Protocol

Journal of PeptideScience

Received: 5 August 2010 Revised: 20 October 2010 Accepted: 1 November 2010 Published online in Wiley Online Library: 3 February 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1336

Scope and limitation of side-chain assisted ligation

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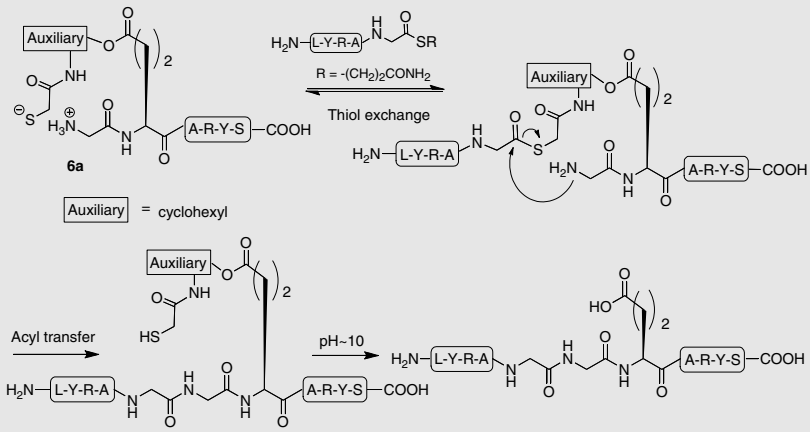
Side-chain assisted ligation is an auxiliary-mediated ligation strategy in which a thiol bearing cyclohexane or cyclopentane is attached to the side-chain of Asp, Glu, Ser or Thr to function in a similar manner to Cys in a native chemical ligation. Following the ligation step, the auxiliary is removed, without product isolation, under alkaline conditions. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide ligation; aminoacyl transfer; protein synthesis; native chemical ligation; removable auxiliary

REACTION SCHEME



GENERAL OPTIMIZED PROCEDURE

General procedure for side-chain assisted ligation: Unprotected peptide segments (1.00 eq of peptide **6a** and 1.20 eq of LYRAG-*SR*) (Reaction Scheme) were dissolved in 0.2 M phosphate buffer (pH 8.0, purged with argon for 10 min) containing 6 M Gn·HCl (denaturated reaction conditions) to a final concentration of 5 mM and incubated at 37 °C. The ligation attained completion after ~8 h and the auxiliary was removed by adjusting the pH of the ligation mixture to pH~10 using 1N NaOH, and was kept for 5 min at rt. The reaction was monitored using analytical reverse-phase HPLC on a C18 column (Jupiter 5 micron, 300A, 150 × 4.60 mm) and a linear gradient (0-90%B) over 15 min. Purification by preparative reverse phase HPLC on a C18 column (Jupiter 10 micron, 300A, 250 × 21.20 mm) using identical gradient afforded the unmodified ligation product **8** in 68 % isolated yield.

Scope and Comments

Native chemical ligation (NCL) continues to lead as the method of choice for chemical and semi-synthesis of proteins [1]. It also remains the basis for the quest of new chemical ligation methods [2–6], including the auxiliary-mediated ligation strategies, which show great potential in chemical protein synthesis [7–12]. We have recently reported a removable auxiliary-assisted cysteine-free ligation method so-called side-chain assisted ligation (SCAL) [13]. SCAL relies on principles similar to sugar-assisted ligation (SAL) [14,15], which is employed in glycopeptide ligation. In SCAL, a thiol bearing cyclohexane or a cyclopentane auxiliary is attached to the side-chain of Asp, Glu, Ser or Thr residues through an ester bond to mimic the function of N-terminal cysteine in NCL. The auxiliary assists ligation by allowing the capture of the thioester peptide through a transthioesterification step followed by a rapid and chemoselective S–N acyl transfer. After completion of ligation, *in situ* hydrolysis of the auxiliary (pH ~10) furnishes the unmodified peptide. Similar to SAL, the addition of one amino acid after the residue to which the auxiliary is attached facilitates the ligation as the reaction proceeds faster through 14/15 membered transition state (TS) compared to the 12-membered TS. The attachment of mercaptoacetic acid as an auxiliary reduces the rate of ligation significantly [13], emphasizing the role of the cyclic auxiliary to

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position the nucleophilic amine in a close proximity to the acyl group.

SCAL is an efficient method for peptide ligation due to the commercial availability of the template, easiness to couple to the side-chain of amino acid and the facile removal of the auxiliary without prior product isolation. The rate-limiting step in the SCAL is S–N acyl transfer; hence, it is possible to perform ligations under low peptide concentrations, which increases its potential in the synthesis of proteins.

Limitations

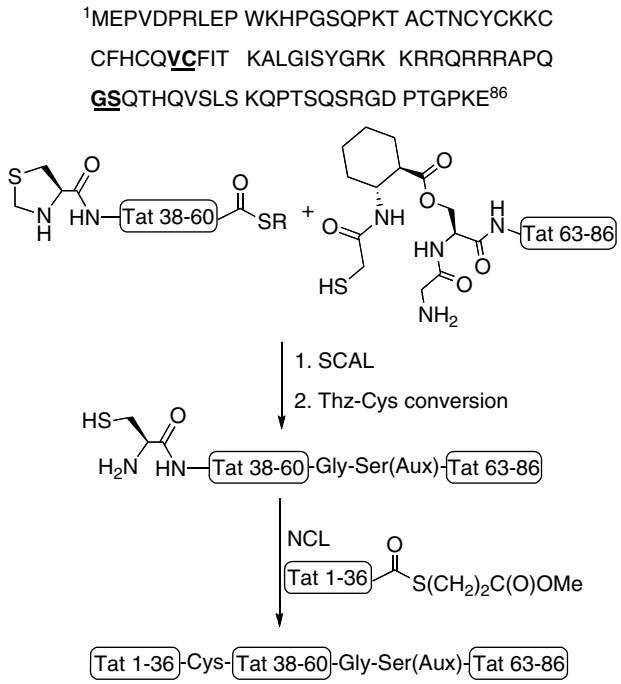
We attempted the total chemical synthesis of the regulatory protein HIV-1 Tat (86 residues) assisted by SCAL [16]. As shown in Scheme 1, SCAL was used to link the middle fragment, HIV-1 Tat(37–60), to the C-terminal fragment, HIV-1 Tat(61–86). The resultant ligation product was exposed to methoxylamine to covert the N-terminal Thz to Cys [17] affording HIV-1 Tat(37–86). Subsequently, employing NCL between fragments HIV-1 Tat(37–86) and HIV-1 Tat(1–36), the N-terminal fragment, yielded the full-length protein with auxiliary. Although the construction of the fragments using SCAL (60% isolated yield for the ligation and Thz removal steps) and NCL (70% isolated) was very successful, the removal of the auxiliary from the polypeptide was not fully accomplished. This could be due to a specific conformation that the polypeptide adopts under these conditions, which could mask the ester bond from saponification. A possible mean to overcome this issue is to use such an auxiliary that would allow ligation at pH 7 and could be detached from the side-chain through preferred intramolecular cyclization at basic pH. For example, an auxiliary that is derived from pipecolic acid would yield quinolizidone (fused bicyclic six-membered ring) in contrary to the cyclohexane-derived auxiliary, which could yield a less preferred seven–six fused bicyclic system. Another limitation is formation of aspartimide by-products, in particular when Asp is used to anchor the auxiliary, which could occur throughout the ligation and/or during the auxiliary removal steps. However, aspartimide formation is considerably reduced when the amino acid subsequent to Asp is sterically hindered. In principle, the use of backbone protection or cleavage of the ligation product with HF would also eliminate this side reaction.

Experimental Procedure

Synthesis of Building Block 4

Synthesis of 2

Compound **1** (1.00 g, 6.38 mmol) was added to a solution of S-trityl-2-mercaptoacetic acid (2.13 g, 6.38 mmol) with HBTU (2.53 g, 6.66 mmol) and DIEA (3.70 ml, 21.05 mmol) in dry DMF (15 ml). The reaction mixture was stirred at room temperature for overnight, diluted with ethyl acetate (40 ml) and washed with water and brine. The organic layer was dried over $MgSO_4$, concentrated and purified over flash column chromatography ($CHCl_3$/MeOH 20 : 1), to give **2** (1.30 g) in 50% yield. R_f 0.73 [$CHCl_3$–MeOH (10 : 1)]; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.92–1.35 (m, 4H), 1.55–1.75 (m, 3H), 1.9 (m, 1H), 2.79 (s, 2H), 3.09–3.20 (m, 2H), 3.28–3.38 (m, 1H), 6.07 (d, J = 6.8 Hz, 1H), 7.18–7.35 (m, 9H), 7.35–7.43 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 23.8, 24.3, 30.9, 34.0, 35.9, 55.9, 68.0, 74.8, 127.1, 128.1, 129.4, 143.9, 170.0; MALDI-TOF (matrix: α-Cyano-4-hydroxycinnamic acid): calcd. for $C_{27}H_{29}NO_2S.Na^+$, [M + Na]$^+$: 454.18 (monoisotope); $C_{27}H_{29}NO_2S.K^+$ [M + K]$^+$: 470.29 (monoisotope), found: 454.20, 470.16.



Scheme 1. The use of SCAL in the synthesis of HIV-1 Tat.

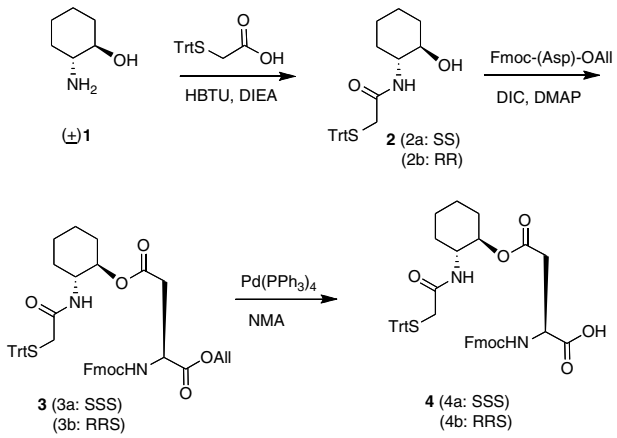
Synthesis of 3

Compound **2** (1.10 g, 2.55 mmol) was added to a solution of Fmoc-Asp-OAll (0.80 g, 2.04 mmol) with DIC (0.35 ml, 2.30 mmol) and catalytic amount of DMAP in dry DCM (20 ml) at 0 °C. The reaction mixture was stirred at room temperature for 12 h. The organic solvent was concentrated and extracted with ethyl acetate (3 × 20 ml). The combined organic layer was washed with brine, dried over $MgSO_4$, concentrated and purified using flash column chromatography (hexane/ethyl acetate 3 : 1) to give 1.20 g of **3** in 72% yield. R_f 0.27 [EtOAc–Hex (1 : 2)]. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.94–1.45 (m, 4H), 1.52–1.99 (m, 4H), 2.70–3.25 (m, 4H), 3.62–3.82 (m, 1H), 4.10–4.27 (m, 2H), 4.27–4.42 (m, 1H), 4.42–4.58 (m, 1H), 4.58–4.75 (m, 3H), 5.23 (d, J = 10.7 Hz, 1H), 5.31 (d, J = 17.1 Hz, 1H), 5.80–5.94 (m, 1H), 7.06–7.64 (m, 19H), 7.57–7.70 (m, 2H), 7.76 (d, J = 7.4 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 23.7, 23.9, 24.1, 30.7, 30.7, 30.7 31.3, 36.1, 36.2, 37.0, 37.0, 47.0, 47.1, 50.2, 50.6, 51.7, 52.2, 66.2, 66.4, 67.3, 67.4, 67.8, 67.9, 75.5, 75.8, 118.7, 118.8, 119.9, 125.2, 125.3, 127.0, 127.6, 127.9, 128.1, 129.5, 131.4, 131.5, 141.2, 143.8, 144.0, 155.9, 156.2, 168.2, 168.3, 170.1, 170.2, 170.6; MALDI-TOF (matrix: α-Cyano-4-hydroxycinnamic acid): calcd. for $C_{49}H_{48}N_2O_7S.Na^+$ [M + Na]$^+$: 831.31 (monoisotope); $C_{49}H_{48}N_2O_7S.K^+$ [M + K]$^+$ (monoisotope): 847.42, found: 831.45, 847.40.

Synthesis of 4

Compound **3** (1.20 g, 1.50 mmol) was dissolved in THF (25 ml); *N*-methylaniline (1.58 ml, 15.00 mmol) and $Pd(PPh_3)_4$ (0.17 g, 0.15 mmol) were added subsequently. The reaction mixture was stirred at room temperature for 1 h. After removing the solvent under reduced pressure, the residue was subjected to column chromatography (MeOH/CH_3Cl 20 : 1) to give **4** in 90% yield (1.03 g). R_f 0.55 [$CHCl_3$–MeOH (10 : 1)]; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.14–1.64 (m, 8H), 2.76–3.24 (m, 4H), 3.79–3.95 (m, 1H), 4.12–4.24 (m, 1H), 4.24–4.38 (m, 2H), 4.55–4.69 (m, 1H),

4.8–4.97 (m, 1H), 5.96–6.14 (m, 1H), 6.44–6.59 (m, 1H), 7.02–7.4 (m, 19H), 7.66–7.70 (m, 2H), 7.75 (d, J = 7.4 Hz, 2H)]; ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.2, 20.5, 22.9, 23.3, 27.3, 27.3, 28.1, 28.3, 36.1, 37.1, 37.3, 47.1, 47.1, 48.9, 49.1, 50.5, 50.5, 67.3, 67.3, 67.8, 72.7, 72.8, 119.9, 125.2, 127.0, 127.6, 128.1, 128.5, 128.6, 129.4, 131.1, 131.9, 132.1, 132.2, 132.3, 141.3, 143.7, 143.7, 144.0, 156.1, 156.1, 168.5, 168.5, 169.9, 173.0, 173.1; MALDI-TOF (matrix: α-Cyano-4-hydroxycinnamic acid): calcd. for $C_{46}H_{44}N_2O_7S.Na^+$ [M + Na]$^+$: 791.28 (monoisotope); $C_{46}H_{44}N_2O_7S.K^+$ [M + K]$^+$: 807.39 (monoisotope), found: 791.39, 807.38.



SPPS of Peptides with an Auxiliary Anchored to Asp or Glu

SPPS of peptides **5a-6a**

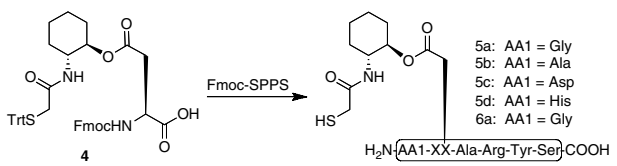
H-Ser(tBu)-2-chlorotrityl resin (0.49 mmol/g) was used for the synthesis of peptides **5a-6a**. Amino acids [including the Fmoc-Asp(auxiliary)-OH and HBTU] were used in fivefold excess of the initial loading of the resin. DIEA was used in tenfold excess. Peptide coupling was performed for 30 min. Fmoc deprotection was achieved by treatment of the resin with 20% piperidine (3 × 3 min).

Cleavage from the resin

A mixture of TFA, triisopropylsilane and water (95 : 2.5 : 2.5) was added to the dried resin peptide. After 2 h, the resin was washed with TFA (4 × 4 ml).

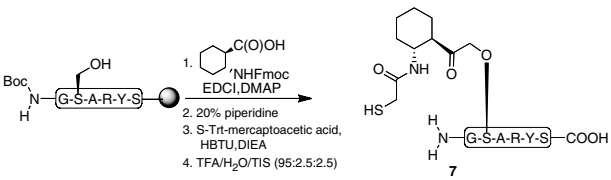
Work-up

The combined solutions were concentrated *in vacuo*. The residue was dissolved in water, purified by preparative reverse-phase HPLC on a C18 column (Jupiter 10 micron, 300A, 250 × 21.20 mm) using a linear gradient (0–90%B) over 20 min (buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile). The fractions were analyzed by MALDI-TOF/MS (RefleX IV, Bruker) (matrix: α-Cyano-4-hydroxycinnamic acid). The desired fractions were collected and lyophilized to give the product in 55–60% isolated yield.



SPPS of Peptides with an Auxiliary Anchored to Ser or Thr

H-Ser(tBu)-2-chlorotrityl resin (0.49 mmol/g) was used for the synthesis of peptide **7**. Amino acids and HBTU were used in fivefold excess of the initial loading of the resin. DIEA was used in tenfold excess. Peptide coupling was performed for 30 min. Fmoc deprotection was achieved by treatment of the resin with 20% piperidine (3 × 3 min). Coupling of unprotected Fmoc-Ser-OH was achieved using DIC and HOBt (each in fivefold excess). Boc-Gly-OH was used as the N-terminal amino acid. Peptide resin was treated with 10% N_2H_4 in methanol (2 × 1.5 h) to hydrolyze any undesired ester on the side-chain of serine. Next, commercially available Fmoc-*trans*-1,2-aminocyclohexane carboxylic acid (Aldrich) was coupled to the side-chain using EDCI (fivefold excess) in the presence of catalytic amount of DMAP in dry DCM. The Fmoc protecting group was then removed with 20% piperidine followed by coupling the S-Trt-mercaptoacetic acid (fivefold excess) using HBTU/DIEA (fivefold/tenfold excess) coupling conditions. Cleavage from the resin and work-up were done as described previously to give the desired product in 60% isolated yield.



Synthesis of Peptide Thioester H_2N-LYRAG-*SR*

MBHA resin (0.59 mmol/g) was washed (5 × DMF, 10 min 10% DIEA/DMF, 5 × DMF). A solution of HBTU (fivefold excess) in DMF was added to 3-(tritylthio)propanoic acid followed by the addition of DIEA (tenfold excess), after 5 min the reaction mixture was added to the resin. After 1 h, the resin was washed with DMF and the trityl was removed using a mixture of TFA, triisopropylsilane and water (90 : 5 : 5) (3 × 1 min). Amino acids and HBTU were used in fivefold excess while DIEA in tenfold excess of the initial loading of the resin. The peptide coupling was performed for 30 min. Boc deprotection was achieved by treatment of the resin with TFA (3 × 1 min).

Cleavage

A mixture of TFMSA, TFA and thioanisole (2 : 8 : 1) was added to the resin. After 1.5 h, the resin was washed with TFA (4 × 4 ml).

Work-up

The combined solutions were concentrated. The residue was dissolved in water, purified by preparative reverse-phase HPLC on a C18 column (Jupiter 10 micron, 300A, 250 × 21.20 mm) using a linear gradient (0–90%B) over 30 min and analyzed by MALDI-TOF/MS (RefleX IV, Bruker) (matrix: α-Cyano-4-hydroxycinnamic acid).

General Procedure for Chemical Ligation

The ligation of unprotected peptide segments was performed as follows: 0.2 M phosphate buffer (pH 8.0) containing 6 M Gn·HCl was degassed with Argon for 10 min before use. Peptides were dissolved (1 eq of peptide auxiliary and 1.20 eq of peptide

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thioester) in the degassed buffer to a final concentration of 5 mM and incubated at 37 °C until the reaction shows completion on analytical HPLC analysis. Before analytical analysis, tris(2-carboxyethyl) phosphine hydrochloride (TCEP) (50 mM) was added to reduce any disulfide bonds. The reaction was monitored using analytical reverse-phase HPLC on a C18 column (Jupiter 5 micron, 300A, 150 × 4.60 mm) and a linear gradient (0–90%B) over 15 min.

General Procedure for Auxiliary Hydrolysis

After the completion of ligation, the pH of the ligation mixture was adjusted to pH ~10 using 1N NaOH and was kept for 5 min at room temperature. The hydrolysis reaction was monitored by analytical HPLC as described above and purified as described for peptides **5a-6a**. The ligation followed by *in situ* removal of auxiliary afforded the desired product in 60–70% isolated yield (ligation product without auxiliary).

Acknowledgement

We are grateful to the Israel Science Foundation for their financial support.

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