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A Fmoc-compatible Method for the Solid-Phase Synthesis of Peptide C-Terminal α-Thioesters based on the Safety-Catch Hydrazine Linker

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Abstract

C-terminal peptide thioesters are key intermediates for the synthesis/semisynthesis of proteins and for the production of cyclic peptides by native chemical ligation. They can be synthetically prepared by solid-phase peptide synthesis (SPPS) methods or biosynthetically by protein splicing techniques. Until recently, the chemical synthesis of C-terminal α-thioester peptides by SPPS was largely restricted to the Boc/Benzyl methodology because of the poor stability of the thioester bond to the basic conditions employed 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 by Fmoc/*t*-Bu chemistry. This method is based on the use of an aryl hydrazide linker, which is totally stable to the Fmoc-SPPS conditions. Once the peptide synthesis has been completed, activation of the linker can be achieved by mild oxidation. This step transforms the hydrazide group into a highly reactive diazene intermediate which can react with different H-AA-SEt to yield the corresponding α-thioester peptide in good yields. This method has been successfully used for the generation of different thioester peptides, circular peptides and a fully functional SH3 protein domain.

Key words: α-thioester peptide, chemical ligation, solid-phase peptide synthesis, Fmoc/tBut, c-Crk N-t SH3 domain, backbone cyclization

Introduction

C-terminal peptides are extremely valuable intermediates for the chemical synthesis of small and medium-sized proteins [1-3] and for the production of cyclic peptides [4-7] by native chemical ligation [8,9]. These mildly activated species are also critical for the construction of topologically [10-14] and backbone engineered [15-17] proteins.

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

The Fmoc–based methodology does not employ such a hazardous reagent. Therefore, synthetic methods compatible with this widely used methodology would greatly complement these approaches, especially in peptides containing functionalities incompatible with Boc-chemistry (i.e. glycopeptides and phosphopeptides). However, the poor stability of the thioester functionality to strong nucleophiles such as the piperidine used for the deprotection of the N^{α}-Fmoc group seriously limits the use of this methodology.

So far, several approaches have been used to overcome this problem. Futaki et al. [28] used an approach where the thioester was formed in solution using a partially protected precursor. Li et al. [29] used a thioester-compatible Fmoc-deprotection cocktail to synthesize an unprotected 25-residue peptide thioester in 24% yield. A similar approach was also used by Clippingdale et al. [30] using in this case the non-nucleophilic base DBU in combination with HOBt. However these approaches are still limited by the residual lability of the thioester to the conditions employed during the Fmoc removal, thus limiting the size of the peptide to be synthesized with good yields.

An alternative approach involving the introduction of the thioester function at the end of the synthesis has been also used by Alsina et al. [31] where the backbone amide linker (BAL) was employed for the synthesis of peptide thioesters using the Fmoc strategy. This approach was used for the synthesis of small peptide thioesters with reasonably good yields, however some racemization was observed during the thiolysis step. Swinnen et al. [32] have also used the stable phenylacetamidomethyl (PAM) [33] and Wang [34] resins to

synthesize thioester peptides by employing EtSH in the presence of Me_2AlCl. This approach was used for the synthesis of a 22-mer peptide thioester with moderate yields [35]. Another method 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 the repetitive exposure to the basic conditions needed for Fmoc removal. However, the linker can be activated by alkylation with diazomethane or idoacetonitrile and then can be easily cleaved with thiols to yield the corresponding thioester. The alkylation step, however, has been shown to alkylate unprotected methionine residues [38].

Although all the available methods for the synthesis of peptide thioesters by the Fmoc-approach have advantages and disadvantages, all of them require either special linkers, resins and/or complicated procedures which seriously limits their general applicability. In the present work we describe a totally novel method for the solid-phase synthesis of peptide thioesters by the Fmoc-approach which addresses all these limitations. This method is based on the use of an aryl hydrazide linker [39-46], which is totally stable to the Fmoc- and Boc-SPPS conditions and yet can be activated for cleavage by mild oxidation once the synthesis is complete.

Results and Discussion

General Scheme. The principle of our approach is based on the use of the base- and acid-stable hydrazine safety-catch linker [42-46]. This linker is totally stable to the conditions employed during the SPPS by either Boc- or Fmoc-approaches [42,43,46] and can be activated by treatment with mild oxidizing agents to provide a reactive acyl diazene intermediate that can react with N- and O-nucleophiles [43]. It is important to note that due to the redox character of S-nucleophiles, acyl diazenes are mainly reduced back to hydrazides in the presence of thiols instead of giving the corresponding thioester with acceptable yields [47].

Based on the early results of Goodman et al. [48] where it was shown that mildly activated α-amino acid *p*-nitrophenyl esters could react very selectively with highly activated carboxylic acid derivatives to form a peptide bond. We reasoned that α-amino acid S-alkyl thioesters could be also potentially used to cleave the extremely reactive acyl diazene even in the presence of the mild activating thioester group. This reaction will

cleave the peptide from the resin yielding the corresponding thioester, and since the hydrazine linker is totally stable to repetitive basic conditions, the peptide thioester could be then assembled by Fmoc-chemistry.

The procedure for our peptide thioester synthesis by Fmoc-chemistry is depicted in Scheme 1 and involves the directly assembly of the peptide on a phenyl-hydrazine resin [49] using standard Fmoc protocols [50]. 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 by adding the corresponding α-amino *S*-alkyl thioester and the fully protected thioester peptide is finally deprotected by using TFA and the appropriate scavengers (see Experimental Section). Note that in the cases where the N-terminal α-amino group needs to be unprotected in the final peptide thioester, the last amino acid should be incorporated as Boc-$^{\alpha}$N-protected during the synthesis. This prevents any potential oxidation of the free amino group during the oxidation step.

Oxidative-Activation of the Resin and Cleavage by α-Amino Thioesters. The first thing that needed to be addressed was how efficient and clean was the cleavage of the activated peptidyl diazene resin by α-amino S-alkyl thioesters. In order to test this new procedure, three small model peptides were synthesized on hydrazinobenzyl AM resin [49] (Table 1). 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. Once the oxidation reaction was complete the activated peptide-resin was then washed and cleaved with 20 equiv. of H-AA-SEt (where AA was either Gly or Ala) [51]. The reaction was quenched by adding acetic acid and the solvent evaporated. Finally the protected peptide thioesters were deprotected by an acidolytic step with TFA. As can be seen in Figure 1 and Table 1, the oxidative-cleavage reaction was extremely clean and efficient in all three peptides. In each case the main product was the corresponding thioester with cleavage yields around 65% [52] (as calculated by resin substitution) and purities around 95% (as calculated by HPLC). As anticipated due to the high reactivity of acyl diazene compounds toward N-nucleophiles, the cleavage reaction was also relatively fast being complete in less than 30 min (Figure 1D). The speed of this reaction was crucial for the success of our approach since allowed to minimize to practically zero the multiple

incorporation of amino thioester residues at the C-terminus of the peptide during the cleavage step.

Epimerization of the C-terminal Amino Acid after the Oxidative Cleavage. Since acyl diazenes are relatively highly activated species towards N- and O-nucleophiles [43], epimerization of the C-terminal residue attached to the resin through oxazolone formation [53] was a real possibility. In order to check that, two diastereomer dipeptides (i.e. LL- and LD Phe-Ala peptides) were assembled on the hydrazine resin following standard Fmoc-protocols. Once assembled, both dipeptides were oxidized with NBS and the corresponding peptidyl diazene was reacted with H-(L)-Ala-OMe in each case. As shown in Figure 2, the HPLC analysis of the crude cleavages for both diastereomer peptides did not reveal any significant epimerization of the middle Ala 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 oxidative-activation step. Although our oxidative cleavage approach for the synthesis of peptide thioesters worked very efficiently in our initial model peptides (Table 1), there was still some potential concern about the stability of peptides containing oxidative-sensitive residues (i.e. Tyr, Trp, Met and Cys) to the oxidation/activation step. In order to test the validity of our method in these situations, several peptides containing these residues were synthesized on the hydrazine residue and then oxidatively cleaved with either H-Ala-SEt or H-Gly-SEt (Table 2). The results, summarized in Figure 2, showed that peptides **4** and **5**, which contained Tyr(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 thioester peptides (Figure 3A and 3B) and insignificant amounts of by-products were detected. This was a gratifying result since the highly activated fenolic and indole rings are known to be very susceptible to halogenation by mildly oxidizing agents like NBS [refs]. In the case of the Tyr residue the t-Bu side chain protecting group was able to protect the aromatic ring from any detectable bromination under the conditions employed. This was probably due to the combination of the steric effect of *t*-butyl group on the 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). It is worth

noting that the use of electrowithdrawing groups has been also reported to protect the fenolic group of Tyr from oxidative halogenation [54]. More striking, however, was the fact that Trp was totally stable to the oxidative cleavage, under our reaction conditions, when protected with the N^{in}-Boc [55] group. In contrast, when peptide **5** was synthesized with no protection on the indole ring the oxidative cleavage with NBS/H-Gly-SEt gave a quite complex crude reaction where different oxidation/bromination products could be easily identified by HPLC/ESMS (results not shown). The protective effect of the N^{in}-Boc group must arise from the electrowithdrawing character of the carbamate moiety which leads to the partial deactivation of the indole ring towards electrophiles.

As expected, Met-containing peptide **6** was completely oxidized to the corresponding sulfoxide during the NBS oxidation step (data not shown), but during the TFA cleavage was reduced if 2% EtSH was added to the cleavage cocktail (Figure 3B). Similarly, Cys(Trt)-containing peptide **7** was also oxidized during the NBS treatment showing a rather complex crude mixture after the TFA deprotection step (data not shown). However, the corresponding thioester peptide **7** could be obtained in good yield if the TFA crude was reduced with EtSH at pH 8.0 for 30 min (Figure 3D). Under these conditions the hydrolysis of the 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 [56]. Hence, Cys-containing peptide **8**, where the N-terminal Cys residue was introduced as Boc-Cys(Npys) [57] during the peptide assembly, 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 residue is not located in this position, the S-StBu group should be used instead. This group is totally compatible with Boc- and Fmoc-strategies and can easily be deprotected by reductive treatment with thiols or phosphines [58].

Finally, our oxidative-cleavage procedure was also used to generate a more complex and longer peptide thioester. Peptide thioester **9**, a 22-mer thioester peptide derived from the

c-Crk *N*-t SH3 domain [59], was prepared to obtain the full synthetic SH3 domain by native chemical ligation (see below). The final crude of the peptide thioester **9** was relatively clean showing only two major peaks (Figure 4F). The major peak (*ca.* 66%) corresponded to the expected peptide thioester **9** as determined by mass spectrometry (calculated molecular weight (average isotope composition) 2762.61 Da; found 2762.16 Da) meanwhile the secondary peak (*ca.* 33%), which eluted earlier in the HPLC chromatogram (Figure 4F), presented a loss of 17 Da versus peptide **9** and it was assigned to be the corresponding 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 [60,61] at the Gly^{12} in peptide **9** (see Table 2). After a single step HPLC purification step, pure peptide **9** was obtained with a modest yield (*ca.* 10%, based on the theoretical resin substitution), however it is important to remark 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. In order to test the suitability of the thioesters generated by this new method thioester peptides **8** and **9** were used for carrying out intramolecular and intermolecular native chemical ligations [8], respectively.

Intramolecular Native Chemical Ligation. Linear precursor peptide thioester **8**, which sequence derives from the tenth type 3 module of Fibronectin (a natural β-strand hairpin) [62], was designed to contain an α-thioester group and a Cys residue at the C- and N-terminal position, 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 TFA crude (Figure 3E) 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 this conditions the backbone cyclization reaction proceeded very fast and efficiently (Figure 4). The reaction was complete in less than 60 min and the major product corresponded to the cyclic peptide **8** as characterized by ES-MS and tryptic digestion (see Experimental Section).

Intermolecular Native Chemical Ligation-Synthesis of functional SH3 protein domain. We used the N-terminal SH3 domain from the c-Crk adaptor protein as a target for the intermolecular native chemical ligation. The amino acid sequence of the c-Crk N-t SH3

protein domain corresponds 134-190 of the c-Crk protein [59]. Retrosynthetic analysis, guided by the structure of the SH3 domain [63], suggested that a functional analogue of the protein domain could be prepared by native chemical ligation between peptide **9** (residues 134-156) and peptide **10** (residues 157-191, CILRIRDKPEEQWWNAE-DSEGKRGMIPVPYVEKYG). Peptide **10** was synthesized using standard Fmoc-protocols on a Rink-amide resin (see Experimental Section). Note that in order 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 PBS. The ligand binding activity of the synthetic SH3 domain was evaluted by using a fluorescence-based titration assay [64]. The equilibrium dissociation constant for binding of the synthetic SH3 domain to the natural proline-rich peptide ligand C3G (peptide **11**) [59] was 0.9 μM (Figure 5B). This value was identical to that reported for the recombinant c-Crk N-t SH3 domain [14].

Conclusions

In summary, we have developed a new complementary method for the facile preparation of peptide thioesters without limitations of size and amino acid composition. Our oxidative-cleavage approach has been shown to be totally compatible with sensitive amino acids if the appropriate protecting groups and oxidative conditions were employed. Also, no detectable racemization was observed during the activation of the hydrazide linker. Finally, our approach does not require special linkers, resins or complicated protocols since it uses commercially available hydrazine resins and the assembly of the peptide chain on the resin is carried out by using standard coupling methods.

Hence, we believe that given the simplicity and efficiency of our approach, this may already prove useful for those cases where the already available methods fail or do not give good yields.

Experimental Section.

Materials and Methods. Fmoc-amino acids, HBTU and 4-Fmoc-hydrazine resin were obtained from Novabiochem (San Diego, CA). Methylene chloride (DCM), N,N-dimethylformamide (DMF) and HPLC-grade acetonitrile (MeCN) were purchased from Fisher. Trifluoroacetic acid (TFA) was purchased from Halocarbon (River Edge, NJ). All other reagents were obtained form Aldrich Chemical Co.

Analytical and semipreparative gradient HPLC were performed on a Hewlett-Packard 1100 series instrument with UV detection. Semipreparative HPLC was run on a Vydac C18 column (10 micron, 10 x 250 mm) at a flow rate of 5 mL/min. Analytical HPLC was performed on a Vydac C18 column (5 micron, 4.6 x 150 mm) at a flow rate of 1 mL/min. Preparative HPLC was performed on a Waters DeltaPrep 4000 system fitted with a Waters 486 tunable absorbance detector using a Vydac C18 column (15-20 micron, 50 x 250 mm) at a flow rate of 50 mL/min. All runs used linear gradients of 0.1% aqueous TFA (solvent A) *vs.* 90% MeCN plus 0.1% TFA (solvent B).^{1}H NMR spectra were obtained at room temperature on a Bruker 400 MHz spectrometer??. Electrospray mass spectrometric analysis was routinely applied to all synthetic peptides and components of reaction mixtures. ESMS was performed on a Applied Biosystems/Sciex API-150EX single quadrupole electrospray mass spectrometer. Calculated masses were obtained using the program ProMac 1.5.3

Glycine *S*-Ethyl Ester, Hydrochloride Salt (H-Gly-SEt·HCl). Boc-Gly-OH (5.0 g, 28.5 mmol) and HOBt·H_2O (4.36 g, 28.5 mmol) were dissolved in DCM (125 mL). HOBt·H_2O (0.87 g, 5.7 mmol). EDC (4.95 mL, 28.5 mmol) and 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 x 250 mL), 1% $NaHCO_3$ (3 x 250 mL)and H_2O (3 x 250 mL), dried with $MgSO_4$ and concentrated in vacuo. The residue (Boc-Gly-SEt) was dissolved in 4 M HCl-dioxane (20 mL) and the deprotection reaction was stirred at room temperature for 90 min. The homogeneous reaction 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 according to analytical RP-HPLC (t_R : 3.49 min using an isochratic at 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: calculated for C_4H_9NOS (average isotope composition) 119.2 Da, found 119.0 ± 0.5 Da.

Alanine *S*-Ethyl Ester, Hydrochloride Salt (H-Ala-SEt·HCl). It was prepared as described above for H-Gly-SEt, but starting with Boc-Ala-OH (5.39 g, 28.5 mmol) to provide 2.2 g (63%) >95.5% pure alanine ethyl thioester according to analytical RP-HPLC (t_R : 6.59 min using an isochratic at 0% B for 2 min and then a linear gradient of 0% to 23%B over 10 min) : ^{1}H NMR (DMSO-d_6) δ 8.56 (br, s, 3H), 4.33 (q, 1H), 3.01 (q, 2H), 1.53 (d, 3H), 1.23 (t, 3H); ESMS: calculated for $C_5H_{11}NOS$ (average isotope composition) 133.2 Da, found 133.0 ± 0.5 Da.

Solid-Phase Peptide Synthesis. All peptides were manually synthesized using the HBTU activation protocol for Fmoc solid-phase peptide synthesis [65] 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 [66]. Side-chain protection was 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 Gly^{12} to minimize aspartimide formation) were used respectively.

Oxidative 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 7 min at room temperature with occasional stirring. The unreacted NBS was then washed with anhydrous DCM (3 x 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 together in DCM (5 mL), and this solution was immediately added to the oxidized peptide resin. The cleavage reaction was kept for 1 h at room temperature. The heterogeneous reaction was then quenched with HOAc (250 μL) and the solvent removed in vacuo. The peptide thioester was deprotected when

necessary with TFA:H_2O:TIS (50:1:1 v/v, 5 mL) for 1-3 h depending of the peptide sequence, except in peptide **6** where TIS was replaced by EtSH as scavenger in the deprotection cocktail. The filtrate from the cleavage was collected, combined with TFA washes (2 x 0.5 mL) of the cleaved peptide resin, concentrated under a stream of N_2, precipitated with cold anhydrous Et_2O (50 mL) and washed with Et_2O (2 x 20 mL). The peptide was then dissolved in buffer A:buffer B (4:1 vol, 5 mL), characterized by HPLC and ESMS and purified.

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

Synthesis of Ac-IAFA-SEt (2). Synthesis (0.1 mmol) was carried out as described above for **1** but H-Ala-SEt·HCl was used for the cleavage instead. The crude peptide was purified by HPLC preparative as described for peptide **1**. The major product was characterized as the desired thioester product by ESMS: calculated for $C_{25}H_{38}N_4O_5S$ (average isotope composition) 506.3 Da, found 506.0 ± 1.0 Da.

Synthesis of H-LFAG-SEt (3). Synthesis (0.1 mmol) was carried as described above for **1**. The last residue was incorporated as Boc-Leu-OH. Oxidative cleavage with NBS and H-Gly-SEt·HCl was carried out as described above. The TFA deprotection step was carried out for 1 h as described above. The major product was characterized as the desired thioester product by ESMS: calculated for $C_{22}H_{34}N_4O_4S$ (average isotope composition) 450.6 Da, found 450.0 ± 1.0 Da.

Kinetics studies of the oxidative cleavage of peptide 3.

Kinetic analyses were performed by analytical HPLC. The Oxidative cleavage for obtaining peptide thioester **3** was performed as described above. During the cleavage reaction with H-Gly-SEt, small aliquots of the supernatant (20 μL) were withdrawn at various time points, treated with 100 μL of TFA for 20 min and then evaporated under a

stream of N_2. The peptide thioester was then solubilized with buffer A:buffer B(2:1 vol., 150 μL), filtrated and analyzed by analytical HPLC. The corresponding half times was calculated by measuring the concentrations of the corresponding thioester peptide and fitting the time course data to the equation: $C_{t,thioester} = C_{0,thioester} \cdot (1-e^{-k \cdot t})$, where $C_{t,thioester}$ is the concentration of thioester peptide at time *t,* $C_{0,thioester}$ is the final concentration of thioester peptide and *k* the corresponding rate constant.

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

Synthesis of H-LYKAA-SEt (4). Synthesis and oxidative cleavage with H-Ala-SEt was performed as described for **2**. The TFA deprotection step was carried out for 1 h as described above. The major product was characterized as the desired thioester product by ESMS: calculated for $C_{29}H_{48}N_6O_6S$ (average isotope composition) 608.8 Da, found 608.0 ± 1.0 Da.

Synthesis of H-LWAG-SEt (5). Synthesis and oxidative cleavage with H-Gly-SEt was perfomed as described for **3**. TFA deprotection was carried out for 1 h as described above The major product was characterized as the desired thioester product by ESMS: calculated for C24H35N5O4S (average isotope composition) 489.6 Da, found 490.0 ± 1.0 Da.

Synthesis of H-LMYKAG-SEt (6). Synthesis and oxidative cleavage with H-Gly-SEt was performed as described for **3**. The TFA deprotection/reduction step was carried out by treatment with TFA:EtSH:H_2O (50:1:1 vol., 5 mL) for 3 h at room temperature as described above. The major product was characterized as the desired thioester product by ESMS: calculated for $C_{33}H_{55}N_7O_7S_2$ (average isotope composition) 726.0 Da, found 725.0 ± 1.4 Da.

Synthesis of H-LCYKAA-SEt (7). Synthesis and oxidative cleavage with H-Ala-SEt was performed as described for **3**. TFA deprotection was achieved with TFA:TIS:H_2O (50:1:1 vol., 5 mL) for 1 h at room temperature as described before. The peptide thioester was then solubilized in buffer A:buffer B (4:1 vol., 5 mL) containing 2% EtSH and the pH was adjusted to 8.0 with 1 M Tris·HCl buffer. The reduction reaction was kept for 30 min at room temperature. The major product was characterized as the desired thioester product by ESMS: calculated for $C_{32}H_{53}N_7O_7S_2$ (average isotope composition) 712.0 Da, found 712.1 ± 0.1 Da.

Synthesis of H-C(Npys)YAVTGKGDSPAAG-SEt (8). Synthesis was carried out as described above with the exception that Boc-Cys(Npys)-OH (375 mg, 1 mmol) was coupled for 45 min as its preformed symmetrical anhydride using DIPC (80 µL, 0.5 mmol) in DCM (4 mL). The oxidative cleavage with NBS/H-Gly-SEt·HCl and the TFA deprotection step were carried was as described above. The major product of the crude material was characterized as the desired thioester product by ESMS: calculated for $C_{61}H_{91}N_{17}O_{21}S_3$ (average isotope composition) 1494.7 Da, found 1494.5 ± 0.7 Da.

Cyclization of H-C(Npys)YAVTGKGDSPAAG-SEt (8). TFA 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 of 7.2 when necessary with concentrated aqueous NaOH solution and then the reaction was initiated by adding EtSH (200 uL). The cyclization 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: calculated 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. 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 DTT solution and analyzed by HPLC. The first order rate constants and the corresponding half times were calculated by measuring the concentrations of the corresponding cyclic specie and fitting the time course data to the equation: $C_{t,cyclic}$ =

$C_{0,cyclic}\cdot(1-e^{-k\cdot t})$, where $C_{t,cyclic}$ is the concentration of cyclic polypeptide at time *t,* $C_{0,cyclic}$ is the final concentration of cyclic peptide and *k* the corresponding rate constant.

Synthesis of Ac-AEYVRALFDFNGNDEEDLPFKKG-SEt (9). Synthesis and acetylation of the N-terminus was performed as described for **1**. The oxidative cleavage with H-Gly-SEt and TFA deprotection step were also carried out as described previously for **3**. The crude material was purified by HPLC semipreparative using a linear gradient of 30-55% B over 30 min. The purified peptide was characterized as the desired thioester product by ESMS: calculated for $C_{125}H_{182}N_{30}O_{39}S$ (average isotope composition) 2761.1 Da, found 2762.2 ± 0.8 Da.

Synthesis of H-CILRIRDKPEEQWWNAEDSEGKRGMIPVPYVEKYG-NH_2 (10). Synthesis was carried out on a Rink-amide resin on a 0.2 mmol scale. After total deprotection and cleavage the crude peptide was purified by HPLC preparative using a linear gradient of 20-40% B over 30 min. The purified peptide was characterized as the desired product by ESMS: calculated for $C_{187}H_{288}N_{52}O_{54}S_2$ (average isotope composition) 4192.8 Da, found 4195.1 ± 1.1 Da.

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 together in 0.2 M sodium phosphate buffer at pH 7.2 containing 5% EtSH in volume. The ligation was allowed to react for 72 h at room temperature. The reaction was then quenched by adding an excess of DTT and the ligated product purified by semiprepartive 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: calculated for $C_{310}H_{464}N_{82}O_{93}S_2$ (average isotope composition) 6891.7 Da, found 6894.1 ± 1.0 Da.

Synthesis of peptide H-WXPPPALPPKKR-NH_2 (11, X stands for 6-aminohexamoic acid). Synthesis was carried out on a Rink-amide resin on a 0.1 mmol scale. After total deprotection and cleavage the crude peptide was purified by HPLC preparative using a linear gradient of 0-70% B over 30 min. The purified peptide was characterized as the desired product by ESMS: calculated for $C_{69}H_{111}N_{19}O_{12}$ (average isotope composition) 1398.8 Da, found 1398.8 ± 1.0 Da.

Fluorescence-based ligand binding assay. The equilibrium dissociation binding constants of synthetic SH3 domain for ligand **11** was measured using a fluorescence-

based titration assay. Experiments were conducted at 25˚ C in a stirred 1 cm-pathlength 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 constants were 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.

Tryptic digestion of cyclic peptide 8. Cyclic polypeptide **8** (*ca*. 60 μg) were dissolved in 50 mM NH_4HCO_3 at pH 8.4 buffer, (1000 μL), along with 10 μL of trypsin-agarose beads (Sigma) and the reaction maintained at 37˚ C. After 50 min. the tryptic digest was subsequently analyzed by analytical HPLC and ESMS. Tryptic fragment obained from cyclic peptide **8**; T1: calculated for $C_{14}H_{26}N_4O_6$ (average isotope composition) 346.4 Da, found 345.0 ± 1.0 Da, T2: calculated for $C_{34}H_{49}N_9O_{14}S$ (average isotope composition) 839.9 Da, found 840.0 ± 1.0 Da. The tryptic map of cyclic peptide 8 gave two peptides because the trypsin used contained some residual chymotrypsic activity.

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Figure Legends

Scheme 1. The principle of the oxidative cleavage approach for obtaining peptide thioesters by Fmoc-chemistry.

Table 1. Cleavage yields, purity and molecular weights of peptide thioesters **1** to **3**.

Table 2. Primary amino acid sequences of different peptides varying in length and complexity used in this study. The side-chain protecting group for sensitive amino acids and the molecular weights for the expected products is also indicated.

Figure 1. HPLC analysis of the crude product obtained by oxidative cleavage with NBS/H-AA-SEt of: (**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 over 30 min was used in each case. (**D**) Kinetic analysis for the formation of peptide thioester **3** by cleavage with NBS/H-Gly-SEt.

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 oxidative cleavage with NBS/H-(L)-Ala-OMe of H-(L)-Phe-(L)-Ala-(L)-Ala-OMe (**A**) and (**B**) H-(L)-Phe-(D)-Ala-(L)-Ala-OMe.

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(tBut)-containing peptide **4**, (**B**) Trp(Boc)-containing peptide **5**, (**C**) Met-containing peptide **6**, (**D**) Cys(Trt)-containing peptide **7** after being reduced for 30 min 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 were a linear gradient of 30-60% buffer B was used instead.

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).

Table 1

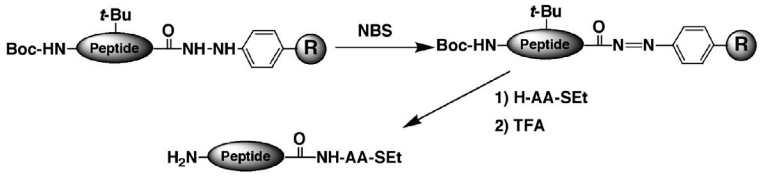
Peptide	Sequence	Mw/ Da		Yield/ %	
1	Ac-IAFG-SEt	492.6[a]	492.0[b]	95[c]	60[d]
2	Ac-IAFA-SEt	506.3[a]	506.0[b]	94[c]	65[d]
3	H-LFAG-SEt	450.0[a]	449.7[b]	95[c]	70[d]

[a] expected; [b] found; [c] based on HPLC purity; [d] based on resin substitution

Table 2

Peptide	Sequence	Mw / Da		Protecting group[a]
4	H-L**Y**KAA-SEt	608.8[b]	608.0[c]	Tyr(t-But)
5	H-L**W**AG-SEt	489.6[b]	490.0[c]	Trp(Boc)
6	H-L**M**YKAG-SEt	726.0[b]	725.0[c]	None
7	H-L**C**YKAA-SEt	712.0[b]	712.1[c]	Cys(Trt)
8	H-**C**YAVTGKDSPAAG-SEt	1494.7[b]	1494.5[c]	Cys(Npys)
9	Ac-AEYVRALFDFN**G**NDEE DLPFKKG-SEt	2761.1[b]	2762.2[c]	(Fmoc-2-hydroxy-4-methylbenzyl)-Gly

[a] Side-chain (peptides **4** to **8**) or backbone (peptide **9**) protecting groups for the underlined residues within the corresponding sequence; [b] expected; [c] found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Peptide
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2) TFA
H2N
Peptide
O
NH-AA-SEt

Scheme 1

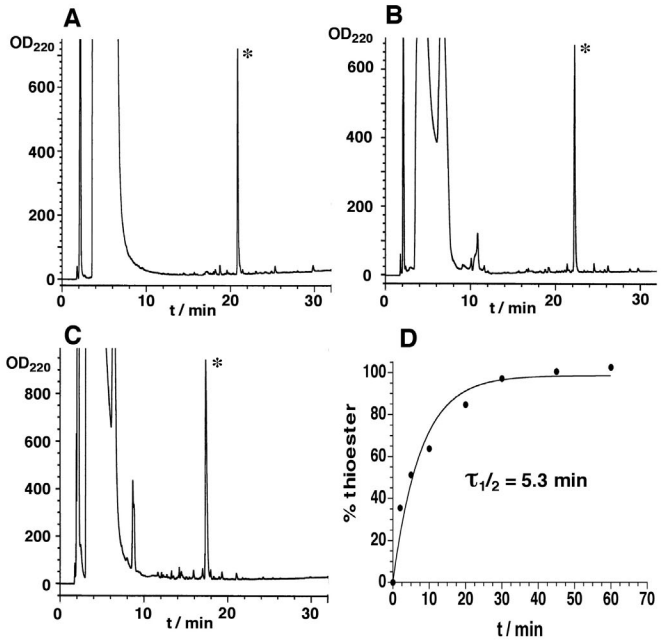
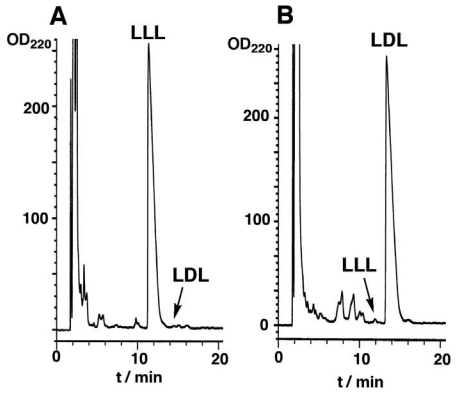


Figure 1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LLL
OD220
200
100
LDL
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Figure 2

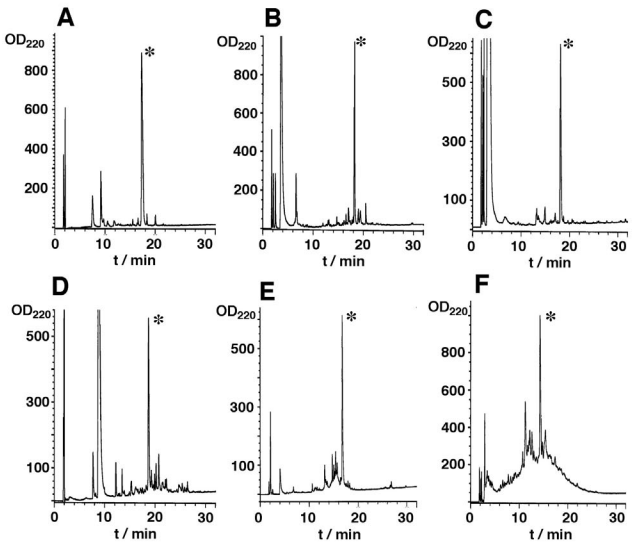


Figure 3

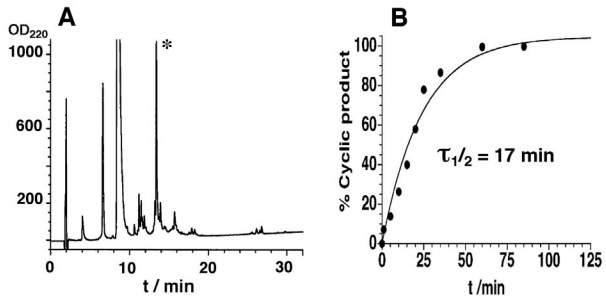
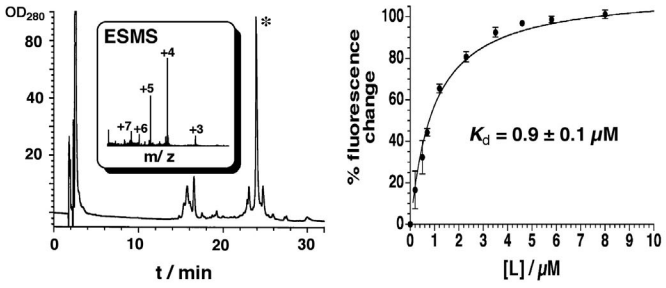

A
OD220
1000
600
200
*
0
10
20
30
t / min
B
% Cyclic product
100
80
60
40
20
0
τ1/2 = 17 min
0
25
50
75
100
125
t /min

Figure 4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40
20
ESMS
+4
+5
+7
+6
+3
m/ z
*
0
10
20
30
t / min
% fluorescence change
100
80
60
40
20
0
K_d = 0.9 ± 0.1 μM
0 1 2 3 4 5 6 7 8 9 10
[L] / μM

Figure 5