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Automated Fmoc-Based Solid-Phase Synthesis of Peptide Thioesters with Self-Purification Effect and Application in the Construction of Immobilized SH3 Domains

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Abstract: Peptide thioesters are important building blocks in the total synthesis of proteins and protein domains via fragment ligation. However, synthetic access of peptide thioesters still is a bottleneck of this powerful ligation chemistry. The commonly used methods for the Fmoc-based synthesis of peptide thioesters involve nonautomated solution steps that have to be performed after the solid-phase assembly of the peptide. Usually, HPLC purification is required. Herein, a method that enables crude peptides to be used in divergent native chemical ligations reactions is described. We present an Fmoc-based solid-phase synthesis of peptide thioesters with self-purification which facilitates access to these important building blocks, since the often cumbersome HPLC purification can be avoided. Fmoc-protected amino acids are coupled on a safety catch sulfonamide resin. The self-purifying effect is achieved through the combination of (a) N-terminal coupling of a cleavable cyclization linker and subsequent backbone-to-side chain cyclization, (b) activation of the sulfonamide linkage by alkylation, (c) thiolysis for the selective detachment of truncation products, and (d) TFA cleavage for the liberation of the desired peptide thioester in unprotected form. We have previously shown a method wherein cyclization was performed after carboxymethylation of the sulfonamide. However, the automation of this method was difficult and side reactions at methionine residues hampered the general applicability. The new design involves peptide synthesis on a modified carboxy-functionalized sulfonamide linker, a substantially milder activation of the sulfonamide bond and the use of monomethoxytrityl as well as 2-phenyl-isopropyl protecting groups. This approach solved the problems with methionine containing peptides and enabled the complete automation of the self-purifying synthesis of peptide thioesters. The study also addressed problems in the synthesis of difficult peptides. Aggregated truncation products can resist extraction and contaminate full-length thioesters obtained after TFA cleavage. It is shown that significant enhancements of the purity were achieved when mild acidic extractions were included in the wash protocols after thiolysis. The potential of the method was demonstrated in the parallel synthesis of 20−40 amino acid long peptide thioesters, which were obtained in excellent purities. The thioesters and cysteinyl peptides were used without purification in the assembly of immobilized SH3 protein domains of SHO1 in yeast. A cysteine scan by native chemical ligation suggested single amino acid to cysteine substitutions that (a) confer useful ligation yields, (b) support correct folding, and (c) sustain the function of the folded protein domain. The chemical synthesis of the SH3-domain of SHO1 succeeded in highest yields when cysteine placements at positions S23, F24, and E36 were avoided. The synthetic SH3 mutants were examined in a binding assay, which indicated that N27C, L30C, and D34C mutations provide functional SH3-domain.

Introduction

Chemically synthesized peptides and proteins are invaluable tools for the studies of biomolecular recognition processes. The advent of robust fragment ligation methods significantly facilitated synthetic access to folded proteins and protein domains. Among the most suitable ligation methods is the native chemical ligation, which involves a peptide thioester and a cysteinyl peptide.[1] The potential to use this reaction for the construction of protein arrays has been recognized; however, the difficulties in the parallel synthesis of peptide thioesters have hampered progress.[2] The chemical Fmoc-based synthesis of peptide thioesters typically proceeds with lower yields than the synthesis of peptide acids and peptide amides.[3−7] Moreover, the involvement of nonautomatable steps and the cumbersome purification steps limit the throughput of thioester synthesis. It is, thus, difficult to apply native chemical ligations in a parallel format

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when the sequences of both peptide segments, the cysteinyl peptide and the peptide thioester, shall be varied.

The commonly used methods for the Fmoc-based synthesis of peptide thioesters involve thioesterification reactions and/or acid treatments that have to be performed after the peptide has been detached from the solid-phase.[3,6−8] Reactions that furnish solid-phase bound peptide thioesters upon acid-induced N → S shift reactions are among the notable exceptions.[5,9] Nevertheless, HPLC-purification usually is required. Recently, we reported a synthesis method, which allows selective detachment of only the full-length peptide thioester and omits the need for HPLC purification.[10] A combination of on-resin macrocyclization via the N-terminus and thiolytic ring-opening at sulfonamide "safety-catch" resin[6,7,11] provided self-purification (Scheme 1A). The major advantage is that the crude materials can be directly used in native chemical ligation reactions. However, our attempts to apply this method in the parallel synthesis of SH3 domains were complicated by side reactions at methionine residues (vide infra). Herein, we describe important advancements of the reported method and we demonstrate the general applicability. The previously published method relied on a sequence of activation−cyclization−thiolysis steps which were not fully adapted for automation. Herein, a new sequence of reactions steps is introduced. This enabled the complete automation of the self-purifying synthesis of peptide thioesters, solved the problems with methionine containing peptides, as well as facilitated the synthesis of difficult peptide thioesters. The practicability of the method was demonstrated in the automated parallel synthesis of peptide thioesters and their subsequent use in the construction of immobilized protein domains. We also present data of a cysteine-scan by native chemical ligation which may help the rapid identification of positions for the introduction of cysteine residues that (a) provide high ligation yields, (b) sustain protein folding without (c) affecting protein−ligand binding.

Results and Discussion

The solid phase synthesis of peptide thioesters with self-purification involves a sequence of steps that, in principle, allow the selective detachment of only the full-length products. The generic protocol includes the coupling of cyclization linker **3a** to peptide **2** which was assembled on a sulfonamide resin **1** by using Fmoc-protected amino acids (Scheme 1A). The cyclization linker provides an initially Aloc-protected amino group which is utilized in a subsequent macrolactamization reaction. The required counterpart, a carboxyl group, is introduced upon alkylation of the *N*-acylsulfonamide in **4** with iodoacetic acid allyl ester **5**. This reaction also activates the *N*-acyl sulfonamide, which in the N-alkylated form is amenable to nucleophilic attack. The Pd(0)-catalyzed removal of the allyl-based protecting groups prepares the stage for the cyclization reaction which provides the macrolactam **7**. Cyclization is followed by thiolytic ring-opening. In this reaction, full-length thioesters are separated from truncation products. Truncated peptides are blocked in the capping steps and are, thus, excluded from the cyclization reaction. Hence, the noncyclic truncation products **8** are released into the solution phase upon thiolysis. The desired peptide thioester remains on solid support in **9** and is liberated upon treatment with trifluoroacetic acid (TFA).

The sequence of activation−cyclization−thiolysis steps described in Scheme 1A allowed the synthesis of peptide thioesters **17** and **18**, which were obtained in higher yield and purity than by conventional synthesis on the sulfonamide resin. For instance, crude peptide thioester **17** (c-Crk, residues 134−156, H-AEYVRALFDFNGNDEEDLPFKKG-SBzl) obtained by the conventional approach was isolated with truncated n-5 product (Figure 1A). The self-purification procedure provided direct access to peptide thioester **17** in high 88% purity and 30% yield (Figure 1B). As a second example, linear synthesis of the difficult peptide segment **18** (BPTI 12−37, H-GPCKARI-IRYNAKAGLCQTFVYGG-SBzl) resulted in a crude material which was contaminated by major amounts of truncation products (Figure 1C). Moreover, HPLC-purification was complicated due to intramolecular thiol-exchange reactions which led to the formation of cyclic thioesters (Figure S1 in Supporting Information). For this reason, **18** was isolated in only 6% yield and in 68% purity after HPLC-purification. In comparison, the activation−cyclization−thiolysis method furnished crude peptide thioester **18** in 18% overall yield and in 72% purity based on HPLC analysis (Figure 1D).[10]

Synthesis of Building Blocks. In the design of the cyclization linker, three criteria were considered: A suitable building block such as **3** should enable (a) facile introduction after the completion of the linear assembly of amino acids, (b) macrocyclization, and (c) detachment of the resin-bound full-length peptide. The synthesis was commenced from commercially available ethyl 4-hydroxybenzoate **19** (Scheme 2). The reaction with iodoacetamide provided the amidomethyloxybenzoate **20** in 90% yield. The subsequent reduction with lithium alumium-hydride furnished the aminoalcohol **21**. For the introduction of the Aloc-protecting group, crude **21** was allowed to react with allyl chloroformate which furnished **22a** in 53% yield over two steps. Finally, the reactive 4-nitrophenyl carbonic acid ester **3a** was synthesized in 96% yield.

Alternatively, the N-Mmt protected cyclization linker **3b** was prepared (Scheme 2). The selective introduction of Mmt required the temporary O-silylation of the hydroxyl group in **21**. The silylation−tritylation−desilylation sequence provided the Mmt-protected aminoalcohol **22b** in 79% yield. Because of the acid sensitivity of the Mmt-protecting group, the final formation of the reactive carbonic acid ester **3b** was performed by using the bis(4-nitrophenyl)carbonate furnishing **3b** rather than the 4-nitrophenyl chloroformate applied in the synthesis of **3a**.

Methionine-Containing Peptides. The synthesis of peptides that contained one or more methionine residues proved difficult.

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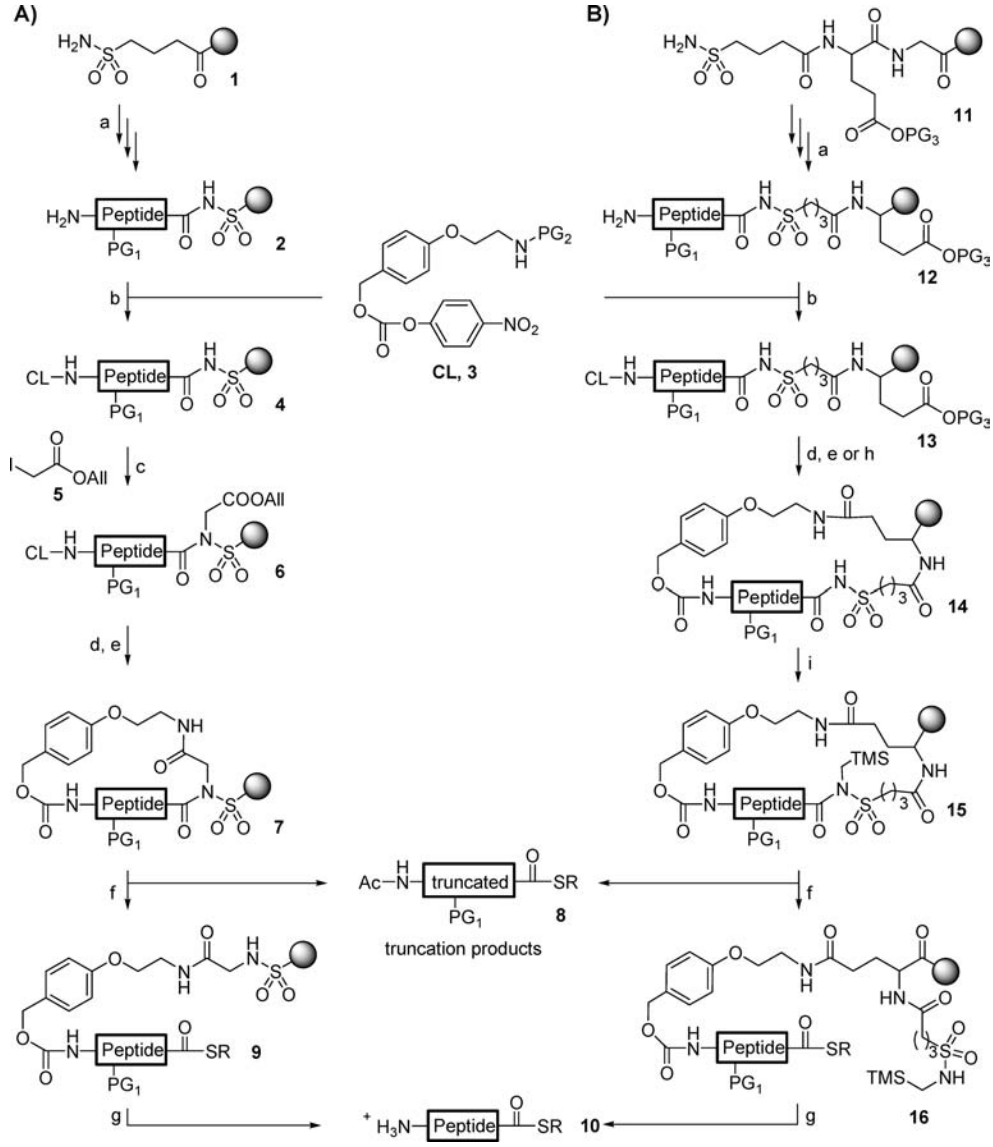
Scheme 1. Fmoc-Based Solid-Phase Synthesis of Peptide Thioesters with Self-Purification: (A) Activation−Cyclization−Thiolysis (ACT) Approach and (B) Cyclization−Activation−Thiolysis (CAT) Approach[a]



[a] Conditions: (a) solid-phase peptide synthesis: Fmoc removal, piperidine/DMF (1:4); 2× 4 min; coupling, 5 equiv amino acid, 4.5 equiv HCTU, 10 equiv NMM, DMF; capping, Ac_2O/2,6-lutidine/DMF (5:6:89); (b) coupling of **3**, 5% NEt_3/DMF; (c) activation, 6 M **5**, 1.5 M *i*Pr_2NEt, DMF; (d) deallylation, 0.02 M $Pd(PPh_3)_4$, 0.2 M *N*,*N*′-dimethylbarbiturate, CH_2Cl_2; (e) cyclization, 0.1 M PyBOP, 0.1 M HOBt, 0.3 M *i*Pr_2NEt, CH_2Cl_2; (f) thiolysis, 2 M RSH, 0.12 M NaSPh, DMF; (g) TFA cleavage; (h) deprotection, 1% TFA/CH_2Cl_2; (i) activation, 1 M $TMSCHN_2$ (*n*-hexane/THF). R = ethyl, benzyl. (All = allyl; Aloc = allyloxycarbonyl; Mmt = monomethyltrityl, PhiPr = 2-phenyl-prop-2-yl (2-phenyl-isopropyl); PG_1 = side-chain protecting groups typically used in Fmoc-based solid-phase peptide synthesis, PG_2 = Aloc or Mmt, PG_3 = All or PhiPr.)

For example, the assembly of peptide **23a** (170−183 of WW-domain of human Yes-kinase associated protein (hYAP)) resulted in a complex mixture of synthesis products wherein **23a** was detected in only 27% purity based on HPLC (Figure 2A).[12] We assumed that the highly concentrated solution of iodoacetic allylester **5** which is required for quantitative alkylation of the sulfonamide bond led to alkylation of methionine residues. Similar problems have been reported for the activation of the *N*-acyl sulfonamide linkage by cyanomethylation.[13] Indeed, the synthesis proceeded smoothly when the methionine was substituted by glutamine in **24** (Figure 2B). In this case, the crude peptide thioester **24** was obtained in 83% purity.

The vulnerability of methionine residues against electrophilic attack called for milder conditions of *N*-acylsulfonamide alkylation. We considered the reaction with trimethylsilyldiazomethane which has been used for the mild detachment of methionine-containing peptides from sulfonamide-resins.[13] To provide the carboxyl group required for macrolactamization, γ-allyl protected glutamic acid was linked to glycine-loaded polystyrene resin. The sulfonamide linker was introduced upon coupling of 3-carboxypropionic sulfonamide to the α-amino function of the glutamic acid (Scheme 1B, **11**). Peptide assembly, coupling of the cyclizationlinker, deallylation, and macrolactamization were performed as described for the previ-

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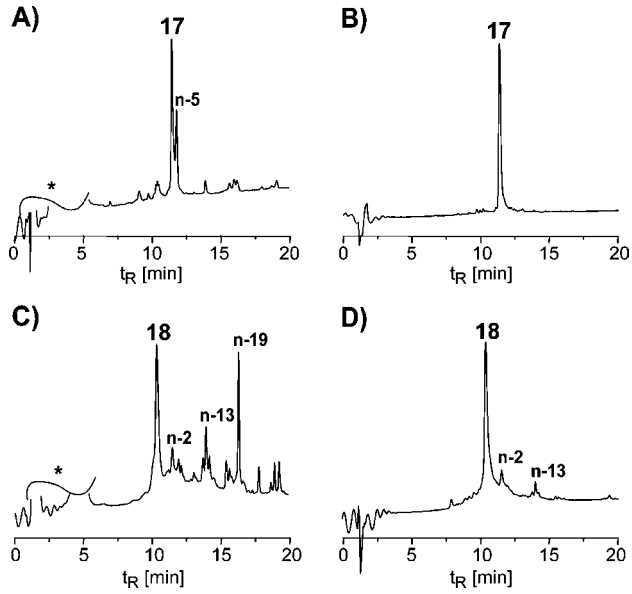
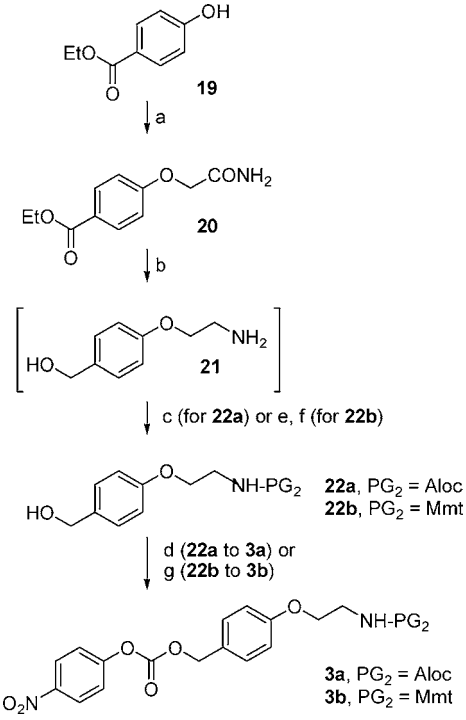


Figure 1. HPLC traces of crude peptide thioesters **17** and **18** obtained by (A and C) conventional approach (*NaSPh and DMF) or (B and D) self-purifying peptide thioester synthesis.

Scheme 2. Syntheses of the N-Aloc-Protected Cyclization Linker **3a** and the N-Mmt-Protected Cyclization Linker **3b**[a]



[a] Conditions: (a) iodoacetamide, NaOH, DMF, 15 h, 90%; (b) 3.5 M suspension of $LiAlH_4$ (THF/toluene), dry THF, 15 h, 76%; (c) Aloc-Cl, NEt_3, 0 °C, 90 min, 53% (reduction + protection); (d) 4-nitrophenyl chloroformate, pyridine, dry CH_2Cl_2, 20 h, 96%; (e) TMS-Cl, iPr_2NEt, dry CH_2Cl_2, reflux, 2 h, then Mmt-Cl, iPr_2NEt, dry CH_2Cl_2, 20 h, 79%; (g) bis(4-nitrophenyl) carbonate, iPr_2NEt, dry CH_2Cl_2, 15 h, 79%.

ous method. For activation of the *N*-acylsulfonamide linkage, macrocycle **14** was treated with trimethylsilyldiazomethane. The thioester was established upon thiolysis of **15**. Acidolytic cleavage was induced with TFA, which released the full-length peptide thioesters **10** into solution. In case of methionine-containing peptides, we also added dimethylsulfide and ammonium iodide for reduction of methionine sulfoxides.[14] This

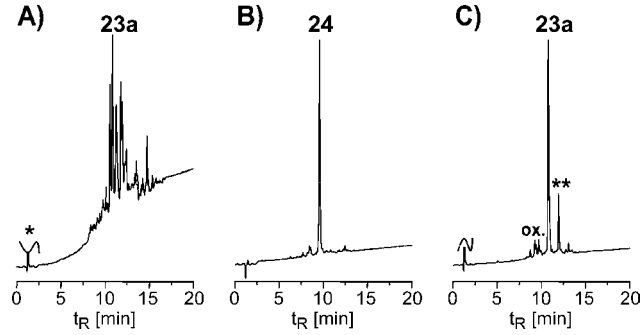


Figure 2. HPLC traces of peptide thioesters **23a** and **24** obtained by (A and B) Fmoc-based synthesis with self-purification (activation−cyclization−thiolysis, *NH_4I) and (C) modified Fmoc-based synthesis with self-purification (cyclization−activytion−thiolysis; *NH_4I; **phenyl thioester; ox, oxidation at methionine; **23**, H-DVPLPAGWEMAKTS-SEt; **24**, H-DVPLPAGWEQAKTS-SEt).

sequence of cyclization−activation−thiolysis steps (rather than activiation−cyclization−thiolysis) provided the peptide thioester **23a** in 31% yield and 75% purity of the crude material (Figure 2C, Table 1). In contrast, the crude material obtained by conventional synthesis had only 35% purity (Figure S2, Table 1). Sulfoxides were identified as main source of impurities. As sodium phenylthiolate was used to accelerate the thiolysis reaction, a phenyl thioester was formed in 15% yield (Figure 2C). This byproduct will also induce the formation of products in native chemical ligation reactions.

The successful assembly of the benzyl peptide thioester **23b** showed that variations of the mercaptane involved in thiolysis are feasible (Table 1). Peptide **25** contained two methionine residues. The improved self-purifying synthesis proved robust and provided crude peptide thioester **25** in 24% yield and 76% purity. The crude material also contained minor amounts of sulfoxides and mixed disulfides with ethanedithiol which was used as scavenger in TFA cleavage (Figure S3A). Larger amounts of byproduct appeared when the solid phase synthesis was performed in the linear fashion at otherwise identical conditions for Fmoc-cleavage, coupling, capping, activation of the sulfonamide linker and subsequent thiolysis (Table 1, Figure S3B). The crude product **25** synthesized by the linear approach required HPLC purification not only because of truncation products, but also because of contaminations with reagents like NaSPh used in thiolysis of acyl−sulfonamide bond.

Difficult Peptides. The 29 residue peptide **26** is derived from the third SH3-domain of RIM-binding protein 2 (RIMB protein 2 (952−980)).[15] Application of the self-purifying synthesis provided **26** in 20% yield and 74% purity. The crude material was contaminated by minor amounts of n-2 truncation product (Figure S4). This was surprising, because the truncation products should elute from the synthesis column during the thiolysis (**15** → **16**) and subsequent washing steps. We assumed that fully protected truncation products can form insoluble aggregates. Their adsorption to the resin may hinder extraction by wash solvents routinely used in solid-phase synthesis. To ensure complete removal of truncation products, we optimized the washing protocol. As model peptide, we chose the segment

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Table 1. Comparison of Conventional Solid-Phase Peptide Thioester Synthesis at the Sulfonamide Resin and Synthesis with Self-Purification

		conventional synthesis		cyclization-activation	
	peptide sequence	purity of crude peptide[a]	yield after purification[b]	purity of crude peptide[a]	yield[b]
23a	DVPLPAGWEMAKTS-SEt	35%	18%	77%	31%
23b	DVPLPAGWEMAKTS-SBzl	–	–	91%	28%
25	AEGEFMRASRCNPNCPMWAGADPA-SEt	58%	36%	76%	24%
26	VSTRRMVALYDYDPRESSPNVDVEAELTF-SBzl	–	–	74%	20%
27	PSGTECIAKYNFHGTAEQDLPF-SBzl	–	–	45%/85%[c]	21%/13%[c]
28	GATAVSEWTEYKTADGK-SBzl	–	–	34%/65%[c]	9%/3%[c]

[a] Purity based on HPLC and detection at 220 nm. [b] Yield is calculated from first amino acid loading. [c] Purity and yield correspond to cyclization−activation approach with optimized extraction protocol.

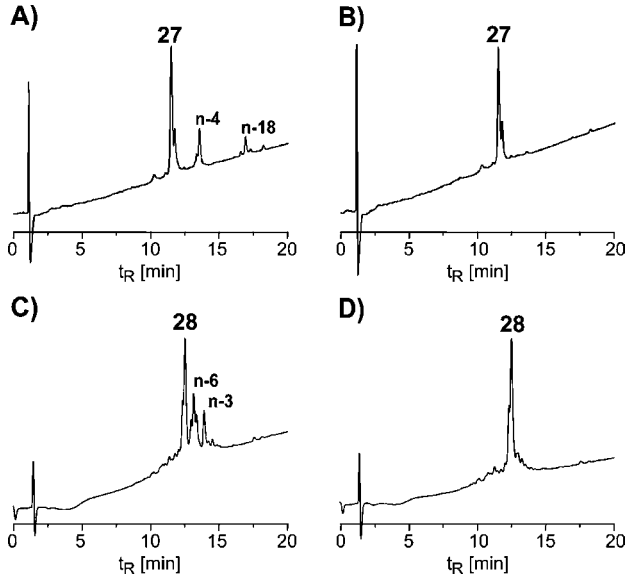

Figure 3. HPLC traces of peptide thioesters **27** and **28** resulting from self-purifying synthesis applying (A and C) a nonoptimized washing procedure after thiolysis. The crude products are contaminated by truncation sequences (**27**, n-4 and n-18, racemization observed; **28**, n-3 and n-6, deleted n-2 observed). (B and D) an optimized extraction protocol after thiolysis. Crude **27** and **28** are not contaminated by N-acylated truncated sequences (**27**, racemization observed; **28**, contamination by deleted n-2).

9−30 of SH3-domain of c-Src kinase (c-Csk) **27**.[16] Here, the problem of adsorption of the truncation product was particularly pressing. The cyclization−activation−thiolysis procedure provided **27** in 21% yield but only with 45% purity (Figure 3A) owing to n-4 and n-18 truncation products. The main product **27** appeared as double peak. Mass spectrometric analysis suggested partial racemization during thiolysis or coupling of the C-terminal phenylalanine with the sulfonamide resin, as previously reported.[17] This problem was not observed in the other peptide syntheses.

The efficiency of the wash protocol applied after the thiolysis step was assessed by means of HPLC analysis of the crude material obtained from the TFA cleavage solution. The original washing procedure involved 10 times repeated extraction by DMF, dichloromethane, methanol, again DMF, and finally dichloromethane, respectively. This led to a crude material which contained 19% n-4 truncation product. We evaluated 15 different solvent systems (Table S1). The use of DMSO, trifluoroethanol, and/or chaotropic salts[18] conferred only marginal improvements to the purity of peptide thioester **27** (Table S1, entries b−i). Aggregation of protected peptides in organic solvents is primarily driven by hydrogen bonding of amide functions. We assumed that protonation of the hydrogen bond acceptors would disrupt the aggregates. Indeed, the amount of truncation product was reduced by 90% when mild acidic extractions (DMSO, CH_2Cl_2/1% TFA, DMF, CH_2Cl_2/1% TFA) were included in the washing protocol (Figure 3B; Table S1, entry k). To prove the generality, we examined the synthesis of a difficult peptide. Recent studies have shown that the WW domain of the formin-binding protein 28 (FBP28) cannot be obtained through conventional methods of solid-phase peptide synthesis.[19] We obtained the FBP28 (1−17) fragment **28** in 9% yield and 34% purity when the original wash procedure was used in the self-purification method.[10] The main impurities derived from a n-2 deletion product and from acetylated truncation products (Figure 3C). The use of the optimized wash protocol conferred a dramatic improvement. HPLC analysis revealed the complete removal of the truncation products (Figure 3D). The nonacetylated n-2 deletion product represented the main impurity. As expected, the synthesis yield remained low (3%). Of note, the purity (65%) reached a level that is sufficient for the direct use of crude **28** in native chemical ligation reactions.

Automated Synthesis. Automated synthesis is user-friendly and a requirement if high-throughput is desired. Many of the synthesis automats used in parallel peptide synthesis rely on open pipetting devices. The exposure of reagents, solvents, and reaction vessels to moisture and oxygen is not strictly excluded. For example, the air sensitivity of $Pd(PPh_3)_4$ required for the removal of the Aloc/Allyl protecting groups suggested changes of the protecting group pattern. The use of *N*-monomethyltrityl(Mmt)-protected cyclization linker **3b** and the 2-phenylisoproyl(PhiPr)-ester for protection of the glutamic acid side chain in resin **11** appeared as a viable alternative. Furthermore, the employment of the volatile dichloromethane shall be avoided in time-consuming reaction steps, in order to avoid drying of the resin. For this reason, the automated macrocyclization (→ **15**) is performed in DMF rather than in dichloromethane. The protocol for automated synthesis was developed by using the minimal sequence of the osteogenic growth peptide (OGP (10−15): GYGFGG, **29**) as target and the *MultiPep RS* parallel

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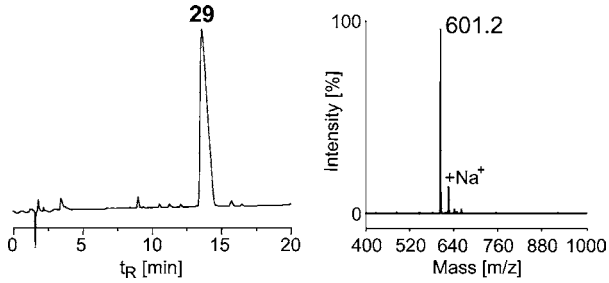

Figure 4. HPLC trace and ESI-MS of peptide thioester **29** obtained by automated self-purifying solid phase synthesis. (For details see Supporting Information).

peptide synthesizer.[20] Each step was optimized independently on the synthesizer while the remaining steps were performed manually. For the assembly of the peptide chain at the sulfamylbutyryl-γ-(phenyl-isopropyl)glutamyl-glycyl-polystyrene resin, we applied a commonly used Fmoc-based peptide synthesis protocol. No significant changes were necessary to achieve the automated coupling of the cyclization linker **3b**. The reaction time was reduced to 2 h and double couplings were employed. The subsequent treatment with 1% TFA in dichloromethane led to the simultaneous removal of the Mmt- and the PhiPr-group. However, this step required special care in order to avoid contaminations of the acidic cleavage solution by DMF. If DMF was used as washing solvent for the dispenser needle, a high excess volume of deprotecting solution was needed to prevent a mixing with DMF within the dispenser tube. Automated macrocyclization proceeded remarkably smoothly. The optimized synthesis protocol was applied in the automated assembly of the ethyl thioester of **29**. The peptide thioester was obtained in 66% yield and high 93% purity (Figure 4).

Parallel Synthesis of Immobilized SH3 Domains and Cysteine Scan. Parallel formats of chemical peptide synthesis, particularly those that proceed on array platforms, have facilitated the high-throughput analysis of protein functions. A number of methods allow the preparation of arrays that display 10−20 residue long peptides.[21] Recently, it has been shown that native chemical ligation provides access to chemically synthesized protein domain arrays.[2] However, only the cysteinyl peptide was subjected to randomization. The involvement of nonautomatable steps and the cumbersome purification have complicated high-throughput sequence variation of the peptide thioester segment. It was our aim to demonstrate a parallel format of the self-purifying synthesis of peptide thioesters. In addition, we wanted to explore whether the divergent native chemical ligation of crude peptide thioesters with crude cysteinyl peptides will provide access to an array of functional protein domains.

Native chemical ligation chemistry requires cysteine residues at the N-terminus of C-terminal peptide fragment. Unfortunately, cysteine is a comparatively rare amino acid and a number of protein domains lack cysteine. For example, only 7 out of the 27 Src homology 3 (SH3) domains of yeast contain cysteine. In such a case, one may either attempt extended native chemical ligation reactions[22] or resume to the substitution of select amino acids by an artificial cysteine. Although the latter approach is probably more common, the identification of a suitable site for the cysteine exchange is not trivial. The artificial cysteine residue should (a) confer useful ligation yields, (b) support correct folding, and (c) sustain the function of the folded protein domain. We expected that an unbiased cysteine scan would facilitate the identification of suitable cysteine exchange sites. This would probably be a valuable asset in those cases where structural data of the targeted protein domain is not available.

We selected the SH3-domain of SHO1 in yeast as a target that lacks native cysteine residues (Figure 5A).[23] It seemed reasonable to synthesize the 62-mer protein by the native chemical ligation of 20−40 amino acid long fragments. Hence, a cysteine walk would involve residues 21−41. The I22C, Q26C, I28C, I35C, and G37C exchanges were excluded in order to avoid problematic Asp−Cys and Glu−Cys ligations.[24] The remaining 16 peptide thioesters were prepared on a parallel peptide synthesizer using the cyclization−activation−thiolysis method described in Scheme 1B. A biotinylated lysine building block was coupled to the N-terminus prior to the coupling of the cyclization linker **3b**. The biotin label was included to facilitate the quantification of immobilized protein domains (vide infra). Interestingly, the obtained yields (3−24%) did not correlate with the length of the synthesized peptide thioesters. In a second run, we changed the positions of the synthesis columns in the parallel synthesizer; however, the yields were reproducible. This indicates sequence specific properties such as a different accessibility to thiolytic cleavage and/or alkylation of the sulfonamide structure. In principle, low yields could also be caused by inefficient macrocyclization reactions. However, we wish to note that macrocyclization is not a strict requirement for "self-purification". Pseudointermolecular cross-linking will confer similar properties. Most importantly, the HPLC analysis of the obtained crude products revealed good to excellent purity (Figure 6). Regardless of their length, the desired peptide thioesters showed up as main compounds. Out of the 16 peptide thioesters examined, only **31l** showed major contaminations. The results suggested that the cyclization−activation−thiolysis method furnishes crude materials that can be used directly in native chemical ligation reactions.

The cysteine peptides **32** were assembled by linear parallel solid-phase peptide synthesis at the rink amide resin. The C-terminal His_6-tag was included to allow immobilization to nickel coated surfaces. The multiple arginine residues required optimization of the acidolytic cleavage (TFA/TIS/Thioanisole/*m*-Cresol (90/4/4/2); 6 h) to avoid incomplete removal of the Pbf side chain protecting group. We deliberately omitted HPLC purification and examined native chemical ligation of crude materials. We reckoned that only the full-length products **32** would be amenable to ligation. In addition, the omission of HPLC purification eliminates a bottleneck and significantly increases the speed of the cysteine scan. The crude cysteine peptides **32** were allowed to react with the corresponding peptide thioester **31**. The ligation reactions were carried out in a

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Figure 5. (A) Native sequence of the SH3-domain of SHO1 (1−62). Within the central sequence region (highlighted in gray), we identified 16 possible ligation sites (framed residues). (B) Cysteine-scan in SH3-domain of SHO1 (1−62). After native chemical ligation, the SH3-domain variants were immobilized to Ni^{2+}-coated well plates via the His_6-tagged C-terminus. The N-terminal biotinylated lysine enables quantification of immobilization by means of HRP-SA recruitment.

degassed phosphate buffer containing 6 M guanidinium hydrochloride and 100 mM NaH_2PO_4 at pH 7.4 at 2 mM concentration of peptides. To maintain reductive conditions and to accelerate the peptide coupling, 20 mM TCEP and 3% thiophenol was included. The reactions were stopped after 24 h by addition of 5% formic acid. The obtained His_6-tagged protein material was immobilized to Ni^{2+}-coated well plates. This facilitated the rapid assessment of synthesis yields as well as functional properties of the SH3-domain variants. After stringent washing, the wells were incubated with a horseradish peroxidase−streptavidin conjugate (HRP-SA). Only in wells with full-length ligation products **30** HRP-SA will be immobilized by the streptavidin−biotin interaction. HRP catalyzes the oxidation of the chromogenic substrate tetramethylbenzidine (TMB) to the colored quinoid compound. The enzymatic reaction was stopped by addition of 1 M H_2SO_4.

The absorbance at 450 nm measured by using a plate reader (Figure 7) was used to calculate the loading (see: Supporting Information). To prevent unspecific binding of the biotinylated peptide thioesters to the well surface, Roche Blocking Reagent was used. Indeed, the absorbance (0.151) measured after mock ligation/immobilization with **31o** in absence of cysteine peptide remained at background level (0.148). Most ligation reactions provided useful loadings. Low signal intensities (absorbance < 0.5) were detected in wells with **30b**, **30c**, and **30l**. Apparently, these ligation reactions were low yielding given the applied reaction conditions. The synthesis of **30b** and **30l** involved ligation at β-branched isoleucine thioesters, which usually proceed slowly. At first glance, it may seem surprising that the Ser−Cys ligation product **30c** was formed in poor yield. However, low signal intensities cannot only be caused by slow ligation reactions, but also by low yields in the synthesis of cysteine peptides. Indeed, the mass spectrometric analysis (Figure S5) exposed that crude cysteine peptide **32c** was the minority compound in a mixture of truncation products. Nevertheless, the results of the absorbance measurements suggested that the ligation chemistry provided full-length SH3 proteins in all tested cases.

We subsequently investigated the functional properties of the immobilized SH3-domain variants in comparison to the recombinant protein. SHO1 represents an osmosensor which mediates activation of the high osmolarity glycerol (HOG) pathway in yeast.[25] This pathway requires recruitment of the SHO1 SH3-domain to a proline rich segment of the MAP kinase kinase PBS2 (90−104; IVNKPLPPLPVAGSS). We used the 5(6)-carboxyfluoresceine (FAM) labeled peptide FAM-βAla-βAla-IVNKPLPPLPVAGSS-NH_2, **33**, to assess the bioactivity of the SH3 cysteine mutants. The immobilized protein-domain variants were allowed to fold in PBS-buffer (pH 7.0) at 4 °C for 15 h. Each immobilized protein domain was incubated with increasing concentrations of **33** for 15 h at 4 °C and FAM fluorescence was determined until saturation was reached. The binding affinities were determined by nonlinear fitting of the obtained binding isotherms (Figure S7). Of note, this analysis is independent of the amount of immobilized protein required that saturation of binding is accomplished. The recombinant GST-SH3-domain was immobilized on a glutathione-coated well plate and analyzed in the same manner. The determined K_d-value for the recombinant protein was 2.2 ± 1.5 μM, which is within reasonable agreement with previously data obtained in solution (K_d = 1.3 μM).[25] Figure 8 lists the relative binding affinities of the SH3-domain variants **30a**−**p** for peptide **33**. Very low relative binding affinities (<5% of wild-type) were measured for cysteine variants **30a** (E21C), **30d** (E25C), **30j** (S33C), and **30p** (K41C). Apparently, cysteine placements at postions 21, 25, 33, and 41 were detrimental to protein folding and/or protein function. At this point, we would like to refrain from a detailed analysis of the binding behavior of the cysteine mutants. The cysteine scan rather served the purpose to rapidly identify suitable positions for the introduction of artificial cysteine residues. This suggested N27C (**30e**), L30C (**30g**), and D34C

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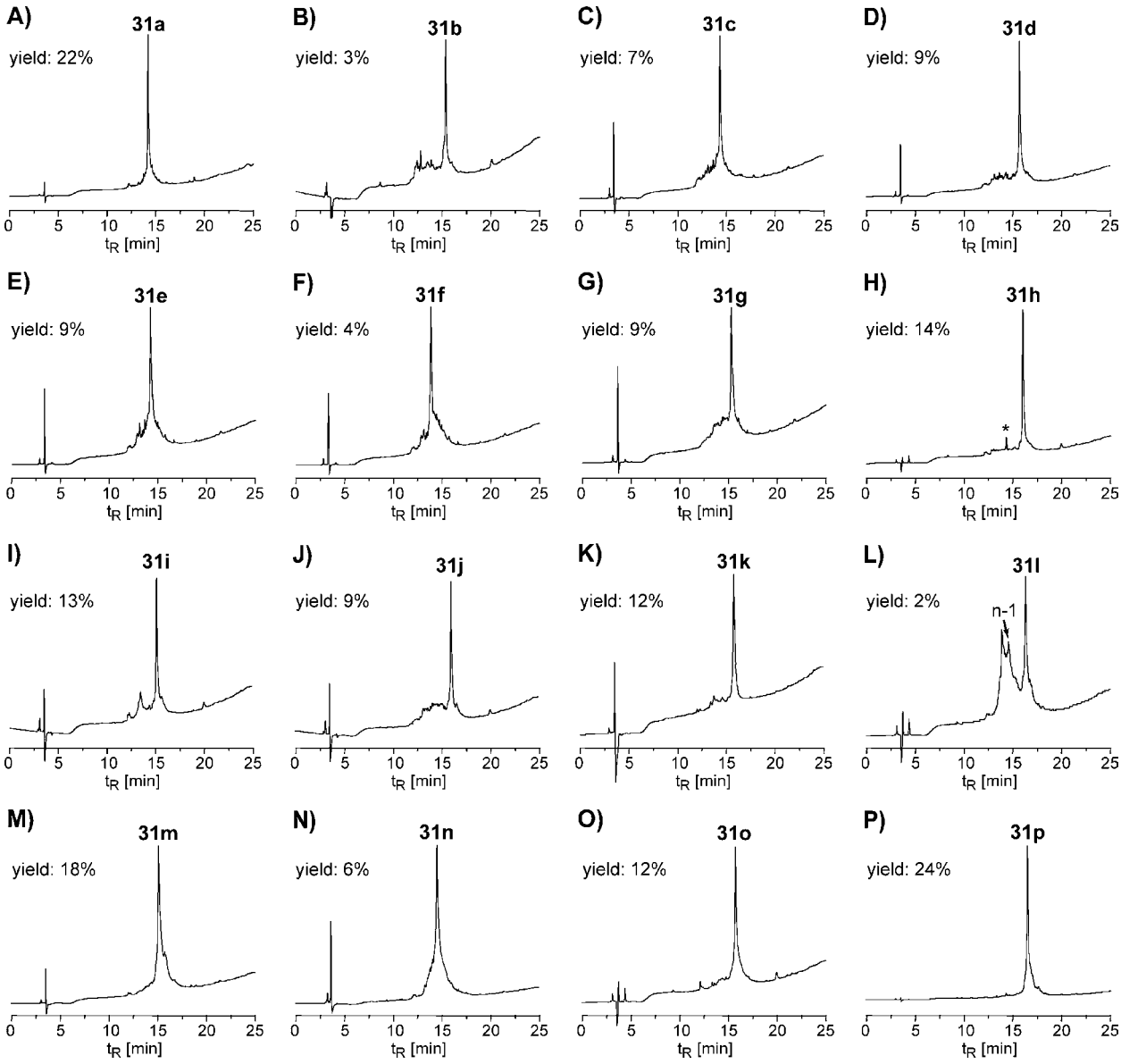

Figure 6. HPLC traces of peptide thioesters **31a**−**p** obtained by automated solid phase synthesis with self-purification according to Scheme 1B.

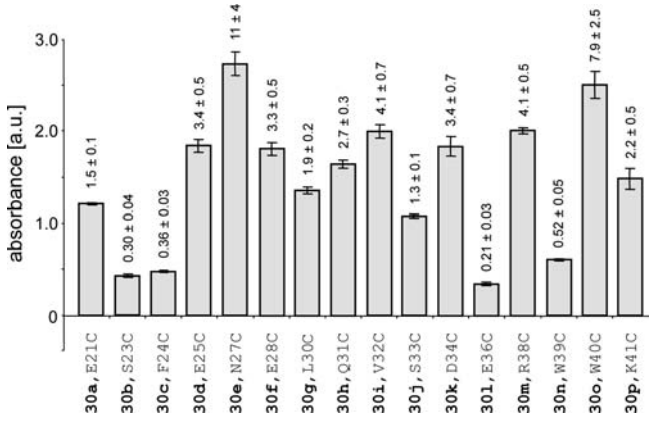

Figure 7. Colorimetric evaluation of native chemical ligation in cysteine-scan of the SH3 domain of SHO1 (λ = 450 nm). The background was measured with 80 nM **31o** in the immobilization step (absorbance: 0.151). The data is presented with background correction and is the mean of three independent experiments. The values at the top of the bars represent the amounts of immobilized SH3-domains in picomoles determined by using the nonlinear fit of a calibration curve (Supporting Information).

(**30k**) variants because these mutations conferred (a) useful synthesis yields while (b) maintaining ~50−100% of the activity of the recombinant wild-type protein. However, it did not escape our notice that the cysteine scan may provide insight into protein function. For example, crystal structure analysis has revealed that K41 is involved in protein domain folding by formation of a salt bridge to D34 (Figure S8). It is, hence, plausible that the K41C mutant **30p** failed to bind the proline-rich peptide **33**. The high binding affinity measured for **30k**, in which the corresponding D34 is replaced by a cysteine, could speak against an essential role of this interaction. However, the neighboring E36 can probably substitute D34 in the salt bridge. Moreover, D34 belongs to a flexible loop-region, whereas K41 is part of a rigid β-sheet-structure. The diminished bioactivity of the E21C mutant **30a** can be correlated to the removal of an essential salt bridge between E21 of the SH3 domain and K3 of the ligand. The highest binding activity (K_d = 2.4 μM, 93% of wild-type activity) was found for the L30C variant **30g**. This seems, at the first glance, surprising because L30 is part of the hydrophobic core (F24, L30,

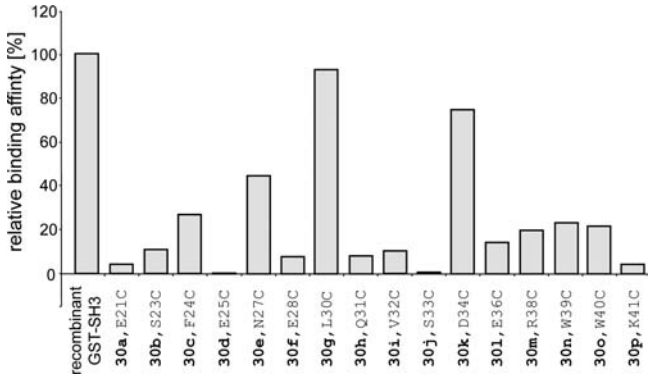

Figure 8. Relative binding affinities of synthetic SH3-domain mutants compared to recombinant GST-SH3-domain of SHO1. The K_d-values were determined by nonlinear regression of saturation binding curves. Conditions: 0.5, 3, 20, 70, and 100 μM FAM-βAla-βAla-IVNKPLPPLPVAGSS-NH_2, **33** in PBS-buffer, 3-fold washing with PBS-buffer. See Supporting Information for details.

V32, and W40), which stabilizes protein structure. However, the hydrophobicity of cysteine (hydrophobicity index 2.5) is comparable with phenylalanine (hydrophobicity index 2.8) and may, thus, be hydrophobic enough to not affect protein folding.

Conclusion

The commonly used methods for the Fmoc-based synthesis of peptide thioesters involve thioesterification reactions and/or acid treatment that have to be performed after the peptide has been detached from the solid-phase. HPLC-purification usually is required. We have developed an approach that avoids time-consuming solution steps and provides crude materials of high purity. The method relies on a sequence of cyclization−activation−thiolysis steps applied at a modified sulfonamide resin. The side chain of a glutamic acid residue, introduced between the sulfonamide linker and the polymeric resin, and an amino-functionalized cyclization linker allow macrolactamization (or pseudointermolecular cross-linking) of the resin-linked peptide. Similar cyclization reactions have previously been used in peptide-inversion protocols.[26] However, the potential for peptide thioester synthesis with self-purification has not been realized. The activation step includes a mild trimethyldiazomethane-induced alkylation of the sulfonamide function, which is fully compatible with nucleophilic amino acids such as methionine. The subsequent thiolysis reaction will cleave the N-alkylated acylsulfonamide and open the macrocycle. This step confers "self-purification" because only the full-length product remains bound to the solid phase while truncation products will be expelled into solution. The desired unprotected peptide thioesters are liberated upon a final TFA treatment, which is performed according to the routine of conventional, linear solid-phase peptide synthesis. The optimization of the key steps (coupling of cyclization linker, deprotection, and macrocyclization) allowed automation of the solid-phase synthesis method. The present study also addressed problems that occurred in the synthesis of difficult peptides. Repeated extractions with slightly acidic organic solvent systems removed impurities arising from fully protected truncation products.

Each step of our cyclization−activation−thiolysis method with self-purification effect can be included in the protocol used for automated synthesis. This distinguishes our method from previous approaches that are based on the reversible attachment of affinity tags to the N-terminus.[27] The use of affinity tags facilitates purification of the peptide. However, the need for special column supports and additional extraction steps have probably prevented the widespread use in solid-phase peptide synthesis.

It was our aim to demonstrate a parallel format of the self-purifying synthesis of peptide thioesters. In addition, we wanted to explore whether an array of protein domains can be prepared in rapid fashion via divergent native chemical ligation reactions of crude peptide thioesters and crude cysteine peptides. We employed a cysteine scan of the SH3-domain of SHO1 in yeast to identify positions for single amino acid to cysteine substitutions that (a) confer useful ligation yields, (b) support correct folding, and (c) sustain the function of the folded protein domain. The automated self-purifying solid-phase synthesis was used to prepare 16 biotinylated peptide thioesters which were obtained in excellent purities and subjected to native chemical ligation with crude His_6-tagged cysteine peptides. Only the full-length ligation products contained both the N-terminal biotin label and the C-terminal His_6 tag. Immobilization to Ni^{2+}-coated well plates and determination of the biotin enabled a rapid assessment of the synthesis yields. The obtained results suggested that the chemical synthesis of the SH3-domain of SHO1 succeeded most readily when cysteine placements at position 23, 24, and 36 are avoided. The data from a binding assay, performed by using a fluorescently labeled proline-rich peptide ligand, indicated that the substitution of residues 27, 30, and 34 by cysteine provides functional SH3-domains.

We propose that the cysteine scan may ensure the fast selection of cysteine mutants for the on-array synthesis of functional protein domains. One may envision that the cysteine scan can simultaneously serve two purposes, because synthesis yields as well as bioactivity of the synthesized protein domain can be assessed. Thus, the cysteine scan may enable a chemical means of single amino acid mutagenesis. Future work will be focused on the on-array synthesis protein domain as well as the synthesis of post-translationally modified protein-arrays.

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Supporting Information Available: Experimental details, analytical data including NMR-spectra, HPLC chromatograms and mass spectra for all peptides (**17**, **18**, **23**−**32**), and measured values of the peptide array synthesis and the binding-assay are reported; complete ref.[21d] This material is available free of charge via the Internet at http://pubs.acs.org.

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