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Fmoc-Based Synthesis of Peptide α-Thioesters Using an Aryl Hydrazine Support†

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C-Terminal peptide thioesters are key intermediates in the synthesis/semisynthesis of proteins and of cyclic peptides by native chemical ligation. They are prepared by solid-phase peptide synthesis (SPPS) or biosynthetically by protein splicing techniques. Until recently, the chemical synthesis of C-terminal α-thioester peptides by SPPS was largely restricted to the use of Boc/Benzyl chemistry due to the poor stability of the thioester bond to the basic conditions required for the deprotection of the N^{α}-Fmoc group. In the present work, we describe a new method for the SPPS of C-terminal thioesters using Fmoc/*t*-Bu chemistry. This method is based on the use of an aryl hydrazine linker, which is totally stable to conditions required for Fmoc-SPPS. When the peptide synthesis has been completed, activation of the linker is achieved by mild oxidation. This step converts the acyl hydrazine group into a highly reactive acyl diazene intermediate which reacts with an α-amino acid alkyl thioester (H-AA-SR) to yield the corresponding peptide α-thioester in good yield. This method has been successfully used to prepare a variety of peptide thioesters, cyclic peptides, and a fully functional Src homology 3 (SH3) protein domain.

Introduction

C-Terminal peptide α-thioesters are key intermediates in the synthesis of small- and medium-sized proteins[1−3] and cyclic peptides[4−7] by native chemical ligation.[8,9] These mildly activated species are also required for the construction of topologically[10−14] and backbone engineered[15−17] proteins.

C-Terminal peptide α-thioesters can be prepared by standard solid-phase peptide synthesis (SPPS) using Boc/benzyl chemistry,[6,18−20] or for larger polypeptide domains and protein domains, using intein-based bacterial expression systems.[3,21,22] Unfortunately, the Boc/benzyl approach requires the use of anhydrous HF which is not well suited for synthesis of phospho-[23,24] and glycopeptides.[25−27]

The Fmoc-based methodology is attractive as it does not employ HF and hence provides the synthesis of phospho- and glycopeptides in good yields. However, the poor stability of the thioester functionality to strong nucleophiles such as piperidine, which is used for the deprotection of the N^{α}-Fmoc group, seriously limits the use of this methodology for the preparation of peptide α-thioesters.

So far, several approaches have been used to overcome this limitation. Futaki et al.[28] used an approach where peptide α-thioesters were prepared in solution using a partially protected precursor. Li et al.[29] used a Fmoc-

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† This paper is dedicated to Professor Bruce Merrifield, inventor of the solid-phase organic synthesis and a continuing inspiration to many.

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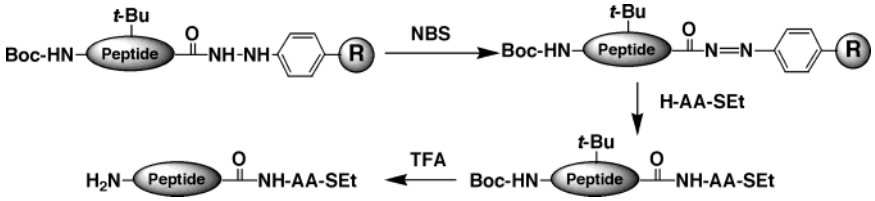
SCHEME 1. Preparation of Peptide α-Thioesters Using an Aryl Hydrazine Support



deprotection cocktail compatible with α-thioesters to synthesize an unprotected 25-residue peptide α-thioester in moderate yield. A similar approach was also used by Clippingdale et al.[30] using in this case a non-nucleophilic base in combination with 1-hydroxybenzotriazole (HOBt).

Alternatively, the introduction of the α-thioester function at the end of a synthesis has been used by Alsina et al.[31] where the backbone amide linker (BAL) was employed for the synthesis of peptide thioesters using an Fmoc-based strategy. This approach was used for the synthesis of small peptide thioesters in good yields. However, some racemization was observed during the thiolysis step. Swinnen et al. used the phenylacetamidomethyl (PAM)[33] and Wang[34] resins to synthesize peptide α-thioesters by employing EtSH in the presence of Me_2AlCl to effect thiolysis of the resin-bound peptide. This approach was used for the synthesis of a 22-residue peptide α-thioester in moderate yield.[35] Another approach developed by Ingenito et al.[36] and Shin et al.[25] involved the use of Kenner's sulfonamide safety-catch linker.[37] This linker is fully stable to repetitive exposure to the basic conditions needed for Fmoc deprotection. When the sulfonamide is alkylated, the peptide resin is activated and easily cleaved with thiols to yield the corresponding peptide α-thioester. However, the use of akylating agents (such as CH_2N_2 or ICH_2CN) has been shown to alkylate unprotected methionine residues.[38] More recently, Brask et al. have introduced a new method for the generation of peptide thioesters using a trithioortho ester linker.[71]

All of the available methods for the synthesis of peptide thioesters by the Fmoc approach have advantages and disadvantages. In the present work, we describe a new strategy for the synthesis of peptide α-thioesters using an Fmoc-based approach. Our method is based on the use of an arylhydrazine linker[39−48] that is totally stable to the conditions of Fmoc- and Boc-SPPS to yield a peptide hydrazide resin. Mild oxidation of the peptide hydrazide affords a peptidyl diazene resin which is used to prepare C-terminal peptide α-thioesters.

Results and Discussion

General Scheme. Our new approach employs a hydrazine safety-catch linker[42−48] that is totally stable to the conditions used during SPPS by either Boc or Fmoc chemistries.[42,43,46−48] The peptide-hydrazine resin is activated by treatment with mild oxidizing agents to provide a reactive acyl diazene intermediate that readily reacts with N- and O-nucleophiles.[43,47,48] S-Nucleophiles, on the other hand, did not cleave the acycl diazene efficiently.[49] Goodman and Stueben[50] demonstrated over 40 years ago that mildly activated α-amino acid *p*-nitrophenyl esters react very selectively with highly activated carboxylic acid derivatives (such as *O*-acyl isoureas) to form peptide *p*-nitrophenyl esters without racemization. We reasoned that α-amino acid *S*-alkyl thioesters should react in an analogous fashion with a highly reactive peptidyl (acyl) diazene in the presence of the mildly reactive alkyl thioester group. Such a reaction should selectively cleave the peptide from the diazene resin furnishing the corresponding C-terminal peptide α-thioester.

Our procedure (Scheme 1) for peptide α-thioester synthesis by Fmoc chemistry involves the direct assembly of the peptide on a phenyl hydrazine resin[51] using standard Fmoc protocols.[52] At the end of the synthesis, the fully protected peptide resin is activated by mild oxidation with *N*-bromosuccinimide (NBS) in the presence of pyridine. The reactive acyl diazene is then cleaved with an α-amino acid *S*-alkyl thioester. Finally, the fully protected peptide α-thioester is deprotected with TFA in the presence of the appropriate scavengers (see the Experimental Section). Note that in the cases where the

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TABLE 1. Cleavage Yields, Purity, and Molecular Weights of Peptide Thioesters 1−3

peptide	sequence	M_w/Da		yield/%	
1	Ac-IAFG-SEt	492.6[a]	492.0[b]	95[c]	60[d]
2	Ac-IAFA-SEt	506.3[a]	506.0[b]	94[c]	65[d]
3	H-LFAG-SEt	450.0[a]	449.7[b]	95[c]	70[d]

[a] Expected. [b] Found. [c] Based on HPLC purity. [d] Based on initial resin substitution.

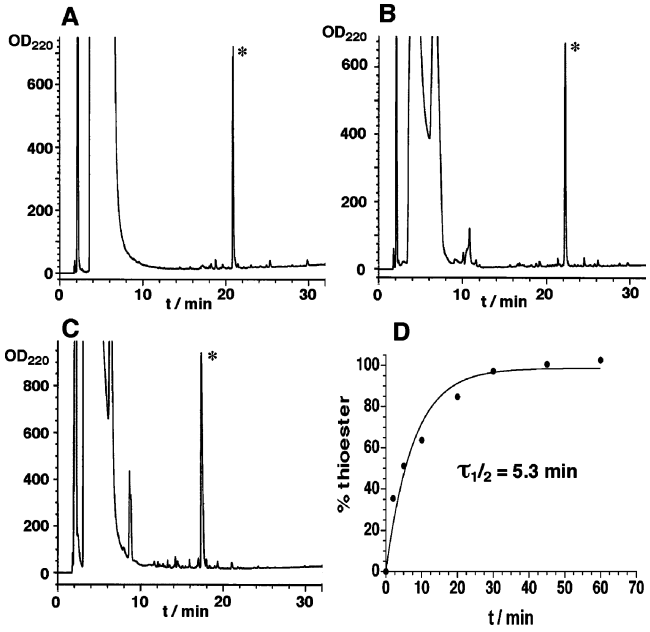

FIGURE 1. HPLC analysis of the crude product obtained by oxidation and cleavage using NBS and H-AA-SEt: (A) peptide **1**, (B) peptide **2**, and (C) peptide **3**. In each case, the asterisk denotes the thioester product. A linear gradient of 0−70% buffer B over 30 min was used in each case. (D) Kinetic analysis for the formation of peptide thioester **3** by oxidation and cleavage with NBS and H-Gly-SEt.

N-terminal α-amino group should be unprotected in the final peptide α-thioester, the last amino acid should be incorporated as the Boc-$^{\alpha}$N-derivative during the synthesis. This prevents the possible oxidation of the free α-amino group during the oxidation step.

Oxidation of the Resin and Cleavage by α-Amino Acid Thioesters. The cleavage of the activated peptidyl diazene resin by α-amino *S*-alkyl thioesters was determined. Three model peptides were synthesized on hydrazinobenzyl AM resin[51] (Table 1), and the protected peptide resins were activated by oxidation with 2 equiv of NBS in the presence of anhydrous pyridine for 10 min at room temperature. When the oxidation reaction was complete, the activated peptide resin was then washed with DCM and cleaved with 20 equiv of H-AA-SEt (where AA was either Gly or Ala).[53] The reaction was quenched with acetic acid and the solvent evaporated. The residue was then deprotected with TFA when necessary. The oxidation and cleavage reactions were clean and efficient with all three peptides (Table 1 and Figure 1). In each case, the main product was the corresponding peptide α-thioester with cleavage yields around 65%[54] and purities around 95% (as calculated by HPLC). The anticipated high reactivity of acyl diazene supports toward N-nucleophiles was confirmed with the completion of the cleavage reactions in less than 30 min (Figure 1). The speed of this reaction was crucial to minimize the multiple incorporation of amino acid thioester residues at the C-terminus of the peptide during the cleavage step.

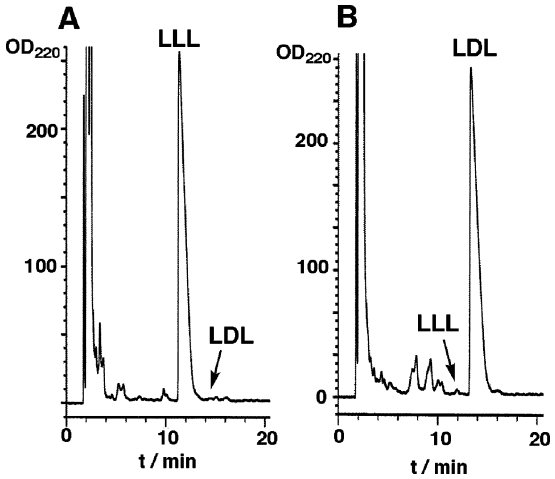

FIGURE 2. Epimerization studies of the C-terminal residue attached to the resin during the activation of the hydrazide linker with NBS. HPLC traces of the crude products for the oxidation and cleavage with NBS and H-L-Ala-OMe of H-L-Phe-L-Ala-L-Ala-OMe (**A**) and H-L-Phe-D-Ala-L-Ala-OMe (**B**).

Epimerization of the C-Terminal Amino Acid after the Oxidative Cleavage. Since acyl diazenes are highly reactive species toward N- and O-nucleophiles,[43,47,48] epimerization of the C-terminal residue attached to the acyl diazene resin through oxazolone formation[55] was investigated.

Two dipeptide diastereomers (LL- and LD-Phe-Ala peptides) were assembled on the hydrazine resin, oxidized with NBS, and then reacted with H-L-Ala-OMe. HPLC analysis of the crude cleavage reactions for both tripeptides (Figure 2) did not reveal significant epimerization of the penultimate residue (less than 0.5%). These results are in good agreement with previous studies where the hydrazine linker has been oxidatively cleaved and no or little racemization was observed.[39,40,46]

Stability of the Peptide Resin to the Oxidation Step. Although our approach for the synthesis of peptide α-thioesters worked well with model peptides (Table 1), there was concern about the stability of peptides containing oxidative-sensitive residues (i.e., Tyr, Trp, Met and Cys) during the oxidation step. Several peptides containing these residues were synthesized on a hydrazine resin, oxidized with NBS, and cleaved with either H-Ala-SEt or H-Gly-SEt (Table 2). The results, summarized in Figure 3, show that peptides **4** and **5** which contain Tyr-

(53) The reactive H-AA-SEt was generated in situ from the corresponding H-AA-SEt·HCl by adding an excess of *N*,*N*-diisopropylethylamine (DIEA) during the cleavage step. Although only peptide thioesters containing either a Gly or Ala at the C-terminal positions were used in this study, it should be noted that other amino acid thioesters could also have been used with the appropriate side-chain protection when required (i.e., trifunctional amino acids). Nonetheless, it is important to note that peptide thioesters containing either an Ala or Gly residue at the C-terminus are the most commonly employed intermediates in native chemical ligation reactions (see ref 19.)

(54) Similar cleavage yields were obtained when propylamine was used as nucleophile to react with the peptidyl diazene resin.

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TABLE 2. Primary Amino Acid Sequences of Peptide Thioesters 4−9 Prepared in This Study[a]

peptide	sequence	M_w/Da		yield[b]/%	protecting group[c]
4	H-L***Y***KAA-SEt	608.8[d]	608.0[e]	90	Tyr(*t*-Bu)
5	H-L***W***AG-SEt	489.6[d]	490.0[e]	80	Trp(Boc)
6	H-L***M***YKAG-SEt	726.0[d]	725.0[e]	85	None
7	H-L***C***YKAA-SEt	712.0[d]	712.1[e]	70	Cys(Trt)
8	H−***C***YAVTGKDSPAAG-SEt	1494.7[d]	1494.5[e]	75	Cys(Npys)
9	Ac-AEYVRALFDFN***G***NDEEDLPFKKG-SEt	2761.1[d]	2762.2[e]	35	(Fmoc-2-hydroxy-4-methylbenzyl)-Gly

[a] The side-chain protecting group for sensitive amino acids and the molecular weights for the expected products is also indicated. [b] Based on HPLC purity. [c] Side chain (peptides **4**−**8**) or backbone (peptide **9**) protecting groups for the underlined residues within the corresponding sequence. [d] Expected. [e] Found.

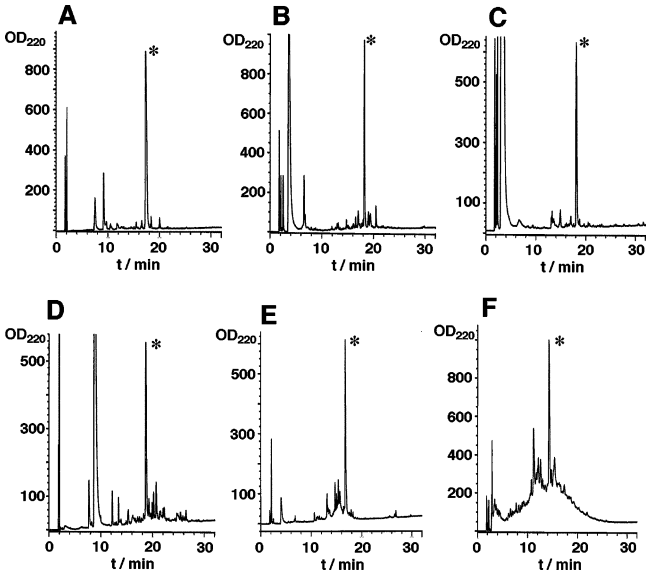

FIGURE 3. HPLC analysis of the crude product obtained by oxidative cleavage with NBS/H-AA-SEt of different peptides varying in length and composition: (A) Tyr(*t*-Bu)-containing peptide **4**, (B) Trp(Boc)-containing peptide **5**, (C) Met-containing peptide **6**, (D) Cys(Trt)-containing peptide **7** after 30 min reduction with EtSH at pH 8.0, (E) Cys(Npys)-containing peptide **8**, and (F) peptide **9**. In each case, the asterisk denotes the thioester product. A linear gradient of 0−70% buffer B over 30 min was used in each case, except in panel **F** where a linear gradient of 30−60% buffer B was used.

(*t*-Bu) and Trp(Boc) residues, respectively, were not affected during the NBS treatment under the conditions used in our study. In both cases, the major product was the expected peptide α-thioester (Figure 3A,B) with minor amounts of byproducts. This was gratifying since phenolic and indole rings are well-known to be very susceptible to halogenation by mildly oxidizing agents such as NBS.[56] In the case of the Tyr residue, the *tert*-butyl side chain protecting group prevented any detectable bromination of the aromatic ring under the conditions employed. This may be due to a combination of the steric effect of *tert*-butyl group on positions 3 and 5 of the phenolic ring and the kinetic control conditions used during the oxidation step (i.e., short reaction times and use of slight excess of oxidizing agent). The alternative use of electron-withdrawing groups has been reported to protect the phenolic group of Tyr from oxidative halogenation.[57] More striking, however, is the fact that Trp totally resisted oxidation under our reaction conditions when protected with the N^{in}-Boc[58] group. In contrast, when peptide **5** was synthesized without protection on the indole ring the oxidation and cleavage with NBS and H-Gly-SEt gave a complex reaction mixture where different oxidation/bromination products could be easily identified by HPLC and ESMS. The protective effect of the N^{in}-Boc group may arise from the electron withdrawing character of the carbamate moiety which leads to the partial deactivation of the indole ring toward electrophiles.[59]

As expected, Met-containing peptide **6** was completely oxidized to the corresponding sulfoxide during the NBS oxidation step, but during the subsequent TFA deprotection step, the sulfoxide was reduced when the reaction was carried out for 3 h at room temperature in the presence of 2% EtSH (Figure 3C). It is interesting to note that Waldmann et al.[47] have recently reported that oxidation of Met-containing peptide-hydrazine resins with Cu(II) salts did not oxidize the Met residue. Unfortunately, these oxidative conditions cannot be employed in the presence of a thioester group due to its lability to transition-metal cations.[68]

Cys(Trt)-containing peptide **7** was also oxidized during the NBS treatment showing a rather complex crude mixture after the TFA deprotection step. However, the desired thioester peptide **7** could be obtained in good yield if the crude TFA cleavage product was reduced with EtSH at pH 8.0 for 30 min (Figure 3D). Under these conditions, the hydrolysis of the α-thioester was minimal. Oxidation of the Cys residue during the activation step, however, could be totally avoided if the thiol group of the Cys residue was protected as a mixed disulfide. Aryl and alkyl mixed disulfides are known to be stable to mild oxidation conditions.[60] Cys-containing peptide **8**, where the N-terminal Cys residue was introduced as Boc-Cys-(Npys),[61] remained totally stable during the oxidation of the hydrazine linker and reduction was not required to obtain the corresponding thioester peptide in good yield (Figure 3E). It is important to note that the Npys protecting group can only be used in those peptides where the Cys residue is at the N-terminal position due to its partial lability to the conditions employed in the Fmoc deprotection step. Thus, in peptides where the Cys

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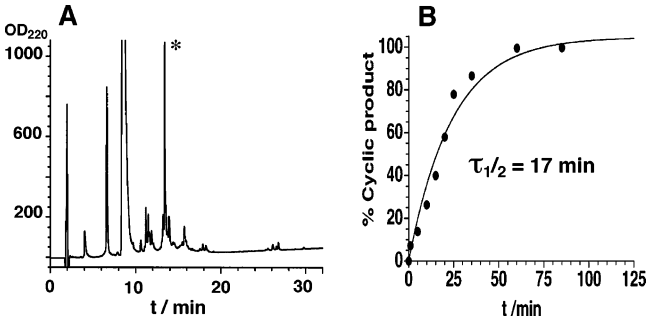

FIGURE 4. Cyclization of peptide **8** by intramolecular native chemical ligation. (A) HPLC analysis of the crude cyclization mixture after 1 h. The cyclic product is marked with an asterisk. HPLC analysis was carried out using a linear gradient of 0−70% buffer B over 30 min. (B) Kinetics for the cyclization of linear precursor peptide **8**.

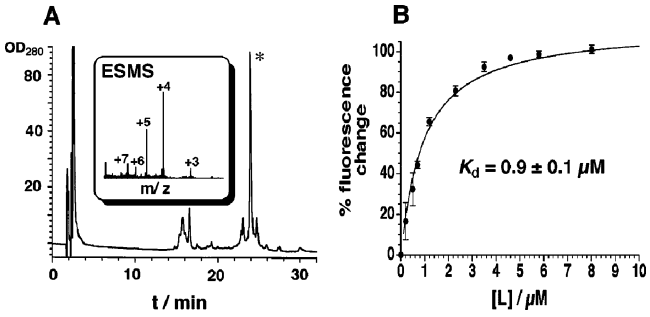

FIGURE 5. Synthesis of the N-terminal SH3 domain of the c-Crk protein adaptor by intermolecular native chemical ligation of peptides **9** and **10**. (A) HPLC analysis of the intermolecular ligation crude after 36 h. Ligated product is marked with an asterisk. Inset, ESMS of ligated SH3 domain, molecular weight (average isotope composition) expected 6891.7 Da, found 6894.1 ± 2.1 Da. (B) Change in fluorescence emission intensity of the ligated SH3 domain upon addition of proline-rich ligand 11 (L).

residue is not located in this position, the S-StBu group should be used.[48] This group is totally compatible with Boc and Fmoc strategies and can easily be deprotected by reductive treatment with thiols or phosphines.[48,62]

Finally, our oxidative-cleavage procedure was also used to generate a more complex and larger peptide thioester. Peptide thioester **9**, a 22-residue thioester peptide derived from the N-terminal SH3 domain of the c-Crk protein adaptor,[63] was prepared to obtain the full synthetic SH3 domain by native chemical ligation (see below). Crude peptide α-thioester **9** was relatively clean showing only two major peaks by HPLC (Figure 3F). The major peak corresponded to the expected peptide thioester **9** as determined by mass spectrometry. The secondary peak (ca. 33% of the first peak) which eluted earlier in the HPLC chromatogram (Figure 3F) presented a loss of 17 Da versus peptide **9**, and it was assigned to be the aspartimide derivative of peptide **9**. Aspartimide formation could be minimized, although not totally avoided, by using the Fmoc-(Fmoc-2-hydroxy-4-methylbenzyl)-Gly derivative[64,65] at ^{12}Gly in peptide **9** (see Table 2). After a single HPLC purification step, pure peptide **9** was obtained with a modest yield (ca. 25%,). However, it is important to note that the synthesis of this fragment by itself was particularly challenging due to the presence of the Asn-Gly sequence, which is prone to form the corresponding aspartimide.

Native Chemical Ligation. To test the suitability of the thioesters generated by this new method, peptides **8** and **9** were used for carrying out intramolecular and intermolecular native chemical ligations.[8]

(1) Intramolecular Native Chemical Ligation. Linear precursor peptide **8**, with a sequence deriving from the tenth type 3 module of fibronectin (a natural β-strand hairpin),[66] was designed to contain an α-thioester group and a Cys residue at the C- and N-terminal positions, respectively. The presence of these two chemical moieties allows the backbone cyclization by intramolecular native chemical ligation.[4,5,7,10] Cyclization of peptide **8** was accomplished by diluting the crude TFA cleavage material in freshly degassed 0.2 M sodium phosphate buffer at pH 7.2 containing 2% EtSH to a final concentration of ca. 200 μM. Under these conditions, the backbone cyclization reaction proceeded quickly and efficiently (Figure 4). The reaction was complete in less than 60 min, and the major product corresponded to cyclic peptide **8** as characterized by ES-MS and tryptic digestion.

(2) Intermolecular Native Chemical Ligation: Synthesis of Functional SH3 Protein Domain. We used the N-terminal SH3 domain from the c-Crk adaptor protein[63] as a synthetic target employing intermolecular native chemical ligation. The amino acid sequence of the c-Crk N-terminal SH3 protein domain corresponds to residues 134−190 of the c-Crk protein. Retrosynthetic analysis, guided by the structure of the SH3 domain,[67] suggested that a functional analogue of the protein domain could be prepared by native chemical ligation between peptide **9** (residues 134−156, Table 2) and peptide **10** (residues 157−191, CILRIRDKPEEQWW-NAEDSEGKRGMIPVPYVEKYG). Peptide **10** was synthesized using an Fmoc protocol on a Rink-amide resin. To facilitate ligation, a Cys residue was introduced at the N-terminus of peptide **10**.

The ligation reaction between peptide **9** and peptide **10** was performed by mixing equimolar amounts of both peptides in 0.2 M sodium phosphate at pH 7.2 containing 2% EtSH. The reaction was shown to be complete in 36 h, as indicated by HPLC analysis of the crude reaction (Figure 5A). The ligation product was by far the main product and could be easily isolated by semipreparative HPLC. Characterization of the product by ES-MS confirmed the identity of the SH3 ligated domain (Figure 5A). The ligated SH3 domain was readily purified by HPLC and refolded by flash dilution in 20 mM sodium phosphate, 100 mM NaCl at pH 7.2. The ligand-binding activity of the synthetic SH3 domain was evaluated using

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a fluorescence-based titration assay.[68] The equilibrium dissociation constant for binding of the synthetic SH3 domain to the natural proline-rich peptide ligand C3G (peptide **11**)[63] was 0.9 μM (Figure 5B). This value is identical to that reported for the recombinant c-Crk N-terminal SH3 domain.[14]

Conclusions

In summary, we have developed a new method for the facile preparation of peptide thioesters without limitations of size and amino acid composition. The oxidation and cleavage reactions have been shown to be totally compatible with sensitive amino acids when the appropriate protecting groups and oxidative conditions are employed. No detectable racemization was observed during the activation and cleavage of the hydrazide linker. This approach does not require special linkers, resins, or complicated protocols as commercially available hydrazine resins are employed and the assembly of the peptide chain is carried out using standard SPPS methods.

The simplicity and efficiency of our approach should complement the already available methods for the synthesis of peptide α-thioesters.

Experimental Section

Glycine *S*-Ethyl Ester, Hydrochloride Salt (H-Gly-SEt·HCl). Boc-Gly-OH (5.0 g, 28.5 mmol) and 1-hydroxybenzotriazole hydrate (HOBt·H_2O; 4.36 g, 28.5 mmol) were dissolved in DCM (125 mL). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 4.95 mL, 28.5 mmol) and *N*,*N*-diisopropylethylamine (DIEA; 5 mL, 28.5 mmol) were added sequentially to the reaction mixture, and the resulting reaction was allowed to stir for 90 min. At this point, ethylthiol (5 mL, 67.5 mmol) was added in one portion and the homogeneous reaction was kept for 4 h at room temperature. The crude reaction mixture was then washed with 1 M aqueous HCl (3 × 250 mL), 1% $NaHCO_3$ (3 × 250 mL), and H_2O (3 × 250 mL), dried over $MgSO_4$, and concentrated in vacuo. The resulting residue (Boc-Gly-SEt) was dissolved in 4 M HCl−dioxane (20 mL) and stirred at room temperature for 90 min. The homogeneous reaction solution was concentrated in vacuo, and the product was precipitated with cold anhydrous Et_2O (50 mL). The precipitate was filtered and dried under vacuum to provide the title product as a white solid (2.1 g, 60%) >99.5% pure glycine ethyl thioester by analytical RP-HPLC (t_R: 3.49 min using an isochratic of 0% B for 2 min and then a linear gradient of 0% to 17%B over 10 min): ^{1}H NMR (DMSO-d_6) δ 8.32 (br, s, 3H), 4.05 (s, 2H), 2.95 (q, 2H), 1.19 (t, 3H); ESMS calcd for C_4H_9NOS (average isotope composition) 119.2 Da, found 119.0 ± 0.5 Da.

Solid-Phase Peptide Synthesis. All peptides were manually synthesized using the HBTU activation protocol for Fmoc solid-phase peptide synthesis[69] on a Rink-amide resin (peptide **10**) or on a 4-Fmoc-hydrazinobenzoyl AM resin (peptides **1** to **9**). Coupling yields were monitored by the quantitative ninhydrin determination of residual free amine.[70] Side-chain protection was employed as previously described for the Fmoc-protocol except for peptides **5**, **6**, **7**, and **9** where Fmoc-Trp-(Boc)-OH, unprotected Fmoc-Met-OH, Boc-Cys(Npys)-OH, and Fmoc-(Fmoc-2-hydroxy-4-methylbenzyl)-Gly-OH (at ^{12}Gly in peptide **9** to minimize aspartimide formation) were used, respectively.

Oxidation and Cleavage of the Hydrazine Linker. The corresponding peptide-hydrazide resin (50 mg, ∼20−30 μmol depending on resin substitution) was swollen in anhydrous DCM for 20 min and drained. *N*-Bromosuccinimide (NBS; 13 mg, 75 μmol) and anhydrous pyridine (25 μL, 310 μmol) were dissolved in anhydrous DCM (5 mL) and then added to the peptide resin. The oxidation reaction was kept for 10 min at room temperature with occasional stirring. Unreacted NBS was removed by washing the peptide resin with anhydrous DCM (3 × 5 mL). Either H-Gly-SEt·HCl (50 mg, 322 μmol) or H-Ala-SEt·HCl (54 mg, 320 μmol) and DIEA (200 μL, 1.1 mmol) were dissolved in DCM (5 mL), and the solution was immediately added to the oxidized peptide resin. The cleavage reaction was kept for 1 h at room temperature. The reaction was then quenched with HOAc (250 μL), and the solvent was removed in vacuo. The peptide thioester was deprotected when necessary with TFA/H_2O/trisisopropylsilane (TIS; 50:1:1 v/v, 5 mL) for 1−3 h, except in peptide **6** where TIS was replaced by EtSH as scavenger in the deprotection cocktail. The filtrate from the cleavage reaction was combined with TFA washes (2 × 0.5 mL) from the cleaved peptide resin and concentrated under a stream of N_2. Precipitation with cold anhydrous Et_2O (50 mL) afforded crude product which was washed with Et_2O (2 × 20 mL). The crude peptide was dissolved in buffer A/buffer B (4:1 vol, 5 mL) and characterized by HPLC and ESMS and further purified by either semi- or preparative HPLC.

Synthesis of Ac-IAFG-SEt (1). The synthesis (0.1 mmol) was carried out on a 4-Fmoc-hydrazinobenzoyl AM resin (0.98 mmol/g) as described above. When the assembly was complete, the Fmoc-N$^\alpha$ protecting group was removed by treatment with 1% DBU and 20% piperidine solution in DMF (5 + 10 min) and then acetylated with Ac_2O/DIEA/DMF (15:15:70) for 10 min. The oxidation with NBS and cleavage with H-Gly-SEt·HCl was carried out as described above. The major product was characterized as the desired thioester product by ESMS: calcd for $C_{24}H_{36}N_4O_5S$ (average isotope composition) 492.6 Da, found 492.0 ± 0.5 Da.

Kinetics Studies of the Cleavage of Peptide 3. Kinetic analyses were performed by analytical HPLC. The oxidation and cleavage for obtaining peptide thioester **3** were performed as described above. Small aliquots of supernatant (20 μL) were withdrawn from the cleavage reaction with H-Gly-SEt at various times, treated with 100 μL of TFA for 20 min, and then evaporated under a stream of N_2. The peptide thioester was solubilized with buffer A/buffer B (2:1 vol., 150 μL), filtered and analyzed by HPLC. The half-life was calculated by measuring the concentrations of the thioester peptide and fitting the time course data to the equation: $C_{t,\text{thioester}} = C_{0,\text{thioester}}(1 - e^{-kt})$, where C is the concentration of thioester peptide at time t, $C_{0,\text{thioester}}$ is the final concentration of thioester peptide and k the rate constant.

Epimerization Studies. The synthesis (0.1 mmol) of L-Phe-L-Ala and L-Phe-D-Ala peptide diastereomers and oxidation with NBS was carried out as described above with the exception that H-L-Ala-OMe·HCl (45 mg, 322 μmol) was used to trap the peptidyl diazene intermediate. The TFA deprotection step was carried out for 1 h as described, and the major product in each case was characterized as the desired tripeptide methyl ester by ESMS: calcd for $C_{16}H_{23}N_3$ (average isotope composition) 321.4 Da, found 321.0 ± 1.0 Da. The two peptide diastereomers were resolved by analytical HPLC using a linear gradient of 10−15% B over 30 min (t_R for LLL and LDL peptides was 12.3 and 13.6 min, respectively).

Cyclization of H-C(Npys)YAVTGKGDSPAAG-SEt (8). The crude peptide **8** (5 mL, ca. 5 μmol) was diluted with 0.2 M Na_2HPO_4 buffer at pH 7.5 (20 mL) to a final concentration ∼200 μM. The final pH was adjusted to 7.2 when necessary with concentrated aqueous NaOH solution, and then the reaction was initiated by adding EtSH (200 μL). The cyclization

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reaction was allowed to proceed for 1 h at room temperature. The major peptide product was then purified by semipreparative HPLC using a linear gradient of 0−50% B over 30 min. The purified product was characterized as the cyclomonomeric product by tryptic digestion and ESMS: calcd for $C_{54}H_{84}N_{16}O_{18}S$ (average isotope composition) 1278.4 Da, found 1278.0 ± 0.1 Da.

Kinetic Studies on Cyclization of Peptide (8). Kinetic analyses were performed by analytical HPLC. The reactions were initiated as described above. Aliquots of the supernatant (50 μL) were withdrawn at various time points, treated with 10 μL of a 50 mM dithiotreitol (DTT) solution, and analyzed by HPLC. The first-order rate constant and the half-life were calculated by measuring the concentrations of the cyclic peptide and fitting the time course data to the equation $C_{t,\text{cyclic}} = C_{0,\text{cyclic}} \cdot (1 - e^{-kt})$, where $C_{t,\text{cyclic}}$ is the concentration of cyclic peptide at time t, $C_{0,\text{cyclic}}$ is the final concentration of cyclic peptide, and k is the rate constant.

Synthesis of c-Crk SH3 Domain by Native Chemical Ligation (Ligation of Peptides 9 and 10). Peptide thioester **9** (1.9 mg, 0.69 μmol) and peptide **10** (3.1 mg, 0.74 μmol) were dissolved in 0.2 M sodium phosphate buffer at pH 7.2 containing 5% EtSH by volume. The ligation was allowed to proceed for 72 h at room temperature. The reaction was then quenched with an excess of DTT and the ligated product purified by semipreparative HPLC using a linear gradient of 20−55% B over 30 min (2.2 mg, 46%). The purified product was characterized as the ligated SH3 domain by ESMS: calcd for $C_{310}H_{464}N_{82}O_{93}S_2$ (average isotope composition) 6891.7 Da, found 6894.1 ± 1.0 Da.

Fluorescence-Based Ligand Binding Assay. The equilibrium dissociation binding constant of synthetic SH3 domain for ligand **11** was obtained using a fluorescence-based titration assay. Measurements were conducted at 25 °C in a stirred 1 cm-path length cell using a Fluorolog III instrument. Excitation was at 300 nm with a 2.5 nm slit, and the fluorescence emission was monitored at 348 nm through a 5 nm slit. The protein concentration was 0.5 μM in a buffer containing 20 mM sodium phosphate, 100 mM NaCl at pH 7.2. The dissociation constant was determined by changes in the fluorescence of the protein solution upon addition of the corresponding peptide ligand at defined concentrations; calculations were made assuming formation of a 1:1 complex.[68]

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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