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Studies Related to the Relative Thermodynamic Stability of C-Terminal Peptidyl Esters of O-Hydroxy Thiophenol: Emergence of a Doable Strategy for Non-Cysteine Ligation Applicable to the Chemical Synthesis of Glycopeptides

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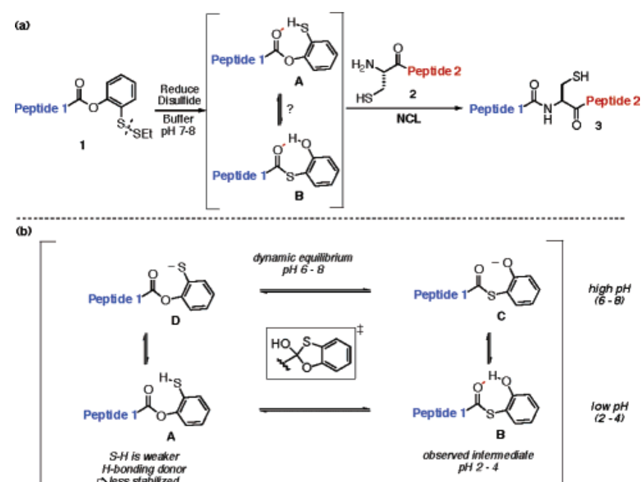
Our laboratory has been studying methods for assembling polypeptides bearing complex oligosaccharide domains at defined sites.[1] Toward this end, we had previously disclosed a method for ligating two glycopeptide fragments, each possessing a fully functionalized carbohydrate domain.[2] A C-terminal glycopeptide fragment is equipped with a phenolic ester bearing a protected *ortho*-thiol moiety (**1**). Disulfide reduction, followed by exposure of the acyl anion system to an N-terminal cysteine-bearing glycopeptide (**2**), leads to formation of a homogeneous glycopeptide (**3**), displaying two different carbohydrate domains (Scheme 1a).

In our initial conception, we had envisioned a possible O → S acyl migration with the formation of a transient thioester intermediate (**A** → **B**). This event would then set the stage for a cysteine-based native chemical ligation (NCL).[3] We had also recognized the possibility of the intervention of more subtle factors. For example, the free *ortho*-thiol group in **A** might well enhance the acylating ability of the O-ester through intramolecular H-bonding.[4] In this interpretation, there need not be a thioester involved in the ligation. Aside from intrinsically interesting mechanistic issues, which drove the study described below, we hoped that a fact-based understanding of the actual pathway could be helpful in broadening the scope and utility of this ligation logic.[5] Accordingly, we set out to examine these and related questions in some detail. Below, we report what were to us some surprising findings. These discoveries led us to explore a promising strategy toward the realization of a cysteine-free NCL that could become broadly applicable to glycopeptide synthesis.

Given the distinctive chemical shift difference between the possible isomeric ester intermediates (e.g., S-ester ∼ 200, O-ester ∼ 170, cf. **A** and **B**), we were hopeful that ^{13}C NMR analysis with a ^{13}C-labeled substrate would provide meaningful insight into the reaction dynamics. Thus, ^{13}C-labeled phenylalanine was incorporated into peptide substrate **4**. Upon addition of tris(2-carboxyethyl)-phosphine hydrochloride (TCEP•HCl, 3 equiv) to **4** in water, disulfide reduction was completed in less than 2 min, and the resulting ^{13}C NMR spectrum was subsequently obtained (Figure 1b). *Surprisingly, only a single peak at 201.5 ppm was observed, corresponding to the thioester (cf.* ***B****)*. It is worthy of note that the pH of the reaction medium had dropped to 2−3 upon addition of TCEP•HCl. Increasing the pH to 4−5 did not change the spectrum significantly (Figure 1c). However, when the pH was increased above 6 (Figure 1d), all of the ^{13}C NMR signals disappeared, indicating a dynamic chemical exchange between the O- and S-acyl species (Scheme 1b).[6] Interestingly, the thioester peak reappeared when the pH of the solution was readjusted to 3 (Figure 1e). The reaction mixture, at pH 7, rapidly consumed cysteine to generate the amide product **5**, with a chemical shift of 172.4 ppm (Figure 1f).

Scheme 1. (a) Previously Disclosed Cysteine-Based Ligation Protocol.[2a] (b) Possible Reaction Intermediates



It is clear from the ^{13}C NMR spectra that the thioester intermediate (**B**) is thermodynamically predominant at low pH (2−4), perhaps due to stabilization imparted by an extra intramolecular H-bonding interaction. When the pH is increased to 6−8, apparently the equilibrium exists in the form of intermediates **C** and **D**, which are exchanging very rapidly. Of these, we would intuitively suppose that **D** is the more stable, based on its "lower energy" acyl linkage and its less basic anionic center. However, this matter has certainly not been established.

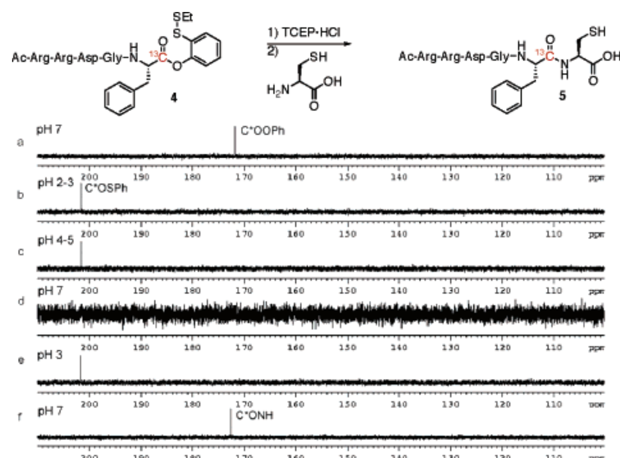


Figure 1. ^{13}C NMR spectra of reaction (125 MHz).

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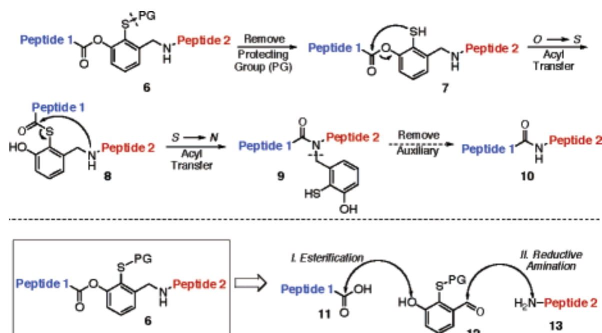
Scheme 2. Design of a Cysteine-Free Ligation Protocol



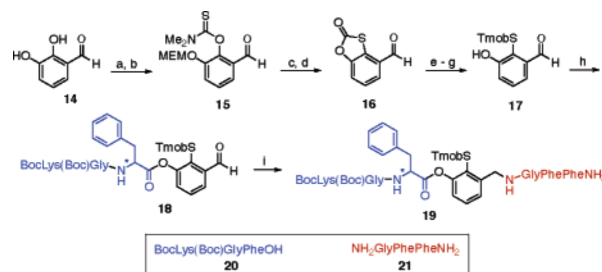
Given the evidence for the emergence of a thioester intermediate, via stoichiometric O → S acyl transfer, the possibility of a modified system, wherein the thioester, generated in situ, would be intercepted by an intramolecular nucleophilic amine group, presented itself.[7] In this way, one could envision developing a useful new peptide ligation method, independent of an N-terminal cysteine, wherein the organizing matrix emerged as a protecting group marking each ligation event.[8]

As is apparent in browsing the generalized Scheme 2, the realization of the proposed method is contingent on two factors. First, it must be possible to construct the two-domain peptide conjugate (cf. **6**) in a facile manner.[9] In addition, both acyl transfers (cf. **7** → **8**, **8** → **9**) must proceed efficiently. With this goal in mind, a novel 1,2,3-trisubstituted aromatic auxiliary unit (**12**) was designed. A convergent two-step procedure was adopted for assembling the conjugate (**6**), wherein the auxiliary unit would first be installed onto the C^{α}-carboxyl terminus of peptide 1 (**11**) through phenol ester coupling. Next, peptide 2 (**13**) would be introduced via reductive amination.

The acid-labile 2,4,6-trimethoxybenzyl group (2,4,6-Tmob) was selected for protection of the thiol group because of its ready removal by TFA[10] and its tolerance of reductive amination conditions. Two appropriately protected peptide fragments, **20** and **21**, were prepared using Fmoc solid-phase peptide synthesis. The auxiliary **17**—itself prepared from **14**—was appended to the C^{α}-carboxyl group of **20** to produce ester **18** (Scheme 3). Although the coupling proceeded in good yield, racemization of the C-terminal phenylalanine presented a significant problem, presumably from oxazolone formation during activation of the C^{α}-carboxyl group (vide infra).[11] Unable to identify conditions by which to suppress racemization, we nonetheless explored the subsequent reductive amination reaction. Thus, aldehyde **18** and peptide **21** were treated with $NaCNBH_3$ and AcOH in MeOH to afford peptide substrate **19** in good yield (>75%). Although compromised by racemization in formation of the O-ester, the overall sequence did accomplish the gross merge we sought.

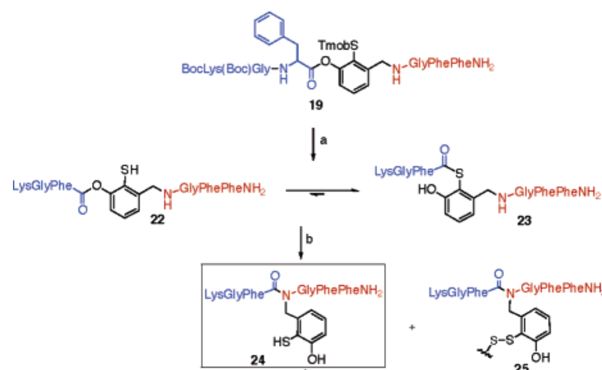
The stage was now set to choreograph the O → S → N acyl transfer (Scheme 4). The actual operation was first triggered by acidic removal of the Tmob blocking group, followed by transfer of the reagents to a proper solvent system in order to promote the desired amide formation. In the event, upon treatment of **19** in 50% TFA in CH_2Cl_2 with 0.5% TIPS as a cation scavenger, the Tmob was completely cleaved within 30 min (along with the other acid-labile protecting groups of the peptide side chains). Next, the solvent was evaporated, and the resultant reaction residue was redissolved in MeOH for LC/MS monitoring. Surprisingly, a notable amount of amide-ligated product **24** had rapidly formed (less than 5 min) in the MeOH solution, which was still fairly acidic due to residual TFA from the prior step. The reaction was complete in under an hour, and the free thiol **24** was obtained along with its corresponding

Scheme 3. Synthesis of Auxiliary **17** and Peptide Conjugate **19**[a]



[a] Key: (a) NaH, MEMCl, DMF, 0 °C to rt, 58%; (b) KOH, $ClCSNMe_2$, THF/H_2O, 0 °C, 92%; (c) reflux, triethyleneglycol dimethyl ether, 74%; (d) 4% TFA/CH_2Cl_2, 95%; (e) NaOH, MeOH/H_2O, 93%; (f) TmobOH, NCS, PPh_3, CH_2Cl_2, 0 °C; (g) K_2CO_3, DMF, two steps, 58%; (h) **20**, EDC, HOBt, DIEA, DMF, 83%; (i) **21**, $NaBH_3CN$, AcOH, MeOH, 77%.

Scheme 4. Peptide Ligation through Consecutive Acyl Migration[a]



[a] Key: (a) 50% TFA/0.5% TIPS, CH_2Cl_2, 30 min; (b) evaporate solvent, redissolve in MeOH, 82%; (c) TCEP·HCl.

Table 1. Ligation Yields of Selected Peptide Substrates

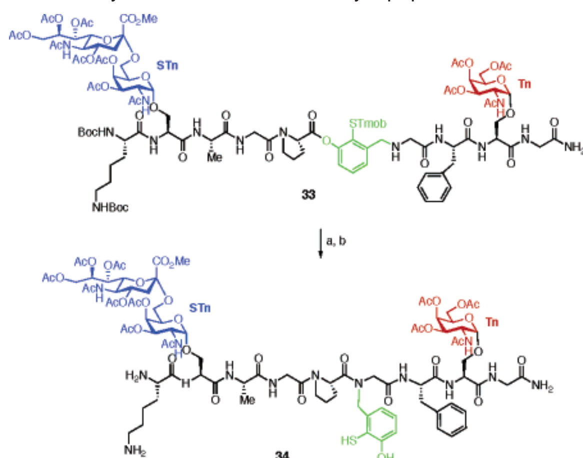
entry	substrates: $peptide_1$ (a) $peptide_2$	reaction conditions[a]	isolated yield (%)
1	**19** BocK(Boc)G**F** (a) **G**FFNH_2	a (1 h)	67[b]
2	**19** BocK(Boc)G**F** (a) **G**FFNH_2	b (1 h)	82
3	**26** BocK(Boc)F**G** (a) **G**FFNH_2	b (30 min)	87
4	**27** BocK(Boc)G**P** (a) **G**FFNH_2	c (8 h)	83
5	**28** BocK(Boc)F**G** (a) **A**FFNH_2	d (4 h)	85
6	**29** BocK(Boc)F**G** (a) **K**(ivDde)FFNH_2	d (12 h)	81
7	**30** BocK(Boc)G**P** (a) **A**FFNH_2	d (24 h)	53
8	**31** BocK(Boc)F**G** (a) **G**FS(Tn)GNH_2	b (30 min)	79
9	**32** BocK(Boc)G**P** (a) **G**FS(Tn)GNH_2	c (8 h)	74
10	**33** BocK(Boc)S(STn)AG**P** (a) **G**FS(Tn)GNH_2	c (10 h)	72

[a] Key: (a) MeOH/H_2O (1:1); (b) MeOH; (c) MeOH, NaH_2PO_4; (d) DMF, NaH_2PO_4. [b] 67% product + 13% hydrolysis side product.

oxidized form, disulfide **25**; the disulfide was easily reduced to **24** upon exposure to TCEP. Although ligation was feasible in aqueous buffer media, hydrolysis of the ester intermediate emerged as a competing reaction under these conditions (Table 1, entry 1).[12] This issue was solved by the use of MeOH as a solvent (entry 2). Overall, this two-step protocol was essentially performed as a one-flask reaction, cleanly providing the desired ligation adduct.

Although an excellent yield was achieved with the Phe−Gly junction, the racemization problem (vide supra) would, of course, limit the practical utility of this ligation method. However, peptide fragments bearing C-terminal glycine or proline residues obviously do not suffer from this type of issue.[13] Indeed, the sterically less demanding Gly−Gly ligation of substrate **26** occurred very rapidly in acidic MeOH (Table 1). In the sterically and electronically more demanding Pro−Gly ligation of substrate **27**, S → N acyl transfer was much slower; however, adding solid NaH_2PO_4 to the MeOH solution significantly increased the acyl transfer rate without leading

Scheme 5. Synthesis of Bidomainal Glycopeptide **34**[a]



[a] Key: (a) 50% TFA/0.5% TIPS, CH_2Cl_2, 35 min; (b) MeOH, NaH_2PO_4, 10 h, 72% (two steps).

to problems of hydrolysis. As expected, excellent ligation yields and racemization suppression were achieved. Interestingly, we found a stable intermediate in the reaction mixture following acidic treatment. Using a ^{13}C-labeled analogue, the intermediate was identified as a thioester. As the S → N acyl transfer proceeded, LC/MS indicated a gradual decrease in the amount of thioester, while the amide product was enhanced correspondingly.

We note that Gly−non-Gly linkages occur frequently in natural peptide sequences. The efficiency of this type of amide ligation was found to be dependent on the substrate and reaction conditions employed. Thus, in substrate **28**, the decreased nucleophilicity and steric hindrance of the secondary amine in alanine resulted in a slow S → N acyl transfer. In the same molecule, the glycine thioester was found to be very prone to hydrolysis under various aqueous conditions. Adding NaH_2PO_4 to the MeOH solution not only noticeably accelerated the desired S → N acyl transfer but also produced a very high level of hydrolytic and even methanolytic side products. Various combinations of solvents and additives were tested, yielding only moderate improvement. Fortunately, by simply dissolving the reaction mixture in DMF after Tmob removal, followed by the addition of a slight excess of NaH_2PO_4, the ligated adducts could be obtained in very good yields with minimal hydrolysis. Under these conditions, ligation of Gly−Lys(ivDde) in substrate **29** and the even more sterically demanding Pro−Ala in **30** was successfully achieved.

Happily, the logic described above could be extended to encompass the synthesis of bidomainal glycopeptides. As listed in Table 1, two substrates, **31** and **32**, were prepared, containing Gly−Gly and Pro−Gly junctions, respectively, each bearing a protected Tn antigen[14] on the N-terminal peptide segment. These compounds were excellent substrates for ligation, proceeding in very good yield. Notably, the differentially diglycosylated substrate **33** (displaying Tn and STn[15]) readily underwent ligation in buffered MeOH to provide the bifunctional glycopeptide **34** in high yield (Scheme 5).

In summary, we have determined the mechanistic basis of our previously described ligation strategy,[2a] through the unexpected finding that ligation progresses via quantitative O → S acyl transfer of the N-terminal coupling partner. On the basis of this finding, we have laid the groundwork for the development of a novel cysteine-free glycopeptide ligation strategy. The method involves a well-orchestrated sequential intramolecular O → S then S → N acyl transfer to deliver the intact amide. While issues of substrate racemization and auxiliary removal remain to be solved, we view this new strategy as a promising platform for future initiatives. The full range of applicability of chemistry-centered glycopeptide ligation "devices" is a matter of continuing interest in our laboratory.

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Supporting Information Available: Experimental procedures and compound characterization data, including LC/MS and NMR data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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