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Site-specific labeling of synthetic peptide using the chemoselective reaction between *N*-methoxyamino acid and isothiocyanate

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Site-specific labeling of synthetic peptides carrying *N*-methoxyglycine (MeOGly) by isothiocyanate is demonstrated. A nonapeptide having MeOGly at its *N*-terminus was synthesized by the solid-phase method and reacted with phenylisothiocyanate under various conditions. In acidic solution, the reaction specifically gave a peptide having phenylthiourea structure at its *N*-terminus, leaving side chain amino group intact. The synthetic human β-defensin-2 carrying MeOGly at its *N*-terminus or the side chain amino group of Lys^{10} reacted with phenylisothiocyanate or fluorescein isothiocyanate also at the *N*-methoxyamino group under the same conditions, demonstrating that this method is generally useful for the site-specific labeling of linear synthetic peptides as well as disulfide-containing peptides. Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: site-specific labeling; *N*-methoxyamino acid; isothiocyanate; human β-defensin

Introduction

Site-specifically modified peptides and proteins are important for the elucidation of their biochemical and biological function. In particular, fluorescent labeling is an often used technique to study the interaction between a peptide or a protein with their binding partner, to analyze the cellular localization, and to create fluorescent biosensors for application [1]. However, one of the problems in these studies has been the difficulty in the site-specific labeling by fluorophores in the polypeptide. Although the chemical synthesis can realize this, the presence of highly hydrophobic probe sometimes makes the folding reaction at the final stage of the synthesis difficult. In particular, peptides with multiple disulfide bonds tend to misfold, when tagged with hydrophobic probes, resulting in the formation of disulfide bond isomers. Therefore, a method to realize site-specific modification after the chemical synthesis and folding is desirable. Along this line, the chemoselectivity of the *N*-alkylaminooxy group with electrophiles, such as succinimidyl ester, activated haloalkanes, has been examined [2,3]. In addition, *N*-alkylaminooxy groups have been extensively studied for the chemoselective glycosylation to create glycoconjugates and glycopeptides [4–11]. These functional groups retain pKa (4–5) significantly lower than that of the α-amino group (about 9.1) and the ε-amino group of Lys residue (about 10.5), which is the basis of the chemoselectivity. Here, we examined the use of *N*-methoxyglycine as a specificity-generating group to introduce probes after the chemical synthesis. If a peptide with *N*-methoxyglycine is synthesized and folded, the *N*-methoxyamino group can be selectively modified with fluorescent probes without disrupting the tertiary structure. One of the unique advantage of *N*-methoxyglycine over another *N*-methoxyamino group is that the α-thiourea structure, which is formed by reaction between *N*-methoxyglycine and arylisothiocyanates such as phenylisothiocyanate (PITC) and fluorescein isothiocyanate (FITC), can be selectively removed through Edman degradation reaction. In this paper, the idea is demonstrated by the selective modification of human β-defensin-2 (hBD2) at the side chain amino group of Lys^{10} as well as terminal amino group.

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Abbreviations: *Boc, t-butoxycarbonyl; DIC, diisopropylcarbodiimide; DIEA, N, N-diisopropylethylamine; DODT, 3,6-dioxa-1,8-octanedithiol; ESI-MS, electrospray-ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; FITC, fluorescein isothiocyanate; GSH, glutathione reduced form; GSSG, glutathione oxidized form; hBD2, human β-defensin-2; HBTU, O-(benzotriazol-1-yl)-N, N, N′, N′-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; NMP, 1-methyl-2-pyrrolidinone; NMR, nuclear magnetic resonance; RPHPLC, reversed-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane; PITC, phenylisothiocyanate.*

Materials and Method

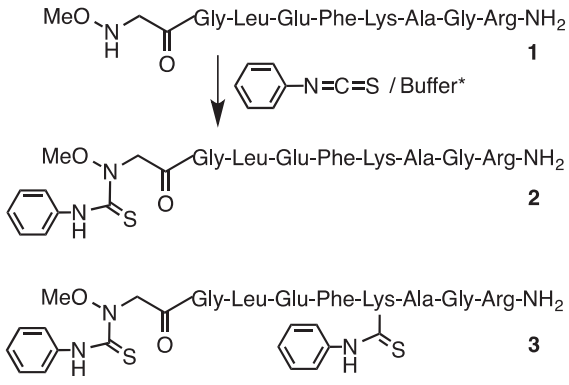
The NMR spectra were recorded using an AV400 spectrometer at 400 MHz (Bruker, MA, USA). The chemical shifts are expressed in ppm downfield from the signal for the internal Me_4Si for solutions in the deuterated solvents. The SPPS of peptide **1** was carried out using a Liberty Blue peptide synthesizer (CEM, NC, USA). The peptide chain assembly for hBD **6** and **7** was carried out by 433A peptide synthesizer (Applied Biosystems, CA). The ESI mass spectra were recorded using a LCQ DECA XP plus spectrometer (ThermoFisher Scientific, MA, USA). The amino acid composition was determined using a LaChrom amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with 6 M HCl at 150 °C for 2 h in an evacuated sealed tube. The content of the peptides in the powders was estimated based on the amino acid analysis. Authentic hBD2 **9b** was purchased from Peptide Institute (Minoh, Japan).

Synthesis of Peptide 1

Starting from Fmoc-CLEAR amide resin (0.48 mmo/g, 0.1 mmol), peptide chain was elongated by Liberty Blue to give Gly-Leu-Glu(OBu^t)-Phe-Lys(Boc)-Ala-Gly-Arg(Pbf)-NH-CLEAR amide resin. Boc-MeOGly-OPfp [10] (0.2 mmol) in DMF was added to the resin, and the mixture was vortexed for 30 min at room temperature. The obtained resin was washed three times with NMP, CH_2Cl_2, ether, respectively, and dried in vacuo to give protected peptide resin (312 mg). The whole resin was treated with TFA containing 2.5% of TIS and 2.5% water (3.0 ml) for 2 h at room temperature. The reaction mixture was filtered, and the filtrate was concentrated by nitrogen stream. The product was precipitated by adding ether and purified by RPHPLC to give peptide **1** (34 μmol, 34% yield). ESI-MS, found: *m/z* 963.8, calcd: 963.5 for $(M+H)^+$. Amino acid analysis: $Glu_{0.98}Gly_{2.13}Ala_1Leu_{1.00}Phe_{0.99}Lys_{1.02}Arg_{1.00}$.

Reaction of Peptide 1 with PITC

Peptide **1** (0.50 μmol) was dissolved in various buffers shown in Scheme 1 (100 μl each), and PITC (1.2 μl, 10 μmol) was added. The solution was kept at room temperature, and an aliquot of the solution was analyzed by RPHPLC at appropriate reaction times.



*Buffer:
A. 10 mM triethylamine in 80% CH_3CN aq. (pH 11.0)
B. 10 mM imidazole in 80% CH_3CN aq. (pH 8.6)
C. 10 mM dimethylaniline in 80% CH_3CN aq. (pH 7.7)
D. 0.5 M acetic acid in 80% CH_3CN aq. (pH 2.9)
E. 0.1% TFA in 80% CH_3CN aq. (pH 1.2)

Scheme 1. Reaction of nonapeptide with PITC in various buffers.

The reaction was terminated after 14 h. Peptide **2**: ESI-MS, found: *m/z* 1098.6, calcd: 1098.5 for $(M+H)^+$. Amino acid analysis: $Glu_{1.05}Gly_{2.13}Ala_1Leu_{1.00}Phe_{1.00}Lys_{1.08}Arg_{0.97}$. Peptide **3**: ESI-MS, obs: 1233.6 (deconvoluted), calcd: 1233.6 for $(M+H)^+$. Amino acid analysis: $Glu_{1.07}Gly_{2.16}Ala_1Leu_{1.01}Phe_{1.02}Lys_{1.02}Arg_{1.05}$.

TFA Treatment of Peptide 2

Peptide **2** (2.0 nmol) was dissolved in TFA containing 5% water (20 μl), and the solution was kept for 10 min at room temperature. After TFA was removed by the nitrogen stream, the residue was purified by RPHPLC. The peak of peptide **2** disappeared, and two new peaks appeared. One peak had the mass number of 876.7, which corresponds well to the theoretical value of Gly-Leu-Glu-Phe-Lys-Ala-Gly-Arg-NH_2 **4** (876.5, $[M+H]^+$). The other peak did not show an intense signal, which might show that this peak was derived from thiohydantoin derivative of methoxyglycine.

Fmoc-Lys(Boc-MeOGly)-OH 5

Fmoc-Lys-OH hydrochloride (740 mg, 1.8 mmol) and Boc-MeOGly-OPfp (450 mg, 1.2 mmol) were dissolved in DMF-CH_2Cl_2 (1 : 1, 10 ml), and DIEA (0.32 ml, 1.8 mmol) was added. After the reaction mixture was stirred for 4 h at room temperature, the solvent was removed in vacuo. The residue was dissolved in ethyl acetate, successively washed with 1 M HCl, H_2O and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel chromatography using $CHCl_3$-methanol-acetic acid (95 : 5 : 1) to give compound **5** (670 mg, 1.2 mmol). R_f 0.19 ($CHCl_3$-methanol-acetic acid 95 : 5 : 1). $[\alpha]_D$ +9.86 (c 1.0, $CHCl_3$). Anal. calcd for $C_{29}H_{37}N_3O_8$ 1/2CH_3COOH: C, 61.53; H, 6.71; N, 7.18, found: C, 61.40; H, 6.41; N, 7.04. ^{1}H-NMR ($CDCl_3$) δ: 7.73 (d, 2H, J = 7.5 Hz), 7.59 (m, 2H, Ar), 7.37 (t, 2H, J = 7.4 Hz, Ar), 7.28 (t, 2H, J = 7.4 Hz, Ar), 6.48 (brt, 1H, J = 5.2 Hz, Lys εN*H*), 5.79 (d, 1H, J = 8.0 Hz, Lys αN*H*), 4.48–4.35 (m, 3H, Ser α*H*, Fmoc CH_2-CH), 4.19 (t, 1H, J = 6.9 Hz, Fmoc CH_2-C*H*), 4.11 (s, 2H, Gly CH_2), 3.68 (s, 3H, OCH_3), 3.26 (m, 2H, Lys εH), 1.87 (brs, 1H, Lys βH), 1.74 (brs, 1H, Lys βH), 1.52 (m, 2H, Lys δH), 1.47 (s, 9H, *t*-Bu), 1.40 (m, 2H, Lys γH). ^{13}C-NMR ($CDCl_3$) d: 127.71 (Ar), 127.09 (Ar), 125.15 (Ar), 119.96 (Ar), 67.03 (Fmoc CH_2-CH), 62.13 (OCH_3), 53.60 (Lys Cα), 52.76 (Gly CH_2), 47.13 (Fmoc CH_2-CH), 39.01 (Lys Cε), 31.70 (Lys Cβ), 28.89 (Lys Cδ), 28.17 ($C(CH_3)_3$), 22.21 (Lys Cγ).

Synthesis of hBD2

Synthesis of peptides was carried out using 433A Peptide Synthesizer by the Fmoc method with HBTU/DIEA activation procedures for preloaded Fmoc-Pro-TrtA-PEG resin (1.0 g, 0.21 mmol/g) (Watanabe Chemical Co., Hiroshima). The coupling of Boc-*N*-methoxyglycine to the *N*-terminus or Fmoc-Lys(Boc-MeOGly)-OH **5** at Lys[10] was carried out by pentafluorophenyl ester or DIC/HOBt method, respectively. After the chain assembly, the resin was treated with TFA–TIS-DODT–H_2O (93/2.5/2.0/2.5) 10 ml/g resin for 2 h. After the removal of TFA by a nitrogen stream, ether was added to form a precipitate, which was washed three times with ether and dried in vacuo. The crude peptide was purified by RPHPLC to obtain the desired reduced form of hBD2. The folding reaction was carried out in 0.1 M ammonium acetate (pH 7.8) in the presence of GSH/GSSG (10 : 100 molar ratio to reduced form of hBD2) and stirred for 24 h at room temperature [12]. After the purification by RPHPLC, the desired hBD2s were obtained.

Journal of PeptideScience

Lys(MeOGly)^{10}-hBD-2 **6**; yield 6.4%, ESI-MS, obs: 4416.1 (deconvoluted), calcd: 4416.3 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{0.94}Thr_{2.46}Ser_{0.81}Glu_{1.02}Pro_{5.21}$ $Gly_6Ala_{1.01}Val_{1.95}Ile_{2.66}$ $Leu_{1.88}Tyr_{0.92}Phe_{0.94}Lys_{4.74}$ $His_{1.13}Arg_{2.01}$.

MeOGly-hBD2 **7**; yield 3.4% based on Pro content on the initial resin, ESI-MS, obs: 4416.1 (deconvoluted), calcd: 4416.3 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{0.97}Thr_{2.51}Ser_{0.84}Glu_{1.01}Pro_{5.21}$ $Gly_6Ala_{1.00}Val_{1.91}Ile_{2.74}Leu_{1.91}Tyr_{0.93}Phe_{0.90}$ $Lys_{4.82}His_{0.96}$ $Arg_{1.97}$.

Selective Labeling of hBD2 with PITC

Synthetic hBD2 carrying MeOGly **6** or **7** was dissolved in 0.5 M acetic acid in 80% CH_3CN aq. at a concentration of 5 mM and PITC (20 eq to hBD2) was added. After the solution was kept overnight at room temperature, the reaction mixture was purified by RPHPLC to obtain the desired PITC-labeled hBD2s.

Lys[PITC-MeOGly]^{10}-hBD2 **8**; yield 71%, ESI-MS, obs: 4551.4 (deconvoluted), calcd: 4551.5 for $(M+H)^+$(average). Amino acid analysis: $Asp_{0.97}Thr_{2.57}Ser_{0.87}Glu_{1.05}$ $Pro_{4.96}Gly_6$ $Ala_{0.98}Val_{1.90}$ $Ile_{2.73}Leu_{1.93}Tyr_{0.94}Phe_{1.04}Lys_{4.91}His_{0.97}Arg_{2.05}$.

PITC-MeOGly-hBD2 **10**; yield 78%, ESI-MS, obs: 4551.4 (deconvoluted), calcd: 4551.5 for $(M+H)^+$(average). Amino acid analysis: $Asp_{0.98}Thr_{2.53}Ser_{0.85}Glu_{1.02}Pro_{5.01}$ $Gly_6Ala_{0.98}$ $Val_{1.94}Ile_{2.72}$ $Leu_{1.94}Tyr_{0.94}Phe_{1.00}Lys_{4.89}His_{0.96}Arg_{1.98}$.

TFA Treatment of PITC-Labeled hBD2 8 and 10

Peptide **8** or **10** was dissolved in 95% aq TFA at a concentration of 1 mM, and the solution was kept at room temperature for 10 min. After the solution was concentrated by nitrogen stream, the residue was precipitated by ether and purified by RPHPLC to obtain native hBD2.

hBD2 (from peptide **8**) **9a**; yield 70%, ESI-MS, obs: 4329.4 (deconvoluted), calcd: 4329.2 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{1.01}Thr_{2.69}Ser_{0.87}Glu_{1.04}Pro_{5.22}$ $Gly_6Ala_{1.03}Val_{2.02}Ile_{2.86}$ $Leu_{2.02}Tyr_{0.99}Phe_{1.15}Lys_{4.95}His_{0.96}Arg_{2.03}$.

hBD2 (from peptide **10**) **9c**; yield 82%, ESI-MS, obs: 4328.8 (deconvoluted), calcd: 4329.2 for $(M+H)^+$ (average). Amino acid analysis: $Asp_{1.02}Thr_{2.64}Ser_{0.83}Glu_{1.03}Pro_{5.01}$ $Gly_6Ala_{1.02}Val_{1.99}Ile_{2.82}$ $Leu_{1.99}Tyr_{0.96}Phe_{1.15}Lys_{4.94}His_{0.98}Arg_{1.94}$.

Selective Labeling of hBD2 by FITC

Synthetic hBD2 carrying MeOGly **6** or **7** was dissolved in 80% CH_3CN aq containing 5 mM acetic acid, and 20 eq of FITC dissolved in DMF was added. After the solution was kept room temperature for 8 h, CH_3CN was briefly removed by a N_2 stream. Ether was added to extract a part of the FITC, and the aqueous phase was purified by RPHPLC to give FITC-labeled hBD2.

Lys[FITC-MeOGly]^{10}-hBD2 **11**; yield 72%, ESI-MS, obs: 4805.5 (deconvoluted), calcd: 4805.7 for $(M+H)^+$(average). Amino acid analysis: $Asp_{0.98}Thr_{2.50}Ser_{0.87}Glu_{1.09}$ $Pro_{5.37}$ $Gly_6Ala_{1.01}Val_{1.95}Ile_{2.71}$ $Leu_{1.92}Tyr_{0.97}Phe_{1.47}Lys_{4.89}His_{0.93}Arg_{1.97}$.

FITC-MeOGly-hBD2 **12**; yield 75%, ESI-MS, obs: 4805.5 (deconvoluted), calcd: 4805.7 for $(M+H)^+$(average). Amino acid analysis: $Asp_{0.98}Thr_{2.48}Ser_{0.85}Glu_{1.05}Pro_{5.17}$ $Gly_6Ala_{1.00}Val_{1.95}Ile_{2.73}$ $Leu_{1.93}Tyr_{0.96}Phe_{1.33}Lys_{4.88}His_{0.92}Arg_{1.92}$.

^{1}H-NMR Measurement of hBD2

hBD2s were dissolved in 5 mM sodium acetate (pH 6.0) containing 10% D_2O at a concentration of 1 mg/ml. The spectrum was obtained suppressing the signal of water by presaturation method.

Results and Discussion

Modification of Model Peptide Carrying MeOGly at its *N*-Terminus

In order to find the optimum condition for the specific labeling of *N*-methoxyamino group, a nonapeptide carrying MeOGly at its *N*-terminus, MeOGly-Gly-Leu-Glu-Phe-Lys-Ala-Gly-Arg-NH_2, **1** was used as a model. For the introduction of fluorescent probe, an isothiocyanate [13] of corresponding fluorophore has often been used. Thus, we first examined the reaction of peptide **1** with PITC as the model reagent. The synthesis of the nonapeptide **1** was carried out using the Fmoc SPPS. The terminal MeOGly was introduced using corresponding pentafluorophenyl ester [10]. After TFA deprotection and purification by RPHPLC, peptide **1** was obtained in 34% yield.

Peptide **1** was then reacted with PITC (20 eq to peptide **1**) as shown in Scheme 1 in various buffers. Under highly basic condition of buffer A, the starting peptide was almost consumed within 8 h and gave peptide **3**, in which both methoxyamino and ε-amino groups were modified by PITC, as a major product (Figure 1). The yield of peptide **3** decreased by decreasing the pH of the buffer, while that of the desired product **2** increased from buffer A to C. Still, the complete selectivity to peptide **2** was not attained under the basic conditions. In contrast, under the acidic conditions (buffer D and E), the complete selectivity to peptide **2** was achieved. The coupling of PITC at the methoxyamino group in peptide **2** was proved by the TFA treatment of peptide **3** for 10 min, which removed the phenylthiocarbamoylmethoxyglycine residue at the *N*-terminus and gave Gly-Leu-Glu-Phe-Lys-Ala-Gly-Arg-NH_2 **4** through the Edman degradation reaction (refer to experimental

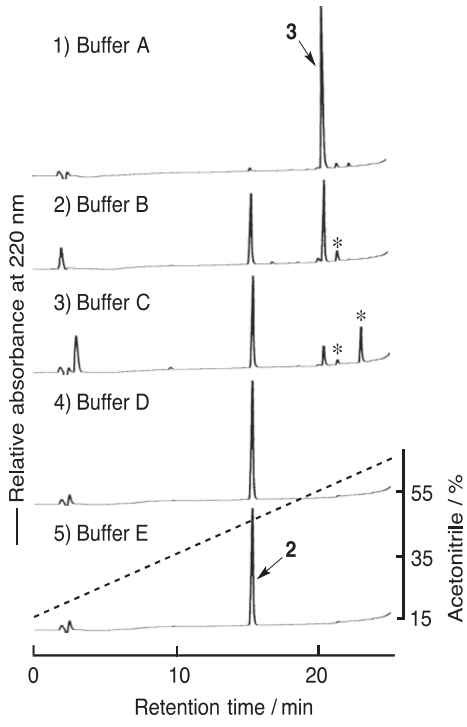


Figure 1. HPLC profiles of the reaction of peptide **1** with PITC in various buffers shown in Scheme 1: 1) buffer A, 2) buffer B, 3) buffer C, 4) buffer D, and 5) buffer E after overnight. Elution conditions: column, Cosmosil 5C18-AR-II (4.6 × 150 mm, Nacalai Tesque, Japan) at a flow rate of 1 ml min^{-1}; eluent, A, 0.1% aqueous TFA, B, 0.1% TFA in acetonitrile. Asterisked peaks are nonpeptides.

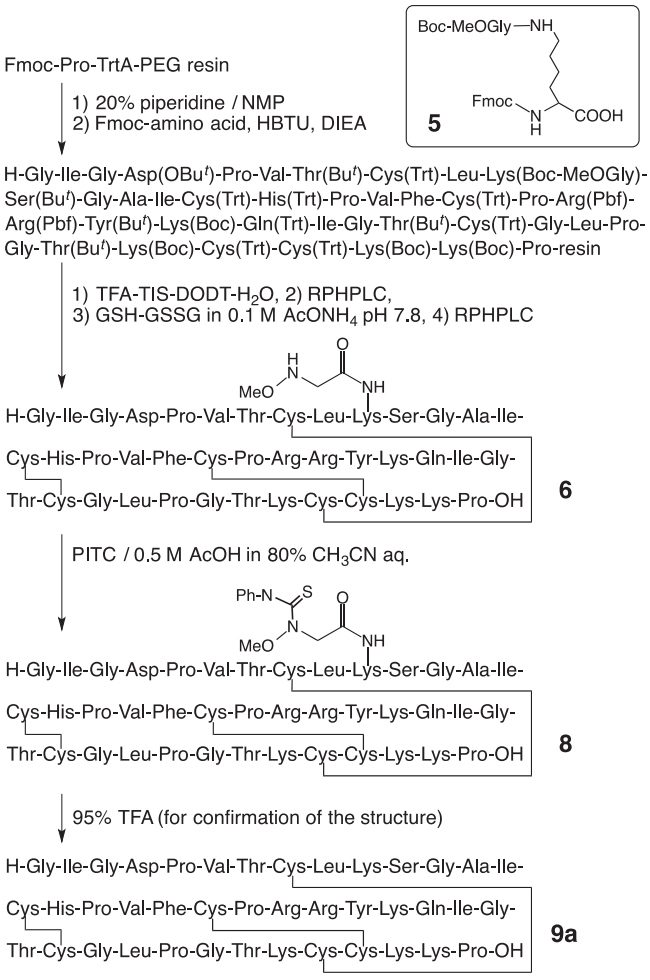
section). If the ε-amino group was modified by PITC, the structure of the peptide would not have been altered. These data demonstrate the high chemoselectivity between the isothiocyanate and the methoxyamino group under acidic conditions.

Application to hBD2

Defensins, diverse members of a large family of cationic host defense peptides, are cysteine-rich peptides and vary in their length, the spacing of their cysteine residues, and their disulfide connectivity [14,15]. In this research, human hBD2 [16] was chosen as a model regarding to properties of having multiple disulfide bonds and lysine residues.

Based on the results in Figure 1, specific modification of the *N*-terminal and side chain amino group of Lys^{10} of hBD2 was performed. In the tertiary structure, Lys^{10} points outward and locates at the end of the helix, which would not cause structural change even after the modification [17]. Scheme 2 shows the procedure in the case of the Lys^{10} modification. Starting from Fmoc-Pro resin, the machine-assisted chain elongation was carried out. Lys^{10} was introduced using Fmoc-Lys(Boc-MeOGly)-OH **5** by DIC-HOBt method. After completion of the chain assembly, the peptide was deprotected by TFA and purified by RPHPLC. The obtained reduced form of hBD2 was then oxidized in the presence of reduced and oxidized form of glutathione (GSH and GSSG) according to the reported procedure [12] for overnight to obtain the desired Lys (MeOGly)^{10}-hBD2 **6** in 6.4% yield after RPHPLC purification. As a same manner, the hBD2 carrying MeOGly at its *N*-terminus **7** was also obtained in 3.4% yield.

The site-specific labeling by PITC was performed in buffer D of Scheme 1, which retains sufficient specificity to methoxyamino group with milder acidity compared to buffer E. As shown in Figure 2, the reaction proceeded efficiently, giving the desired Lys [PITC-MeOGly]^{10}-hBD-2 **8** after overnight reaction. To confirm whether the Lys^{10} was specifically labeled without disrupting tertiary structure, peptide **2** was treated with 95% TFA-H_2O for 10 min and the obtained native hBD2 **9a** was compared on RPHPLC and ^{1}H-NMR with the authentic hBD2 **9b**. As shown in Figure 2 4) and 5), both hBD2 showed the same retention time on RPHPLC. In addition, they showed quite similar ^{1}H-NMR spectrum (Figure 3),



Scheme 2. Synthetic route of PITC-modified hBD2 **9a**.

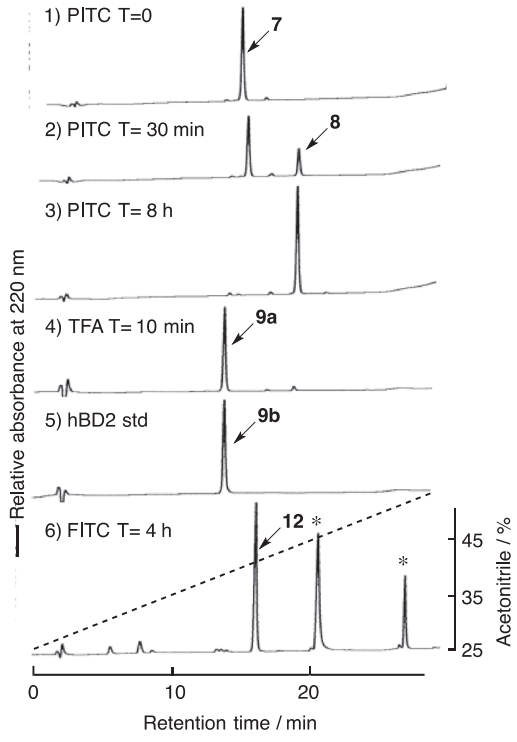


Figure 2. HPLC profiles of the reaction of [Lys(MeOGly)^9]-hBD2 **7** and [Lys(MeO(PITC)Gly)^9]-hBD2 **8**: 1) **7** with PITC 0 min, 2) 30 min, 3) overnight, 4) **8** with TFA 10 min, 5) authentic sample of hBD2 **9b**, and 6) **7** with FITC 4 h. Elution conditions: column, Cosmosil 5C18-AR-II (4.6 × 150 mm, Nacalai Tesque, Japan) at a flow rate of 1 ml min^{-1}; eluent, A, 0.1% aqueous TFA, B, 0.1% TFA in acetonitrile.

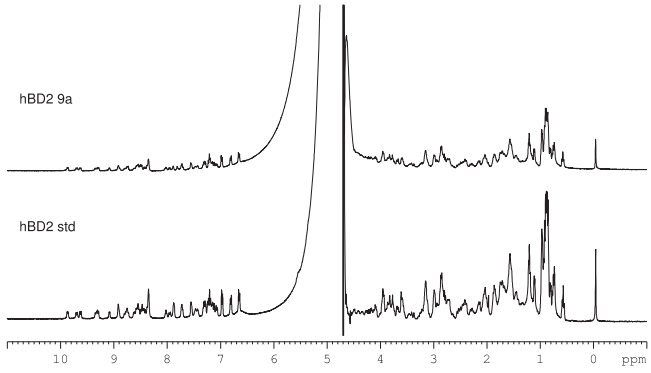


Figure 3. ^{1}H-NMR spectra of hBD2 **9a** and authentic hBD2 **9b**.

Journal of PeptideScience

demonstrating that the specific labeling was realized without disrupting its tertiary structure.

Finally, the specific labeling of hBD2 carrying MeOGly with fluorescent dye, FITC, was performed under the same conditions as in the case of PITC labeling (see experimental and supporting information). The reaction also proceeded specifically and efficiently giving the desired FITC-labeled hBD2s in comparable yields as those of PITC-labeled ones. ^{1}H-NMR spectrum (supporting information) showed that the FITC-labeling does not disturb the tertiary structure of hBD2, as the signals were well dispersed as in the case of PITC-labeling, indicating the success of specific labeling. These FITC-labeled peptides were stable during RPHPLC purification using aqueous acetonitrile containing 0.1% TFA as an eluent and also stable to neutral phosphate buffer. These peptides will be used for the analysis of the interaction between hBD2 and biological membrane as well as for the functional analysis of hBD2.

Conclusions

The result in this study shows that if MeOGly residue is introduced during the SPPS, the obtained peptide can be specifically modified by isothiocyanate reagent at the *N*-methoxyamino group. From the results of PITC and FITC modification of hBD2, it is clear that the peptides with multiple disulfide-bonds can be selectively modified without disrupting their tertiary structure. In principle, this method realizes that peptides with various modifications and fluorescent probes can be synthesized from a single peptide with MeOGly residue, which will be valuable for the functional analysis of various bioactive peptides.

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