Ninhydrin as a reversible protecting group of amino-terminal cysteine

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Abstract: The proximity of the α-amino and β-thiol of α-amino terminal-cysteine (NT-Cys) residues in peptides imparts unique chemical properties that have been exploited for inter- and intra-molecular ligation of unprotected peptides obtained through both synthetic and biological means. A reversible protecting group orthogonal to other protection strategies and reversible under mild conditions would be useful in simplifying the synthesis, cleavage, purification and handling of such NT-Cys peptides. It could also be useful for the sequential ligation of peptides. To this end, we explored tri-one chemistry and found that ninhydrin (indane-1,2,3 trione) reacted readily with cysteine or an NT-Cys-containing peptide on- or off-resin at pH 2–5 to form Ninhydrin-protected Cys (Nin-Cys) as a thiazolidine (Thz). The Thz ring, protecting both the amino and thiol groups in Nin-Cys, completely avoids the formylation and Thz side reactions found during hydrofluoric acid (HF) cleavage when N-π-benzylxymethyl histidine groups are present. Nin-Cys is stable during coupling reactions and various cleavage conditions with trifluoroacetic acid or HF, but is deprotected under thiolytic or reducing conditions. These properties enable a facile one-step deprotection and end-to-end-cyclization reaction of Nin-Cys peptides containing C-terminal thioesters.

Abbreviations: APCI, atmospheric pressure chemical ionization; Boc, t-Butoxycarbonyl; CT, C-terminal; DBU, 1, 8-diazabicyclo[5,4,0]undec-7-ene; DCM, dichloromethane; DIEA, N,N-disopropylethylamine; DMF, dimethylformamide; Dmt, dimethylthiazolidinecarboxylate; EDT, ethanediitol; ES-MS, electrospray mass spectrometry; Fmoc, florenylmethoxycarbonyl; GnHCl, guanidine hydrochloride; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HF, hydrofluoric acid; His(Bom), N-π-benzylxymethyl histidine; HOBt,
1-hydroxybenzotriazole; LC-MS, liquid chromatography mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MPS, 3-mercaptopropionsulfonic acid; Nin-Cys, ninhydrin-protected cysteine; NMP, N-methyl pyrrolidine; NT, N-terminal; Ochx, cyclohexylox; MeBzl, 4-methylbenzyl; RP-HPLC, reverse-phase high-pressure liquid chromatography; SPPS, solid-phase peptide synthesis; TCEP, tris-carboxymethylphosphine; TFA, trifluoroacetic acid; Thz, thiazolidine; Tos, tosyl; Xan, xanthyl.

N-terminal cysteine (NT-Cys) residues feature two vicinal nucleophiles, β-thiol and α-amine, which confer chemical reactivity unique among biopolymers. This reactivity has recently been exploited for the chemoselective ligation and cyclization of unprotected peptides [1]. Presently, a popular method involves a peptide containing a carboxyl terminal (CT) thioester moiety, which can react with a peptide containing an NT-Cys residue under mild, aqueous conditions to form a ligated product (2–6). Peptides containing both NT-Cys and CT-thioester can also be used to form cyclic peptides (5,7–13). In both cases, the resulting peptides regenerate a cysteine at the ligation site. Pseudoproline ligation methods have also been developed for segment ligation (14–16) and cyclization (17,18) of NT-Cys peptides through imine capture and the formation of thiazolidine (Thz) intermediates.

A biosynthetic extension of ligation chemistry involving NT-Cys is found in the naturally occurring protein splicing mediated by embedded proteases, known as inteins, which catalyze the cleavage and formation of peptide bonds (19,20). The proposed mechanism of protein splicing involves both thioester (or ester) intermediates and NT-Cys (or Ser/Thr) moieties. Inteins have since been utilized for peptide-protein ligation (22–24), protein–protein ligation (25–28) and protein cyclizations [27]. NT-Cys residues have been found to play an important role in the biological function of several proteins, including the sonic hedgehog [29,30] and cholesteryl ester transfer proteins [31].

The application of NT-Cys-based ligation and cyclizations has led to an expansion of protocols for preparing peptide thioesters. A common method involves t-Butoxy-carbonyl [Boc]-protecting group chemistries via a thioester linkage to the resin. A problem encountered in Boc synthesis is the incompatibility of NT-Cys residues with the preferred side chain-blocking group for histidine, benzyloxymethyl [Bom]. Hydrogen fluoride cleavage of N-π-benzyloxymethyl histidine [His[Bom]] produces a molecule of formaldehyde that reacts in situ with NT-Cys to form a stable Thz adduct (32). Scavengers such as cysteine and resorcinol have been shown to minimize the formation of the Thz (33), but are incapable of completely suppressing this side reaction (34). Cysteine as a scavenger also raises the possibility of thioester exchange with the target thioester during cleavage work-up and purification.

It is known that ninhydrin [1,2,3-indanetriene monohydrate] reacts with primary amines to form the chromophore Ruhman’s purple [35]. This reaction was first adapted for qualitative monitoring of solid-phase peptide synthesis by Kaiser [36], and was later adapted for more quantitative monitoring by Sarin et al. [37]. Although very effective for most peptide resins, several groups have shown that this reaction does not work for peptide resins containing an NT-cysteine (38–40). During the reaction, the β-thiol of an NT-Cys adds to the Schiff’s base intermediate trapping both molecules as a spirothiazolidine (39,41). The product of this reaction is reminiscent of the 2,2-dimethylthiazolidine-4-carboxylic acid [Dmt-OH] formed from acetone and cysteine (42). Kemp and Carey have used this molecule as a protected NT-Cys for peptide ligation by prior thiol capture (43).

The condensation product of ninhydrin and cysteine [1,3-dione-4′-carboxy-spiro[indane-2,2′-thiazolidin]1]], ninhydrin-protected cysteine 1 [Nin-Cys], is a literature compound (39). We show here that Nin-Cys can be attached to peptide resins using standard chemistries. The Thz structure formed with the ninhydrin effectively protects NT-Cys residues during both trifluoroacetic acid (TFA) and hydrofluoric acid (HF) cleavage of His[Bom]-containing peptides. Following cleavage, Nin-Cys is removed under mild thiolytic or reducing conditions. Using this protection strategy, we synthesized a 40-residue peptide thioester and performed a one-step Nin-Cys deprotection and peptide cyclization using an end-to-end ligation. Additionally, ninhydrin was used for site-specific modification of NT-Cys residues on unprotected peptides. Our results demonstrate that ninhydrin protection of NT-Cys, is useful for improving the synthesis of cyclic peptides, and has potential for the development of new methods for the sequential ligation of peptides.

Results

Coupling of Nin-Cys-OH to peptide resins

Figure 1 displays a general scheme for the synthesis and coupling of Nin-Cys-OH to a peptide resin. An efficient
approach would be a direct coupling of Nin-Cys-OH to the α-amine of a peptide resin without protection on the secondary amine of the Thz ring. A lack of reactivity with activated amino acids was reported for the less sterically hindered Dmt \(^\text{43}\) and we anticipated that acylation of Nin-Cys would be slow. In model studies, Nin-Cys-OH was activated with \(2-(1\text{H-benzo}t\text{riazol-1-yl})-1,1,3,3\)-tetramethyluronium hexafluorophosphate (HBTU) and coupled for 20 min to tripeptide resins containing NT-glycine, -methionine, and -isoleucine, respectively. In all cases, the HPLC elution profiles of the peptide products show that the couplings proceeded efficiently with minimal sequential additions of Nin-Cys (Fig. 2). It is significant to note that even when coupling to isoleucine, the Nin-Cys attached cleanly with no Nin-Cys dimer formation observed under our reaction conditions. These results indicate that the spiroindanedione moiety of Nin-Cys-OH sufficiently protects the secondary amine from further acylation reaction while not preventing the coupling of Nin-Cys-OH to bulky residues such as isoleucine. Nin-Cys-OH can therefore be coupled to peptide resins without the need for protecting the secondary amine-Thz group. It should be noted that when coupling reactions were allowed to proceed >30 min, peptides containing dimers or trimers of Nin-Cys (<2%) were observed.

**HF cleavage of Nin-Cys peptides**

To demonstrate that ninhydrin can effectively protect NT-Cys from formylation side reactions in the presence of His(Bom) during HF cleavage, a model decapeptide Nin-CYLLH\(_6\) [Nin-CYLLHHHHHH-OH] was synthesized. This poly-histidine-containing peptide was employed as an antigen for raising antibodies against the poly-histidine epitope of His-tagged proteins, and hence was a suitable peptide for model studies. During model peptide synthesis, the resin was evenly divided before coupling of the final cysteine residue such that Nin-Cys-OH could be attached to one portion as a control reaction, and Boc-Cys[MeBzl] to the other half to determine the extent of the formylation side reactions. Liquid chromatography mass spectrometry (LC-MS) analysis of the crude peptides following HF cleavage with 10% p-cresol as the only scavenger showed that >50% the Boc-Cys[MeBzl]-peptide formed Thzs with the formaldehyde generated [Thz-Cys adduct: obs. \([M + 2\text{H}]^{2+} = 673.27\), calc. \([M + 2\text{H}]^{2+} = 673.2\)], whereas none of the control Nin-Cys peptides were modified [obs. \([M + 2\text{H}]^{2+} = 738.29\), calc. \([M + 2\text{H}]^{2+}\) Nin-CYLLH\(_6\) = 738.30] (see Fig. 3). A single-ion scan of the crude Nin-Cys peptide also failed to show evidence of a Thz adduct [data not shown]. These results confirm that the Nin-Cys group is compatible with HF cleavage conditions and completely protects NT-Cys from His[Bom]-mediated Thz side reaction.

**Deprotection of Nin-Cys protected peptides**

We tested three different methods [Fig. 4] for deprotecting Nin-CYLLH\(_6\). The first method involved subjecting the Nin-Cys peptide to treatment with an excess of cysteine at pH 7.7 and 23 °C. Under these conditions, Nin-CYLLH\(_6\) was completely deprotected in 30 min [Fig. 4A and B]. Evidently, the Thz is in equilibrium with starting materials (ninhydrin and cysteine) and the complete deprotection of Nin-Cys peptides is driven by mass action. Furthermore, in initial studies we were able to remove the ninhydrin group from Nin-CGYY while attached to resin using cysteine O-methylester in dimethylformamide (DMF) and N,N-diisopropylethylamine (DIEA) [data not shown].
Although cysteine was effective for deprotection, it was not acceptable for thioester-containing peptides because the cysteine will form an amide bond with a thioester. Thus, 3-mercaptopropionic acid (MPS) was explored as a potential deprotecting agent because the reaction of MPS with thioesters simply generates another (useful) thioester. Treatment with an excess of MPS at pH 7.7 was nearly as effective as cysteine at removing the Nin-Cys group (Fig. 4C). The inability of MPS to form a cyclic adduct with ninhydrin is overcome by using a large excess of the reagent.

Finally, we found that elemental zinc, which is known to reduce α-substituted ketones by reductive elimination, is
also useful as a Nin-Cys deprotection reagent. This elimination reaction is most efficient when the leaving group is nearly perpendicular to the plane of the ketone as is the case with the geometry of the spiro ring system of Nin-Cys. As an example, Nin-CYLLH₆ was completely deprotected using 10% TFA/water and zinc dust for 1 h [Fig. 4D].

**Cyclization of a Nin-Cys protected peptide thioester**

To broaden the scope of Nin-Cys as a protecting group, we attached Nin-Cys-OH to a thioester-containing peptide. The peptide, termed E₂-SR (a 40-residue, thioester-containing sequence corresponding to the second extracellular loop of chemokine receptor CCR₅), was synthesized using standard Boc chemistries. The sequence of the peptide synthesized was CGKTRSQRKHLHYTCSSHPFYSQYQFWKNFQTLKIVKG [underlined residues corresponded to those double coupled during stepwise synthesis]. This sequence contains five additional residues not found in the sequence of the receptor. The NT CGK and C-terminal KG residues were added to increase solubility and allow for the end-to-end cyclization of the peptide through the reaction of the NT-Cys with a C-terminal thioester as has been described by our laboratory and others [2,3,5,44]. The Nin-Cys was attached manually at the final step of the assembly of E₂-SR [Fig. 5]. Following HF cleavage, the deconvoluted mass for the Nin-E₂-thioester peptide on electrospray mass spectrometry (ES-MS) was 5087.5 (calc. 5087.79, Fig. 5B). Cleavage of an E₂-SR peptide containing no ninhydrin protection resulted in formylation of a significant portion of the peptide at the NT-Cys yielding a Thz ring structure [calc. [M + H]⁺ = 4957.7, obs. [M + H]⁺ = 4957.8].

The Nin-E₂-SR peptide was cyclized using a one-step deprotection and cyclization reaction mediated by an excess of MPS [Fig. 6]. Monitoring by reverse-phase high-pressure liquid chromatography (RP-HPLC) shows that under these conditions the Nin-E₂-SR was approximately 80% deprotected in <10 min and the end-to-end cyclization was complete after 2 h [Fig. 7]. The MPS serves to deprotect the Nin-Cys and also acts to scavenge any free ninhydrin that may be generated over the course of the reaction. Interestingly, cyclization of the E₂ thioester peptide in buffer containing 6 M guanidine hydrochloride (GnHCl) was much less efficient than without GnHCl. That cyclization is more efficient in the absence of denaturant has been observed with other peptides [13] and is consistent with E₂-SR adopting native structure in aqueous conditions. We note that this cyclized E₂ peptide represents one of the largest cyclic peptides synthesized to date by chemical means [8]. Furthermore, the ease with which it was obtained using the ninhydrin protecting group establishes the usefulness of this protecting group.
for synthesis of cyclic peptides using NT-Cys-based chemistries.

**Selective protection of NT-cysteine residues on unprotected peptides**

Considering the much higher nucleophilicity of thiol groups at acidic pH compared with amines \(^{33,45}\) and other functional groups found on unprotected peptides, we postulated that it might be possible to specifically derivatize the NT-Cys of unprotected peptides with ninhydrin. Indeed, it has been shown that at pH 5.5 and room temperature the major reaction product of cysteine and ninhydrin is Nin-Cys \(^{33,38,45}\). In the same studies, it was shown that the reaction of ninhydrin with primary amines like glycine could be minimized at pH < 5. Thus, we tested the reactivity of several peptides with varying ninhydrin concentrations at pH 2–5 to determine the selectivity of Thz formation.
between an NT-Cys containing peptides and ninhydrin without modifying other residues (Table 1). As expected, the NT-Cys-containing peptides were readily modified with ninhydrin at pH 2–5 as determined by RP-HPLC and by Ellman’s reaction to detect unreacted peptide (46). The matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry of these products confirmed that a Thz ring system was formed in each case. Also, the observed one-to-one stoichiometry of the modification shows that no other residues on these peptides were modified.

We were concerned that NT serine or threonine residues might react with ninhydrin under these conditions to form oxazolidine ring structures. To address this, two model peptides, SIGSLAK and TRKI-amide were incubated with ninhydrin and neither of these peptides was modified under the conditions used for modifying NT-Cys residues. Another concern was the possible reaction of ninhydrin with NT α-amino groups or the ε-amino group on lysine, however, no reaction with either of these groups was observed in our model studies.

In the case of GRGDSPC, reactivity was observed between the C-terminal cysteine residue and ninhydrin. In this case, addition of thiol to ninhydrin appears to be an equilibrium with <40% of the peptide observed to be modified after 24 h in 0.5 mM ninhydrin. Furthermore, the adduct, which we presume is a hemithioketal, was unstable during handling and reverted back to starting peptide before a MALDI-TOF mass spectrum could be obtained.

These results demonstrate that using 0.5–2 mM ninhydrin at pH 2–5 and reaction times of 2 h or less, peptides with no internal cysteines can be site-specifically modified at NT-Cys residues. Also, ninhydrin adducts formed with C-terminal cysteine residues and internal cysteine residues are unstable, and will most likely fall apart during work up and purification processes.

**Discussion**

One of the most direct applications for ninhydrin protection of NT-Cys is in protecting NT-Cys from formylation during HF cleavage when His(Bom) groups are present. His(Bom) offers the advantage of protecting the π-nitrogen of the imidazole ring, giving the least risk for racemization during coupling reactions (47). However, the use of His(Bom) poses significant problems as the formaldehyde generated during HF cleavage will react with NT-Cys to form a stable Thz adduct (32). Although scavengers can be used to suppress this side reaction (33), even the most effective scavenger, cysteine, is unable to completely circumvent this problem (34). Our results show that Nin-Cys is completely stable to high HF conditions and effectively protects against Thz formation without the need for additional scavengers. As Nin-Cys is easily deprotected following cleavage, this protection strategy simplifies the use of His(Bom) in sequences containing NT-Cys residues.

Thioester ligation chemistries have become very popular for the synthesis of cyclic peptides (5,7–13). Most thioester peptides reported thus far were synthesized via Boc chemistry, although several florenylmethoxycarboxyl (Fmoc)-based strategies have been reported (48,49). Under

**Table 1. Reactivity of peptides with various concentrations of ninhydrin at pH 2–5**

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* Reaction occurred at C-terminal cysteine residue.

Peptide concentrations were 100 μM, reaction times were 2 h. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry was used to verify that the reaction of ninhydrin with NT-Cys residues resulted in a thiazolidine ring structure in each case.

+, <50% of total peptide reacted; ++, >95% of peptide reacted; –, no reaction observed by analytical reverse-phase high-pressure liquid chromatography; ND, not determined.

appropriate conditions, NT-Cys will react with the thioester resulting in a native cysteine bond. We have shown here that a 40 residue, Nin-Cys-containing peptide can be deprotected and cyclized in an efficient one-pot reaction. Nin-Cys simplifies the synthesis of cyclic peptides in two ways. First, it allows for the use of His(Bom) without the need for additional scavengers during cleavage. This eliminates the need for further purifications prior to the cyclization reaction and eliminates the need for other less effective histidine protecting groups. Secondly, it allows for the control of the cyclization reaction, as Nin-Cys remains protected until the addition of a thiol group to initiate deprotection and cyclization. Muir and colleagues have developed methods for the on-resin cyclization of peptides through thioester ligation \cite{5}. These methods require the deprotection of the peptide with HF prior to cyclization. For these methods and others, Nin-Cys offers a unique and flexible means of reversibly protecting NT-Cys residues.

In our model studies we have demonstrated that ninhydrin can be used to site-specifically modify or protect NT-Cys on unprotected peptides in aqueous conditions \cite{1}. We see no serious side reactions of ninhydrin with any residues under the conditions studied. Considering that we have also shown that the ninhydrin can be easily removed, our results offer the possibility for performing site-specific modifications on unprotected peptides and proteins that contain NT-Cys residues. These chemistries could prove useful for protecting the NT-Cys residues of proteins expressed using intein systems \cite{5,21,19} or in the reversible protection of proteins such as sonic hedgehog \cite{29,30} and cholesteryl ester transfer protein \cite{31} which require NT-Cys residues for their biological function.

Another potential extension of these chemistries is the use of ninhydrin to attach peptides or proteins to solid supports. If ninhydrin could be incorporated onto a solid phase, our studies here show that at pH values under 5 it should be possible to attach unprotected peptides or proteins containing NT-Cys residues to such a support because of the specificity of the reaction. Such a method could also prove very useful for the purification of proteins and peptides produced using intein expression systems.

In summary, we have shown that ninhydrin can be used effectively to protect NT-Cys residues during synthesis and cleavage in TFA or HF and that this protection extends to sequences containing His(Bom) residues without the need for additional scavengers. Nin-Cys can be deprotected from peptides using mild reducing conditions, and in the case of thioester peptides, the products will cyclize under the same conditions allowing for a one-step deprotection and cyclization. Ninhydrin can also be used to site-specifically modify and protect NT-Cys residues on unprotected peptides and potentially on proteins as well. Our studies show the effectiveness of ninhydrin as a reversible protecting group for NT-Cys and offer possibilities for the sequential ligation of peptides, the dual modifications of peptides and proteins and the reversible and chemoselective attachment of peptides and proteins to solid supports.

**Experimental Procedures**

**HPLC and mass spectrometry**

Electrospray mass spectra were obtained on a Sciex AT-TOF LC-MS \cite{PE Biosystems, Foster City, CA, USA} using an electrospray ion source and processed using Sciex Biomulti-view software. Masses obtained through deconvolution are those calculated for the uncharged species. MALDI-TOF spectra were obtained on a Voyager Elite spectrophotometer from Perseptive Biosystems running in either reflector or linear modes. Dihydroxybenzoic acid was the matrix generally used for smaller molecules \cite{500–1500 mu}, while sinnapinic acid was used for larger peptides.

Analytical HPLC was performed on a Rainin analytical system equipped with a model UV-C Dynamax detector \cite{Rainin Instrument Co., Woburn, MA, USA} and interfaced with a Macintosh IIsi computer \cite{Apple Computer Inc., Cupertino, CA, USA}. Dynamax [Rainin Instrument Co.] system software [version 1.4.5] was used to obtain and analyze the data. All analytical runs were performed at a flow rate of 1 mL/min using either a Waters symmetry C-18 RP column \cite{4.6 × 250 mm} or a Vydac C-18 RP peptide and protein column \cite{4.6 × 150 mm}. Semi-preparative purifications were performed at a flow rate of 5 mL/min on a Vydac C-18 RP column \cite{10 × 250 mm}. UV absorbance was monitored at 220 or 280 nm. In all cases, buffer A consists of 0.1% TFA in water and buffer B consists of 0.085% TFA in acetonitrile.

**Peptide synthesis**

All automated peptide syntheses were performed on ABI model 431A or 430 synthesizers \cite{Synthassist version 2.0 software}. All coupling reagents, resins, and Fmoc/Boc amino acids were purchased from Nova Biochem or Applied
Biosystems [Foster City, CA, USA]. All solvents were from Applied Biosystems.

Fmoc syntheses were run at 0.2 mmol scale using preloaded HMP resins and HBTU/DIEA activation [Fast Moc]. A conventional monitor previous peak algorithm was employed during the piperidine deprotection step for completeness via conductivity, and based on this, conditional deprotection [2% DBU/2% piperidine/96% NMP] followed by double couplings were performed as necessary. Cleavage and deprotection reactions were accomplished in 87.5% TFA/5% phenol/2.5% EDT/5% water (Reagent K).

For Boc chemistry, syntheses were run at 0.5 mmol scale using HBTU/DIEA activation, neat TFA deprotection, and in situ neutralization [50] except for Nin-CYL-LH₆ where DCC activation was used. Benzyl based side chain protection was used except for the following residues: Gln[Xan], Asn[Xan], Asp[Ochx], Glu[Ochx], His[Bom] and Arg[Tos]. Double couplings were performed for all glutamine, asparagine and arginine residues, and other sequences predicted to hinder coupling. Cleavages for all glutamine, asparagone and arginine residues, and Nin-CYS-OH was coupled to the resin as described previously. To the other half, Cys(MeBzl) was coupled using the synthesizer. Both resins were cleaved in HF with 10% p-cresol for 1 h. The peptides were precipitated in ether, washed twice with ether and then lyophilized from 10% acetic acid. The crude peptides were analyzed by LC-MS [see Fig. 3] and then purified on a semi-prep C-18 RP-HPLC [10 x 250 mm] column using a gradient of 10-50% B over 40 min. Purified peptide was analyzed by C-18 RP-HPLC, MALDI-TOF and ES-MS [see Results].

The synthesis of Nin-Cys-OH was performed as described by Prota and Ponsiglione [39]. L-Cysteine-HCl [527 mg (3 mmol)] was dissolved in 10 mL water. While stirring, 534 mg (3 mmol) of ninhydrin was added to the solution. The solution was covered with foil and stirred at 23 °C for 1 h. The resulting precipitate was filtered and recrystallized from ethanol. The product was dried under vacuum. The melting point [uncorrected]: 166 °C (lit. 167 °C); analytical RP-HPLC (5-60% B, 40 min) showed 99% purity; APCI-MS: expected mass = 263.2, observed mass = 263.1). On resin coupling of Nin-Cys-OH was performed manually. Nin-Cys-OH was activated with HBTU/1-hydroxybenzotriazole [HOBt] and immediately added to the resin. The mixture was agitated for 20 min, filtered, and washed successively with DMF, dichloromethane (DCM) and methanol.

Synthesis of mercaptopropionamide-MBHA resin (MPA-MBHA)

To 28.7 mL of 0.5 M HOBt/HBTU in DMF was added 14.35 mL of 2 M DIEA followed by 5 g [14.35 mmol] of 3-[S(trityl)]-mercaptopropionic acid [Bachem, King of Prussia, PA, USA]. The solution was added to 5 g of MBHA resin (0.8 mmol/g) and agitated for 30 min. The resin was washed with several volumes of DMF, DCM and methanol in succession and dried in vacuo. A ninhydrin assay [36,37] was used to confirm that the coupling had gone to completion.

Synthesis of Nin-CYLLH₆

Nin-CYLLH₆ was synthesized using standard automated Boc chemistries with DCC and HOBt activation. All six histidine residues were Bom protected. Following coupling of the tyrosine residue, half of the resin was removed and Nin-Cys-OH was coupled to the resin as described previously. To the other half, Cys(MeBzl) was coupled using the synthesizer. Both resines were cleaved in HF with 10% p-cresol for 1 h. The peptides were precipitated in ether, washed twice with ether and then lyophilized from 10% acetic acid. The crude peptides were analyzed by LC-MS [see Fig. 3] and then purified on a semi-prep C-18 RP-HPLC [10 x 250 mm] column using a gradient of 10-50% B over 40 min. Purified peptide was analyzed by C-18 RP-HPLC, MALDI-TOF and ES-MS [see Results].

Deprotection of Nin-Cys peptides

For all solution-phase deprotections, the peptides were diluted to a final concentration of 50 μM. For deprotecting peptides with cysteine, the peptides were first dissolved in 100 μL of phosphate buffer (200 mM, pH 7.6) containing 6 M GnHCl and then added to 900 μL of HEPES buffer (200 mM, pH 7.7). To this was added 24.2 mg of cysteine and 10 μL of 1 M tris-carboxymethylphosphine (TCEP) for final concentrations of 200 and 10 mM respectively. The solution was shaken and time points were analyzed by analytical RP-HPLC and MALDI-TOF mass spectrometry. The same procedure was used for deprotections with MPS using 200 mM MPS [35 mg/mL] in place of cysteine. For deprotecting in Zn/TFA, the peptides were dissolved in 1 mL of 10% TFA in water and then added to 200 mg of zinc dust. The reaction was always ventilated since
hydrogen gas is produced. Again, time points were assayed by analytical HPLC and MALDI-TOF mass spectrometry. Solid phase deprotections of Nin-Cys peptide resins were accomplished by washing 10 mg resin [0.2 mmol/g] with DMF, adding 1 mL of cysteine-O-methyl ester in DMF (1 mM) and 175 μL of DIEA [1 mM final concentration] and agitating for 30 min. The resins were then washed with DMF, DCM and methanol.

**Reaction of test peptides with ninhydrin**

Peptides were reacted with ninhydrin at various concentrations and pH. In each case, the peptide was dissolved in the appropriate buffer and a volume of ninhydrin (100 mM) was added to reach the desired concentration. Ellman’s reaction [46] was performed by diluting 100 μL aliquots of the test reaction with 850 μL of phosphate buffer [pH 7.5] and 50 μL of 3 mM DTNB and measuring the absorbance at 412 nm on a Shimadzu UVmini-1240 spectrophotometer [Shimadzu Scientific Instruments Inc., Columbia, MD, USA]. Analytical RP-HPLC (Vydac C-18 column) and MALDI-TOF mass spectrometry were used for all reactions to determine the extent of reaction and to characterize any products observed. ZipTips [Millipore, Bedford, MA, USA] containing C-18 packing were used to desalt aliquots prior to MALDI-TOF mass spectrometry. The peptide concentrations were all 100 μM as estimated by weight or peptide content (as provided by American Peptide Co., Inc., Sunnyvale, CA, USA). For those peptides purchased from American Peptide Co., purity was checked by HPLC, and in all cases the peptides were used without further purification.

**Ninhydrin + CLRRASLG**

Also known as Cys-Kemptide, CLRRASLG was purchased from American Peptide Co. and was used as a model NT-cysteine peptide. It was mixed with ninhydrin in all conditions studied from pH 2 to 5 and ninhydrin concentrations from 100 μM to 10 mM [see Table 1]. Although the rate of reaction varied with conditions, in all cases the reaction went to completion as determined by Ellman’s assays and RP-HPLC (2–40% B, 40 min, 220 nm) yielding peptides with a Thz ring structure as determined by MALDI-TOF mass spectrometry ([M + H]+ calc. = 1018.2, [M + H]+ obs. = 1018.1].

**Ninhydrin + CGYGDKKKRKVG**

CGYGDKKKRKVG [American Peptide Co.] was reacted with ninhydrin (0.5, 2 and 10 mM) at pH 2 and in all cases the reaction went to completion. Analytical RP-HPLC (2–22% B, 30 min, 280 nm) and Ellman’s assays confirmed that over 99% of the starting peptide was derivatized, while MALDI-TOF mass spectrometry showed formation of Thz ring adduct ([M + H]+ calc. = 1519.8, [M + H]+ obs. = 1520.2].

**Ninhydrin + GRGDPC**

GRGDPC [American Peptide Co., Inc.] was reacted with ninhydrin at pH 2–5 at 0.5 and 10 mM ninhydrin. After 2 h, the extent of reaction was between 30 and 40% by HPLC (2–40% B, 40 min, 220 nm). The derivatized peptide had a retention time of 26.3 min [underivatized peptide = 11.4 min], but MALDI-TOF mass spectrometry revealed the [M + H]+ of the derivatized peptide to be 692.0 ([M + H]+ calc. for underivatized peptide = 691.9). After 1 h, the amount of derivatized peptide observed by RP-HPLC did not vary significantly with time.

**Ninhydrin + other peptides (GTHKSEIAFHR, SIGSLAK, WAVGHPF, NRVYVHPF and TRKI-amide)**

GTHKSEIAFHR was reacted with ninhydrin under all conditions studied. Analytical RP-HPLC (2–40% B, 40 min, 220 nm) showed no detectable modification of the peptide. SIGSLAK [American Peptide Co., Inc.] showed no reaction with ninhydrin as observed by RP-HPLC (2–40% B, 40 min, 220 nm). WAVGHPF [American Peptide Co., Inc.] showed no reaction after 2 h with 0.5 mM ninhydrin at pH 2–5 or with 2 mM ninhydrin at pH 2–3 as assayed by RP-HPLC (10–50% B, 40 min, 280 nm). NRVYVHPF and TRKI-amide were reacted with 10 mM ninhydrin for up to 20 h at pH 2 and showed no sign of derivatization as assayed by RP-HPLC (NRVYVHPF: 10–50% B, 40 min, 280 nm; TRKI-amide: 2–22% B, 30 min, 220 nm).

**Cyclization of Nin-E2- and E2-thioester peptides**

Nin-E2-SR [150 μg] was dissolved in 0.5 mL of 60% acetonitrile and then 0.4 mL of this peptide solution was added
to 0.6 mL of HEPES buffer [200 mM, pH 7.7]. To this was added 8.9 mg of MPS and 5 μL of 1 M TCEP for final concentration of 50 and 5 mM respectively. The solution was shaken at room temperature. Time points were assayed by RP-HPLC using a 40-min gradient of 20–50% B and absorbance was monitored at 220 nm [see Fig. 7]. The final deprotected cyclic peptide was assayed by MALDI-TOF mass spectrometry ([M + H]+ calc. = 4841.6, [M + H]+ obs. = 4841.5).

References


