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Rapid Synthesis of Acyl Transfer Auxiliaries for Cysteine-Free Native Glycopeptide Ligation

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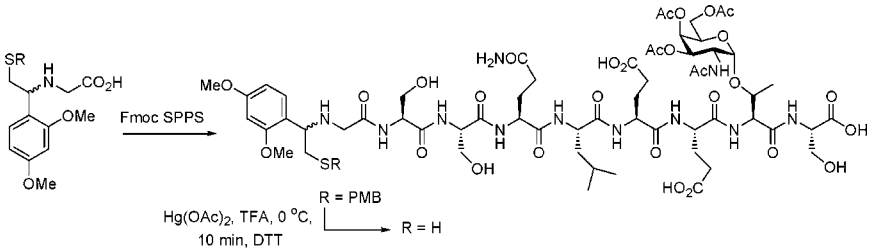
ABSTRACT



Rapid, facile routes to the TFA-cleavable 4,5,6-trimethoxy-2-mercaptobenzyl and 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl classes of auxiliaries for cysteine-free native chemical ligation are described. Rapid synthesis, coupled with mild cleavage conditions will undoubtedly broaden the utility of such auxiliaries, particularly where chemically fragile peptide modifications such as glycosylation are present.

The pioneering research of Kent[1] and Tam[2] in the development of chemoselective peptide ligation strategies has facilitated the total synthesis of hundreds of proteins.[3] However, the requirement for an N-terminal cysteine residue is considered a limitation of the native chemical ligation (NCL) methodology, and consequently cleavable thiol auxiliaries have been developed.[4] Most, however, required multistep syntheses and/or exposure to harsh conditions such as HF during synthesis or to facilitate auxiliary removal, which is not compatible with some posttranslational modifications such as glycosylation. We became interested in using the TFA−cleavable auxiliaries developed by Offer et al.[4f] and Botti et al.[4a] since, in our recent research, concerning the application of NCL to the assembly of O-linked glycoprotein GlyCAM-1, we had observed that a peptide containing both an N-terminal cysteine residue and an internal cysteine residue (introduced to facilitate a prior ligation) failed to give a ligation product until the internal cysteine was capped.[5] We then hoped to devise an alternative strategy employing a removable auxiliary to circumvent any problem arising from the presence of the internal thiol while, at the same time, developing an auxiliary synthesis that was more compatible with glycopeptide chemistry and that would allow convenient incorporation of auxiliaries as amino acid "cassettes" (Scheme 1) using standard Fmoc solid-phase peptide synthesis (SPPS). We focused on auxiliary-linked glycine conjugates since X-Gly[4d,g] ligation junctions seem most useful, particularly with the 1-phenyl-2-mercaptoethyl class of auxiliary,[4g] and these would adequately permit investigation of our preferred Leu-Gly and Ser-Gly ligation junctions.

A key aim was to employ suitably labile protecting groups (R, Scheme 1) for the thiol functionality such as *S*-*p*-methoxybenzyl (PMB), *S*-*o*-nitrobenzyl (ONB), and *S*-trityl

(1) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science* **1994**, *266*, 776.
(2) Liu, C.-F.; Tam, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6584.
(3) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923.
(4) (a) Botti, P.; Carrasco, M. R.; Kent, S. B. H. *Tetrahedron Lett.* **2001**, *42*, 1831. (b) Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996**, *118*, 5891. (c) Clive, D. L. J.; Hisaindee, S.; Coltart, D. M. *J. Org. Chem.* **2003**, *68*, 9247. (d) Low, D. W.; Hill, M. G.; Carrasco, M. R.; Kent, S. B. H.; Botti, P. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 6554. (e) Offer, J.; Dawson, P. E. *Org. Lett.* **2000**, *2*, 23. (f) Offer, J.; Boddy, C. N. C.; Dawson, P. E. *J. Am. Chem. Soc.* **2002**, *124*, 4642. (g) Marinzi, C.; Offer, J.; Longhi, R.; Dawson, P. E. *Bioorg. Med. Chem* **2004**, *12*, 2749.

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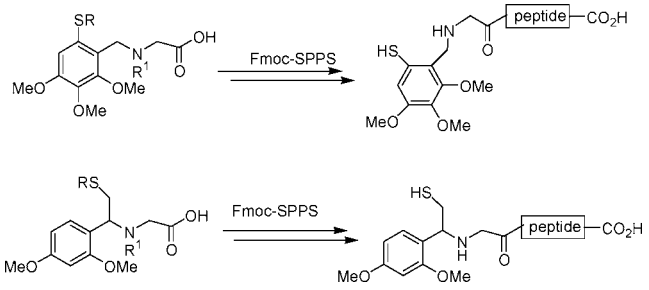
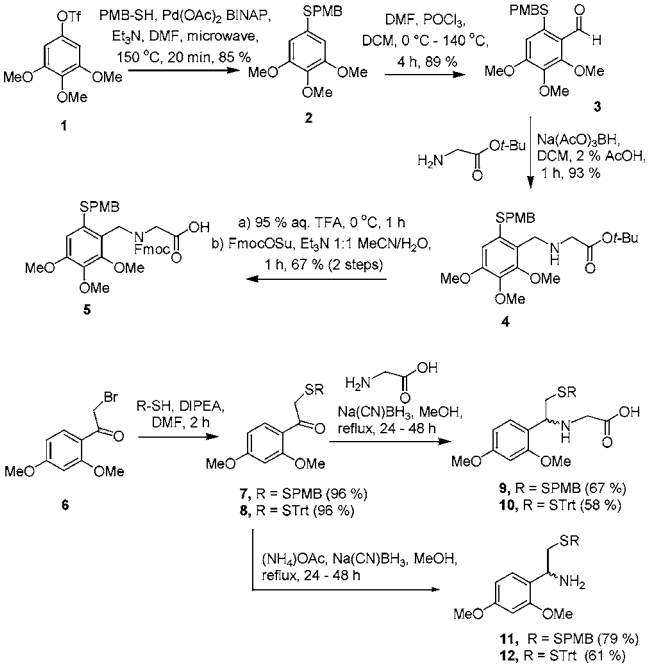
Scheme 1. Strategy for Introduction of Glycine-Linked 4,5,6-Trimethoxy-2-mercaptobenzyl and 1-(2,4-Dimethoxyphenyl)-2-mercaptoethyl Auxiliary Cassettes



(Trt) since reaction conditions employed for their removal have been shown to be compatible with glycopeptide synthesis.[4g,5,6] For the synthesis of glycine-linked 4,5,6-trimethoxy-2-mercaptobenzyl auxiliary **5** (Scheme 2), we

Scheme 2. Synthesis of Auxiliary-Linked Glycine Cassettes



introduced the SPMB-protected thiophenolic moiety using triflate **1** and PMB-thiol in a single step using the palladium-catalyzed coupling chemistry developed by Buchwald and Hartwig.[7]

Initially, we were disappointed to find that reported conditions for palladium-catalyzed aryl C−S bond formation using various substituted aryl triflates[8] failed to give appreciable quantities of thioether **2** with our substrate (**1**). However, this reaction occurred readily upon heating for 20 min at 150 °C in a microwave reactor. The commercially available aryl bromide could also be employed in place of **1**, but the yields were consistently lower (24−44%) under comparable reaction conditions. ONB-thiol failed to provide access to ONB-protected thiophenol under our optimized microwave conditions, and the *S*-trityl protecting group was not investigated at this stage as it was unlikely to survive the subsequent reaction conditions. Following Vilsmeier formylation,[4f] aldehyde **3** was conjugated directly to glycine using H-Gly-O*t*Bu (utilizing the *tert*-butyl ester to ease purification of the product) via reductive amination employing sodium triacetoxyborohydride as reductant in excellent yield.[9] The auxiliary−glycine conjugate **4** was then prepared for standard SPPS via TFA-mediated cleavage of the *tert*-butyl ester followed by Fmoc protection of the secondary amine. Synthesis of protected 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl auxiliaries **9** and **10** was completed in only two steps. PMB- and trityl-protected thiol moieties were introduced by the action of the corresponding thiols on commercially available bromoacetophenone **6** to afford thioethers **7** and **8** in excellent yields. Glycine was then introduced via reductive amination using sodium cyanoborohydride as a reductant in refluxing methanol.[10] This route was investigated since under identical conditions for the preparation of **4** ($H_2NCH_2CO_2tBu$, $Na(AcO)_3BH$, AcOH/DCM), only reduction of the ketone was observed. Furthermore, only the glycine-linked auxiliary **4** was amenable to the *tert*-butyl deprotection and Fmoc protection cycle (**9** was resistant to Fmoc protection). We reasoned that, if the increased steric bulk proximal to the amino group of **9** was precluding Fmoc protection, then Fmoc protection might not be required at all during peptide synthesis. Consequently, **5** and **9** or **10** were introduced directly (using 3−5 equiv) to preassembled peptides corresponding to selected GlyCAM-1 protein fragments (Table 1) with no evidence of multiply coupled species arising from the use of **9** in SPPS. Interestingly, auxiliaries **11** and **12** of the type commonly employed in the submonomer approach (through subsequent reaction with bromoacylated peptides)[4] could also be conveniently prepared under identical reaction conditions employing ammonium acetate in place of glycine in the reductive amination. After Fmoc deprotection and cleavage from the resin of **5**-linked peptide **13** (Table 1), the PMB protecting group was efficiently removed using excess $Hg(OAc)_2$ in 10% aqueous AcOH followed by the addition of DTT to a final concentration of 5% w/v. **9**-linked peptides (**14** and **15**) were more resistant to such treatment, but the SPMB group was readily cleaved upon exposure to $Hg(OAc)_2$ in neat TFA for 10 min at 0 °C followed by dilution to 10% aqueous

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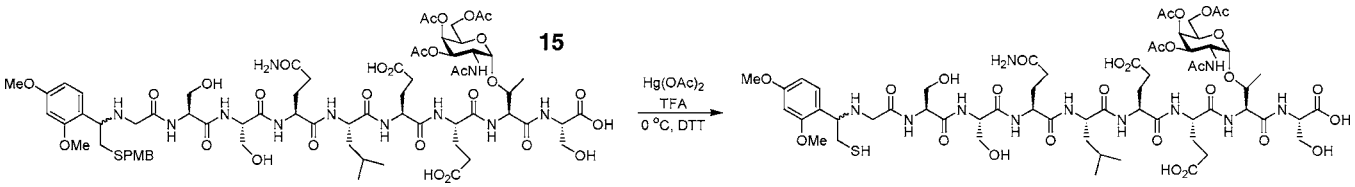
(8) Zheng, N.; McWilliams, J. C.; Fleitz, F. J.; Armstrong, J. D., III; Volante, R. P. *J. Org. Chem.* **1998**, *63*, 9606.

(9) H_2N-Ala-O*t*Bu was also introduced under identical conditions in 96% yield.

(10) Williams, R. E.; Ehrlich, P. P.; Zhai, W.; Hendrix, J. *J. Org. Chem.* **1987**, *52*, 2615.

Table 1. Synthesis and Ligation Reactions of Auxiliary-Linked GlyCAM-1 Fragments[11]



	protected peptide−auxiliary conjugates			free thiol[a]			ligation product		
		m/z (calcd)	*m/z* (obsd)	*m/z* (calcd)	*m/z* (obsd)	thioester for model ligation[b]	*m/z* (calcd)	*m/z* (obsd)	auxiliary removed (Y/N)
13[c]	**5**-GlyCAM-1 (109−132)	2782.1	2782.2	2662.0	2661.7	AEEE**L**-SBn	3233.7	–[d]	N
						GLR**G**-SBn	3045.6	3046.0	Y
14	**9**-GlyCAM-1 (109−132)	2766.1	2766.4	2646.1	2645.0	AEEE**L**-SBn	2029.6	–[d]	N
15	**9**-GlyCAM-1 (75−83)	1582.6	1582.9	1462.5	1462.7	GLR**S**-SBn	1845.9	–[d]	N
						GlyCAM-1(1−77**S**)-SMESNA	9905.2	–[d]	N
						GLR**G**-SBn	1875.9	1876.6	Y
16[e]	**10**-GlyCAM-1 (75−83)	–	–	1462.5	1462.7	–	–	–	–

[a] After removal of SPMB or STrt protecting groups. [b] Prepared according to ref 12, and ref 13 for bacterially derived thioester. [c] Fmoc removed prior to cleavage from resin. [d] Only a trace amount of product was observed after 48 h by LC-MS and was not isolated. [e] TFA-mediated cleavage from the resin affords the fully unprotected auxiliary-linked glycopeptide, which is identical to deprotected **15** and was not tested independently in ligation reactions.

TFA and addition of DTT to a final concentration of 5% w/v (Figure 1).

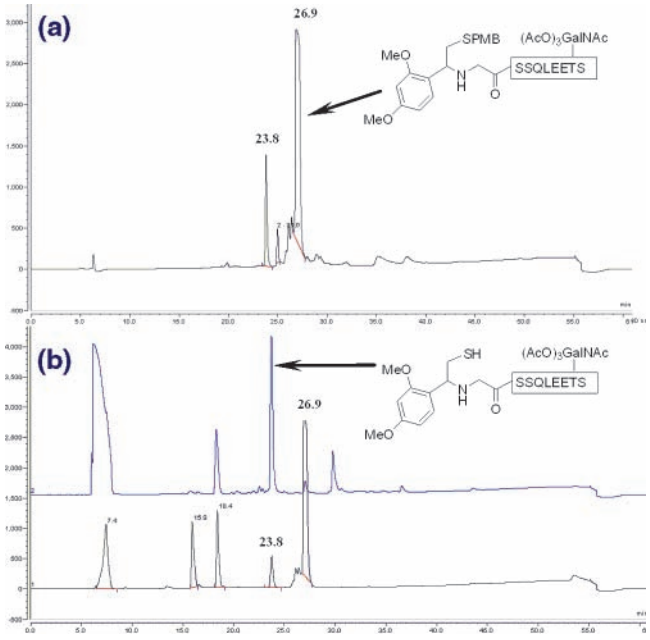


Figure 1. HPLC purification and SPMB deprotection of glycopeptide **15**. (a) TFA-cleaved **15** eluting at 26.9 min. Slight cleavage of SPMB under the resin cleavage conditions gives rise to fully unprotected material at 23.8 min; (b) SPMB cleavage from **15** in 10% AcOH, $Hg(OAc)_2$, 0 °C−rt, 5% DTT, 1 h (black trace) and in neat TFA at 0 °C, 10 min (blue trace) shows almost complete conversion.

With deprotected conjugates **13**−**15** in hand, we aimed to evaluate their ability to perform native chemical ligation reactions at our preferred Leu−Gly and Ser−Gly junctions, thus further probing the generality of the auxiliary-mediated ligation reaction with scaffolds arising from **5** and **9**. Model experiments were indeed required since there have been no published reports of the use of these particular scaffolds in cysteine-free NCL at Leu−Gly or Ser−Gly junctions.[4] For ligation reactions, AEEEL-SBn and GLRS-SBn thioesters were prepared so as to model the GlyCAM-1 Leu^{108}−Gly^{109} and Ser^{74}−Gly^{75} ligation junctions, respectively, using established methodology.[12]

Ligation reactions at peptide concentrations of approximately 3 mM were conducted in 6 M guanidine hydrochloride, 200 mM sodium phosphate buffer; pH 8.0, containing 2% w/v mercaptoethanesulfonic acid (MESNA) and 20 mM tris-carboxyethylphosphine (TCEP). The reactions were monitored by LC-MS. Initially, minor peaks attributable to the ligation products between AEEEL-SBn and **13** or **14** (PMB removed) were observed; however, these species did not accumulate over time and so we concluded that they were likely to be the transthioesterified yet unrearranged starting materials. A further ligation between fully deprotected glycopeptide **15** and GLRS-SBn thioester was also unsuccessful, as was ligation between unprotected **15** and a readily available bacterially derived peptide-thioester (corresponding to GlyCAM-1 residues 1-77, which also terminates in a serine thioester). Again only traces of product were observed after 48 h. This bacterially derived peptide-thioester had success-

(11) See Supporting Information for full details of peptide sequences, experimental procedures, and spectra.

(12) Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684.

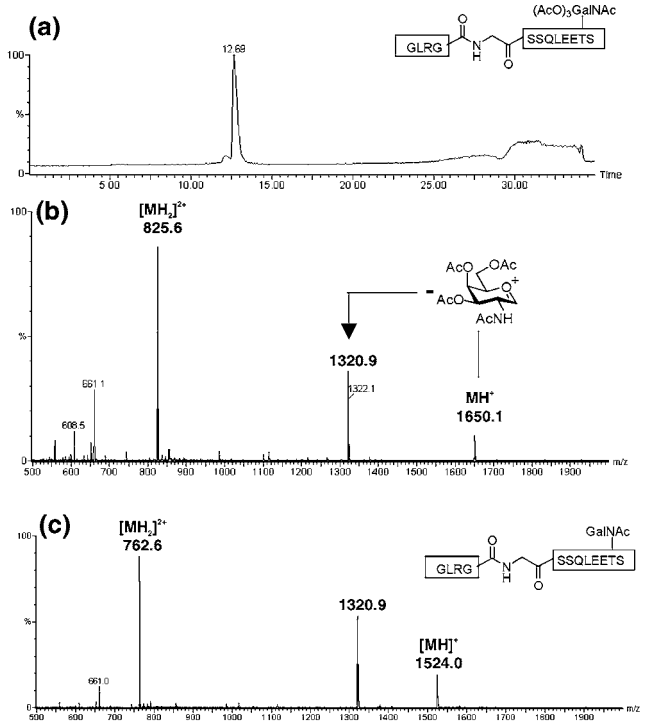


Figure 2. Cleavage of auxiliaries after Gly−Gly native glycopeptide ligation reactions. (a) LC-MS trace for purified ligated glycopeptide product after auxiliary removal. (b) MS of glycopeptide product (calcd mass = 1649.4 Da, showing characteristic fragmentation). (c) Treatment with hydrazine hydrate (2% v/v, 4 h) removes acetate esters on monosaccharide (calcd mass for deacetylated glycopeptide = 1524.4 Da).

fully participated in several NCL reactions previously with glycopeptides bearing a cysteine residue at the N-terminus.[5,13] These findings were in stark contrast to those obtained when a GLRG-SBn thioester (the glycine analogue of GLRS-SBn) was employed in ligation reactions. Reactions between GLRG-SBn thioester and unprotected **13** or **15** were virtually complete within 48 h.[11] The ligation products were readily purified by semipreparative reverse-phase HPLC, and the auxiliaries were removed upon treatment with 95% TFA for 3 h (Figure 2). The acetyl esters employed as protecting groups for the carbohydrate hydroxyl groups during SPPS were finally removed with 2% v/v hydrazine hydrate in 10 mM sodium phosphate buffer, which also confirmed the presence of a stable amide-linked ligation product.

In summary, we have developed particularly rapid and facile routes to two popular classes of TFA-cleavable acyl transfer auxiliaries for cysteine-free NCL. In both cases, deprotection of a *p*-methoxybenzyl thioether facilitated rapid liberation of the thiol functionality under conditions compatible with glycopeptide synthesis. Trityl-protected auxiliaries **10** and **12** could also be prepared in only two steps and incorporated into synthetic glycopeptides, though yields for the reductive amination of the trityl-protected precursors were consistently lower than those for the analogous SPMB-protected compounds. Additionally, we applied a relatively mild reductive amination protocol for the apparently problematic[4c] introduction of the amine functionality of auxiliaries **9**−**12**. Furthermore, unprotected glycine can participate in the reductive amination and the auxiliary−glycine conjugates obtained can be coupled directly to synthetic peptides without further protecting group manipulations. Auxiliary introduction and cleavage were shown to be compatible with the presence of glycosidic linkages and to function in cysteine-free NCL reactions across Gly−Gly ligation junctions. Unfortunately, both classes of auxiliary failed to deliver ligation products at Leu−Gly junctions and the 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl auxiliary failed to deliver a ligation product at Ser−Gly junctions, which may limit their use in our studies unless amino acid substitutions in the GlyCAM-1 protein backbone are tolerated. We believe, however, that the rapid synthesis, coupled with mild cleavage conditions will undoubtedly broaden the utility of such auxiliaries in favorable cases, particularly where sensitive peptide modifications such as glycosylation are present.

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Supporting Information Available: Full synthetic procedures for the preparation of all compounds and data for characterization of all products. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) Macmillan, D.; Bertozzi, C. R. *Tetrahedron* **2000**, *56*, 9515.