin (0.1 mmol) was used as solid-phase support. Fmoc-HBA (0.3 mmol Fmoc-HBA, 0.15 mmol DIC, and 0.01 mmol DMAP) was attached to resin through symmetrical anhydride method to get *N*-Fmoc-4-hydrazino-benzoyl Wang resin. Fmoc-Cys(Trt)-OH/Fmoc-Cys(Acm)-OH and other Fmoc-protected amino acids were then coupled to the hydrazine resin using standard Fmoc protocols. At the end of the synthesis, the fully protected peptide resin was activated by mild oxidation with $Cu(OAc)_2$ (0.5 mmol) in the presence of pyridine and acetic acid [7]. The reactive acyl diazene was then cleaved with H_2O. Finally, the fully protected

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peptide acid was deprotected with TFA in the presence of the appropriate scavengers (TFA/thioanisole/water/phenol/EDT, 82.5 : 5 : 5 : 5 : 2.5 v/v) [6,8–10].

HPLC Analysis Procedure

The peptides were dissolved in water and analyzed using reverse-phase gradient elution with a Shimadzu C18 reversed phase column (5 μm, 150 mm × 4.6 mm). A linear gradient of mobile phase ~20–80% B in 20 min (mobile phase A: water with 0.1% TFA and mobile phase B: acetonitrile with 0.1% TFA) at a flow rate of 1 ml/min, and UV detection at 214 nm were utilized.

Table 1. ESI-mass data of *C*-terminal cysteine-containing peptide acids

Peptide	Expected mass (Da)	Found (*m/z*)	Observed mass (Da)	Diff. (Da)
Lyp-1	994.2	$[M+1]^{+}$ 1045.7 $[M+2]^{2+}$ 523.6 $[M+3]^{3+}$ 349.4	1045.2	51
M-08	937.1	$[M+2]^{2+}$ 494.9	987.8	50.7
Somatostatin	1639.9	$[M+2]^{2+}$ 846.8 $[M+3]^{3+}$ 564.5	1691.0	51.1

Results and Discussion

Identification of the 3-(1-Piperidinyl)-Alanine Adducts

Three linear peptides (Figure 1) with *C*-terminal cysteine were synthesized on Wang resin using the Fmoc/tBu orthogonal protection strategy. Occurrence of the side reaction was diagnosed by a peak of 51 Da higher in mass spectrometry (Table 1). In addition, we have used microwave energy to enhance Fmoc-based SPPS effectively. It represents an efficient way to drive both the deprotection and coupling reactions to completion [11–13]. However, it should be remarked that microwave irradiation could dramatically enhance the formation of 3-(1-piperidinyl)-alanine at the *C*-terminus and made the by-product the main product. The HPLC comparison of crude products from the standard and microwave-assisted condition reveals the enhancement of microwave irradiation to the side reaction in SPPS (Figure 2).

In addition, we tried to prepare a model tetrapeptide of H-Phe-Phe-Gly-Cys-OH under microwave irradiation using standard Fmoc-based SPPS on Wang resin. The resulting product was characterized by NMR spectroscopy and mass spectrometry, which indicated that the product containing 3-(1-piperidinyl)-alanine residue was the dominating product (Figure 3b) and no expected H-Phe-Phe-Gly-Cys-OH could be obtained under microwave irradiation. We also prepared lyp-1 on Rink amide resin which could be treated by TFA-mediated cleavage yielding a peptidyl amide, and lyp-1-Gly-OH on Wang resin, in which the *C*-terminal of the peptide (lyp-1) was extended by glycin (Figure 4). Under microwave irradiation, both peptides were obtained with high yield and purity (characterization data available in supporting information).

Based on the identification of the side product and all the phenomena above, we considered that β-elimination of the protected sulfhydryl moiety was governed by the acidity of the proton of the α-carbon. The acidity of the proton was mainly influenced by the carboxyl group of the *C*-terminal cysteine residue anchoring to the resin. Attachment of the first residue on hydroxymethyl-based Wang resin and aminomethyl-based Rink amide resin forming an amino acid ester and amino acid amide, respectively, explains the influence of the acidity of α-proton adjacent to the substituent. Furthermore, the lability of the protected sulfhydryl leaving group as an oxygen (ester) substituent has more potential electron-withdrawing inductive effect than nitrogen (amide) substituent (Figure 5).

Efficient Synthesis of *C*-Terminal Cysteine Peptides

For the efficient and rapid synthesis of *C*-terminal cysteine peptides, a flexible solid-phase technology is required. Such a strategy should solve the problem of 3-(1-piperidinyl)-alanine residue formation of *C*-terminal cysteine residue in the presence of a base.

Here, we describe a successful development of the SPPS method that prevents the formation of the cysteine-derived side product described earlier. The strategy employs Fmoc-HBA building block as an oxidation-labile linker for anchoring to the common commercially available Wang resin. Although hydrazine linkers have been used several times in SPPS (see below), its application in the assembly of peptides with *C*-terminal cysteine residues to prevent Cys-derived side reactions has not been described so far.

Peptides can be assembled directly on the solid support via the use of aryl hydrazine linker under standard Fmoc protocols. When the synthesis is completed, the fully protected peptide hydrazide is activated by mild oxidation with $Cu(OAc)_2$ in the presence of pyridine and acetic acid. The resulting acyl diazene resin is then cleaved with H_2O. The fully deprotected peptide acid is finally obtained by treatment with TFA (Scheme 2).

Aryl hydrazine was first used in peptide synthesis as a carboxylic-protecting group, which could be converted into a highly reactive

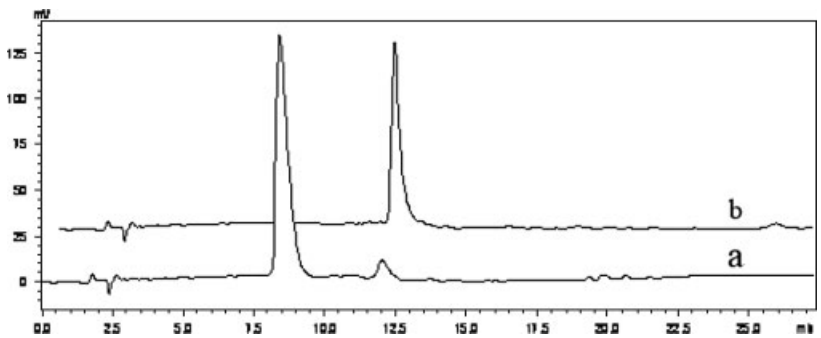


Figure 2. HPLC comparison of products from the standard (a) and microwave-assisted condition (b).

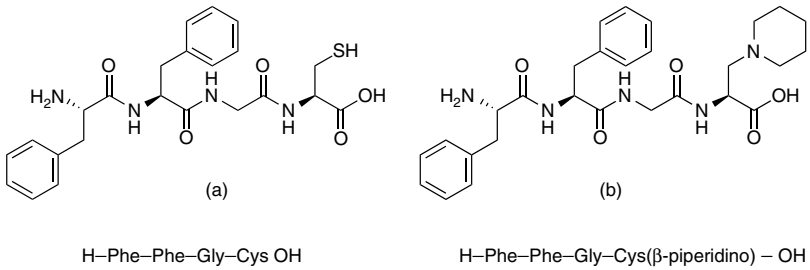
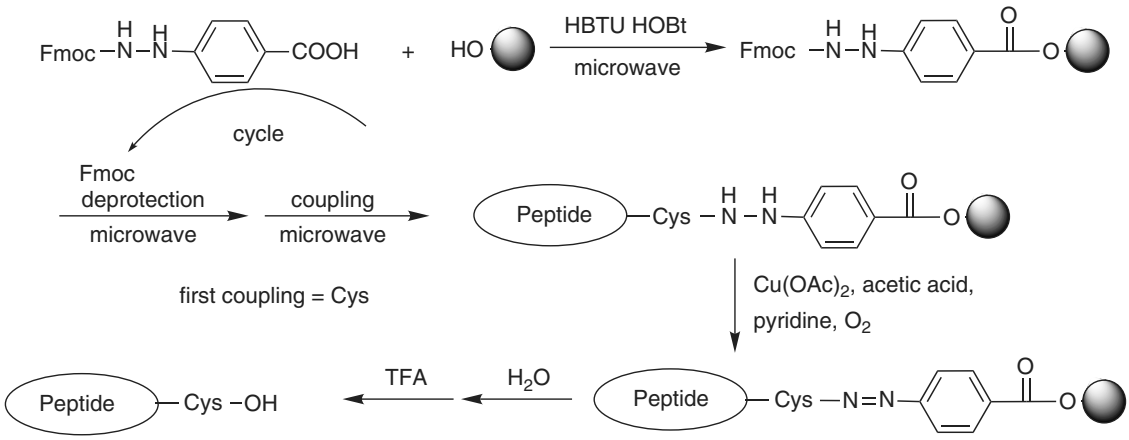


Figure 3. Structure of expected tetrapeptide (a) and resulting product (b).



Scheme 2. General procedure of the peptide synthesis using aryl hydrazine linker.

H-Cys-Gly-Asn-Lys-Arg-Thr-Arg-Gly-Cys-OH

Lyp-1

H-Cys-Gly-Asn-Lys-Arg-Thr-Arg-Gly-Cys-NH_2

Lyp-1 amide

H-Cys-Gly-Asn-Lys-Arg-Thr-Arg-Gly-Cys-Gly-OH

Lyp-1-Gly-OH

Figure 4. Lyp-1 and its derivatives.

acyl diazene intermediate followed by heterolytic elimination of nitrogen with the addition of H_2O to give a carboxylic acid, benzene, and nitrogen [14].

Millington *et al*. developed aryl hydrazine as linker for SPPS, which was cleavable under mild oxidative conditions to give peptides with *C*-terminal acid, amide, or ester functionalities [6]. It has been reported that peptidyl diazenes were free of racemization during cleavage with nucleophiles [15–17]. Furthermore, Woo *et al*. have shown that mild oxidation of peptide hydrazides with NBS was totally compatible with oxidation-sensitive residues (i.e. Tyr, Trp, and Cys) when the appropriate protecting groups and oxidative conditions were employed [15]. And for Met-containing peptide, Cu(II) salts oxidation did not oxidize the Met residue.

In our microwave-assisted solid-phase synthesis of *C*-terminal cysteine peptides procedure [18,19], Wang resin was used as solid-phase support. Fmoc-HBA was attached to resin through symmetrical anhydride method to get *N*-Fmoc-4-hydrazino-benzoyl Wang resin. Fmoc-Cys(Trt)-OH/Fmoc-Cys(Acm)-OH and other Fmoc-protected amino acids were then coupled to the hydrazine resin using standard Fmoc protocols. At the end of the synthesis, the fully protected peptide resin was activated by mild oxidation with $Cu(OAc)_2$ in the presence of pyridine and acetic acid. The reactive acyl diazene was then cleaved with H_2O. Finally, the fully protected peptide acid was deprotected with TFA in the presence of the appropriate scavengers. It should be noted that the last amino acid should be incorporated as the $^{\alpha}$ N-Boc-derivative during the synthesis. This prevented the possible cyclization of the free α-amino group with reactive acyl diazene intermediate [9]. All peptides (linear somatostatin, lyp-1, and M-08) were synthesized under microwave irradiation. The crude peptides were analyzed by RP-HPLC and their molecular weights were evaluated by ESI-MS spectra.

Owing to the electron-donating effect of nitrogen atom of aryl hydrazine linker, the acidity of α-proton adjacent to the linker was reduced, which stabilized the sulfhydryl-protected side-chain moiety. And meanwhile, the aryl hydrazine linker was totally stable to the conditions used by Fmoc chemistry during microwave-assisted SPPS. As the aryl hydrazine linker was activated in the condition of oxidation, there was concern about the stability of peptides containing oxidation-sensitive residues (i.e. Trp and Cys). Our approach for the synthesis of *C*-terminal cysteine peptides seems to work well also for the assembly of other model peptides.

Conclusions

Here, we identified the formation of 3-(1-piperidinyl)-alanine in the microwave-assisted synthesis of *C*-terminal cysteine peptides, corroborated the mechanism of the side reaction, and developed an efficient synthesis of *C*-terminal cysteine peptides using an aryl

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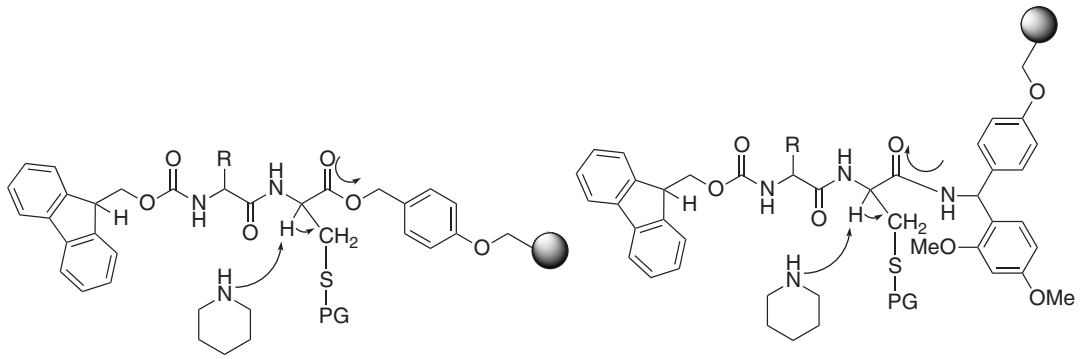

Figure 5. Acidity comparison of α-proton influenced by the solid support.

hydrazine linker. The oxidation and cleavage reactions have been shown to be totally compatible with sensitive amino acids when the appropriate protecting groups and oxidation conditions were employed.

Acknowledgements

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Supporting information

Supporting information may be found in the online version of this article.

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