Peptide Ligation-Desulfurization Chemistry at Arginine**

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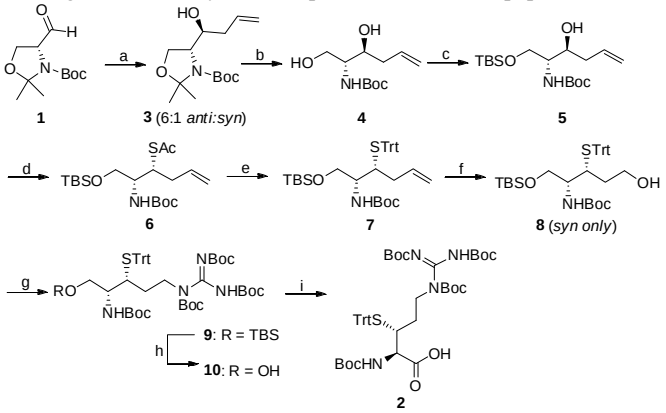
Native chemical ligation[1] remains the most efficient and widely used peptide ligation methodology for the synthesis of homogeneous proteins and glycoproteins.[2] The method utilizes an N-terminal cysteine (Cys) residue on a peptide to facilitate an initial transthioesterification reaction with a C-terminal peptide thioester, followed by a rapid S→N acyl shift to generate an amide bond in a chemoselective manner. To expand the repertoire of ligation chemistry, significant effort has been aimed toward the development of post-ligation desulfurization chemistry.[3] This concept was first investigated by Yan and Dawson, who demonstrated the desulfurization of Cys to alanine (Ala) *via* hydrogenation following the ligation event.[4] Recently, this research area has been greatly accelerated through the advent of a metal free dethiylation (MFD) reaction by Wan and Danishefsky.[5] This methodology enables rapid desulfurization of ligation products in aqueous media to produce native peptide and protein products in excellent yields and has been employed in the total chemical synthesis of several complex proteins and glycoproteins to date.[6] Further expansion of the native chemical ligation-desulfurization concept has recently been made possible through synthetically-derived thiol-containing amino acids.[7] The utility of these building blocks in ligation-desulfurization chemistry has been demonstrated through the assembly of large peptides and proteins facilitated by thiol-derived amino acids at the ligation junction.[7e, 7f, 7h, 7j, 7k, 8]

Despite the significant advances in ligation-desulfurization chemistry, we reasoned that a general synthetic strategy toward thiol substituted amino acids from a common starting material, which currently does not exist, would enable further expansion of the ligation-desulfurization methodology to peptide and protein synthesis. We have recently demonstrated a seven step synthesis of β-selenol phenylalanine from commercially available Garner's aldehyde (**1**).[9] In our exploration of Garner's aldehyde as a general starting point for the synthesis of amino acids bearing reactive thiol and selenol auxiliaries, we have developed an efficient and selective route to a suitably protected β-thiol arginine (Arg) derivative **2**, which is presented herein. We also demonstrate the utility of **2** in ligation-desulfurization chemistry in a variety of model systems, and ultimately in a kinetically controlled ligation[10]-desulfurization sequence to access a homogeneous glycopeptide oligomer.

Synthesis of β-thiol Arg building block **2** began with the addition of allyltributyltin to Garner's aldehyde[11] to generate the allyl alcohol **3** in 80% yield as an inseparable 6:1 mixture of *anti*:*syn* diastereomers.[12] Deprotection of the hemiaminal under acidic conditions followed by TBS protection afforded **5**. The crucial sulfur moiety was installed by activation of **5** as the mesylate followed by subsequent S_N2 inversion using potassium thioacetate to provide **6** as predominantly the *syn*-diastereomer. The thioacetate was unmasked before converting to *S*-trityl(Trt)-protected compound **7** in excellent yield, to facilitate eventual incorporation into peptides *via* solid-phase peptide synthesis (SPPS). The allyl moiety was oxidatively cleaved with OsO_4 and $NaIO_4$ to generate the aldehyde followed by immediate reduction to the primary alcohol **8** using $LiBH_4$ in 50% yield over the two steps. At this stage the *syn*-diastereomer could be separated from the minor *anti*-diastereomer. A Mitsunobu reaction with *N,N′,N″*-tri-Boc-guanidine enabled installation of the protected Arg side-chain in 80% yield. Deprotection of the TBS ether *via* treatment with tetrabutyl-ammonium fluoride (TBAF) afforded alcohol **10**. Finally, oxidation to the carboxylic acid was accomplished using a two-step oxidation procedure employing Dess-Martin periodinane (DMP) to generate the aldehyde, followed by a Pinnick oxidation to yield the protected building block **2**, ready for incorporation into model peptides.



Scheme 1. Synthesis of β-thiol Arg (**2**) from Garner's aldehyde (**1**). a) Allyltributyltin, $BF_3{\cdot}OEt_2$, CH_2Cl_2, -78 °C, 3 h, 80%; b) *p*-TsOH, 1,4-dioxane, rt, 3 h, 82%; c) TBS-Cl, Et_3N, DMAP, CH_2Cl_2, rt, 16 h, 87%; d) 1. MsCl, Et_3N, CH_2Cl_2, 0 °C, 20 min, 2. KSAc, DMF, 50 °C, 5 h, 65% over 2 steps; e) 1. NaOMe, MeOH, rt, 5 min, 2. Trt-OH, $BF_3{\cdot}OEt_2$, Et_2O, rt, 45 min, 76% over two steps; f) 1. OsO_4, $NaIO_4$, 2,6-lutidine, H_2O/1,4-dioxane (3:1, v/v), rt, 2 h, 2. $LiBH_4$, THF, rt, 40 h, 50% over 2 steps; g) *N,N′,N″*-tri-Boc-guanidine, PPh_3, DIAD, 30 °C, 10 min, 80%; h) TBAF, THF, rt, 1.5 h, 96%; i) 1. DMP, CH_2Cl_2, rt, 2 h, 2. $NaClO_2$, NaH_2PO_4, 1-methylcyclohexene, *t*BuOH/THF/H_2O (1:7:2, v/v/v), rt, 20 min, 37% over 2 steps.

Building block **2** was next incorporated into the model resin-bound pentapeptide **11** which was assembled by standard Fmoc-strategy SPPS (Scheme 2, see Supporting Information for synthetic details). Owing to the sterically hindered nature of **2**, considerable optimization was required to find suitable conditions for a high yielding coupling to the resin-bound peptide. The use of DIC/HOAt, PyBOP or HATU led to incomplete reactions and substantial guanylation by-product formation in the case of HATU couplings

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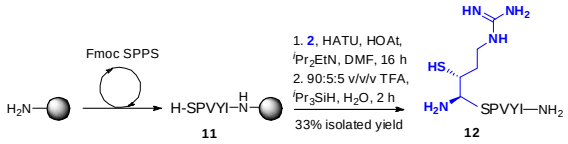
(see Supporting Information). However, optimized conditions using a slight excess of **2** (1.2 eq.), HATU (1.15 eq.), HOAt (12.0 eq.), and *N,N*-diisopropylethylamine (2.4 eq.) in DMF provided the desired peptide **12** in good yield following acidolytic cleavage from the resin and HPLC purification. Peptide thioesters bearing a range of C-terminal amino acids [glycine (G), alanine (A), phenylalanine (F), serine (S) and valine (V)] were also prepared using a previously reported method[13] to serve as coupling partners with **12**.



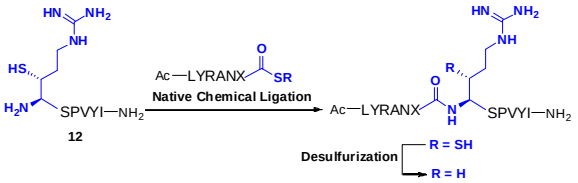
Scheme 2. Fmoc-SPPS of peptide **12** bearing a β-thiol Arg residue.

Ligation reactions between peptide **12** and each thioester were carried out under typical native chemical ligation conditions[1, 14] [6 M guanidine hydrochloride (Gn•HCl), 100 mM Na_2HPO_4, 50 mM tris-(2-carboxyethyl)phosphine (TCEP) in the presence of 2 vol.% thiophenol at 37 °C and pH 7.2-7.4] (Table 1). Reactions were conducted for 24 h in all cases. Gratifyingly, after this time, all reactions proceeded to completion to provide the desired ligation products in excellent yields after HPLC purification (75-94%, Table 1). Importantly, an excellent yield (79%) was also achieved for the sterically demanding valine thioester (Entry 5, Table 1). Extensive evaluation of the ligation kinetics of peptide **12** with each peptide thioester (Figure 1) enabled determination of the second order rate constants[15] for each reaction (see Table 1 and Supporting Information). The results of this study indicate that the rate of reaction at the N-terminal thiolated Arg residue is heavily dependent on the nature of the C-terminal residue of the peptide thioester coupling partner, with reactions at the more sterically demanding thioesters (e.g. V) proceeding much slower than their comparably unhindered counterparts (e.g. G). The non-linear rate observed in the first 5 min of each reaction is likely owing to the slow rate of trans-thioesterfication of alkyl thioesters with thiophenol.[16] It should be noted that the overall trend of ligation rates observed here is consistent with previously reported rate studies for native chemical ligation at Cys performed by Dawson and co-workers.[14] In order to compare the rate of ligation at Arg in our model system with that of Cys, native chemical ligation of H-CSPVYI-NH_2 with the model glycine thioester was conducted (see Supporting Information). Remarkably, peptide **12** reacted at a similar rate (k = 6.50 ± 0.53) compared with the peptide posessing an N-terminal Cys residue (k = 5.47 ± 0.64) despite the extra steric demand at the ligation junction.

Following these rate studies, the isolated ligation products were subjected to desulfurization using the MFD methodology[5] (VA-044, TCEP and glutathione[7c] at 65 °C). Interestingly, in comparison to desulfurization reactions at Cys, reactions at Arg were sluggish. The slower rate of desulfurization *via* MFD may be owing to the ability of the guanidine moiety of the Arg side chain to act as an intramolecular radical trap.[17] Nonetheless, these reactions provided native peptide products in good to excellent yields (66-91%, Table 1) following purification by reversed-phase HPLC.

Having successfully demonstrated the scope and applicability of the Arg-mediated ligation reactions, we next aimed to apply the synthetic methodology to a biologically relevant peptide target, namely a glycopeptide corresponding to a fragment of the extracellular domain of mucin 1 (MUC1) a glycoprotein which is heavily over-expressed and abberantly glycosylated in a host of epithelial tumors. The extracellular domain of MUC1 consists of multiple copies of a 20 amino acid variable number tandem repeat (VNTR) sequence (RPAPGSTAPPAHGVTSAPDT) which, in tumor cells, is decorated with truncated carbohydrate markers, known as the tumor-associated carbohydrate antigens (TACAs). Given the over-expression and unique glycosylation of the VNTR in epithelial cancers, glycopeptides mimicking these structures have been the subject of intense research efforts as potential tumor vaccine candidates.[18]

Table 1. Ligation reactions of peptide **12** with a range of peptide thioesters together with peptide desulfurization.



Entry	Peptide thioester (X =)	Ligation yield[a]	Second Order Rate Constant, k ($M^{-1}s^{-1}$)	Desulfurization yield[a]
1	G	94%	6.50 ± 0.53	72%
2	A	75%	0.67 ± 0.12	91%
3	F	77%	3.73 ± 0.82	75%
4	S	81%	0.36 ± 0.06	69%
5	V	79%	0.0053 ± 0.0007	66%

[a] Isolated yields; Reaction conditions: **Ligation:** 5 mM buffer (6 M Gn•HCl, 100 mM Na_2HPO_4, 50 mM TCEP), 2 vol.% PhSH, 37 °C, pH 7.2-7.4, 24 h. **Desulfurization:** 200 mM VA-044, 6 M Gn•HCl, 100 mM Na_2HPO_4, 500 mM TCEP, 40 mM glutathione, 65 °C, 16 h.

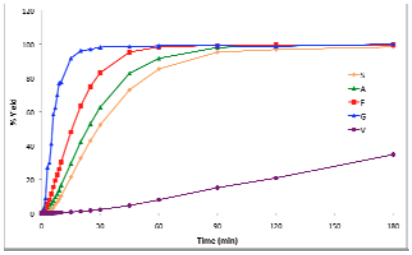


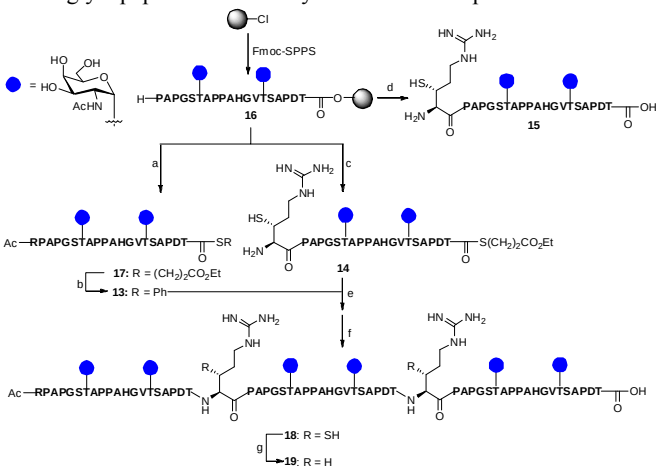
Figure 1. Rates of ligation between **12** and a variety of peptide thioesters (Ac-LYRANX-$S(CH_2)_2CO_2Et$, X = G, A, F, S, V).

We envisaged the use of a three component one-pot, kinetically controlled ligation,[10a] facilitated by a thiolated Arg moiety reacting with two different Thr thioesters, to assemble a glycopeptide possessing three copies of the VNTR and six copies of a monosaccharide TACA (the T_N antigen). Kinetically controlled ligations at Cys, as reported by Kent and co-workers,[10a] take advantage of the innate difference in reactivity between alkyl and aryl thioesters to facilitate rapid construction of the target while minimizing intermediate purification steps. This technique has recently been successfully employed in the construction of complex proteins, including human lysozyme,[19] HIV-1 protease[20] as well as a wild-type erythropoietin glycoprotein bearing four glycans.[6c] In spite of the comparatively sluggish rate of native chemical ligation at Thr thioesters,[14] we were hopeful that the kinetically controlled ligation using Thr acyl donors would proceed smoothly with limited cyclization and oligomerization by-products, based upon observations reported by Bang and co-workers for a kinetic ligation

with Val thioesters.[10b] We therefore envisaged that the MUC1 VNTR would be an ideal candidate for a kinetic ligation reaction.

Our initial synthetic targets were three 20 amino acid MUC1 VNTR glycopeptides, each containing two copies of the T_N antigen, required for the proposed kinetically controlled ligation: (1) glycopeptide thioester **13** bearing a C-terminal thiophenyl thioester, (2) bifunctional glycopeptide **14**, possessing an N-terminal β-thiol Arg residue and a C-terminal alkyl thioester moiety and (3) glycopeptide **15**, displaying an N-terminal β-thiol Arg residue (Scheme 3). All three targets were prepared in a divergent manner from resin bound glycopeptide **16**, synthesized on 2-chloro-trityl chloride resin *via* standard Fmoc-SPPS (Scheme 3**,** see Supporting Information). Glycopeptide thioester **13** was prepared by first coupling a native Arg residue to resin bound **16** followed by Fmoc-deprotection and acetylation of the N-terminus. The glycopeptide was cleaved from the resin using hexafluoroisopropanol (HFIP) to retain all side-chain protecting groups before thioesterifying with ethyl-3-mercaptopropionate using conditions described by Kajihara and co-workers.[21] Treatment with an acidic cleavage cocktail followed by HPLC purification provided alkylthioester **17** in 16% yield based on the original resin loading. The resultant alkyl thioester was subsequently treated with excess thiophenol to facilitate thioester exchange to the more reactive thiophenyl thioester **13** in 56% yield along with 40% recovered starting material. Bifunctional glycopeptide **14** was synthesized by coupling β-thiol Arg building block **2** to **16** before cleavage from the resin using 30 vol.% HFIP in DCM. Thioesterification followed by global acidic deprotection afforded **14**, bearing the thiol Arg building block and a C-terminal alkyl thioester, in 13% yield based on the original resin loading. Finally, **15**, possessing a β-thiol Arg moiety at the N-terminus and a C-terminal carboxylic acid was synthesized by coupling **2** to **16** followed by resin cleavage and side chain deprotection, affording peptide **15** in 11% yield.

With the three requisite MUC1 glycopeptide fragments in hand, we next embarked on the kinetically controlled ligation reaction (Scheme 3). Thiophenyl thioester **13** was first reacted with bifunctional fragment **14** at a final concentration of 2 mM in ligation buffer (6 M Gn•HCl, 100 mM Na_2HPO_4, 50 mM TCEP) at room temperature and pH 6.6. The reaction was conducted in the absence of an external thiol catalyst to prevent transthioesterification of the alkyl thioester moiety of fragment **14**, and thereby limit the formation of cyclization and oligomerization by-products. The reaction was monitored by LC-MS until consumption of the limiting glycopeptide **14** (t = 16 h), at which point fragment **15** was added along with 2 vol.% thiophenol to facilitate activation of the alkyl thioester and enable ligation to the β-thiol Arg moiety of **15**. The second ligation reaction proved to be substantially slower than the first, reaching completion after 31 h. This observed difference in time scale suggests that one of the key factors in determining the overall rate of ligation is transthioesterification to form the more reactive aryl thioester. Indeed, similar results have been observed by Alewood and co-workers for ligation at proline thioesters.[15b] Importantly, the low reactivity of the alkyl thioester may have significant implications for the selectivity of the reaction; we were pleased to find that the two ligations took place with no observable cyclization or oligomerization byproducts and afforded the 60 amino acid glycopeptide **18** in 43% isolated yield (ca. 65% yield per ligation). Desulfurization of **18**, using MFD[5, 7c] successfully afforded the doubly-desulfurized product **19**, a native 7 kDa glycopeptide comprised of three copies of the MUC1 VNTR. The slower rate of desulfurization observed in the model systems was again evident here. In order for the reaction to reach completion, additional dosing with VA-044 and glutathione was required (see Supporting Information). Nonetheless, we were pleased to isolate native glycopeptide **19** in 38% yield after HPLC purification.



Scheme 3. Synthesis of MUC1 fragments **13**, **14** and **15** and assembly of MUC1 VNTR glycopeptide **19** *via* a kinetically controlled ligation-desulfurization sequence. a) 1. Fmoc-Arg(Pbf)-OH, PyBOP, NMM, DMF, 1 h, 2. 10 vol.% piperidine/DMF, 3 min, 3. 10 vol.% Ac_2O/pyridine, 3 min, 4. 30 vol.% HFIP/DCM, 2 h, 5. ethyl-3-mercaptopropionate, PyBOP, $^i Pr_2EtN$, DMF, -30 °C, 2.5 h, 6. 85:5:5:5 (v/v/v/v) TFA, $^i Pr_3SiH$, thioanisole, H_2O, 2 h, 16% isolated yield; b) 6 M Gn•HCl/100 mM Na_2HPO_4, 2 vol.% PhSH, rt, 18 h, 56% isolated yield; c) 1. **2**, HATU, HOAt, $^i Pr_2EtN$, DMF, 16 h, 2. 30 vol.% HFIP/DCM, 2 h, 3. ethyl-3-mercaptopropionate, PyBOP, $^i Pr_2EtN$, DMF, -30 °C, 2.5 h, 4. 85:5:5:5 (v/v/v/v) TFA, $^i Pr_3SiH$, thioanisole, H_2O, 2 h, 13% isolated yield; d) 1. **2**, HATU, HOAt, $^i Pr_2EtN$, DMF, 16 h, 2. 90:5:5 (v/v/v) TFA, $^i Pr_3SiH$, H_2O, 2 h, 11% isolated yield; e) **13** + **14**, 2 mM buffer (6 M Gn•HCl/100 mM Na_2HPO_4, 50 mM TCEP), pH 6.6, rt, 16 h; f) **15**, 2 vol.% PhSH, rt, 31 h, 43% isolated yield over 2 steps; g) 200 mM VA-044, 6 M Gn•HCl, 100 mM Na_2HPO_4, 500 mM TCEP, 40 mM glutathione, 65 °C, 32 h, 38% isolated yield.

In summary, we have developed a method for peptide ligation-desulfurization chemistry at Arg through the synthesis of protected, β-thiol Arg building block **2** from Garner's aldehyde. The synthetic route described here may serve as a general pathway to access other β-thiol amino acids in the future. The scope of ligation at Arg was demonstrated through reaction with a variety of C-terminal peptide thioester coupling partners, and through extensive analysis of the ligation kinetics. This established that the steric nature of the thioester moiety strongly influences the rate of the Arg-mediated ligation in a similar manner to native chemical ligation at Cys. Furthermore, we have successfully utilized the β-thiol Arg-based ligation in a one-pot, three-component kinetically controlled ligation to rapidly assemble a homogeneous 7 kDa MUC1 VNTR glycopeptide in good yield. Future work in our laboratory will aim to exploit this technology in the total synthesis of other proteins, including a library of other MUC1 glycopeptide oligomers for subsequent incorporation into cancer vaccine candidates.

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Arg-uably efficient

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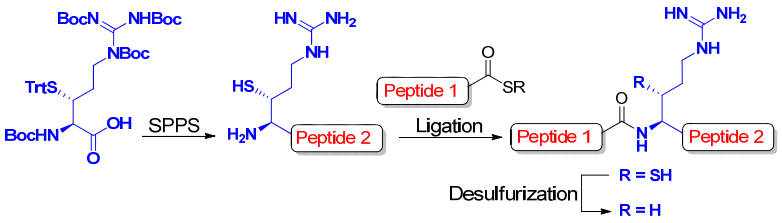
Peptide Ligation-Desulfurization Chemistry at Arginine



The synthesis of a novel β-thiol arginine (Arg) building block has enabled efficient ligation-desulfurization chemistry at a new ligation junction. The utility of this amino acid in ligation chemistry is demonstrated through reactions and kinetic studies with a range of peptide thioesters. Finally, application of the method is highlighted in a one-pot kinetically controlled ligation to rapidly generate a 7 kDa MUC1 glycopeptide.