Late-stage diversification strategy for the synthesis of peptide acids and amides using hydrazides

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Abstract

Aim: Modification of the C-terminus of a peptide to improve its properties, particularly after constructing the peptide chain, has great promise in the development of peptide therapeutics. This study discusses the development of a late-stage diversification method for synthesizing peptide acids and amides from hydrazides which can serve as a common precursor.

Methods: Peptide hydrazides were synthesized solely by using conventional solid-phase peptide synthesis (SPPS). Hydrazides were subjected to oxidation by potassium peroxymonosulfate (Oxone) to afford carboxylic acids. Azidation of hydrazides using sodium nitrite ($NaNO_2$) under acidic conditions, followed by the addition of β-mercaptoethanol (BME), could also be used to generate carboxylic acids. For the preparation of peptide amides, azides that can be prepared from hydrazides were reacted with ammonium acetate (NH_4OAc) or tris(2-carboxyethyl)phosphine (TCEP)·hydrochloride (HCl) to develop the products through ammonolysis or a Staudinger reaction, which produces iminophosphorane from an azide and a phosphine. The antimicrobial activity of modelin-5 derivatives synthesized from the corresponding hydrazides was evaluated by the colony count of *Escherichia coli* (*E. coli*) after treatment with the peptides.

Results: Oxone oxidation yielded the corresponding acids rapidly although oxidation-prone amino acids were incompatible. Azidation and subsequent treatment with BME afforded peptide acids an acceptable yield even in sequences containing amino acids that are prone to oxidation. Both methods for conversion of hydrazides to amides were found to afford the desired products in good yield and compatibility. The conditions that were developed were adapted to the synthesis of modelin-5 derivatives from the corresponding hydrazides, yielding late-stage production of the desired peptides. The amides of the resulting peptide showed more potent activity against *E. coli* than the acid form, and the most potent activity was observed from the hydrazide.

Conclusions: The developed protocols allow hydrazides to be converted to acids or amides, enabling late-stage diversification of peptide C-terminal residues.

Keywords

Introduction

Peptide therapeutics play a role in drug development because of the unique properties of peptides, which include high specificity, good efficacy, and low immunogenicity when compared to small molecule drugs and biologics [1, 2]. To develop a peptide-based drug, chemical peptide synthesis and subsequent modifications on the sequence and terminal structures are steps that are essential to improve its pharmacological properties after the first step, the discovery of potential peptide therapeutics.
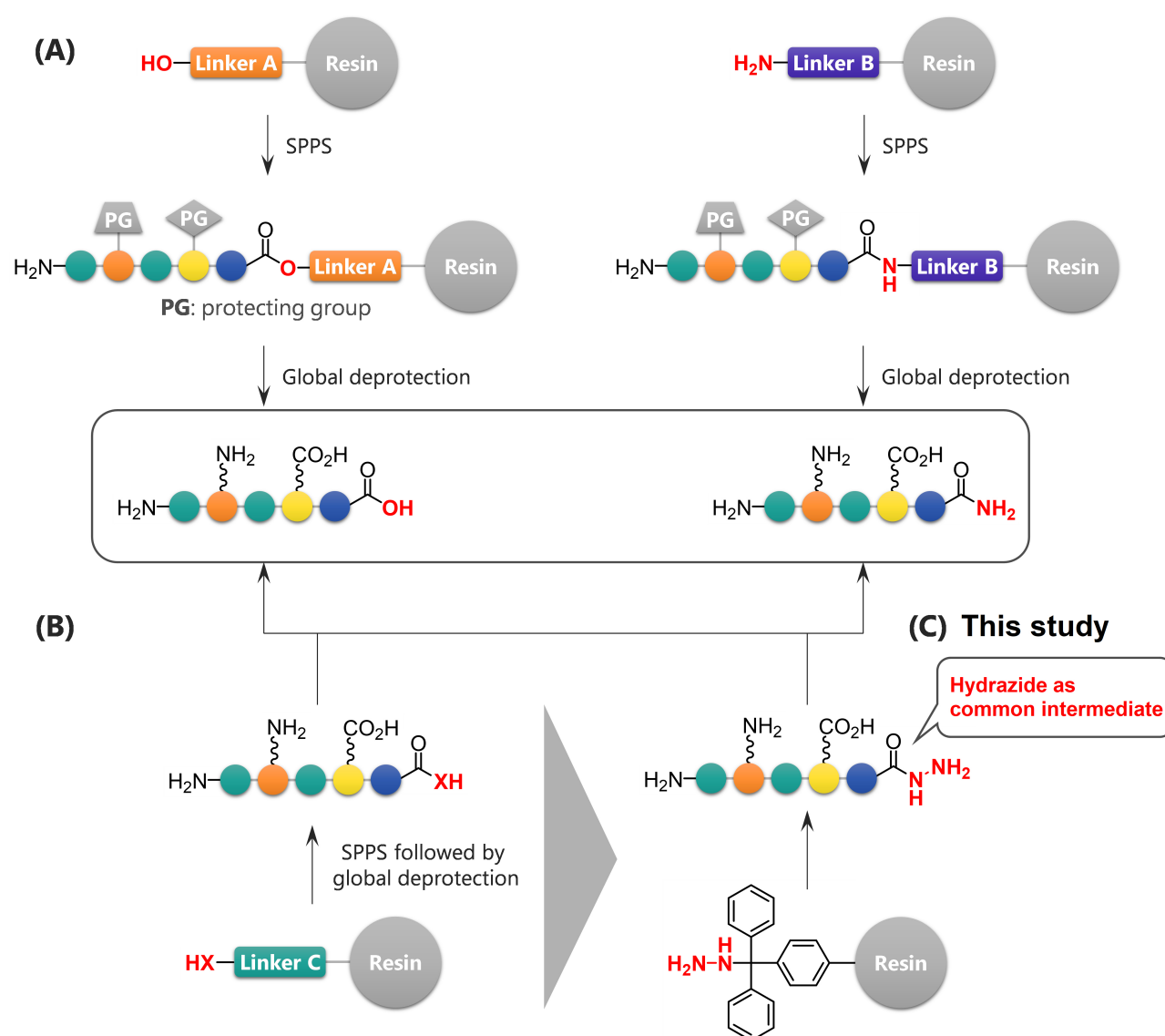
Figure 1. Synthesis of C-terminally modified peptides. (A) Synthesis of C-terminally modified peptides using different linkers; (B) late-stage diversification of C-terminally modified peptides; (C) hydrazide-mediated late-stage synthesis of carboxylic acids or amides

Modification of the C-terminus of a peptide can significantly affect its bioactivity, stability, or three-dimensional conformations [3]. Even relatively minor structural changes, such as α-amidation of the C-

terminal moiety, often significantly affect the peptide properties. For this reason, many strategies to access C-terminally modified peptides have been developed. The most widely used strategy is based on the selection of linkers prior to solid-phase peptide synthesis (SPPS) [4, 5]. This is a reliable strategy but the desired functional groups must be incorporated at the beginning of the synthesis because the peptides are usually synthesized beginning at the C-terminus (Figure 1A). Therefore, this type of strategy requires a significant synthetic effort to create a peptide library containing diverse C-terminal residues.

Late-stage modification strategies, which can lead to new analogs of a parent structure, have been recognized as useful methods that may allow the performance of only a single SPPS to construct the peptide sequence and complete the subsequent transformation of its C-terminus to generate various analogs (Figure 1B). The key to the success of this strategy in peptides is achieving the chemoselective and site-selective transformation, particularly in the absence of any protecting groups on reactive functional groups. Consequently, hydrazide was used as a chemoselective handle at the C-terminus (Figure 1C). This study focused on transforming hydrazides to carboxylic acids and amides because both of these C-terminal structures are common in bioactive peptides, often significantly affecting their structure and activity. Oxidation of a hydrazide will convert it to a carboxylic acid [6–10]. Treatment with β-mercaptoethanol (BME) can generate peptide acids from the corresponding thioesters, which can be prepared from unprotected hydrazides [11–13]. Ammonolysis [14] or the Staudinger reaction [15] of the peptide azides which in turn can be prepared from hydrazides are two potential methods for the preparation of peptide amides. Here a systematic and comprehensive evaluation of the relevant transformations is described and their utility by synthesizing modelin-5 derivatives including an artificial antimicrobial peptide is demonstrated [16].

Materials and methods

Materials

All commercially available reagents and protected amino acids were purchased and used without further purification. Rink amide ChemMatrix, trityl (Trt)-OH ChemMatrix, and HMPB ChemMatrix were purchased from Biotage Japan Ltd. Dry dichloromethane (DCM), dry N,N-dimethylformamide (DMF), methanol (MeOH), sodium nitrite (NaNO$_2$), disodium hydrogen phosphate, copper (II) sulfate, 5-hydrate (CuSO$_4$·5H$_2$O), N,N-diisopropylethylamine (DIPEA), and ammonium acetate (NH$_4$OAc) were purchased from KANTO Chemical Co., Inc. 9-Fluorenylmethyloxycarbonyl (Fmoc) carbazate, 2-iodoxybenzoic acid (IBX), N-bromosuccinimide (NBS), trifluoroacetic acid (TFA), triisopropylsilane (TIS), BME, 4-dimethylaminopyridine (DMAP), piperidine, and m-cresol were purchased from Tokyo Chemical Industry Co., Ltd. DMF, diethyl ether (Et$_2$O), acetonitrile (CH$_3$CN), potassium peroxymonosulfate (Oxone) and tris(2-carboxyethyl)phosphine (TCEP)·hydrochloride (HCl) were purchased from Fuji Film Wako Pure Chemical Industries, Ltd. Thioanisole, lysogeny broth (LB), Miller, and guanidine (Gn)·HCl were purchased from NACALAI Tesque, Inc. N,N'-Diisopropylcarbodiimide (DIPCI) and Fmoc-$_D$-alanine (Ala)-OH·H$_2$O were purchased from Watanabe Chemical Industries Ltd. Ethyl cyanohydroxyiminoacetate (OxymaPure) was purchased from Merck KGaA. Thionyl chloride was purchased from Kishida Chemical Co., Ltd. Fmoc-$_L$-Ala-OH·H$_2$O and Fmoc-methionine (Met)-OH were purchased from Bachem AG. Fmoc-cysteine (Cys)(Trt)-OH, Fmoc-glutamic acid (Glu)(Ot-Bu)-OH, Fmoc-phenylalanine (Phe)-OH, Fmoc-glycine (Gly)-OH, Fmoc-histidine (His)$tert$-butoxycarbonyl (Boc)-OH, Fmoc-isoleucine (Ile)-OH, Fmoc-lysine (Lys)(Boc)-OH, Fmoc-leucine (Leu)-OH, Fmoc-proline (Pro)-OH, Fmoc-arginine (Arg)2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf)-OH, Fmoc-serine (Ser)(t-Bu)-OH, Fmoc-threonine (Thr)(t-Bu)-OH, Fmoc-valine (Val)-OH, Fmoc-tyrosine (Tyr)(t-Bu)-OH, and Fmoc-tryptophan (Trp)(Boc)-OH were purchased from CEM corporation. Fmoc-Cys acetamidomethyl (Acm)-OH was purchased from Peptide Institute, Inc.

LCMS and preparative HPLC

Liquid chromatography-mass spectrometry (LCMS) analyses were carried out on a Waters Alliance high-performance liquid chromatography (HPLC) system with Acquity single quadrupole (SQ) Detector (ESI-SQ). For HPLC separations, a Cosmosil 5C$_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 mm × 250 mm, flow

rate 1.0 mL/min) and a Cosmosil 5C$_{18}$-AR-II preparative column (Nacalai Tesque, 20 mm × 250 mm, flow rate 8.0 mL/min) were employed, and the eluted products were detected by ultra-violet (UV) at 220 nm. A solvent system consisting of 0.1% TFA aqueous solution (aq; *v/v*, solvent A) and 0.1% TFA in CH$_3$CN (*v/v*, solvent B) was used for HPLC elution.

Synthesis of model peptide hydrazides, H-LYRA-Xaa-NHNH$_2$ (1a–q)

The hydrazine-incorporated resin was prepared using previously reported methods [17]. Trt-OH ChemMatrix resin was swollen in dry DCM and treated with thionyl chloride (10 equiv) at room temperature (rt) for 2 h. After being washed with dry DCM and dry DMF, the resulting resin was swollen in dry DCM. To the resin was added a solution of Fmoc carbazate (4 equiv) and DIPEA (10 equiv) in dry DMF (same volume as DCM) dropwise at 0℃. The reaction mixture was shaken at rt overnight, then MeOH was added. After 10 min, the resin was filtered and washed successively with DMF, water, DMF, MeOH, and Et$_2$O. The loading was confirmed by quantification of the Fmoc group [18].

A manual synthesis of Fmoc-SPPS was conducted as follows: (coupling) Fmoc-protected amino acid, OxymaPure, and DIPCI (4 equiv each) were reacted in DMF (0.3 mol/L), 1 h, rt; (Fmoc removal) was achieved with 20% (*v/v*) piperidine in DMF for 10 min at rt. After the elongation, the resulting resin was treated with TFA-TIS-H$_2$O [95:2.5:2.5 (*v/v*), 50 μL/1 mg resin] at rt. After 2 h, Et$_2$O was added to the mixture to give a precipitate which was collected by centrifugation and thoroughly washed with Et$_2$O. The LCMS data of the crude materials are shown in Figures S1–18.

Oxidative conversion of hydrazides

The elongated resin containing 1 (5 mg, approximately 1.5 μmol) was deprotected through the protocol described above and the resulting crude material was dissolved in 50% CH$_3$CN-H$_2$O (200 μL) containing an oxidant (30 μmmol, 20 equiv of CuSO$_4$·5H$_2$O; 7.5 μmmol, 5.0 equiv for Oxone, IBX, and NBS), and the mixture was allowed to react at rt. The reaction progress was monitored by LCMS (Figures S19–37).

BME-mediated conversion of hydrazides

The resin of elongated 1 (5 mg, approximately 1.5 μmol) was deprotected through the protocol described above. The resulting crude material was dissolved in 300 μL of a low pH buffer (6 mol/L Gn·HCl, 0.2 mol/L Na phosphate, pH 3), then 30 μL of 0.5 mol/L NaNO$_2$ in a low pH buffer was added to the solution at –10℃ [19]. After 30 min reaction at –10℃, 1 mol/L BME in a high pH buffer (6 mol/L Gn·HCl, 0.2 mol/L Na phosphate, pH 8, 300 μL) was added to the mixture. The pH was adjusted to pH 8.9 and the reaction was allowed to stand for 30 min at rt. The reaction progress was analyzed by LCMS (Figures S38–54).

Ammonolysis of peptidyl azides derived from hydrazides

The resin of elongated 1 (5 mg, approximately 1.5 μmol) was deprotected through the protocol described above. The resulting crude material was dissolved in 40 μL of a low pH buffer (6 mol/L Gn·HCl, 0.2 mol/L Na phosphate, pH 3), then 10 μL of 1 mol/L NaNO$_2$ aq was added to the mixture at –10℃. After 30 min reaction at –10℃, 8 mol/L NH$_4$OAc (pH 8.3, 150 μL) was added to the mixture. The reaction was allowed to stand for 30 min at rt and the reaction progress was analyzed by LCMS (Figures S55–72).

Amidation of peptidyl azides by the Staudinger reaction

The reaction via azidation in an acidic aq was performed as follows. The resin of elongated 1 (5 mg, approximately 1.5 μmol) was deprotected using the method described above, and the resulting crude material was dissolved in a low pH buffer (6 mol/L Gn·HCl, 0.1 mol/L phosphate, pH 3, 100 μL). To the solution was added 10% (*w/w*) NaNO$_2$ aq (5 μL) at –10℃. After 30 min at –10℃, 100 μL of phosphate buffer (0.1 mol/L, 6 mol/L Gn·HCl, 0.2 mol/L TCEP·HCl, pH 3) was added. The mixture was allowed to react for 30 min at rt, the reaction progress being analyzed by LCMS (Figures S73–90).

The azidation in TFA solution was performed as follows (Figure S91 and Table S1) [20]. The resin bearing elongated 1 (5 mg, approximately 1.5 μmol) was treated with TFA-TIS-H$_2$O-*m*-cresol-thioanisole

[80:2.5:2.5:5:10 (*v/v*), 50 μL/1 mg resin] at rt. After a 2-h reaction, 10% (*w/w*) NaNO$_2$ aq (5 μL) was added to the mixture at –10℃. The solution was allowed to react for 30 min at –10℃ and then cold Et$_2$O was added to give a precipitate. This crude product was dissolved in 50% CH$_3$CN-H$_2$O (200 μL) containing TCEP·HCl (2 equiv) and the mixture was allowed to stand at rt for 30 min. The reaction progress was analyzed by LCMS (Figures S92–109).

Evaluation of epimerization

To monitor the epimerization, the amino acids were coupled by DIPCI and DMAP for carboxylic acids or Rink amide ChemMatrix resin for amides. Then, epimers with C-terminal D-amino acids were synthesized by standard manual Fmoc SPPS. The resulting epimers were used to determine the HPLC conditions for the separation (Figures S110 and 111).

Synthesis of modelin-5 derivatives

Hydrazide-incorporated resin (0.31 mmol/g) was used for the preparation of the hydrazide (9). On this resin, the peptide was elongated using an automated Fmoc SPPS procedure with a Biotage Initiator$^+$ Alstra peptide synthesizer with a 10 mL open-type vial, coupling with Fmoc-protected amino acids, OxymaPure, and DIPCI (4 equiv each) in DMF (0.5 mol/L), for 5 min at 75℃ under microwave irradiation; Fmoc removal was accomplished with 20% (*v/v*) piperidine in DMF for 3 min at 50℃ under microwave irradiation. The resulting resin was treated with TFA-TIS-H$_2$O [95:2.5:2.5 (*v/v*); 50 μL/1 mg resin] at rt for 2 h. The resin in the reaction mixture was removed by filtration and then the filtrate was concentrated in a stream of air. The crude product was dissolved in 0.1% TFA aq (3 mL) and the aqueous phase was washed with Et$_2$O. The crude peptide was purified by preparative HPLC with a linear gradient of solvent B in solvent A, 22–32% over 60 min to give the peptide hydrazide (9; 20 mg from 103 mg of protected resin, 34% isolated yield, Figure S112).

For the preparation of the peptide acid (10), the crude 9, prepared as described above (from 102 mg of protected resin, 23 μmol) was dissolved in 3 mL of 50% CH$_3$CN-H$_2$O with Oxone (116 μmol, 5 equiv), after which the solution was incubated at 37℃ for 30 min. The crude peptide was purified by preparative HPLC with a linear gradient of solvent B in solvent A, 22–32% over 60 min to give the purified peptide (10; 18 mg, 32% isolated yield, Figure S113).

For the preparation of the peptide amide (11), the crude 9, prepared from 99 mg (23 μmmol) of protected resin, as described above was dissolved in 1.5 mL of 6 mol/L Gn·HCl, 0.1 mol/L phosphate (pH 3). After the addition of 0.1 mL of 10% (*w/w*) NaNO$_2$ aq at –10℃, the mixture was allowed to react for 30 min at the same temperature. To the solution was added 1.5 mL of a phosphate buffer (0.1 mol/L, 6 mol/L Gn·HCl, 0.2 mol/L TCEP·HCl, pH 3), and then the reaction mixture was incubated at rt for 30 min. The crude peptide was purified by preparative HPLC with a linear gradient of solvent B in solvent A, 22–32% over 60 min to give the peptide amide (11; 21 mg, 37% isolated yield, Figure S114).

Evaluation of antimicrobial activity

Escherichia coli (*E. coli*) W3110 [21] was grown on LB [1% (*w/v*) tryptone, 0.5% (*w/v*) yeast extract, and 1% (*w/v*) NaCl] containing 1.5% agar, and colonies were inoculated in 5 mL LB medium at 200 rpm at 37℃ for approximately 4 h (OD$_{600}$ = 1.6). The bacterial cells were collected by centrifugation (6,000 × *g*, 10 min, 4℃) and washed with 1 mL of a 25% strength Ringer's solution three times through centrifugation (6,000 × *g*, 5 min, 4℃). The bacterial cells were suspended with Ringer's solution and diluted to adjust the OD$_{600}$ to 0.5. A 100 μL sample of the resulting suspensions was mixed with 100 μL of synthesized modelin-5 derivatives (9–11) in Ringer's solution to give a final peptide concentration of 500 μmol/L. This mixture was incubated in an orbital shaker (100 rpm, 37℃) for 1 h. The bacterial suspension was inoculated onto LB agar plates and incubated at 37℃. After 14 h incubation, the colony forming unit (CFU) was calculated.

Results

To examine the feasibility of converting peptide hydrazides to carboxylic acids, the model peptide (H-LYRAG-NHNH$_2$, 1a) was treated with several oxidants (Figure 2 and Table 1). Although copper (II)

sulfate (CuSO$_4$) was effective in converting an N-alkyl hydrazide peptide to carboxylic acid [6, 7], it produced the corresponding carboxylic acid (2a) only in a low yield (entry 1). Oxone oxidation proceeded smoothly within 30 min (entry 2). Treatment with IBX or NBS consumed the starting material but failed to generate the desired product (entries 3 and 4). The plausible mechanism of Oxone-mediated transformation of hydrazides to acids is given in Figure 3 [22].
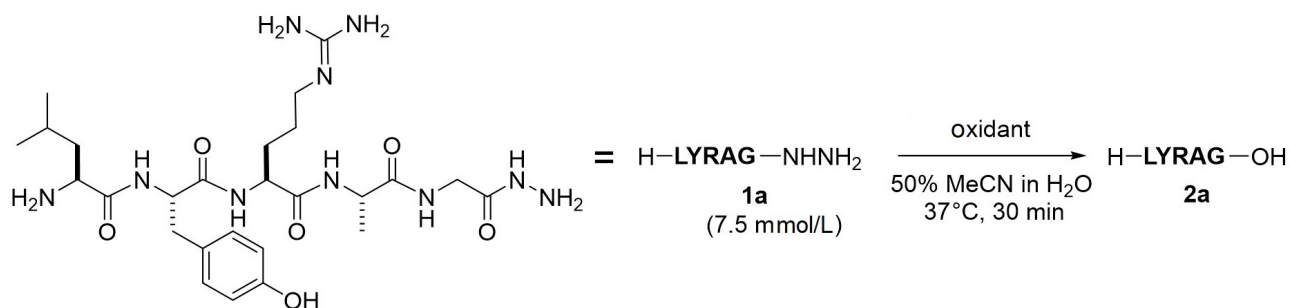

Figure 2. Direct oxidation of peptide hydrazides

Table 1. Optimization of oxidant

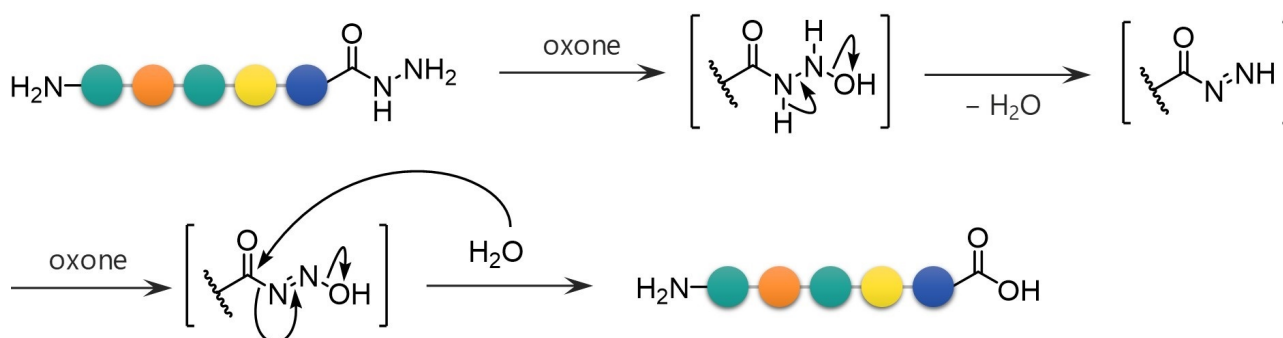| Entry	Oxidant	Equiv	pH	HPLC purity of 2a (%)[a]
1	CuSO$_4$·5H$_2$O	20	3.8	11
2	Oxone	5	2.1	93
3	IBX	5	1.8	< 1
4	NBS	5	4.4	< 1

[a] detected at 220 nm

Figure 3. Plausible mechanism of Oxone-mediated transformation of hydrazides to acids

Formation of azides from hydrazides followed by a reaction with BME is another approach for the conversion of hydrazides to carboxylic acids. Unprotected hydrazides can be transformed into peptidyl azides with the aid of NaNO$_2$ under acidic conditions. In the presence of thiols [19], azides readily afford the corresponding thioesters. Thioesters of BME are formally hydrolyzed through the intramolecular *S*-to-*O* acyl transfer followed by thiirane formation, releasing the appropriate carboxylate (Figure 4) [11]. This reaction sequence has been used for the epimerization-free synthesis of peptide acids with a C-terminal Cys [12], but has not been systematically and comprehensively evaluated.

To evaluate the scope of these two methods to generate carboxylic acids, peptide hydrazides bearing amino acids at the C-terminus were prepared. The exceptions were aspartic acid (Asp), asparagine (Asn), and glutamine (Gln) because of the synthetic difficulty due to intramolecular cyclization [19]. Using oxidation by Oxone, the desired carboxylic acids were obtained with 63–87% purity (Figure 5, Table 2). Oxidation-prone amino acids, namely Cys, Met, and Trp, were however incompatible with this condition. A separate protocol using BME after azide formation succeeded in the transformation of hydrazides to the corresponding carboxylic acids in 60–95% yield. Ile and Pro required a longer reaction time to completely convert the intermediate thioester to the acid (entries 4 and 7). Unlike the reaction conditions of the Oxone
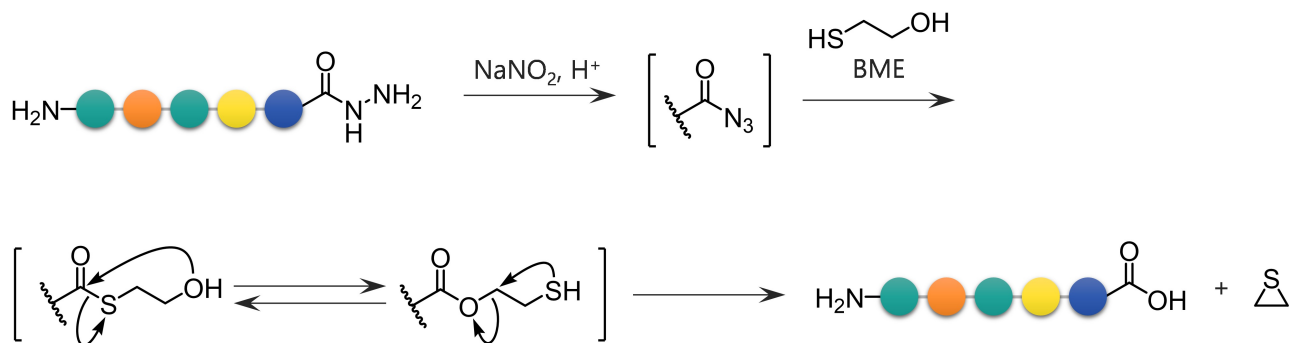
Figure 4. BME-mediated formal hydrolysis of peptidyl azides preparable from unprotected hydrazides

reaction, the BME-mediated reaction was applicable to the peptides with C-terminal Cys, Met, or Trp (entries 11, 13, and 18). The epimerization of C-terminal amino acids (Ala) was suppressed during the reactions (epimer ratio > 99:1, Figure S110).
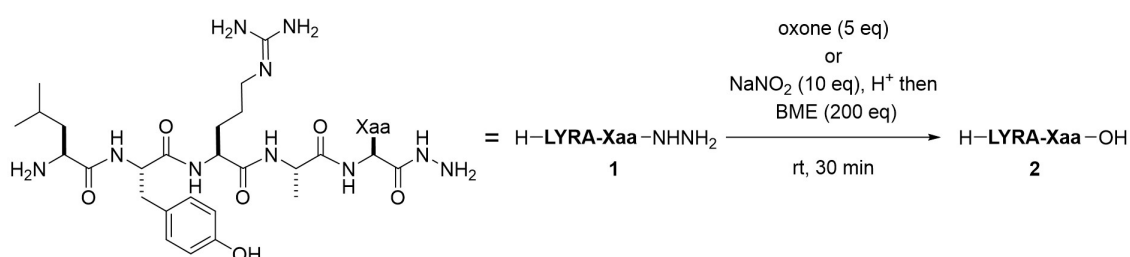

Figure 5. Conversion of hydrazides to carboxylic acids

Table 2. Scope of the C-terminal amino acids in conversion to carboxylic acids

Entry	Xaa	Product	HPLC purity of 2 (%)[a]	
			Oxone	BME
1	Gly	2a	84	82
2	Ala	2b	82	82
3	Val	2c	77	76
4	Ile	2d	63	61 (74)[b]
5	Leu	2e	80	79
6	Phe	2f	87	89
7	Pro	2g	77	65 (87)[b]
8	Ser	2h	82	81
9	Thr	2i	81	91
10	Glu	2j	81	95
11	Cys	2k	< 1	66
12	Cys(Acm)	2k'	< 1	-
13	Met	2l	< 1	80
14	Tyr	2m	81	87
15	His	2n	81	85
16	Lys	2o	76	75
17	Arg	2p	84	60
18	Trp	2q	< 1	86

[a] detected at 220 nm; [b] after the addition of BME, a 20 h reaction was performed. -: not examined

To convert a peptide hydrazide to the peptide amide, two methods were used: 1) Azidation followed by ammonolysis, and 2) azidation followed by the Staudinger reaction (Figure 6). After preliminary experiments, peptide amides were found to be formed by ammonolysis of peptidyl azides using 6 mol/L NH$_4$OAc in a slightly basic buffer. Similarly, the Staudinger reaction of peptidyl azides using 0.1 mol/L TCEP·HCl as a water-soluble phosphine gave the desired amide within 30 min.
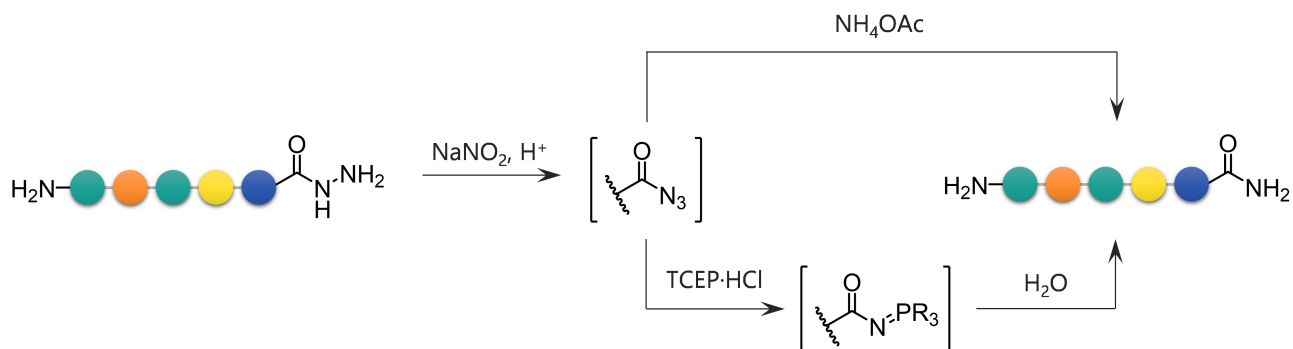
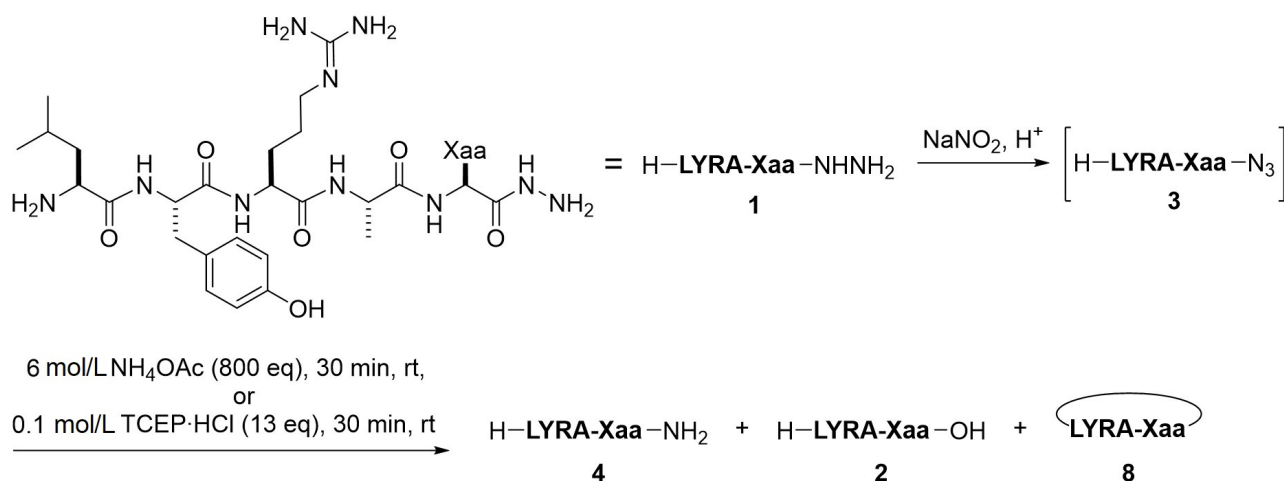**Figure 6.** Synthesis of peptide amides from hydrazides

6 mol/L NH$_4$OAc (800 eq), 30 min, rt,
or
0.1 mol/L TCEP·HCl (13 eq), 30 min, rt

H−**LYRA-Xaa**−NH$_2$ + H−**LYRA-Xaa**−OH + **LYRA-Xaa**
4　　　　　　　　　　　　**2**　　　　　　　　　**8**

Figure 7. Conversion of hydrazides to amides

Table 3. Scope of the C-terminal amino acids in conversion to amides

Entry	Xaa	Product	HPLC purity (%)[a]				
			Ammonolysis			**Staudinger reaction**	
			4	**2**	**8**	**4**	**2**
1	Gly	2a	86	5	6	94	6
2	Ala	2b	80	4	9	84	7
3	Val	2c	82	2	8	91	1
4	Ile	2d	91	< 1	< 1	92	< 1
5	Leu	2e	74	5	13	94	4
6	Phe	2f	87	6	< 1	91	6
7	Pro	2g	90	1	4	86	2
8	Ser	2h	79	3	9	93	4
9	Thr	2i	71	3	13	91	3
10	Glu	2j	< 1	77	< 1	4 (74)[b]	87
11	Cys	2k	39[c]	< 1	< 1	26	7
12	Cys(Acm)	2k'	75	9	10	93	< 1
13	Met	2l	80	6	9	87	4
14	Tyr	2m	83	8	2	85	5
15	His	2n	45	39	4	68 (83)[b]	28
16	Lys	2o	86	5	6	87	6
17	Arg	2p	91	6	< 1	91	5
18	Trp	2q	82	8	< 1	80	8

[a] detected at 220 nm; [b] azidation was performed in TFA solution instead of acidic aqueous condition; [c] disulfide dimer of 2k was observed
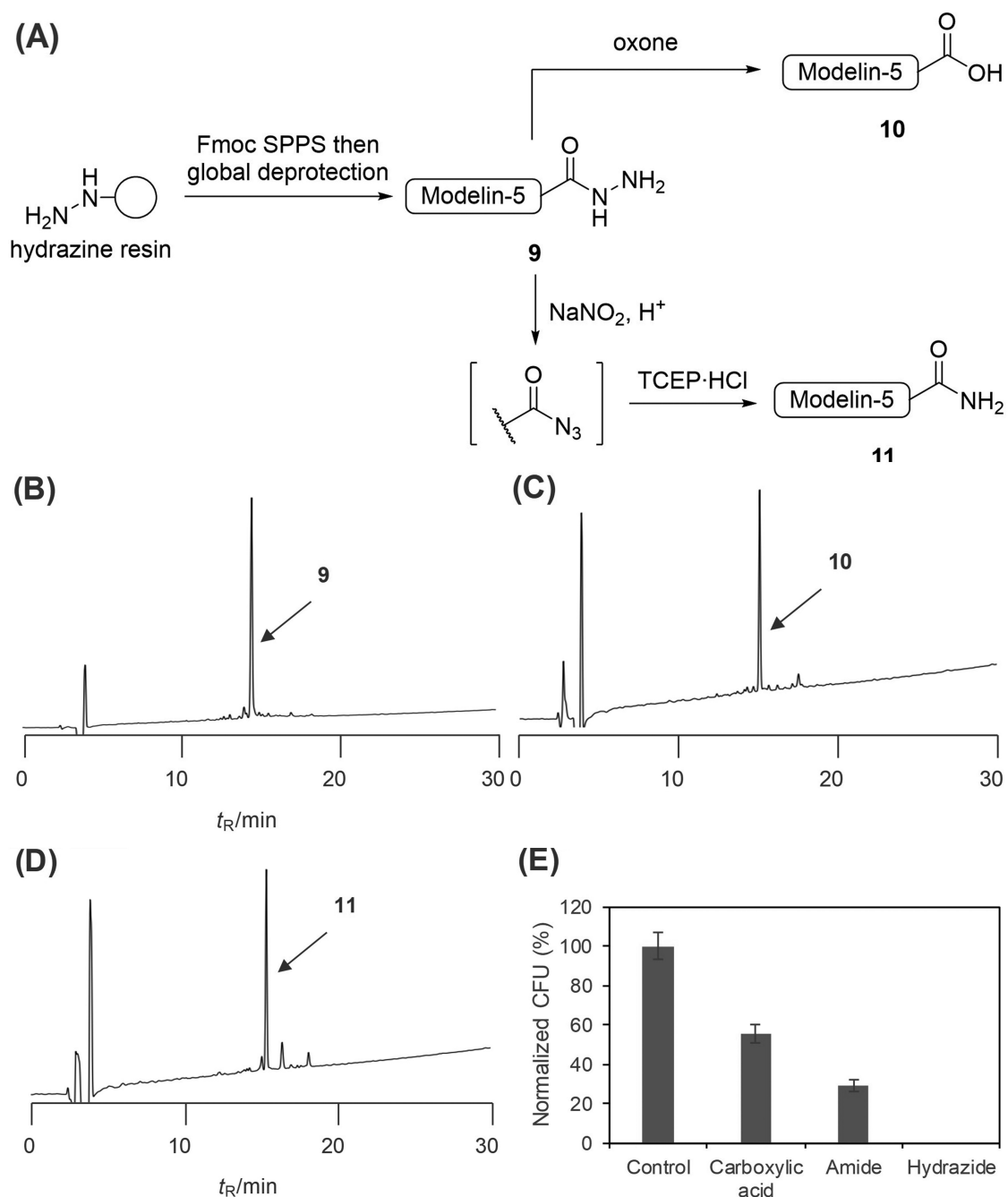
Figure 8. Synthesis and evaluation of modelin-5 derivatives. (A) Synthetic scheme of modelin-5 derivatives from hydrazide; crude HPLC charts of (B) modelin-5-CONHNH$_2$; (C) modeline-5-COOH; or (D) modelin-5-CONH$_2$; (E) antimicrobial activity of modelin-5 derivatives against *E. coli*. Error bars represent the standard deviation (*n* = 3). Retention time (t_R): 14.5 min for peptide hydrazide (9), 15.1 min for peptide acid (10), and 15.4 min for peptide amide (11). HPLC conditions: Cosmosil 5C$_{18}$-AR-II analytical column with the linear gradient of MeCN/0.1% aqueous TFA (10:90–60:40 over 30 min) at flow rate 1.0 mL/min, detected at 220 nm.

With the optimal conditions in hand, the scope of these conditions with respect to various C-terminal amino acids was examined (Figure 7 and Table 3). In the NH$_4$OAc-mediated ammonolysis, the desired amides (4) were obtained together with the hydrolyzed products (2) and compounds with molecular weight 17 less than these, probably because of the intramoleculearly cyclized peptides (8). In the case of C-terminal Glu and His, significant amounts of the hydrolyzed peptide (2) were obtained (entries 10 and 15). The peptide with a C-terminal Cys residue afforded a low yield because of the formation of a disulfide-related byproduct, which was suppressed by using Acm protection for the thiol (entries 11 and 12).

The procedure using the Staudinger reaction also proceeded smoothly, yielding the peptide amides (4) in acceptable yields (80–94%) except for sequences containing Glu-, His-, or Cys-. Glu- or His-containing peptides afforded a low yield of the desired products, but 87% and 28% of the hydrolyzed product (2) were

observed (entries 10 and 15). When performing the azide formation in a TFA cocktail [20] instead of under aqueous conditions, the hydrolysis was suppressed, giving the desired amides in 74% for Glu and 83% for His. The thiol group of Cys-containing peptide was oxidized to the sulfinic acid while the side reaction was suppressed using Cys(Acm) as with the ammonolysis condition (entries 11 and 12). Azidation in TFA followed by the Staudinger reaction was also effective in generating the corresponding amides from hydrazides, but in some cases, amines, oxazolidinones, or thiazolidinones were observed as a main byproduct formed through Curtius rearrangement followed by hydrolysis or cyclization of the resulting isocyanate (Table S1). The peptides having Ala at the C-terminus showed no epimerization in the transformation to amides (epimer ratio > 99:1, Figure S111).

The applicability of the developed late-stage diversification strategies was validated by synthesizing modelin-5 derivatives (H-KLAKKLAKLAKLAKAL-X; X = OH, NH_2, or $NHNH_2$), an artificial antimicrobial peptide (Figure 8A). Modelin-5 is known to have varied antimicrobial potencies depending on its C-terminal structure; in particular, modelin-5-$CONH_2$ is more potent than modelin-5-COOH [23]. The modelin-5 sequence was elongated on a hydrazine-incorporated resin through the conventional Fmoc SPPS. The standard cleavage of the elongated peptide afforded the modelin-5-$CONHNH_2$ (9) in 34% isolated yield (Figure 8B). The global deprotection of the peptidyl resin followed by oxidation by Oxone smoothly yielded the modeline-5-COOH (10) in 32% isolated yield from the resin loading (Figure 8C). The modelin-5-$CONH_2$ (11) was synthesized in 37% yield through the azidation of the corresponding hydrazide followed by the Staudinger reaction (Figure 8D).

Finally, the antimicrobial potency against *E. coli* of the synthesized modelin-5 derivatives was evaluated. The cells were incubated with 500 μmol/L of each peptide derivative for 1 h. The treated cells were plated and then incubated at 37°C for 14 h, after which the number of colonies was counted (Figure 8E). As has been reported [23], the potency of the amide derivative (11) was higher than that of the carboxylic acid (10). Surprisingly, the hydrazide-type derivative (9) showed the most potent antimicrobial activity against *E. coli* but the mechanism that underlies such potency differences was unclear.

Discussion

Derivatization of peptides is an important method to control and improve their properties. Modification of the C-terminus of bioactive peptides has important implications for their potency. Late-stage diversification is a practical concept to create libraries with diversity, although it requires a chemoselective toolbox to apply it to peptides because peptides have various types of functional groups. In this study, simple and practical protocols were developed for the late-stage transformation of peptide C-terminus residues from hydrazides to the corresponding peptide acids and amides. Oxone oxidation yielded carboxylic acids directly from hydrazides without any additional transformations although some oxidation-prone amino acids showed low compatibility with this reaction. Chemoselective azidation of hydrazides followed by the addition of BME also succeeded in yielding the carboxylic acids via a thioester intermediate. This method is compatible with oxidation-prone amino acids. Different kinds of reaction sequences have been attempted for some specific examples. The same procedure, hydrazide activation by acetylacetone followed by BME addition [13], azidation followed by hydrolysis in basic aqueous conditions have been used [12, 24], but to the best of our knowledge, such a systematic and comprehensive evaluation has not been completed to date.

Chemoselective azidation of hydrazides and subsequent ammonolysis or a Staudinger reaction also succeeded in the late-stage amide synthesis although a C-terminal Cys must be protected. C-terminal Glu hampers the efficient amide synthesis probably due to the intramolecular anhydride formation followed by hydrolysis. This undesired reaction was suppressed by using a protocol other than a low pH aqueous buffer, to generate a peptide azide in TFA, but the versatility of the TFA condition was not so strong and side reactions involving Curtius rearrangement occurred more frequently. These results suggest that it is important to choose appropriate reaction conditions according to the components and sequence of peptides.

A general strategy to obtain both acid and amide peptides requires double SPPS using a resin with different linkers. In contrast, our protocols allow the performance of only a single SPPS to obtain both peptides. For example, the synthesis of modelin-5-COOH and -CONH$_2$ using different linkers requires a total of 64 steps (16 couplings and 16 removals of N$_\alpha$ protection for each SPPS). In comparison, late-stage transformation reduces the number of synthetic steps to 34 (32 steps for peptide elongation and two steps for the transformation) and additionally produces a hydrazide as another component to enrich the library. This benefit was confirmed by evaluating the antimicrobial activity of the modelin-5 derivatives, leading to the unexpected discovery that modelin-5-CONHNH$_2$ showed the most potent activity. Considering the antimicrobial peptide with C-terminal hydrazide, Li et al. [24] reported that the antimicrobial spectrum of Chex1-Arg20, an antimicrobial peptide, was broadened by incorporation of the hydrazide. They hypothesized that the broadened spectrum of activity may be due to the stability against bacterial enzyme attacks being improved in the hydrazide. Since modelin-5-CONH$_2$ has a potent ability to kill *Bacillus subtilis* via membranolytic modes of action [25], the hydrazide might also improve the stability against bacterial enzymes. Efforts to exploit the mechanism and influence of C-terminal hydrazide modification into the antimicrobial activity are in progress in our laboratory.

Abbreviations

Acm: acetamidomethyl

Ala: alanine

aq: aqueous solution

Arg: arginine

BME: β-mercaptoethanol

Boc: *tert*-butoxycarbonyl

CH$_3$CN: acetonitrile

CuSO$_4$·5H$_2$O: copper (II) sulfate, 5-hydrate

Cys: cysteine

DCM: dichloromethane

DIPCI: *N,N'*-diisopropylcarbodiimide

DMF: *N,N*-dimethylformamide

E. coli: *Escherichia coli*

Et$_2$O: diethyl ether

Fmoc: 9-fluorenylmethyloxycarbonyl

Glu: glutamic acid

Gly: glycine

Gn: guanidine

HCl: hydrochloride

His: histidine

HPLC: high-performance liquid chromatography

IBX: 2-iodoxybenzoic acid

Ile: isoleucine

LB: lysogeny broth

LCMS: liquid chromatography-mass spectrometry

Leu: leucine

Lys: lysine

MeOH: methanol

Met: methionine

$NaNO_2$: sodium nitrite

NBS: *N*-bromosuccinimide

NH_4OAc: ammonium acetate

Oxone: potassium peroxymonosulfate

OxymaPure: ethyl cyanohydroxyiminoacetate

Phe: phenylalanine

Pro: proline

rt: room temperature

Ser: serine

SPPS: solid-phase peptide synthesis

TCEP: tris(2-carboxyethyl)phosphine

TFA: trifluoroacetic acid

Thr: threonine

TIS: triisopropylsilane

Trp: tryptophan

Trt: trityl

Tyr: tyrosine

Val: valine

Supplementary materials

The supplementary material for this article is available at: https://www.explorationpub.com/uploads/Article/file/100823_sup_1.pdf.

Declarations

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Author contributions

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

References

1. Wang L, Wang N, Zhang W, Cheng X, Yan Z, Shao G, et al. Therapeutic peptides: current applications and future directions. Sig Transduct Target Ther. 2022;7:48.
2. Muttenthaler M, King GF, Adams DJ, Alewood PF. Trends in peptide drug discovery. Nat Rev Drug Discov. 2021;20:309–25.
3. Arbour CA, Mendoza LG, Stockdill JL. Recent advances in the synthesis of C-terminally modified peptides. Org Biomol Chem. 2020;18:7253–72.
4. Alsina J, Albericio F. Solid-phase synthesis of C-terminal modified peptides. Pept Sci. 2003;71:454–77.
5. Moss JA. Guide for resin and linker selection in solid-phase peptide synthesis. Curr Protoc Protein Sci. 2005;40:18.7.1–19.
6. Sato K, Tanaka S, Wang J, Ishikawa K, Tsuda S, Narumi T, et al. Late-stage solubilization of poorly soluble peptides using hydrazide chemistry. Org Lett. 2021;23:1653–8.
7. Fabre B, Pícha J, Vaněk V, Buděšínský M, Jiráček J. A CuAAC–hydrazone–CuAAC trifunctional scaffold for the solid-phase synthesis of trimodal compounds: possibilities and limitations. Molecules. 2015;20:19310–29.
8. Srinivas R, Reddy BVS, Yadav JS, Ramalingam T. An efficient and selective conversion of hydrazides into esters and acids. J Chem Res. 2000;2000:376–7.
9. Takale BS, Telvekar VN. Selective oxidation of hydrazides using *o*-iodoxybenzoic acid to carboxylic acids, esters, and aldehydes. Chem Lett. 2010;39:546–7.
10. Cheung HT, Blout ER. The hydrazide as a carboxylic-protecting group in peptide synthesis. J Org Chem. 1965;30:315–6.
11. Gates ZP, Stephan JR, Lee DJ, Kent SBH. Rapid formal hydrolysis of peptide-ᵅthioesters. Chem Commun. 2013;49:786–8.
12. Zuo C, Yan BJ, Zhu HY, Shi WW, Xi TK, Shi J, et al. Robust synthesis of C-terminal cysteine-containing peptide acids through a peptide hydrazide-based strategy. Org Biomol Chem. 2019;17:5698–702.
13. Liang LJ, Chu GC, Qu Q, Zuo C, Mao J, Zheng Q, et al. Chemical synthesis of activity-based E2-ubiquitin probes for the structural analysis of E3 ligase-catalyzed transthiolation. Angew Chem Int Ed. 2021;60:17171–7.
14. Tsuda S, Masuda S, Yoshiya T. Solubilizing trityl-type tag to synthesize Asx/Glx-containing peptides. Chembiochem. 2019;20:2063–9.
15. Staudinger H, Meyer J. Über neue organische phosphorverbindungen III. Phosphinmethylenderivate und phosphinimine. Helv Chim Acta. 1919;2:635–46. German.
16. Bessalle R, Gorea A, Shalit I, Metzger JW, Dass C, Desiderio DM, et al. Structure-function studies of amphiphilic antibacterial peptides. J Med Chem. 1993;36:1203–9.

17. Huang YC, Chen CC, Li SJ, Gao S, Shi J, Li YM. Facile synthesis of C-terminal peptide hydrazide and thioester of NY-ESO-1 (A39-A68) from an Fmoc-hydrazine 2-chlorotrityl chloride resin. Tetrahedron. 2014;70:2951–5.

18. Eissler S, Kley M, Bächle D, Loidl G, Meier T, Samson D. Substitution determination of Fmoc-substituted resins at different wavelengths. J Pept Sci. 2017;23:757–62.

19. Fang GM, Li YM, Shen F, Huang YC, Li JB, Lin Y, et al. Protein chemical synthesis by ligation of peptide hydrazides. Angew Chem Int Ed. 2011;50:7645–9.

20. Sato K, Tanaka S, Yamamoto K, Tashiro Y, Narumi T, Mase N. Direct synthesis of N-terminal thiazolidine-containing peptide thioesters from peptide hydrazides. Chem Commun. 2018;54: 9127–30.

21. Hayashi K, Morooka N, Yamamoto Y, Fujita K, Isono K, Choi S, et al. Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. Mol Syst Biol. 2006;2:2006.0007.

22. Kulkarni PP, Kadam AJ, Desai UV, Mane RB, Wadgaonkar PP. A simple and efficient oxidation of hydrazides to N,N'-diacylhydrazines using Oxone® in an aqueous medium. J Chem Res. 2000;2000: 184–5.

23. Dennison SR, Phoenix DA. Influence of C-terminal amidation on the efficacy of modelin-5. Biochemistry. 2011;50:1514–23.

24. Li W, Tailhades J, Hossain MA, O'Brien-Simpson NM, Reynolds EC, Otvos L, et al. C-Terminal modifications broaden activity of the proline-rich antimicrobial peptide, Chex1-Arg20. Aust J Chem. 2015;68:1373–8.

25. Dennison SR, Hauß T, Badiani K, Harris F, Phoenix DA. Biophysical investigation into the antibacterial action of modelin-5-NH$_2$. Soft Matter. 2019;15:4215–26.