Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Self-Purifying Solid-Phase Synthesis of Peptide Thioesters

Franziska Mende and Oliver Seitz

Institut für Chemie, Humboldt-Universität zu Berlin, Brook-Taylor-Str. 2, 12489 Berlin, Germany

Content:

1 Materials and methods

Fmoc-protected amino acids were purchased from Senn Chemicals (Dielsdorf, Switzerland). Resins and the sulfonamide linker were obtained by Novabiochem (Schwalbach, Germany). Coupling reagents were aquired by Iris Biotech (Marktredwitz, Germany) and Novabiochem. Furthermore chemicals were purchased from Acroc Organics, Sigma-Aldrich, Merck and ABCR. Water was purified with a Milli-Q Ultra Pure Water Putification System. TLC was performed with Merck Silica Gel 60 F254 plates. ^{1}H- and ^{13}C-NMR spectra were recorded with Bruker DPX 300 spectrometer. The signals of the residual protonated solvents ($CDCl_3$ and DMSO-d_6) were used as reference signal. High resolution mass spectra (HRMS) were measured with a Finnigan LTQ FT (ESI+) spectrometer.

Manual solid phase synthesis was performed by using 2 mL polyethylene syringe reactors (from MultSynTech) equipped with a fritted disc. Automated linear solid-phase Fmoc-synthesis was performed by using an Intavis ResPep parallel synthesizer equipped with micro scale columns.

Analytical HPLC was performed by using an Agilent 1100 series instrument (column: Machery-Nagel Gravity C18 A 3µ 125/4) and solvents A (98.9% H_2O, 1% acetonitrile, 0.1% formic acid) and B (98.9% acetonitrile, 1% H_2O, 0.1% formic acid) in a linear gradient (3% B - 50% B in 20 min at 55°C) with a flow rate of 1 mL/ min. Detection was achieved with a UV-VIS-detector at wavelength λ=210 nm.

Analytical HPLC measurements for reaction monitoring of native chemical ligation were performed by using a Merck-Hitachi Elite LaChrom chromatograph (column: Varian Polaris C18 A 5µ 250/4) and solvents A (98.9% H_2O, 1% acetonitrile, 0.1% TFA) and B (98.9% acetonitrile, 1% H_2O, 0.1% TFA) in a linear gradient (3% B – 80% B in 20 min at 55°C) with a flow rate of 1 mL/ min. Detection was achieved with a UV-VIS-detector at wavelength λ=220 nm.

Crude peptides obtained by conventional synthesis protocols were purified by semi preparative HPLC, performed on an Agilent 1100 series instrument (column: Varian Polaris C18 A 5µ 250/ 100) using A (98.9% H_2O, 1% acetonitrile, 0.1% TFA) and B (98.9% acetonitrile, 1% H_2O, 0.1% TFA) in a linear gradient (3% B - 50% B in 20 min at 55°C) with a flow rate of 6 mL/ min. Detection of the signals was achieved with a UV-VIS-detector at wavelength λ=210 nm.

MALDI-TOF mass spectra were recorded with a Voyager-DE^{TM} Pro Biospectrometry Workstation of PerSeptive Biosystems (matrix: dihydroxy benzoic acid and sinapinic acid).

2 Synthesis of 4´-(2-*Aloc*-aminoethoxy)benzyl-4-nitrophenyl carbonate (1)

Synthesis of ethyl 4-(carbamoylmethoxy)benzoate

To a solution of 1 g (6.02 mmol) ethyl 4-hydroxybenzoate in 20 mL DMF was added 250 mg NaOH (1 eq, 6.02 mmol). After stirring at room temperature for 1 h 1.34 g iodoacetamide (1.2 eq, 7.22 mmol) was transferred to the solution. The reaction reached completion after stirring for 20 h at room temperature. For work-up 60 mL of ethyl acetate was added which was followed by two washings with 20 mL H_2O and subsequent drying over anhydrous $MgSO_4$. After removing the solvent *in vacuo* the solid was recrystallized in ethyl acetate to give a white crystalline solid in 90% yield (1.2 g, 5.42 mmol). TLC: R_f=0.26 (ethyl acetate). EI-MS: *m/z*=223.1 ($C_{11}H_{13}NO_4$ [M^+], calculated 223.1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ[ppm]=1.37 (t, *J*=7.1; 3H, C**H**$_3$CH$_2$OC(O)); 4.33 (q, *J*=7.1; 2H, C**H**$_2$OC(O)); 4.54 (s, 2H, C**H**$_2$C(O)NH$_2$); 6.29 (s, 1H, N**H**$_2$); 6.55 (s, 1H, N**H**$_2$); 6.94 (d, *J*=9.0, 2H, aromatic H-3, H-5); 8.02 (d, *J*=9.0, 2H, aromatic H-2, H-6). ^{13}C-NMR (75 MHz, $CDCl_3$): δ[ppm]=14.31 ($C_{p,}$, **C**H$_3$CH$_2$OC(O)); 60.85 (C_s, **C**H$_2$OC(O)); 66.95 (C_s, **C**H$_2$C(O)NH$_2$); 114.18 (2C_t, aromatic C-3, C-5); 124.44 (C_q, aromatic **C**C(O)OCH$_2$CH$_3$); 131.75 (2C_t, aromatic C-2, C-6); 160.47 (C_q, aromatic **C**OCH$_2$C(O)NH$_2$); 165.97 (C_q, ester); 170.35 (C_q, amide)

Synthesis of allyl 2-(4-(hydroxymethyl)phenoxy)ethyl carbamate

To a solution of 1 g (4.48 mmol) 4-(carbamoylmethoxy)benzoate in absolute THF (50 mL) at -50°C was added dropwise 9.26 mL (3.6 eq, 32.4 mmol) of a 3.5 M suspension of $LiAlH_4$ in THF/ toluene. Thereafter, the reaction mixture was allowed to warm to room temperature and was stirred overnight. Addition of saturated Na_2SO_4 solution (2 mL) at 0°C resulted in the formation of $Al(OH)_3$ as white precipitate. The solid was separated by filtration and washed with water. The filtrate was concentrated to 100 mL. The same volume of 1,4-dioxane and, subsequently, 0.75 mL (5.35 mmol, 1.2 eq.) triethylamine were added. At 0°C 0.65 mL (5.35 mmol, 1.2 eq.) allyl chloroformate was added dropwise. After stirring at 0°C for 90 min the reaction

mixture was extracted (5x 20 mL ethyl acetate). The combined organic solutions were dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The crude product was purified by flash chromatography on silica gel. (hexane/ ethyl acetate (1:1)) to afford 53% (600 mg, 2.39 mmol) of allyl carbamate as a white solid. TLC: R_f=0.23 (hexane/ ethyl acetate (1:1)). EI-MS: *m/z*=251.3 ($C_{13}H_{17}NO_4$ [M^+], calculated 251.1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ[ppm]= 1.97 (s, 1H, O**H**); 3.50 (q, *J*=5.4, 2H, C**H**$_2$NH); 3.94 (t, *J*=5.4, 2H, CH_2CH_2NH); 4.49 (d, *J*=5.4, 2H, C**H**$_2$CH=CH_2), 4.53 (s, 2H, C**H**$_2$OH); 5.14 (dd, *J*=1.5, 10.5, 1H, CH_2=CHC**H**$_2$); 5.23 (dd, *J*=1.8, 17.1, 1H, C**H**$_2$=$CHCH_2$); 5.83 (m, 1H, C**H**=CH_2); 6.79 (d, *J*=8.7, 2H, aromatic H-2, H-6); 7.20 (d, *J*=8.7, 2H, aromatic H-3, H-5). ^{13}C-NMR (75 MHz, $CDCl_3$): δ[ppm]= 40.48 (C_s, **C**H_2NH); 64.81 (C_s, **C**H_2CH=CH_2); 65.67 (C_s, **C**H_2OH); 66.89 (C_s, **C**H_2CH_2NH); 114.40 (2C_t, aromatic, C-2, C-6), 117.78 (C_s, **C**H_2=$CHCH_2$); 128.61 (2C_t, aromatic, C-3, C-5), 132.67 (C_q, aromatic, **C**CH_2OH); 133.61 (C_t, **C**H=CH_2); 156.27 (C_q, **C**(O)OCH_2CH=CH_2), 157.96 (C_q, aromatic, **C**OCH_2CH_2NH)

Synthesis of 4´-(2-*Aloc*-aminoethoxy)benzyl-4-nitrophenyl carbonate

To a solution of 400 mg (1.6 mmol) allyl-2-(4-(hydroxymethyl)phenoxy)ethyl carbamate in 10 mL anhydrous CH_2Cl_2 at 0°C was added 156.7 μL (1.2 eq, 1.92 mmol) pyridine. A solution of 391.6 mg (1.1 eq, 1.74 mmol) 4-nitrophenylchloroformate in 8 mL anhydrous CH_2Cl_2 was added dropwise. After stirring at room temperature for 20 h the solvent was removed *in vacuo*. The residue was dissolved in diethylether. Unsoluble pyridinium salt was removed by filtration. The filtrate was then concentrated to dryness and the resulting solid was recrystallized (Et_2O/ hexane) to afford 96% (680 mg, 1.54 mmol) of carbonate as a white solid. TLC: R_f=0.67 (hexane/ ethyl acetate (1:1)). HRMS: *m/z*=439.1112 ($C_{20}H_{20}N_2O_8$ [M^+], calculated 439.1112). ^{1}H-NMR (300 MHz, $CDCl_3$): δ[ppm]= 3.61 (q, *J*=5.1, 2H, C**H**$_2$NH); 4.05 (t, *J*=5.1, 2H, C**H**$_2CH_2$NH); 4.58 (d, *J*=5.4, 2H, C**H**$_2$CH=CH_2); 5.21 (dd, *J*=0.9, 8.7, 1H, C**H**$_2$=$CHCH_2$), 5.23 (s, 2H, C**H**$_2$OH); 5.31 (dd, *J*=1.5, 17.1, 1H, C**H**$_2$=$CHCH_2$); 5.92 (m, 1H, C**H**=CH_2); 6.91 (d, *J*=8.7, 2H, aromatic, H-3, H-5); 7.38 (d, *J*=8.7, 2H, aromatic, H-2, H-6); 7.36 (d, *J*=9.0, 2H, aromatic (NP), H-2, H-6); 8.26 (d, *J*=9.0, 2H, aromatic (NP), H-3, H-5). ^{13}C-NMR (75 MHz, $CDCl_3$): δ[ppm]= 40.64 (C_s, **C**H_2NH); 65.91 (C_s, **C**H_2CH=CH_2); 67.15 (C_s, **C**H_2CH_2NH); 70.99 (C_s, **C**H_2OH); 114.79 (2C_t, aromatic, C-3, C-5), 118.04 (C_s, **C**H_2=$CHCH_2$); 121.95 (2C_t, aromatic (NP), C-2, C-6), 125.45 (C_s, aromatic (NP), C-3,

C-5); 126.94 (C_q, aromatic, **C**CH_2O); 130.87 ($2C_t$, aromatic, C-2, C-6); 132,87 (C_t, **C**$H=CH_2$); 145.51 (C_q, aromatic (NP), **C**NO_2), 152.61 (C_q, **C**$(O)OC_6H_4NO_2$), 155.69 (C_q, **C**$(O)OCH_2CH=CH_2$); 156.43 (C_q, aromatic, **C**OCH_2CH_2NH); 159.30 (C_q, aromatic (NP), **C**$OC(O)OCH_2C_6H_4$)

3 Synthesis of iodoacetic allylester (4)

The synthesis of iodoacetic acid was performed by reaction of allyl trichloroacetimidate and iodoacetic acid as described in literature.[1-3]

4 Solid Phase Peptide Synthesis

4.1 Synthesis protocols

Manual solid-phase synthesis according to the Fmoc/ tBu-strategy

Fmoc cleavage: DMF/piperidine (4:1, 1000 μL) was added to the resin. After 5 min, the procedure was repeated once. The resin was washed (5x 1 mL DMF, 5x 1 mL CH_2Cl_2, 5x 1 mL DMF).

Coupling: 4 eq (corresponding to resin loading) amino acid derivative was dissolved in DMF (final concentration 0.1 M) and then preactivated for 4 min by addition of HCTU (3.6 eq), HOBt (4 eq) and DIPEA (8 eq). The solution was then added to the resin. After 30 min, the resin was washed (5x 1 mL DMF, 5x 1 mL CH_2Cl_2, 5x 1 mL DMF).

Capping: Ac_2O/ pyridine (9:1, 1 mL) was added to the resin. After 5 min the resin was washed (5x 1 mL DMF, 5x 1 mL CH_2Cl_2, 5x 1 mL DMF).

Automated solid-phase synthesis according to the Fmoc/ tBu-strategy

Automated solid-phase synthesis was performed in 5 μmol and 10 μmol scales. The amounts of reagents in the described synthesis protocol correspond to 5 μmol scale.

Fmoc cleavage: DMF/piperidine (4:1, 300 μL) was added to the resin. After 2 min, the procedure was repeated once. The resin was then washed with DMF (2x 320 μL, 3x 250 μL, 2x 200 μL).

Coupling: A preactivation vessel was charged with a 0.5 M TCTU solution in DMF (5.4 eq corresponding to resin loading), a 4 M NMM solution in DMF (12 eq) and a 0.4 M amino acid derivative solution in NMP (6 eq). After 2 min the solution was

added to the resin. After 30 min, the resin was washed with DMF (2x 200 μL). In synthesis of longer peptides coupling step was repeated as of 15th reaction cycle.

Capping: Ac_2O/2,6-lutidine/DMF (5:6:89, 200 μL) was added. After 2 min the resin was washed with DMF (3x 200 μL).

At the end of linear solid phase peptide assembly, resin was washed with DMF (2x 600 μL, 3x 500 μL, 2x 400 μL) and with ethanol (3x 400 μL).

4.2 Optimization of self-purifying peptide thioester synthesis at sulfonamide-trityl resin

General procedure

Synthesis of double linker resin: 100 mg of trityl resin was allowed to swell for 1 h in 1 mL anhydrous CH_2Cl_2. A solution of 1.2 eq 4-sulfamylbutanoic acid (final concentration 0.4 M in anhydrous CH_2Cl_2) and 4.8 eq DIPEA was added to the resin. After 4 h the resin was washed (5x 1 mL CH_2Cl_2/ MeOH/ DIPEA (17:2:1), 5x 1 mL CH_2Cl_2, 5x 1 mL DMF, 5x 1 mL CH_2Cl_2) and dried *in vacuo*.

Preloading of 4-sulfamylbutyryl-trityl resin: Resin loading was performed with the Fmoc-protected glycine (ca. 0.4 mmol/g) according to the standard protocol (see: NovaBiochem Catalog 2006/ 2007).

Solid-phase peptide synthesis at the sulfonamide-trityl resin: As model sequence for optimization of synthesis strategy the minimal sequence of the osteogenic growth peptide Gly-Tyr-Gly-Phe-Gly-Gly was chosen. Solid-phase synthesis was carried out as described. The cyclizationlinker was introduced to resin-bound peptide as glycine conjugate.

Cleavage of peptidylsulfonamide from trityl resin: Resin-bound peptide was treated with diluted TFA (1% in CH_2Cl_2, 1 mL/ 100 mg resin, 2 min). The solution was filtered and subsequently quenched in pyridine/ MeOH (1:9, 200 μL/ 100 mg resin). This procedure was repeated eight to ten times. The combined fitrates were concentrated *in vacuo*. Subsequent precipitation in diethylether furnished the fully protected peptidylsulfonamide.

Optimization of activation of acyl-sulfonamide bond

The N-acetylated peptide was synthesized in 5 μmol scale as described above. Activation was performed with 25 eq, 50 eq and 100 eq of iodoacetic allylester dissolved in DMF to final concentrations of 2 M, 4 M and 8 M. After filtration through a plug of basic aluminium oxide 10 eq, 15 eq and 25 eq of DIPEA were added.

Thereafter, the solution was transferred to resin-bound peptide. The reaction was always performed under protection from light. After 24 h resin was washed (5x 1 mL DMF, 10x 1 mL CH_2Cl_2). After cleavage from trityl resin a mixture of activated and non-activated peptidylsulfonamide was isolated. The product was dissolved in acetonitril/ H_2O (1:1) and then characterized by HPLC and MALDI-TOF-MS measurements. Activated peptidylsulfonamide: HPLC: t_R=25.38. MALDI-TOF-MS: *m/z*=925.5 ($[M+Na]^+$, calculated 925.0). $C_{41}H_{55}N_7O_{14}S$ (MW=901.35); Non-activated peptidylsulfonamide: HPLC: t_R=21.61. MALDI-TOF-MS: *m/z*=827.4 ($[M+Na]^+$, calculated 826.9). $C_{36}H_{49}N_7O_{12}S$ (MW=803.32).

Yields of activated peptidylsulfonamide:	25 eq iodoacetic allylester	53%
	50 eq iodoacetic allylester	70%
	100 eq iodoacetic allylester	94%

Optimization of deallylation and subsequent macrocyclization

Deallylation by addition of dimethyl amino borane complex and subsequent macrocyclization: The peptide was synthesized in 5 μmol scale. After activation of acyl-sulfonamide bond, resin was washed twice with anhydrous degassed CH_2Cl_2. Under argon, a saturated solution of $Me_2NH \cdot BH_3$ in degassed CH_2Cl_2 was transferred to the resin-bound peptide. Furthermore, a 0.02 M solution of 1 eq $Pd(PPh_3)_4$ in anhydrous and degassed CH_2Cl_2 was added. After 10 min the resin was washed (5x 1 mL CH_2Cl_2, 5x 1 mL DMF, 5x 1 mL deg. CH_2Cl_2) and the procedure was repeated once for 20 min. Finally, resin was washed (5x 1 mL CH_2Cl_2, 5x 1 mL DMF, 2x 1 mL dioxane/ H_2O (9:1), 1x MeOH, 5x 1 mL DMF, 5x 1 mL. CH_2Cl_2). Subsequently, resin was treated twice with 8 eq PyBOP, 8 eq HOBt and 24 eq DIPEA (final concentration 0.1 M in CH_2Cl_2, 1 h) to form the desired macrocycle. After cleavage from solid support crude peptidylsulfonamide was characterized by MALDI-TOF-MS. The desired macrocycle and the tertiary amide formed by reaction of $Me_2NH \cdot BH_3$ and deprotected carboxylic group were detected (Figure S1 A). Peptidylsulfonamide macrocycle: MALDI-TOF-MS: *m/z*=1018.5 ($[M+Na]^+$, calculated 1018.1). $C_{46}H_{58}N_8O_{15}S$ (MW=994.37). By-product: MALDI-TOF-MS: *m/z*=1063.5 ($[M+Na]^+$, calculated 1063.1). $C_{48}H_{65}N_9O_{15}S$ (MW=1039.43).

Deallylation by addition of N,N'-dimethylbarbiturate (DMB) and subsequent macrocyclization: After peptide assembly in 5 μmol scale and subsequent activation of acyl-sulfonamide bond, resin was washed twice with anhydrous degassed CH_2Cl_2.

Under argon, a 0.2 M solution of DMB in degassed CH_2Cl_2 was transferred to the resin-bound peptide. Furthermore, a 0.02 M solution of 1 eq $Pd(PPh_3)_4$ in anhydrous degassed CH_2Cl_2 was added. After 30 min resin was washed (5x 1 mL CH_2Cl_2, 5x 1 mL DMF, 5x 1 mL deg. CH_2Cl_2) and the procedure was repeated once. Finally, resin was washed (5x 1 mL CH_2Cl_2, 5x 1 mL DMF, 2x 1 mL dioxane/ H_2O (9:1), 1x MeOH, 5x 1 mL DMF, 5x 1 mL. CH_2Cl_2). The deprotection was followed by macrocyclization under the described conditions. After cleavage from solid support, crude peptidylsulfonamide was characterized by MALDI-TOF-MS. By use of DMB as scavenger in deallylation only the desired macrocycle was detected (Figure S1 B). Peptidylsulfonamide macrocycle: MALDI-TOF-MS: *m/z*=1018.5 ($[M+Na]^+$, calculated 1018.1). $C_{46}H_{58}N_8O_{15}S$ (MW=994.37).

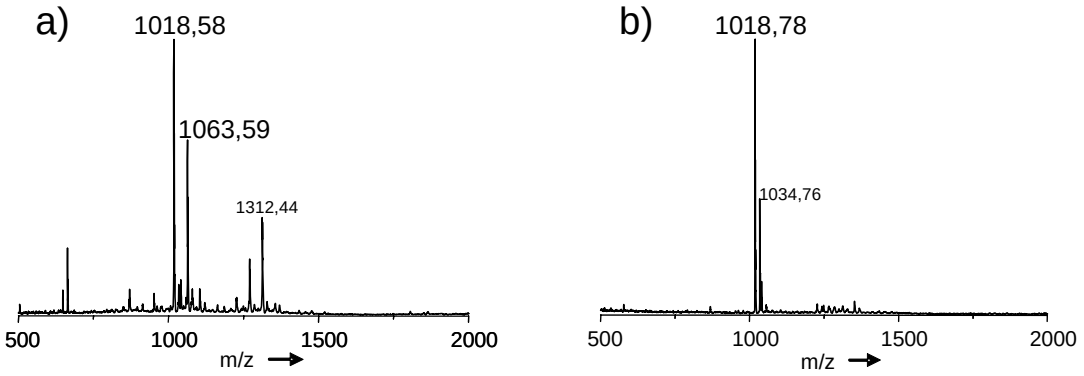

Figure S1. MALDI-TOF-MS of peptidylsulfonamide obtained after deallylation and macrocyclization when using A) $Me_2NH \cdot BH_3$ or B) N,N-dimethylbarbiturate as allyl scavenger.

Thiolysis of activated acyl-sulfonamide bond

Thiolysis was performed with ethylmercaptane according to Pessi's thioester synthesis strategy furnishing the resin-bound peptide thioester sulfonamide conjugate.[4] After cleavage from trityl resin the product was dissolved in acetonitril/ H_2O (1:1) and then characterized by HPLC and MALDI-TOF-MS measurements. Yield: 87%. HPLC: t_R=23.37. MALDI-TOF-MS: *m/z*=1079.5 ($[M+Na]^+$, calculated 1079.4). $C_{48}H_{64}N_8O_{15}S_2$ (MW=1056.39).

4.3 Conventional peptide thioester synthesis at the sulfonamide "safety-catch" resin

General procedure

Preloading of 4-sulfamylbutyryl resin: Resin loading was performed with the Fmoc-protected amino acids (ca. 0.2 mmol/g) according to the standard protocol (see: NovaBiochem Catalog 2006/ 2007).

Solid-phase peptide synthesis at the sulfonamide resin: Solid-phase synthesis was carried out as described above. Last coupled amino acid was N-Boc protected for blocking of the N-terminus until final cleavage of the peptide.

Activation and thiolysis of acyl-sulfonamide bond: Activation with iodoacetonitril and subsequent thiolysis of the sulfonamide bond were performed according to Pessi's thioester synthesis strategy.[4, 5]

Deprotection of amino acid side-chains: The isolated crude peptide was treated with a mixture of TFA/m-cresol/H_2O/EDT/TIS (85:5:5:2.5:2.5, 2 h). The TFA solution was then concentrated *in vacuo* following by precipitation in diethylether. After reprecipitation the crude product was characterized by HPLC- and MS-measurements, purified by semi preparative HPLC and then lyophilized.

Synthesis of ^{H_2N}*Gly-Tyr-Gly-Phe-Gly-Gly*^{COSEt} (**13**)

Peptide sequence was assembled via manual solid-phase Fmoc-synthesis in 30 μmol scale. The peptide thioester was synthesized according to the general procedure in 27% yield (5.5 mg, 9.2 μmol) after purification. HPLC: t_R=8.58. ESI-MS: *m/z*=601.3 ($[M+H]^+$, calculated 601.4). $C_{28}H_{36}N_6O_7S$ (MW=600.35).

Synthesis of ^{H_2N}*Leu-Asn-Glu-Leu-Asp-Ala-Asp-Glu-Gln-Ala-Asp-Leu*^{COSBzl} (**14**)

The peptide sequence was assembled via automated solid-phase Fmoc-synthesis in 5 μmol scale. The peptide thioester was synthesized according to the general procedure in 8% yield (0.6 mg, 0.4 μmol) after purification. HPLC: t_R=13.73. ESI-MS: *m/z*=1451.5 ($[M+H]^+$, calculated 1451.6). $C_{62}H_{94}N_{14}O_{24}S$ (MW=1450.6).

Synthesis of ^{H_2N}*Gly-Pro-Cys-Lys-Ala-Arg-Ile-Ile-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly*^{COSBzl} (**15**)

The peptide sequence was assembled via automated solid-phase Fmoc-synthesis in 5 μmol scale. The peptide thioester was synthesized according to the general

procedure in 6% yield (0.9 mg, 0.3 μmol). During purification cyclic thioesters caused by intramolecular thiol exchange were formed (Figure S2). So, peptide was obtained in only 68% purity. HPLC: t_R=10.36. MALDI-TOF-MS: *m/z*=3001.2 ([M+H]$^+$, calculated 3002.5). $C_{140}H_{208}N_{36}O_{32}S_3$ (MW=3001.49).

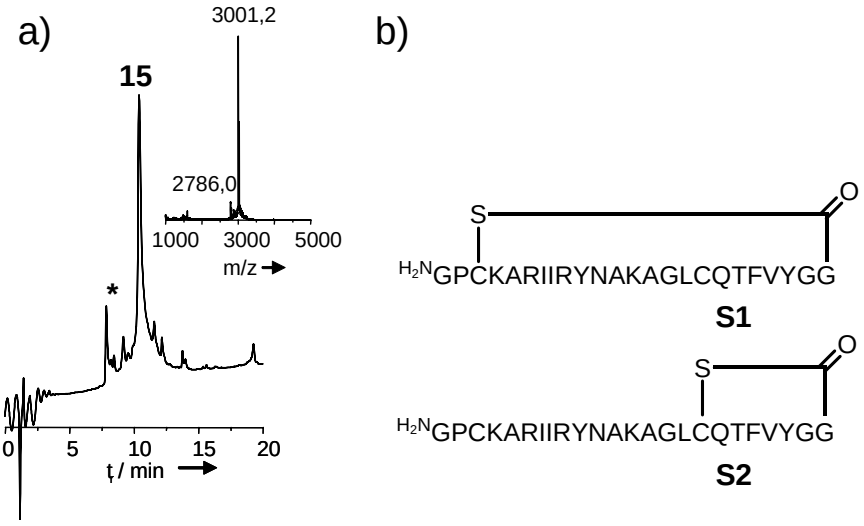

Figure S2. a) HPLC trace of 15 after HPLC purification of crude material obtained by linear synthesis (* cyclic thioesters); b) cyclic peptide thioesters formed by intramolecular thiol exchange.

Synthesis of ^{H_2N}Gly-Ala-Thr-Ala-Val-Ser-Glu-Trp-Thr-Glu-Tyr-Lys-Thr-Ala-Asp-Gly-Lys^{COSBzl} (**16**)

The peptide elongation was performed via automated solid-phase Fmoc-synthesis in 5 μmol scale. The peptidethioester was synthesized according to the general procedure. Desired peptide thioester could only be detected in traces in HPLC and ESI-MS. HPLC: t_R=8.57. ESI-MS: *m/z*=1919.8 ([M+H]$^+$, calculated 1919.9). $C_{86}H_{126}N_{20}O_{28}S$ (MW=1918.9).

Synthesis of ^{H_2N}Ala-Glu-Tyr-Val-Arg-Ala-Leu-Phe-Asp-Phe-Asn-Gly-Asn-Asp-Glu-Glu-Asp-Leu-Pro-Phe-Lys-Lys-Gly^{COSBzl} (**17**)

The peptide sequence was assembled via automated solid-phase Fmoc-synthesis in 5 μmol scale. The peptide thioester was synthesized according to the general procedure in 13% yield (1.8 mg, 0.7 μmol) after purification and 95% purity. HPLC: t_R=11.47. MALDI-TOF-MS: *m/z*=2782.5 ([M+H]$^+$, calculated 2781.3). $C_{128}H_{182}N_{30}O_{38}S$ (MW=2780.29).

4.4 Self-purifying peptide thioester synthesis at the sulfonamide "safety-catch" resin

General procedure

Preloading of 4-sulfamylbutyryl resin: Resin loading was performed with Fmoc-protected amino acid (ca. 0.2 mmol/g) according to the standard protocol (see: NovaBiochem Catalog 2006/ 2007).

Solid-phase peptide synthesis at the sulfonamide resin: Solid-phase synthesis was carried out as described above.

*Coupling of cyclization linker **1**:* After removal of Fmoc-group by treatment with DMF/ piperidine (4:1, 2x 5 min) and subsequent washing (5x 1 mL DMF, 5x 1 mL CH_2Cl_2, 5x 1 mL DMF) resin-bound peptide was reacted with 10 eq cyclization linker **1** (final concentration 0.1 M in DMF/ NEt_3 (95:5), 15h) and washed (5x 1 mL DMF, 5x 1 mL CH_2Cl_2, 5x 1 mL DMF). The resin was treated with pyridine/ Ac_2O (9:1, 1 mL, 5 min) followed by washing (5x 1 mL DMF, 5x 1 mL CH_2Cl_2, 5x 1 mL DMF).

Activation of acyl-sulfonamide bond: 100 eq of iodoacetic allylester (corresponding to resin-bound amounts of sulfonamide linker) were dissolved in DMF to a final concentration of 8 M. After filtration through basic aluminium oxide 25 eq DIPEA was added. The solution was transferred to the resin. During reaction, the syringe reactor was protected from light to avoid formation of hydroiodic acid. After 24 h resin was washed (5x 1 mL DMF, 5x 1 mL CH_2Cl_2, 5x 1 mL DMF).

Removal of Aloc- and Allyl-protecting groups: The resin was washed twice with anhydrous degassed CH_2Cl_2. Under argon, a 0.2 M solution of 10 eq N,N-dimethylbarbiturate in degassed CH_2Cl_2 was transferred to the resin-bound peptide. A 0.02 M solution of 1 eq $Pd(PPh_3)_4$ in anhydrous and degassed CH_2Cl_2 was added. After 30 min the resin was washed (5x 1 mL CH_2Cl_2, 5x 1 mL DMF, 5x 1 mL deg. CH_2Cl_2) and the procedure was repeated once. Finally, resin was washed (5x 1 mL CH_2Cl_2, 5x 1 mL DMF, 2x 1 mL dioxane/ H_2O (9:1), 1x MeOH, 5x 1 mL DMF, 5x 1 mL. CH_2Cl_2).

Macrocyclization: The resin was treated with 8 eq PyBOP, 8 eq HOBt and 24 eq DIPEA (final concentration 0.1 M in CH_2Cl_2). After 60 min the procedure was repeated once. The resin was washed (5x 1 mL CH_2Cl_2, 10x 1 mL DMF).

Thiolysis of acyl-sulfonamide bond: Thiolysis was performed with ethyl- or benzylmercaptane according to Pessi's thioester synthesis strategy.[4]

Final cleavage of peptide thioester from solid support and deprotection of amino acid side-chains: The resin-bound peptide was washed (5x 1 mL DMF, 10x 1 mL CH_2Cl_2). A mixture of TFA/ m-cresol/ H_2O/ EDT/ TIS (85:5:5:2.5:2.5, 2 h) was added. The TFA solution was then concentrated *in vacuo* following by precipitation in diethylether. The precipitation was repeated once. The crude product was characterized by HPLC- and MS-measurements and then lyophilized.

Synthesis of ^{H_2N}*Gly-Tyr-Gly-Phe-Gly-Gly*^{COSEt} (**13**)

The peptide sequence was assembled via manual solid-phase Fmoc-synthesis in 30 μmol scale. The peptidethioester was synthesized according to the general procedure in 38% yield (6.8 mg, 11.4 μmol) after purification. HPLC: t_R=8.59. ESI-MS: *m/z*=601.3 ($[M+H]^+$, calculated 601.4). $C_{28}H_{36}N_6O_7S$ (MW=600.35).

Synthesis of ^{H_2N}*Gly-Tyr-Gly-Phe-Gly-Gly*^{COSEt} *by enforcing formation of truncation product*

The peptide sequence was assembled via manual solid-phase Fmoc-synthesis in 10 μmol scale. In the last coupling step reduced amounts of cyclizationlinker-glycine conjugate (1 eq corresponding to resin loading) and coupling reagents (0.9 eq HCTU, 1 eq HOBt and 2 eq DIPEA) were used to force truncation product formation. As comparison, the peptide was also synthesized by conventional approach. As last amino acid derivative Boc-Gly was coupled under substoichiometric conditions (1 eq Boc-Gly, 0.9 eq HCTU, 1 eq HOBt and 2 eq DIPEA) to enforce formation of the truncation product n-1. The peptidethioester was synthesized according to the general procedure. In case of conventional method truncation product n-1 could be detected (Figure S3). Self-purifying method: ^{H_2N}*Gly-Tyr-Gly-Phe-Gly-Gly*^{COSEt}: HPLC: t_R=8.52. ESI-MS: *m/z*=601.3 ($[M+H]^+$, calculated 601.4). $C_{28}H_{36}N_6O_7S$ (MW=600.35); ^{H_2N}*Tyr-Gly-Phe-Gly-Gly*^{COSEt}*:* not detected. Conventional synthesis: ^{H_2N}*Gly-Tyr-Gly-Phe-Gly-Gly*^{COSEt}: HPLC: t_R=8.56. ESI-MS: *m/z*=601.3 ($[M+H]^+$, calculated 601.4). $C_{28}H_{36}N_6O_7S$ (MW=600.35); ^{H_2N}*Tyr-Gly-Phe-Gly-Gly*^{COSEt}*:* HPLC: t_R=13.36. ESI-MS: *m/z*=586.2 ($[M+H]^+$, calculated 586.3). $C_{28}H_{35}N_5O_7S$ (MW=585.34).

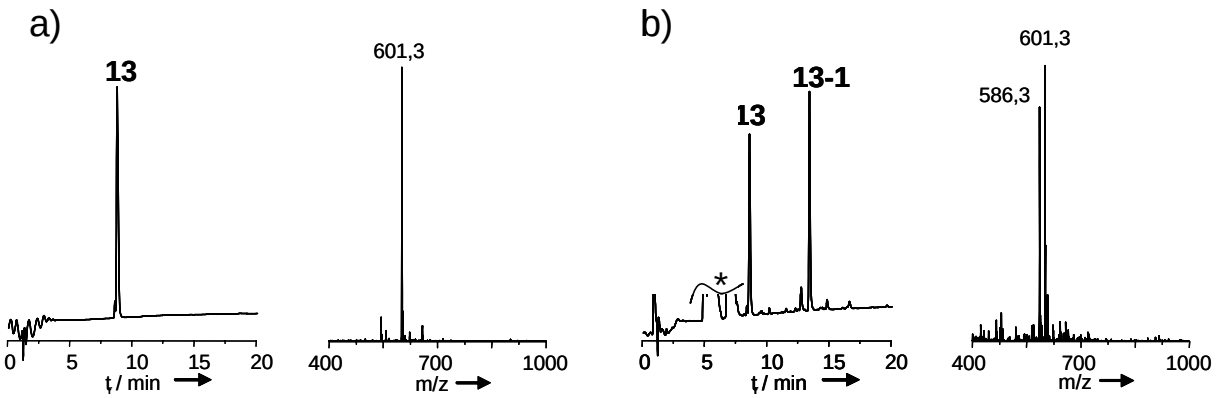

Figure S3. HPLC traces of crude thioester obtained by A) self-purifying peptide thioester synthesis and B) conventional synthesis.

Synthesis of ^{H_2N}*Leu-Asn-Glu-Leu-Asp-Ala-Asp-Glu-Gln-Ala-Asp-Leu*^{COSBzl} (**14**)

The peptide sequence was assembled via automated solid-phase Fmoc-synthesis in 5 μmol scale. The peptidethioester was synthesized according to the general procedure in 19.3% yield (1.4 mg, 0.97 μmol) in 99% purity. HPLC: t_R=13.61. ESI-MS: *m/z*=1451.6 ($[M+H]^+$, calculated 1451.6). $C_{62}H_{94}N_{14}O_{24}S$ (MW=1450.6).

Synthesis of ^{H_2N}*Gly-Pro-Cys-Lys-Ala-Arg-Ile-Ile-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly*^{COSBzl} (**15**)

The peptide sequence was assembled via automated solid-phase Fmoc-synthesis in 5 μmol scale. The peptidethioester was synthesized according to the general procedure. Complete removal of formed truncation products was difficult. To reduce their content it was important to employ intensive washing after thiolysis (10x 1 mL DMF, 10x 1 mL CH_2Cl_2, 10x 1 mL DMF, 10x 1 mL DMF (60°C), 10x 1 mL CH_2Cl_2) The peptide thioester was obtained in 18.7% yield (2.8 mg, 0.93 μmol) and in 77% purity. HPLC: t_R=10.38. MALDI-TOF-MS: *m/z*=3003.9 ($[M+H]^+$, calculated 3002.5). $C_{140}H_{208}N_{36}O_{32}S_3$ (MW=3001.49).

Synthesis of ^{H_2N}*Gly-Ala-Thr-Ala-Val-Ser-Glu-Trp-Thr-Glu-Tyr-Lys-Thr-Ala-Asp-Gly-Lys* ^{COSBzl} (**16**)

The peptide elongation was performed via automated solid-phase Fmoc-synthesis in 5 μmol scale. The peptidethioester was synthesized according to the general procedure. Complete removal of formed truncation products was difficult. To reduce their content it was important to employ intensive washing after thiolysis (10x 1 mL

DMF, 10x 1 mL DCM, 5x 1 mL DMF, 3x 1 mL Gn HCl, 10x 1 mL DMF, 10x 1 mL DCM, 5x 1 mL DMF, 10x 1 mL H_2O, 5x 1 mL MeOH, 10x 1 mL DMF, 10x 1 mL DCM) The peptide thioester was obtained in ~3% yield and in 54% purity. HPLC: t_R=8.59. ESI-MS: *m/z*=1919.8 ($[M+H]^+$, calculated 1919.9). $C_{86}H_{126}N_{20}O_{28}S$ (MW=1918.9).

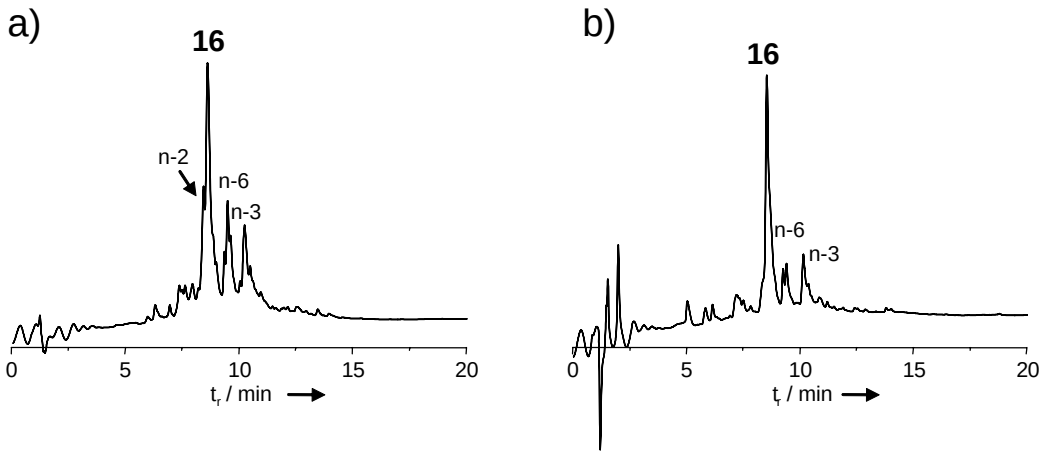

Figure S4. HPLC traces of **16**: A) no Gn · HCl washings after thiolysis step, B) Gn · HCl washings after thiolysis step.

Synthesis of ^{H_2N}*Ala-Glu-Tyr-Val-Arg-Ala-Leu-Phe-Asp-Phe-Asn-Gly-Asn-Asp-Glu-Glu-Asp-Leu-Pro-Phe-Lys-Lys-Gly*^{COSBzl} (**17**)

The peptide sequence was assembled via automated solid-phase Fmoc-synthesis in 5 μmol scale. The peptidethioester was synthesized according to the general procedure in 30.2% yield (4.2 mg, 1.51 μmol) and 98% purity. HPLC: t_R=11.35. MALDI-TOF-MS: *m/z*=2782.2 ($[M+H]^+$, calculated 2781.3). $C_{128}H_{182}N_{30}O_{38}S$ (MW=2780.29).

4.5 Solid-phase peptide synthesis at rink-amide resin

General procedure

Preloading of rink-amide resin: Resin loading was performed with the Fmoc-protected amino acids (ca. 0.2 mmol/g) according to the standard protocol (see: NovaBiochem Catalog 2006/ 2007).

Solid-phase peptide synthesis at the rink-amide resin: Automated solid-phase synthesis was carried out as described above.

Synthesis of cysteine peptides by using Fmoc-on/Fmoc-off approach: The N-terminal Fmoc-protected peptide was cleaved from the resin (TFA/ m-cresol/ H_2O/ EDT/ TIS (85:5:5:2.5:2.5), 2 h) and further extracted with TFA (2x 0.2 mL). The

combined TFA solutions were concentrated *in vacuo* followed by precipitation in diethylether. The N-Fmoc-protected crude product was purified by semi preparative HPLC. After removal of the eluent *in vacuo*, the Fmoc-group was cleaved by treatment with 300 μL piperidine/ H_2O (1:1, 5 min). Deprotection was followed by acidification with TFA (300 μL) and subsequent precipitation in diethylether. After repeated reprecipitation the peptide was dissolved in acetonitril/ H_2O (1:2, 0.1% TFA) and again purified by semi preparative HPLC. After removal of the eluent the pure product was lyophilized and characterized by HPLC and MALDI-TOF-MS measurements.

Synthesis of ^{H_2N}*Cys-Ile-Leu-Arg-Ile-Arg-Asp-Lys-Pro-Glu-Glu-Gln-Trp-Trp-Asn-Ala-Glu-Asp-Ser-Glu-Gly-Lys-Arg-Gly-Met-Ile-Pro-Val-Pro-Tyr-Val-Glu-Lys-Tyr-Gly*^{CONH_2} (**18**)

The cysteine peptide was assembled via automated solid-phase Fmoc-synthesis in 10 μmol scale in 6.6% yield (2.8 mg, 0.67 μmol). HPLC: t_R=16.30. MALDI-TOF-MS: *m/z*=4193.0 ($[M+H]^+$, calculated 4193.7). $C_{187}H_{288}N_{52}O_{54}S_2$ (MW=4192.73).

5 Synthesis of N-terminal SH3-domain of *c*-Crk protein by native chemical ligation

Synthesis of ^{H_2N}*Ala-Glu-Tyr-Val-Arg-Ala-Leu-Phe-Asp-Phe-Asn-Gly-Asn-Asp-Glu-Glu-Asp-Leu-Pro-Phe-Lys-Lys-Gly-Cys-Ile-Leu-Arg-Ile-Arg-Asp-Lys-Pro-Glu-Glu-Gln-Trp-Trp-Asn-Ala-Glu-Asp-Ser-Glu-Gly-Lys-Arg-Gly-Met-Ile-Pro-Val-Pro-Tyr-Val-Glu-Lys-Tyr-Gly*^{CONH_2} (**19**)

The peptide thioester and the cysteine peptide of N-terminal SH3-domain of c-Crk protein (residues 134-191) were dissolved in 80 μL (c=1 mM) in a degassed phosphate buffer (6 M GnHCl, 100 mM NaH_2PO_4, pH 7.5). To accelerate ligation benzylmercaptane (1%), thiophenol (3%) and TCEP (20 mM) were added. The reaction vial was placed in a shaker at 25°C. HPLC-monitoring showed that ligation nearly reached completion after 15 h. Subsequent HPLC purification furnished the SH3-domain in 53% yield. HPLC: t_R=17.40. MALDI-TOF-MS: *m/z*=6851.5 ($[M+H]^+$, calculated 6850.6). $C_{308}H_{462}N_{82}O_{92}S_2$ (MW=6849.59).

6 Abbreviations

Aloc	allyloxycarbonyl
Boc	*tert*-butoxycarbonyl
Cbz	benzyloxycarbonyl
CL	cyclization linker
DHB	dihydroxybenzoic acid
DIPEA	diisopropylethylamine
DMB	N,N-dimethylaminobarbiturate
DMF	dimethylformamide
eq	equivalents
Fmoc	9-fluorenylmethoxycarbonyl
HCTU	O-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxy-benzotriazole
HPLC	high performance liquid chromatography
MALDI	matrix assisted laserdesorption ionization
MS	mass spectrometry
NMP	1-methylpyrrolidine
NMR	nucleic resonance spectroscopy
NP	nitrophenyl
OGP	osteogenic growth peptide
PMB	*para*-methoxybenzyl
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
SPPS	solid-phase peptide synthesis
TCTU	O-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFA	trifluoro acetic acid
TOF	time-of-flight

7 Literature

[1] F. Cramer, K. Pawelzik, H. J. Baldauf, *Chem. Ber.* **1958**, 1049-1054.

[2] F. Cramer, N. Hennrich, *Chem. Ber.* **1961**, *94*, 976-989.

[3] G. Kokotos, A. Chiou, *Synthesis* **1997**, 168-170.

[4] R. Ingenito, E. Bianchi, D. Fattori, A. Pessi, *J. Am. Chem. Soc.* **1999**, *121*, 11369-11374.

[5] B. J. Backes, J. A. Ellman, *J. Org. Chem.* **1999**, *64*, 2322-2330.