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Aziridine-2-carboxylic Acid-Containing Peptides: Application to Solution- and Solid-Phase Convergent Site-Selective Peptide Modification

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Abstract: The development of a method for site- and stereoselective peptide modification using aziridine-2-carboxylic acid-containing peptides is described. A solid-phase peptide synthesis methodology that allows for the rapid generation of peptides incorporating the aziridine residue has been developed. The unique electrophilic nature of this nonproteinogenic amino acid allows for site-selective conjugation with various thiol nucleophiles, such as anomeric carbohydrate thiols, farnesyl thiol, and biochemical tags, both in solution and on solid support. This strategy, combined with native chemical ligation, provides convergent and rapid access to complex thioglycoconjugates.

Introduction

Carbohydrate and lipid moieties introduced via the post-translational modification of proteins are key determinants of their structure and biological activity.[1] Numerous biological processes, such as immunity and cell signaling, are controlled by complex glycoconjugates, generated by the addition of carbohydrate moieties to side chains of serine, threonine, and asparagine residues.[2] Lipoproteins, exemplified by the Ras proteins, are key regulators of eukaryotic cell growth.[3] As a result of their biological and therapeutic significance, determination of the functional roles of these post-translationally introduced functionalities has been a focus of extensive research.[1d,4] Obtaining homogeneous samples of conjugates necessary for such studies has been a major challenge. For example, natural glycoproteins are often expressed in many different glycoforms;[2] similarly, lipidated proteins cannot be obtained from yeast and bacterial expression systems.[1a,d] Therefore, efficient methods for the preparation of homogeneous modified proteins and peptides are needed.

Traditional synthetic approaches for the generation of homogeneous glycopeptides rely on the formation of fully protected glycosylated amino acid building blocks and their subsequent incorporation into peptides, mainly via solid-phase peptide synthesis.[5] These methods suffer from loss of valuable glycosylated intermediates in each iteration of the solid-phase synthesis. Furthermore, lack of convergence limits application of these methods in the rapid preparation of various modified peptides and proteins. To overcome these limitations, alternate convergent ligation approaches have been developed. These strategies rely on the late-stage coupling of preformed peptide/protein and carbohydrate components, where chemoselectivity results from the conjugation of carbohydrate nucleophiles with unique electrophilic sites incorporated in peptide and protein substrates, providing both native glycopeptides and their mimetics.[6] Peptide electrophiles could be generated either via modification of existing amino acid residues (e.g., Cys[6i,j] and Asp[6h,l]), or via incorporation of non-proteinogenic amino acids (e.g., dehydroalanine,[6c,f] ketones,[6a,g] cyclic sulfamidates,[6d] and halogenated residues[6m]). Alternatively, chemoselectivity in carbohydrate−peptide conjugations has been realized through the application of a copper(I)-catalyzed [3 + 2] cycloaddition between azide and alkyne functionalities.[7] In analogy to glycopeptides, lipopeptides have been prepared both by incorporation of preformed cysteine derivatives into a growing

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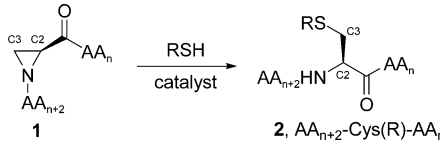
Scheme 1



peptide chain[8] and by site-selective alkylation of this nucleophilic residue with farnesyl halides as electrophiles.[9]

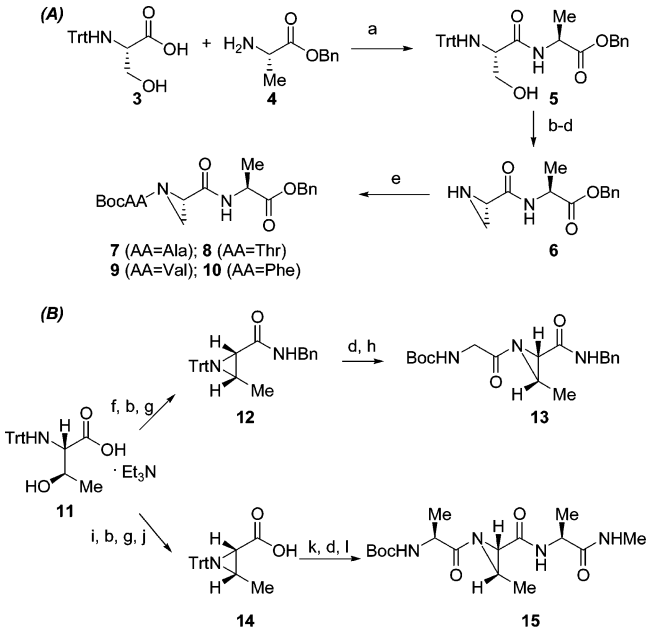
The electrophilic aziridine functionality represents a valuable synthetic building block due to its ability to undergo ring-opening reactions with a wide range of nucleophiles.[10] This reactivity profile of the aziridine moiety has prompted the preparation of aziridine-2-carboxylic acid (Azy)[11] as well as short peptides incorporating this subunit.[12] However, these pioneering procedures suffer from low yields of the desired Azy-containing peptides, as well as the incompatibility of the required protecting groups with established solid-phase peptide synthesis protocols. Furthermore, to date only di- and tripeptides incorporating the Azy moiety have been prepared. Clearly, a more versatile and rapid route to aziridine-2-carboxylic acid containing peptides would be valuable, given their potential to serve as electrophiles in peptide conjugations with appropriate nucleophiles. Indeed, nucleophilic ring-opening of the Azy moiety with simple thiol nucleophiles has been carried out in the presence of a catalytic amount of boron trifluoride etherate[13] or stoichiometric amounts of thiobenzoic acid.[14] In the context of longer peptides, these acidic promoters are likely to be inefficient given the presence of multiple Lewis basic sites, and thus, these methods are not applicable to the conjugation of elaborate Azy-containing peptides.

Herein, we report details of our efforts to develop a new approach to site- and stereoselective peptide modification using aziridine-2-carboxylic acid-containing peptides **1** (Scheme 1).[15] It was anticipated that, in the presence of a suitable catalyst, thiol addition to the less sterically hindered C3-carbon of the aziridine moiety would provide the desired cysteine adduct **2**. This approach allows for the late-stage coupling of preformed aziridine-2-carboxylic acid-containing peptides with complex thiol moieties such as lipids, carbohydrates, and biochemical tags.

Results and Discussion

Synthesis of Aziridine-2-carboxylic Acid-Containing Peptides and Conjugation with Thiol Nucleophiles. The preparation of short aziridine-containing peptides for exploratory investigations of thiol conjugation followed a strategy based on the early work of Okawa and Nakajima.[12b] Tripeptides containing a central Azy moiety were prepared by initial coupling of *N*-trityl-protected serine **3** with alanine benzyl ester **4** (Scheme 2A). The resulting dipeptide **5** was reacted with methanesulfonyl chloride and subsequently treated with triethylamine in THF to afford the corresponding *N*-tritylaziridine-containing dipeptide. Trityl removal followed by acylation of the free aziridine in **6** with various Boc-protected amino acids led to the generation of tripeptides **7**−**10** that possess a central aziridine moiety.

Scheme 2 [a]



[a] Reagents and conditions: (a) BOP, ^{i}Pr$_2$NEt, CHCl$_3$ (92%); (b) MsCl, Et$_3$N, CH$_2$Cl$_2$; (c) Et$_3$N, THF (91% over two steps); (d) TFA, MeOH, CHCl$_3$ (66% for **6**); (e) BocAAOH, BOP, ^{i}Pr$_2$NEt, CHCl$_3$ (88% for **7**, 76% for **8**, 81% for **9**, 89% for **10**); (f) BnNH$_2$, EDC·HCl, HOBt, CH$_2$Cl$_2$ (96%); (g) Et$_3$N, DME (79% for **12** over two steps); (h) BocGlyOH, BOP, ^{i}Pr$_2$NEt, CHCl$_3$ (86% over two steps); (i) BnBr, THF (80%); (j) H$_2$, Pd/BaSO$_4$, THF (97% over three steps); (k) HAlaNHMe, BOP, ^{i}Pr$_2$NEt, CHCl$_3$ (77%); (l) BocAlaOH, BOP, ^{i}Pr$_2$NEt, CHCl$_3$ (99%).

Similarly, two 3-MeAzy-containing peptides were prepared. 3-MeAzy-containing dipeptide **13** was synthesized starting from *N*-tritylthreonine **11** (Scheme 2B). Following the initial protection of the carboxylic acid moiety in the form of a benzylamide, formation of trityl-3-MeAzy was achieved via a two-step sequence, consisting of conversion of the alcohol to mesylate and closure of the aziridine ring with triethylamine to generate trityl-protected aziridine **12**. Trityl deprotection and acylation with BocGlyOH allowed for the generation of 3-MeAzy-containing dipeptide **13**. For the synthesis of tripeptide **15** possessing a central 3-MeAzy moiety, Trt-3-MeAzyOH monomer **14**, prepared from *N*-tritylthreonine **11** in four steps,[12c,16] was coupled with alanine *N*-methylamide (Scheme 2B). Removal of the trityl group and subsequent acylation with BocAlaOH provided the desired BocAla-3-MeAzy-AlaNHMe tripeptide **15** in good overall yield.

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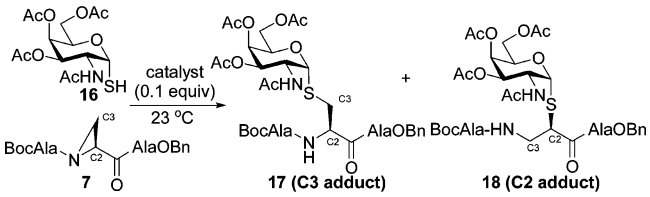
Table 1. Conjugation of α-SH-GalNAc with Azy Tripeptide

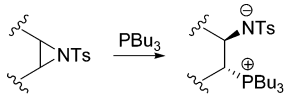


entry	catalyst	solvent (time)	yield, % (**17**:**18**)
1	PBu_3	CH_3CN (20 h)	81 (4.2:1.0)
2	PBu_3[a]	CH_3CN (4 h)	78 (4.5:1.0)
3	$Et_2PCH_2CH_2PEt_2$	CH_3CN (8 h)	68 (4.3:1.0)
4	Me_2PhP	CH_3CN (33 h)	44 (3.7:1.0)
5	Et_3N	CH_3CN (24 h)	66 (3.7:1.0)
6	DABCO	CH_3CN (19 h)	37 (3.3:1.0)
7	DBU	CH_3CN (4 h)	78 (4.5:1.0)
8	DBU	DMF (10 h)	80 (3.1:1.0)
9	DBU	DME (3 h)	79 (4.6:1.0)
10	DBU	$CHCl_3$ (11 h)	83 (6.3:1.0)

[a] Reaction performed in the presence of 1 equiv of catalyst.

To evaluate nonacidic conditions for the conjugation of aziridine-2-carboxylic acid-containing peptides with thiols with an initial focus on the generation of glycopeptides, various catalysts were screened for the coupling of the aziridine-containing tripeptide BocAlaAzyAlaOBn (**7**) with the C1 thio analogue of the tumor-associated T_N antigen (**16**) (Table 1). It was anticipated that the aziridine moiety positioned between two amino acid residues in **7** would be a good mimic of the Azy functionality within longer peptides. Furthermore, the GalNAc-derived C1 α-thiol **16** is a core carbohydrate unit found in various α-O-linked, mucin-related glycopeptides.[2] To allow for the generation of thioisosteres of these glycopeptides, it was critical not only to find appropriate conjugation conditions suitable for the addition of carbohydrate-thiol nucleophiles to the less sterically hindered C3 carbon of the Azy moiety but also to assess the stereochemical consequences at the anomeric position of the carbohydrate coupling partner.

On the basis of reports by Hou and co-workers[17] that phosphines are efficient catalysts in the reaction of aziridines derived from cyclohexene and styrene with simple thiol nucleophiles (thiophenol, *tert*-butyl thiol, and *p*-methylbenzylthiol), the thio-T_N antigen **16** was reacted with the tripeptide **7** in the presence of *n*-Bu_3P (0.1 equiv; Table 1, entry 1) in acetonitrile at 23 °C. The corresponding carbohydrate−peptide conjugates were obtained in good yield, favoring adduct **17** (resulting from the addition of thiol to the C3 position of the Azy moiety, Cys-like adduct) over its constitutional isomer **18** (resulting from the thiol adding to the C2 position, β^2-HGly-like adduct).[18] While addition of a stoichiometric amount of *n*-Bu_3P resulted in a faster reaction (entry 2), the ligated products were obtained in similar yield and selectivity. Use of bisphosphines, such as 1,2-bis(diethylphosphino)ethane (entry 3), did not offer any advantage in the conjugation reaction, as the products were obtained in diminished yield. Finally, use of Me_2PhP (entry 4) led to a marked decrease in isolated yield, demonstrating that introduction of an electron-withdrawing substituent, even in the form of a single aryl group, significantly decreases the efficiency of the ligation. Despite the high yield observed in the conjugation reaction with *n*-Bu_3P as the catalyst, its oxidative instability makes it a nonideal catalyst for this transformation.[19] The initial mechanistic rationale by Hou et al.[7a] as to the role of the phosphine in the ring opening of *N*-tosyl aziridines **19** is that of the formation of a phosphonium intermediate **20** (eq 1). This



(1)

intermediate serves as base initiator that leads to the generation of thiolate, capable of aziridine thiolysis. If the phosphine simply initiated a base-catalyzed process, it was anticipated that a similar outcome could be obtained with tertiary amine catalysts. To test this hypothesis, the conjugation reaction was performed in the presence of a substoichiometric amount of triethylamine (entry 5),[20] which did promote the transformation, albeit only in moderate yield and with diminished regioselectivity. Further screening of tertiary amine bases revealed that DABCO (entry 6) is not an efficient catalyst in this reaction, as significant decomposition of the carbohydrate coupling partner was observed and the conjugated products were isolated in a low 37% yield. However, an increase in both yield and selectivity was obtained with DBU. In the presence of 0.1 equiv of DBU in acetonitrile, conjugated products were obtained in 78% yield, favoring the desired cysteine conjugate over the undesired β-homoglycine adduct in a ratio of 4.5:1, in a kinetically controlled ligation process (entry 7).[21] Variations in solvent revealed that chloroform provides the optimal medium for this transformation (entry 10), affording conjugated products in a combined yield of 83%, further favoring the cysteine adduct (ratio 6.3:1.0). In all of these cases, ligation proceeds with complete retention of the α-anomeric configuration of the carbohydrate nucleophile, and the (*R*) configuration at the α-carbon of the newly formed L-cysteine derivative is secured. Efficient regio- and stereoselective conjugation of α-thioGalNAc **16** with aziridine-containing peptide **7** demonstrates the applicability of this conjugation approach to the generation of thio-linked glycopeptides. The importance of these thioglycopeptides is highlighted by reports of their enhanced stability toward both chemical and enzymatic degradation, as compared with their O-linked counterparts.[22]

The ligation protocol employing DBU as a catalyst was applied to the conjugation of different thiol nucleophiles and Azy-containing peptides (Table 2). Both ethanethiol and the more sterically demanding *tert*-butyl thiol reacted efficiently with BocAlaAzyAlaOBn (**7**) in the presence of DBU (0.1 equiv) at 23 °C to afford tripeptide−thiol conjugates in high yield, strongly favoring the product resulting from the addition of thiol to the C3 position of the Azy moiety (Table 2, entries 1 and 2).

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(18) The minor β^2 amino acid adduct was obtained as a single diastereomer, which is presumably formed via invertive displacement at the aziridine α-carbon.

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(20) Et_3N (0.2 equiv) was previously used as a catalyst in aminolysis of *N*-Ns-Azy-O^{t}Bu with benzylamine: Turner, J. J.; Sikkema, F. D.; Filippov, D. V.; van der Marel, G. A.; van Boom, J. H. *Synlett* **2001**, 1727−1730.

(21) The major product remained unchanged after resubjection to the reaction conditions.

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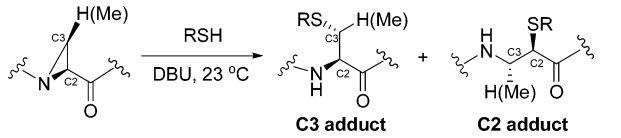
Table 2. Conjugation of Thiols with Aziridine-containing Peptides



C3 adduct C2 adduct

entry	RSH	peptide	solvent (time)	yield, % (C3:C2 adduct)
1	EtSH	**7**	CH_3CN (4 h)	85 (20:1)[a]
2	^{t}BuSH	**7**	CH_3CN (8 h)	84 (C3 only)[a]
3	**16**	**8**	$CHCl_3$ (3 h)	93 (5.3:1)[a]
4	**16**	**9**	$CHCl_3$ (46 h)	89 (13:1)[a]
5	**16**	**10**	$CHCl_3$ (19 h)	90 (16:1)[a]
6	EtSH	**13**	$CHCl_3$ (2 h)	36 (C3 only)[b]
7	EtSH	**15**	$CHCl_3$ (15 h)	61 (C3 only)[c]
8	**16**	**13**	$CHCl_3$ (18 h)	65 (C3 only)[b]
9	**16**	**15**	$CHCl_3$ (15 h)	43 (C3 only)[b]

[a] Reaction performed in the presence of 0.1 equiv of DBU. [b] Reaction performed in the presence of 1 equiv of DBU. [c] Reaction performed in the presence of 0.5 equiv of DBU.

Similarly, thio-T_N antigen **16** was conjugated with the Azy-containing peptides **8**−**10** in high yields (Table 2, entries 3−5). In all cases, the constitutional isomer resulting from the addition of the thiol to the less sterically hindered C3 carbon of the aziridine was strongly favored.[15]

Encouraged by these results, we explored an extension of this ligation methodology to threonine-derived methylaziridine-carboxylic acid- (3-MeAzy-) containing peptides. Reaction of these peptide electrophiles with carbohydrate thiol nucleophiles would allow for the generation of thio analogues of threonine glycosylation sites found in numerous mucin glycopeptides.[2a] To test the possibility of conjugation of 3-MeAzy-containing peptides with thiols, ethanethiol was reacted with BocGly-3-MeAzyNHBn dipeptide (**13**) in the presence of a stoichiometric amount of DBU (Table 2, entry 6).[23] The conjugate resulting from the addition of thiol to the C3 position of the 3-MeAzy moiety was obtained in a low 36% yield, although no product resulting from the addition of thiol to the C2 position of the aziridine was observed. The major byproduct in this transformation, H-3-MeAzyNHBn, is a result of deacylation of the aziridine via addition of thiolate to the acyl moiety of glycine.[24] It was presumed that this side reaction could be suppressed if a peptide substrate with a more sterically demanding acyl substituent on the aziridine nitrogen were used. Indeed, when tripeptide **15**, incorporating Ala instead of Gly on the aziridine group, was reacted with ethanethiol in the presence of DBU (0.5 equiv, entry 7), the desired conjugation product was isolated in 61% yield. Reactions of the C1-thio analogue of T_N antigen (**16**) with 3-MeAzy-containing peptides were also investigated (entries 8 and 9). In the presence of DBU (1 equiv), the dipeptide glycoconjugate was obtained in 65% yield while the tripeptide−carbohydrate adduct was prepared in a modest 43% yield. In both instances, the peptide−carbohydrate coupling proceeded with retention of the α-anomeric configuration of the carbohydrate coupling partner, generating thio analogues of the threonine-derived glycosylation sites as a single diastereoisomer and constitutional isomer. Under these conjugation conditions, the 3-MeAzy peptide electrophiles have significantly attenuated reactivity toward thiol nucleophiles compared with Azy-containing peptides, likely a result of the increased steric bulk of the methyl substituent.

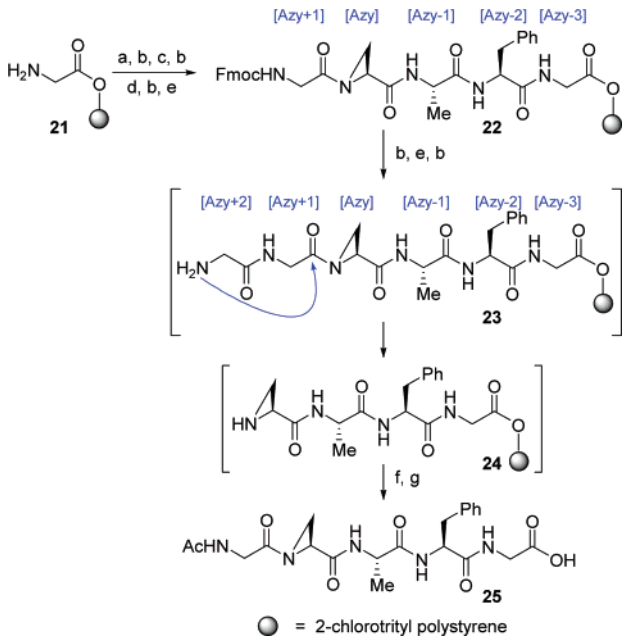
While these initial experiments established the feasibility of base-catalyzed Azy thiolysis, further investigations into the conjugation of thiol nucleophiles with more complex aziridine-containing peptides were impeded by the lack of an efficient method to prepare more elaborate peptide electrophiles. Present solution-phase synthesis of Azy-containing peptides requires introduction and multistep elaboration of TrtSerOH prior to further peptide chain extension. Clearly, an improved method was needed for the rapid generation of diverse Azy-containing peptide electrophiles.

Solid-Phase Synthesis of Azy-Containing Peptides. Given the encouraging results for the conjugation of short Azy-containing peptides with thiol nucleophiles, extension of the scope of this methodology to more diverse peptides was investigated. An Fmoc-based solid-phase peptide synthesis (SPPS) strategy was adopted for the preparation of longer Azy-containing peptides, on the basis of the observation that the acylated Azy moiety is highly unstable in the presence of acids, thereby precluding the possibility of Boc-based SPPS.[12b] The acid sensitivity of the Azy residue was also considered when choosing the appropriate linker for the solid-phase synthesis. For this purpose, polystyrene-attached 2-chlorotrityl linker was selected as it can be cleaved under mildly acidic conditions (20% hexafluoro-2-propanol in dichloromethane)[25] found to be compatible with the acylated Azy moiety. This allowed for the cleavage of aziridine−peptides from the resin and assessment of coupling efficiencies during the course of SPPS. In addition to the acid instability of the Azy moiety, this subunit is also labile to standard basic Fmoc deprotection conditions (20% piperidine in DMF), which were found to affect both aziridine ring opening and deacylation. To avoid these side reactions, 1% DBU in DMF was used for Fmoc deprotection during the iterative cycles of solid-phase synthesis.[26]

In accordance with these parameters, solid-phase synthesis was carried out starting with 2-chlorotrityl polystyrene resin preloaded with glycine (**21**), to which FmocPheOH was coupled by use of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as the activating agent and *N*-methylmorpholine (NMM) as a base in DMF (Scheme 3). After removal of Fmoc with 1% DBU in DMF, FmocAlaOH was incorporated, followed successively by FmocAzyOH, and FmocGlyOH to form pentapeptide **22** with high efficiency. However, attempts to extend this Azy-containing peptide by the coupling of FmocGlyOH at the [Azy + 2] site, followed by Fmoc removal and attempted coupling of the AcGlyOH at the [Azy + 3] position, were unsuccessful. Upon removal of the resin, pentapeptide AcGlyAzyAlaPheGlyOH (**25**) was obtained as the major product. Its formation could be rationalized by the intramolecular N-deacylation of the Azy moiety by the amine liberated upon removal of Fmoc from the amino acid in the [Azy + 2] position, followed by the acylation of the resulting free aziridine **24** with AcGlyOH (Scheme 3). This problem was particularly significant in those peptides where an amino acid with a sterically undemanding side chain was

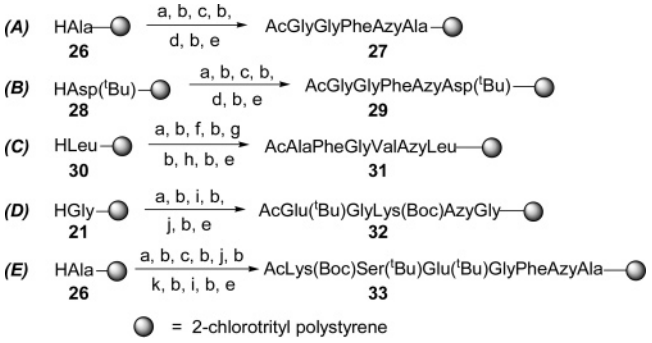
(23) Low conversion to the desired product was observed after prolonged reaction time when 0.1 equiv of DBU was used in this reaction.
(24) A similar mode of reactivity of acylated aziridines was previously observed by Okawa in the reaction of acylated Azy-containing peptides with primary amines (ref 12a).
(25) Bollhagen, R.; Schmiedberger, M.; Barlos, K.; Grell, E. *J. Chem. Soc., Chem. Commun.* **1994**, 2559−2560.
(26) Wade, J. D.; Bedford, J.; Sheppard, R. C.; Tregear, G. W. *Pept. Res.* **1991**, *4*, 194−199.

Scheme 3 [a]



[a] Reagents and conditions: (a) FmocPheOH, HBTU, NMM, DMF; (b) DBU, DMF; (c) FmocAlaOH, HBTU, NMM, DMF; (d) FmocAzyOH, HBTU, NMM, DMF; (e) FmocGlyOH, HBTU, NMM, DMF; (f) AcGlyOH, HBTU, NMM, DMF; (g) $(CF_3)_2CHOH$:CH_2Cl_2 (20% v/v).

Scheme 4 [a]



[a] Reagents and conditions: (a) FmocAzyOH, HBTU, NMM, DMF; (b) DBU, DMF; (c) FmocPheOH, HBTU, NMM, DMF; (d) FmocGlyGlyOH, HBTU, NMM, DMF; (e) Ac_2O, Pyr; (f) FmocValOH, HBTU, NMM, DMF; (g) FmocPheGlyOH, HBTU, NMM, DMF; (h) FmocAlaOH, HBTU, NMM, DMF; (i) FmocLys(Boc)OH, HBTU, NMM, DMF; (j) FmocGlu(tBu)-GlyOH, HBTU, NMM, DMF; (k) FmocSer(tBu)OH, HBTU, NMM, DMF.

incorporated in the [Azy + 1] position. Indeed, the susceptibility of aziridinyl amides toward deacylation is reinforced by an elongated C(O)−N bond compared with non-aziridine-derived amides due to reduced amide−imidate resonance.[27] To circumvent this problem, a strategy involving the simultaneous introduction of a dipeptide unit incorporating both the [Azy + 2] and [Azy + 3] residues was envisioned. Intramolecular deacylation of the aziridine functionality would be disfavored as this route would avoid a six-membered transition state. Indeed, successful extensions of the Azy-containing peptide backbone were achieved with this strategy (Scheme 4). Through multiple applications of this solid-phase synthesis protocol, a series of Azy-containing peptides were rapidly prepared, including those possessing functionalized residues such as glutamic acid, serine, and lysine (**29**, **32**, **33**). Analytical samples of all of the prepared peptides were obtained via cleavage from the resin with hexafluoro-2-propanol in dichloromethane (20% v/v). ^{1}H NMR and ESI-MS analysis showed the crude peptides to be >80% pure.

Thiol Ligation on Solid Support. Successful incorporation of the Azy moiety via SPPS was followed by investigation of the ability of these compounds to undergo site-selective conjugation with various thiols. The polymer-supported peptides (Table 3) were treated with an excess of thiol (5−10 equiv) in the presence of DBU (1 equiv) in DMF at 23 °C. Following the ligation, detachment from the resin was accomplished with either hexafluoro-2-propanol or TFA, which effected the concomitant removal of the acid-labile amino acid side-chain protecting groups. Each of the peptide conjugates was purified by preparative RP-HPLC to afford a net yield of product as calculated over the multistep sequence commencing with the single amino acid preloaded resin.

Initial couplings were performed with simple alkanethiols. Ethanethiol as well as the sterically demanding *tert*-butyl thiol was efficiently conjugated with polymer-supported pentapeptides **27** and **29** to provide conjugates **38**−**40** (Table 3, entries 1−3), each in a nine-step sequence starting with alanine- or aspartic acid-preloaded resins **26** and **28**. High yields and regioselectivities in these transformations established the feasibility of the ligation on solid support as the average yield for these couplings was at least 89% per step. Incorporation of more complex alkanethiols included a biotin tag, commonly used to probe peptide-receptor/antibody interactions.[28] Thus aziridine-containing pentapeptide **27** (entry 4), as well as the more complex heptapeptide **33** (entry 5) were conjugated with the biotin thiol **34** to provide the desired cysteine adducts in good yields (94% and 89% per step, respectively, over 9- and 13-step sequences, starting from **26**). Moreover, the allylic thiol moiety of farnesyl thiol **35** was also efficiently coupled with the aziridine-containing peptides **27** and **31**, affording lipopeptides **43** and **44** with an average yield of at least 90% per step, starting with the single amino acid preloaded resin (entries 6 and 7). The importance of a convergent synthetic strategy for the preparation of these peptides is highlighted by the involvement of farnesylated proteins in gene expression, apoptosis, and remodeling of the actin cytoskeleton.[29] Compared to the frequently used cysteine alkylation protocol with farnesyl halides, this late-stage convergent coupling between the Azy moiety and farnesyl thiol, in which the respective roles of nucleophile and electrophile are reversed, surmounts the commonly encountered problems of cysteine oxidation as well as overalkylation of peptide substrates.[1a,d]

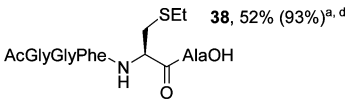
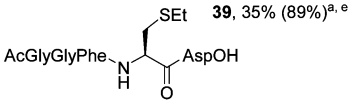
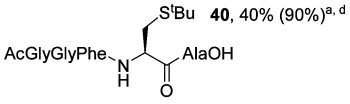
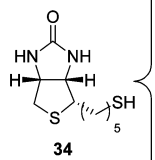
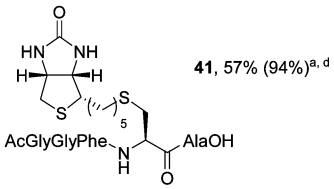
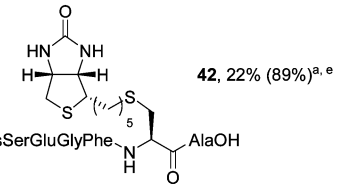
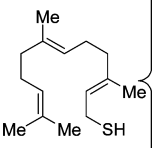
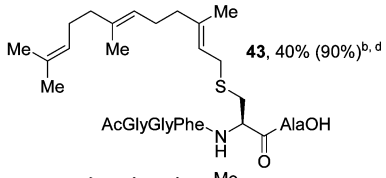
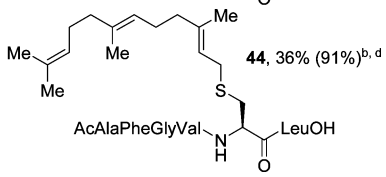
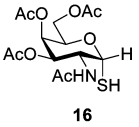
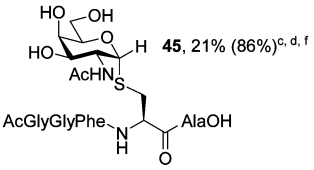
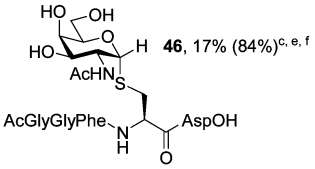
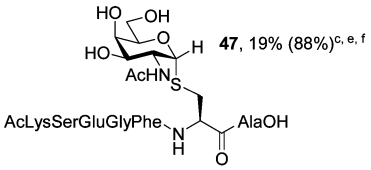
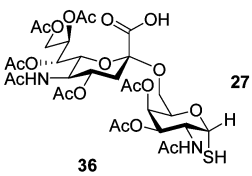
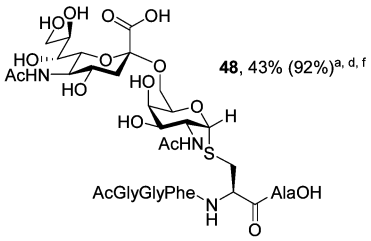
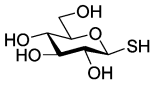
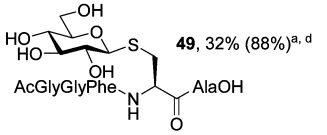
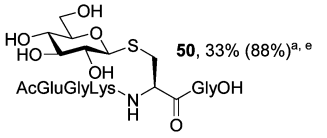
The solid-supported aziridine ligation protocol is also useful for the preparation of thioglycopeptides. The thio-T_N antigen **16** was reacted with polymer-supported pentapeptides **27** and **29** to give, after release of thioglycopeptide from the resin, acetate deprotection, and RP-HPLC purification, the thioglycopeptides **45** and **46** in 21% and 17% yield, respectively, each over a 10-step sequence (entries 8 and 9, Table 3). Similar efficiency was observed when heptapeptide **33** (entry 10) was

(27) Shao, H.; Jiang, X.; Gantzel, P.; Goodman, M. *Chem. Biol.* **1994**, *1*, 231−234.

(28) Fu, P.; Layfield, S.; Ferraro, T.; Tomiyama, H.; Hutson, J.; Otvos Jr., L.; Tregear, G. W.; Bathgate, R. A. D.; Wade, J. D. *J. Peptide Res.* **2004**, *63*, 91−98.

(29) (a) Barbacid, M. *Annu. Rev. Biochem.* **1987**, *56*, 779−827. (b) Bos, J. L. *Cancer Res.* **1989**, *49*, 4682−4689.

Table 3. On-Bead Conjugation of Azy Peptides with Thiols

entry	thiol	peptide	peptide conjugate; yield from **26**, **28** or **30**; (yield/step)
1	EtSH	**27**	 **38**, 52% (93%)[a, d]
2	EtSH	**29**	 **39**, 35% (89%)[a, e]
3	^{t}BuSH	**27**	 **40**, 40% (90%)[a, d]
4	 **34**	**27**	 **41**, 57% (94%)[a, d]
5		**33**	 **42**, 22% (89%)[a, e]
6	 **35**	**27**	 **43**, 40% (90%)[b, d]
7		**31**	 **44**, 36% (91%)[b, d]
8	 **16**	**27**	 **45**, 21% (86%)[c, d, f]
9		**29**	 **46**, 17% (84%)[c, e, f]
10		**33**	 **47**, 19% (88%)[c, e, f]
11	 **36**	**27**	 **48**, 43% (92%)[a, d, f]
12	 **37**	**27**	 **49**, 32% (88%)[a, d]
13		**32**	 **50**, 33% (88%)[a, e]

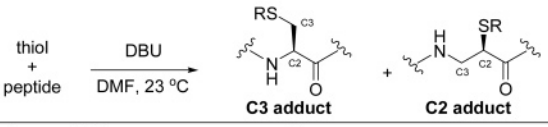
[a] DBU, DMF, 23 °C. [b] DBU, $CHCl_3$, DMF, 23 °C. [c] DBU, DMF, 60 °C. [d] $(CF_3)_2CHOH/CH_2Cl_2$ (20% v/v). [e] TFA/CH_2Cl_2/$^{i}Pr_3SiH$/$HS(CH_2)_2SH$, 10:10:1:1. [f] NaOMe, MeOH, pH 8.5.

reacted with thiol **16** to provide glycoconjugate **47** (19% yield over 14 steps, average of 88% per step).[30] In addition, the thio analogue of the mucin-related ST_N antigen **36**, which incorporates a ketal glycosidic linkage between sialic acid and galactosamine as well as a free carboxylic acid functionality, reacted smoothly with the pentapeptide **27**, affording the disaccharide-pentapeptide conjugate **48** in 43% yield over a 10-step sequence, an efficiency of 92% per step (entry 11). The complete retention of the α-anomeric configuration in the coupling of the thio-T_N and ST_N carbohydrates punctuates the potential of this approach to prepare and study glycosylated S-mucin peptide fragments, especially in light of reports highlighting the critical role of the core GalNAc α-anomeric stereochemistry in properly organizing the peptide backbone of the mucin glycopeptide motif.[31] Finally, the anomeric thiol of unprotected carbohydrates, exemplified by the *β*-thioglucopyranose **37**, is also amenable to this conjugation approach. Azy-containing pentapeptides **27** and **32**, when subjected to conjugation with this nucleophile, provided the desired α-amino acid adducts in 32% and 33% yields, each over a 9-step

(30) Reactions in entries 8−10 were carried out by heating the reaction mixture to 60 °C to allow for faster reaction. A decrease in the yields of the isolated products in these reactions may be attributed to the thermal instability of the 2-chlorotrityl linker.

(31) (a) Liang, R.; Andreotti, A. H.; Kahne, D. *J. Am. Chem. Soc.* **1995**, *117*, 10395−10396. (b) Live, D. H.; Williams, L. J.; Kuduk, S. D.; Schwarz, J. B.; Glunz, P. W.; Chen, X.-T.; Sames, D.; Kumar, R. A.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3489−3493. (c) Schuman, J.; Qiu, D.; Koganty, R. R.; Longenecker, B. M.; Campbell, A. P. *Glycoconjugate J.* **2000**, *17*, 835−848. (d) Coltart, D. M.; Royyuru, A. K.; Williams, L. J.; Glunz, P. W.; Sames, D.; Kuduk, S. D.; Schwarz, J. B.; Chen, X.-T.; Danishefsky, S. J.; Live, D. H. *J. Am. Chem. Soc.* **2002**, *124*, 9833−9844. (e) Schuman, J.; Campbell, A. P.; Koganty, R. R.; Longenecker, B. M. *J. Pept. Res.* **2003**, *61*, 91−108.

Table 4. On-Bead Conjugation of Azy Peptides with Thiols



entry	thiol	peptide	C3: C2 adduct
1	EtSH	AcValAzyAla**Ala**Gly—● (**51**)	1.0:5.6[a]
2	EtSH	AcValAzySer(ᵗBu)**Ala**Gly—● (**52**)	1.0:4.4[a]
3	**35**	AcValAzyAla**Ala**Gly—● (**51**)	1.0:5.2[a]
4	**16**	AcValAzySer(ᵗBu)**Ala**Gly—● (**52**)	1.0:1.4[a]
5	EtSH	AcValAzyAla**Pro**Gly—● (**53**)	5.8:1.0[a]
6	**16**	AcValAzyAla**Pro**Gly—● (**53**)	3.7:1.0[a, b]
7	EtSH	AcValAzyAla**Sar**Gly—● (**54**)	3.7:1.0[a]

[a] $(CF_3)_2CHOH/CH_2Cl_2$ (20% v/v). [b] NaOMe, MeOH, pH 8.5.

sequence starting from **26** and **21**, respectively (entries 12 and 13). During the course of the ligation, the β-anomeric configuration of the carbohydrate thiol remained unchanged. High levels of chemoselectivity for the addition of thiol to the Azy moiety in these transformations eliminate the need for protection of carbohydrate peripheral hydroxyl functionalities. These examples demonstrate not only the high efficiency of the Azy incorporation during the course of SPPS but also the tolerance of this residue to multiple rounds of amino acid couplings and Fmoc deprotections during the course of SPPS.

Regioselectivity of Azy Opening as a Function of Peptide Structure. During the course of investigation of on-bead ligation with additional peptide substrates in which the Azy moiety was further removed from the solid support, variations in regioselectivity were observed (Table 4). When ethanethiol was reacted with resin-supported AcValAzyAlaAlaGly pentapeptide **51** (Table 4, entry 1), the ethylthio-β^2-homoglycine-containing peptide, resulting from thiol addition to C2, was observed as the major product (C3:C2 adduct = 1.0:5.6).[32] Comparable levels of regioselectivity favoring thiol addition to the more sterically hindered C2 carbon of the aziridine moiety were observed in the reaction of ethanethiol and farnesyl thiol (**35**) with the resin-bound AcValAzySer(ᵗBu)AlaGly (**52**) and AcValAzyAlaAlaGly (**51**) pentapeptides as well (entries 2 and 3).[33] A similar, although less marked, effect was observed in the conjugation of peptide **52** with T_N antigen **16**, in which two constitutional isomers were generated in the ratio 1.0:1.4, slightly favoring the product resulting from thiol addition to the C2 carbon of the aziridine (entry 4).[34] However, when ethanethiol was reacted with AcValAzyAlaProGly pentapeptide **53** (entry 5), which differs from **51** only in the replacement of Ala with Pro in the [Azy − 2] position, the C3 adduct was obtained as the major product (C3:C2 adduct = 5.8:1.0). Interestingly, the reaction rates for these two ligations (entries 1 vs 5) were also appreciably different. While the reaction of EtSH (10 equiv) with the polymer-supported AcValAzyAlaAlaGly pentapeptide (**51**) (entry 1) reached completion in less than 24 h, reaction of pentapeptide AcValAzyAlaProGly (**53**) with EtSH achieves only 75% conversion after 6 days (entry 5) under the same conditions.[35] A similar trend in ligation regioselectivity and reaction rate was also evident in the conjugation of carbohydrate-derived thiol **16** with the resin-attached Pro-containing peptide AcValAzyAlaProGly (**53**) (entry 6, ~60% conversion, 6 days; C3:C2 adduct = 3.7:1.0).[36] These data imply that the nature of the amino acid in the [Azy − 2] position may be a critical determinant of the regioselectivity in the aziridine ring-opening process. One obvious difference between Ala- and Pro- containing peptides is the absence of an NH group in the [Azy − 2] position in the latter. Indeed, reaction of ethanethiol with the polymer-supported pentapeptide AcValAzyAlaSarGly (**54**) (entry 7), which also possesses a tertiary amide group in the [Azy − 2] position (Sar = sarcosine = *N*-methylglycine), provided a mixture of conjugated products favoring the C3 adduct (C3:C2 adduct = 3.7:1.0), an outcome similar to that of the otherwise identical proline-containing peptides. The potential significance of the [Azy − 2] NH group in enhancing rate and facilitating addition of nucleophile to the C2 position may be attributed to an inductive activation of the α-carbon of the Azy residue resulting from the involvement of its carbonyl moiety in a hydrogen bond with the amide proton of the [Azy − 2] amino acid in a γ-turn-like conformation.[37] Alternatively, similar carbonyl activation via interchain hydrogen bonding[38] could arise from high resin loadings.[39] However, direct evidence for these hypotheses has yet to be secured, as no significant change in regioselectivity was observed when ligations were performed in the presence of polar additives such as DMSO and ethylenecarbonate.[40] Alternatively, given that insertion of Pro and Sar into peptides is known to inhibit peptide aggregation,[41] the regiochemical outcomes in Table 4 may arise from more subtle conformational effects caused by the [Azy − 2] residue. Studies are underway to further explore these intriguing aspects of the reactivity of various Azy-containing peptide substrates, as well as the influence that this residue has on the conformational properties of the resulting peptides.

C-Terminal Extension of Glycopeptides via Native Chemical Ligation. Given the observed variations in regioselectivity of aziridine opening in peptides with multiple residues C-

(32) In these reactions, the ratio of products was determined by NMR analysis of the crude product mixture. In instances when the analysis of crude NMR data did not allow an accurate estimate of this ratio, products were analyzed by HPLC-ESI.

(33) When the resin-bound AcValAzySer(ᵗBu)AlaGly pentapeptide was swelled in DMF containing 20% DMSO (v/v) and then reacted with EtSH and DBU, the same product distribution was observed.

(34) A similar distribution of products was observed in the reaction of these two coupling partners in 10% DMSO in DMF (v/v) and in the presence of 2 M ethylenecarbonate in DMF.

(35) A substantial decrease in the ligation rate for the peptides possessing *N*-alkyl amino acids in the [Azy-2] position was observed for all of the tested peptides.

(36) While the expected glycosylated cysteine adduct was the major component of the crude mixture, two other minor carbohydrate-containing products were observed after cleavage from the solid support. Upon removal of *O*-acetates with sodium methoxide in methanol, a C3:C2 adduct ratio of 3.7:1.0 was obtained (HPLC-ESI).

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(39) Peptides **51** and **52** were prepared starting from glycine-preloaded 2-chlorotrityl polystyrene resin **21** with a loading of 0.78 mmol/g.

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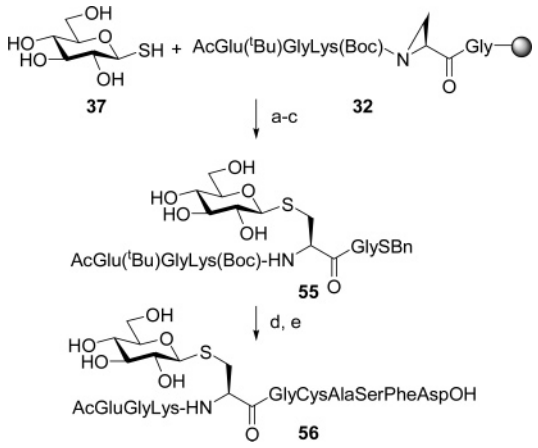
Scheme 5 [a]



[a] Reagents and conditions: (a) DBU, DMF; (b) $(CF_3)_2CHOH/CH_2Cl_2$ (20% v/v); (c) BnSH, EDC·HCl, HOBt, DMF (44% over 10 steps from **21**; 92%/step); (d) TFA/CH_2Cl_2/$^iPr_3SiH$/$HS(CH_2)_2SH$, 10:10:1:1 (88%); (e) HCysAlaSerPheAspOH, Gn·HCl, PhSH, sodium phosphate buffer, pH 7.5 (83%).

terminal to the aziridine moiety (Table 4), direct application of the thiol−aziridine ligation strategy to construct internal homogeneous cysteine conjugates of extended peptide substrates may be hampered by the formation of constitutional isomers. This potential complication notwithstanding, it is clear that the ligation of thiols with aziridine-containing peptides possessing Azy as the second amino acid from the resin proceeds with exceptionally high efficiency and regio- and stereoselectivity for the cysteine conjugates (Table 3, entries 1−13). In fact, the convergency of this strategy is by no means compromised when it is applied to the preparation of more complex peptide conjugates given the prospect of further extension at the C-terminus via subsequent peptide ligation. Native chemical ligation[42] proceeds under mild conditions and is compatible with the presence of glycosylated amino acids,[6h,43] although ligation efficiency in the presence of a bulky glycosylated residue in proximity to the C-terminal coupling fragment has heretofore not been assessed. Thus, unprotected β-thioglucopyranose **37** was reacted with aziridine-containing pentapeptide **32** (Scheme 5), prepared according to the standard iterative procedure (vide supra). Following the carbohydrate−peptide ligation, the resin was removed by treatment with hexafluoro-2-propanol in dichloromethane (20% v/v), and the glycopeptide conjugate was then condensed at the C-terminus with benzylthiol,[44] providing the desired glycopeptide thioester **55** in characteristically high overall yield (44% over 10 steps from **21**, ∼92% per step). Deprotection of the side-chain protecting groups in thioester **55** was accomplished with TFA in the presence of ethanedithiol and tri-*iso*-propyl silane. This generated the corresponding fully deprotected glycopeptide thioester, which was subsequently purified by RP-HPLC. The ligation of AcGluGlyLysCys(β-Glucose)Gly-SBn (**55**) and HCysAlaSerPheAspOH, prepared and purified by the standard solid-phase synthesis protocol, was performed in sodium phosphate buffer (100 mM, pH 7.5), containing 6 M guanidine hydrochloride and thiophenol (4% v/v). The ligation was complete in less than 3 h, and the product was purified by RP-HPLC to afford glycosylated decapeptide **56** in 83% yield. The successful preparation of **56**, commencing with the conjugation of unprotected carbohydrate thiol **37** and aziridine-containing peptide **32**, followed by the extension of the peptide backbone with the cysteine-containing peptide, demonstrates the utility of this sequential ligation approach in the convergent assembly of complex peptide thioconjugates.

Conclusion

The development of a novel method for site- and stereoselective peptide modification based on incorporation of the aziridine-2-carboxylic acid moiety into peptides has been described. To allow for the rapid construction of diverse Azy-containing peptides, FmocAzyOH has been prepared and incorporated into peptides via a modified Fmoc-based solid-phase synthesis protocol. The unique electrophilic nature of this nonproteinogenic residue allows for site-selective conjugation with thiol nucleophiles in a highly selective aziridine ring-opening process. A wide range of thiol nucleophiles have been coupled with Azy-containing peptides, both in solution and on solid support. This method is applicable to the site-specific labeling of peptides with valuable biochemical probes and is amenable to the preparation of posttranslationally modified peptides, such as farnesylated peptides and thioglycoconjugates derived from either protected or unprotected carbohydrate nucleophiles. This methodology, combined with native chemical ligation, also allows for the generation of complex thioglycoconjugates in which the stereochemical integrity of the carbohydrate donor and the α-carbon stereocenter of the amino acid is preserved. Present studies are focused on delineation of the factors governing the regioselectivity of the process and application of this methodology for the generation of stereodefined β-amino acids. This facile route to Azy-containing peptides and the efficiency with which these substrates undergo ligation also shows promise for the convergent synthesis of multivalent glycopeptide displays and application in functional proteomics studies.

Experimental Section

General. All reactions were performed in dry modified Schlenk (Kjeldahl shape) flasks fitted with a glass stopper under positive pressure of argon, unless otherwise noted. Air- and moisture-sensitive liquids were transferred via syringe. Organic solutions were concentrated by rotary evaporation below 30 °C at ca. 25 Torr. Flash column chromatography was performed on 230−400 mesh silica gel. Thin-layer chromatography was performed on glass plates precoated to a depth of 0.25 mm with 230−400 mesh silica gel impregnated with a fluorescent indicator (254 nm). When necessary, solvents were degassed by the freeze-pump thaw method (>3 cycles).

Dichloromethane, toluene, acetonitrile, 1,2-dimethoxyethane, and tetrahydrofuran were purified by passage through two packed columns of neutral alumina under an argon atmosphere. Chloroform, pyridine, diisopropylethylamine, and triethylamine were distilled from calcium hydride at 760 Torr. Methanol was distilled from magnesium oxide at 760 Torr. DMF was dried over activated 4 Å molecular sieves. Millipore water was used for reactions in an aqueous solvent or cosolvent.

Infrared (IR) spectra were obtained on a Perkin-Elmer Spectrum BX spectrophotometer referenced to a polystyrene standard. Data are presented as frequency of absorption (reciprocal centimeters). Proton and carbon-13 nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR)

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spectra were recorded on a Varian 400, a Varian 500, or a Varian Inova 500 NMR spectrometer; chemical shifts are expressed in parts per million (δ scale) downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent [$CHCl_3$, δ 7.27; HDO, 4.80; C_6D_4HN, 7.22; CHD_2OD, 3.31; $DCON(CD_3)CHD_2$, 2.75]. Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and/or multiple resonances), coupling constants in Hertz (Hz), and integration.

RP-HPLC purification and analyses were carried out on a Rainin HPLC system (model SD 200), equipped with UV absorbance detector (model UV 1), at a wavelength of 220 nm. The products were analyzed and purified on Vydac C18 columns (analytical, 250 × 4.6 mm; preparative, 250 × 22 mm). Products were eluted in 0.1% TFA in acetonitrile/water in a linear gradient optimized for every compound at flow rates of 1 mL min^{-1} for analytical and 8 mL min^{-1} on the preparative column.

General Procedure for Conjugation with Azy-containing Peptides in Solution: Reaction of BocAlaAzyAlaOBn with EtSH. DBU (1.0 μL, 0.006 mmol, 0.1 equiv) was added to a solution of BocAlaAzy-AlaOBn **7** (27 mg, 0.06 mmol, 1 equiv), and ethanethiol (6 μL, 0.08 mmol, 1.20 equiv) in acetonitrile (315 μL) at 23 °C. Formation of a white precipitate was observed after 12 min. The resulting solution was stirred at this temperature for 3.5 h and then concentrated in vacuo. The residue was purified by silica gel flash chromatography (25% ethyl acetate in dichloromethane) to afford the C3 adduct (27 mg, 85%), as a white solid and C2 adduct (0.4 mg, 1%), as a white solid (crude ^{1}H NMR ratio of C3:C2 adduct = 20:1).

BocAlaCys(Et)AlaOBn. Mp = 53 °C; R_f = 0.30 (33% ethyl acetate in dichloromethane); ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.39−7.33 (m, 5 H), 7.26 (br s, 1H), 7.06 (d, J = 6.4 Hz, 1H), 5.18 (ABq, J_{AB} = 12.3 Hz, 2H), 4.96 (br s, 1H), 4.59 (pentet, J = 7.2 Hz, 1H), 4.54 (m, 1H), 4.13 (m, 1H), 3.08 (dd, J = 14.8 and 2.5 Hz, 1H), 2.77 (dd, J = 14.0 and 7.1 Hz, 1H), 1.46 (s, 9H), 1.44 (d, J = 7.1 Hz, 3H), 1.39 (d, J = 7.3 Hz, 3H), 1.26 (t, J = 7.5 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ = 172.9, 172.4, 170.1, 156.0, 135.5, 128.8, 128.6, 128.4, 80.7, 67.4, 52.4, 50.9, 48.7, 33.5, 28.5, 26.5, 18.2, 18.0, 14.9; FTIR (neat film) 3317, 1737, 1680, 1645, 1532, 1166, 850 cm^{-1}. HRMS (FAB)$^+$ m/z calcd for $C_{23}H_{36}N_3O_6S$ $[M + H]^+$, 482.2325; found, 482.2318.

BocAlaβ^2EtS-HGlyAlaOBn. R_f = 0.17 (33% ethyl acetate in dichloromethane); ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.50 (d, J = 6.98 Hz, 1H), 7.40−7.34 (m, 5H), 7.18 (br s, 1H), 5.22 (ABq, J_{AB} = 12.2 Hz, 2H), 5.04 (d, J = 4.3 Hz, 1H), 4.66 (pentet, J = 7.7 Hz, 1H), 4.03 (pentet, J = 7.7 Hz, 1H), 3.93 (m, 1H), 3.55 (m, 2H), 2.56 (q, J = 7.4 Hz, 2H), 1.52 (d, J = 7.2 Hz, 3H), 1.44 (s, 3H), 1.32 (d, J = 7.0 Hz, 3H), 1.23 (t, J = 7.5 Hz, 3H); FTIR (neat film) 3291, 1651, 1546, 1037 cm^{-1}. HRMS (ESI)$^+$ m/z calcd for $C_{23}H_{36}N_3O_6S$ $[M + H]^+$, 482.2325; found, 482.2325.

General Procedure for Conjugation with 3-MeAzy-containing Peptides in Solution: BocGly-3-MeCys(Et)NHBn. DBU (13.0 μL, 0.087 mmol, 1.0 equiv) was added to a solution of BocGly-3-MeAzyNHBn (29.2 mg, 0.084 mmol, 1.0 equiv) and ethanethiol (52.2 μL, 0.840 mmol, 10.0 equiv) in chloroform (420 μL) at 23 °C. The resulting clear solution was stirred at this temperature for 2 h and then concentrated in vacuo. The residue was purified by silica gel flash chromatography (40% ethyl acetate in dichloromethane) to afford the C3 conjugation adduct (13 mg, 36%) as a colorless oil. R_f = 0.37 (50% ethyl acetate in dichloromethane); ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.42 (m, 1H), 7.36−7.24 (m, 5H), 7.19 (d, J = 7.5 Hz, 1H), 5.22 (m, 1H), 4.60 (dd, J = 7.7 and 2.6 Hz, 1H), 4.54 (dd, J = 14.7 and 6.2 Hz, 1H), 4.39 (dd, J = 14.7 and 5.3 Hz, 1H), 3.80 (m, 2H), 3.60 (m, 1H), 2.62 (m, 1H), 2.49 (m, 1H), 1.41 (s, 9H), 1.20 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 169.5, 169.3, 156.7, 138.0, 128.8, 128.2, 127.6, 81.0, 56.9, 45.0, 43.9, 41.3, 28.4, 26.0, 18.4, 15.0; FTIR (neat film) 3305, 2976, 2930, 1650, 1518, 1455, 1367, 1250, 1169 cm^{-1}. HRMS (ESI)$^+$ m/z calcd for $C_{20}H_{32}N_3O_4S$ $[M + H]^+$, 410.2114; found, 410.2132.

General SPPS Procedure: AcGlyGlyPheAzyAla-2-Chlorotrityl Resin (27). Alanine-preloaded 2-chlorotrityl resin **26** (228 mg, 0.15 mmol, 1 equiv; loading 0.64 mmol/g) was swollen in CH_2Cl_2/DMF (30% v/v) in a solid-phase peptide synthesis reaction vessel (see Ace Glass) for 1 h. After swelling, the resin was rinsed with DMF (10 × 2 mL). FmocAzyOH (190 mg, 0.6 mmol, 4 equiv), HBTU (227 mg, 0.6 mmol, 4 equiv), and 0.4 M NMM in DMF (6 mL, 2.4 mmol, 16 equiv) were premixed in a vial and added to the resin. Nitrogen was bubbled through the reaction vessel for 2.5 h, after which the resin was rinsed with DMF (10 × 2 mL). To ensure complete reaction, the coupling was repeated (FmocAzyOH, 93 mg, 0.3 mmol, 2 equiv; HBTU, 114 mg, 0.3 mmol, 2 equiv; 0.4 M NMM/DMF, 3 mL, 1.2 mmol, 8 equiv; reaction time 1 h). The resin was subsequently rinsed with DMF (10 × 2 mL), and Fmoc was removed with 1% DBU/DMF (6 mL, 0.4 mmol, 2.7 equiv) for 1 min and then rinsed with DMF (10 × 2 mL). Fmoc deprotection was repeated twice in the same manner. FmocPheOH (236 mg, 0.6 mmol, 4 equiv), HBTU (228 mg, 0.6 mmol, 4 equiv), and 0.4 M NMM/DMF (6 mL, 2.4 mmol, 16 equiv) were premixed in a vial and added to the reaction vessel containing resin. Nitrogen was bubbled through for 2.5 h, the resin was rinsed with DMF (10 × 2 mL), and the coupling was repeated to ensure complete acylation of the secondary amine within the Azy residue (FmocPheOH, 234 mg, 0.6 mmol, 4 equiv; HBTU, 227 mg, 0.6 mmol, 4 equiv; 0.4 M NMM/DMF, 6 mL, 2.4 mmol, 16 equiv; reaction time 1 h). The resin was subsequently rinsed with DMF (10 × 2 mL), and Fmoc was removed with 1% DBU/DMF (6 mL, 0.4 mmol, 2.7 equiv) for 1 min and then rinsed with DMF (10 × 2 mL). Fmoc deprotection was repeated twice in the same manner. FmocGlyGlyOH (219 mg, 0.6 mmol, 4 equiv), HBTU (230 mg, 0.6 mmol, 4 equiv), and 0.4 M NMM/DMF (6 mL, 2.4 mmol, 16 equiv) were premixed in a vial and added to the reaction vessel containing resin. Nitrogen was bubbled through for 2 h, and the resin was rinsed with DMF (10 × 2 mL). Fmoc was removed with 1% DBU/DMF (6 mL, 0.4 mmol, 2.7 equiv) for 1 min, and the resin was rinsed with DMF (10 × 2 mL). Fmoc deprotection was repeated twice in the same manner. A solution of Ac_2O in pyridine (1:3 v/v; 15.5 mL, 37.5 mmol, 250 equiv) was then added to the SPPS reaction vessel with the resin, through which nitrogen was bubbled for 15 min. Following acetylation, the resin was washed with DMF and CH_2Cl_2 and stored at −10 °C under Ar until it was used for ligations. A portion of the product was cleaved from the resin with 20% (v/v) hexafluoro-2-propanol (HFIPA) in dichloromethane. ^{1}H NMR analysis of a crude product showed ca. 85−90% purity for the desired peptide. ^{1}H NMR (500 MHz, DMF-d_7) δ = 8.64 (d, J = 6.9 Hz, 1H), 8.49 (br s, 1H), 8.33 (t, J = 5.5 Hz, 1H), 8.21 (t, J = 6.0 Hz, 1H), 8.18 (d, J = 7.2 Hz, 1H), 7.33−7.21 (m, 5H), 4.68 (m, 1H), 4.39 (pentet, J = 7.1 Hz, 1H), 3.89−3.76 (m, 4H), 3.30 (dd, J = 5.6 and 3.2 Hz, 1H), 3.25 (dd, J = 14.3 and 4.5 Hz, 1H), 3.01 (dd, J = 14.0 and 9.3 Hz, 1H), 2.65 (dd, J = 5.6 and 2.0 Hz, 1H), 2.45 (dd, J = 3.2 and 1.9 Hz, 1H), 1.96 (s, 3H), 1.41 (d, J = 7.2 Hz, 3H). HRMS (ESI)$^+$ m/z calcd for $C_{21}H_{28}N_5O_7$ $[M + H]^+$, 462.1989; found, 462.1992.

General Procedure for Conjugation with Aziridine-containing Peptides on Solid Support. Method A: AcGlyGlyPheCys(Et)AlaOH (38). DBU (2.4 μL, 0.016 mmol, 1 equiv) was added to a suspension of AcGlyGlyPheAzyAla-2-chlorotrityl resin **27** (32 mg, 0.016 mmol, 1 equiv) and ethanethiol (12 μL, 0.16 mmol, 10 equiv) in DMF (650 μL) at 23 °C. The resulting suspension was stirred at this temperature for 16 h, then the resin was washed with CH_2Cl_2 (25 × 1.5 mL), and the crude product mixture was cleaved from the resin with 20% (v/v) HFIPA/CH_2Cl_2 (9 mL, 15 min, 23 °C). Concentration in vacuo and purification of the residue by preparative RP-HPLC (10% CH_3CN in H_2O to 70% CH_3CN in H_2O + 0.1% TFA, in 50 min) provided conjugate **38** (4 mg, 52% from HAla-preloaded resin **26**; 9 steps, 93%/step), as a white solid. R_t (10% CH_3CN in H_2O to 70% CH_3CN in H_2O + 0.1% TFA, in 50 min, analytical column) = 14.60 min. ^{1}H NMR (500 MHz, CD_3OD) δ = 8.35 (t, J = 5.4 Hz, 1H), 8.20 (d, J = 7.3 Hz, 1H), 8.16 (d, J = 8.0 Hz, 1H), 8.13 (d, J = 7.6 Hz, 1H), 7.27−

7.19 (m, 5 H), 4.63 (ddd, J = 9.6, 7.9, and 5.1 Hz, 1H), 4.52 (m, 1H), 4.36 (pentet, J = 7.5 Hz, 1H), 3.80 (m, 4H), 3.21 (dd, J = 14.0 and 5.1 Hz, 1H), 3.04 (dd, J = 13.9 and 5.2 Hz, 1H), 2.97 (dd, J = 14.0 and 9.6 Hz, 1H), 2.80 (dd, J = 14.0 and 9.0 Hz, 1H), 2.59 (qd, J = 7.5 and 1.7 Hz, 2H), 2.02 (s, 3H), 1.41 (d, J = 7.4 Hz, 3H), 1.24 (t, J = 7.3 Hz, 3H). HRMS (ESI)$^+$ m/z calcd for $C_{23}H_{34}N_5O_7S$ [M + H]$^+$, 524.2179; found, 524.2186.

Method B: AcGlyGlyPheCys(Et)AspOH (39). DBU (4.4 μL, 0.03 mmol, 1 equiv) was added to a suspension of AcGlyGlyPheAzyAsp-(^{t}Bu)-2-chlorotrityl resin **29** (52 mg, 0.03 mmol, 1 equiv) and ethanethiol (22 μL, 0.29 mmol, 10 equiv) in DMF (1.2 mL) at 23 °C. The resulting suspension was stirred at this temperature for 22 h, and then the resin was filtered and washed with dichloromethane (25 × 1.5 mL). The ligation product was cleaved from the resin by treatment with TFA/CH_2Cl_2/^{i}Pr$_3$SiH/HS(CH$_2$)$_2$SH (10:10:1:1, 10 mL) for 1.5 h at 23 °C. The resin was filtered off, and the filtrate was coevaporated sequentially with toluene (2 × 4 mL), followed by cold Et_2O (5 mL). The resulting white precipitate was washed with hexanes (3 × 2 mL), and cold Et_2O (3 × 1 mL). Purification of the residue by preparative RP-HPLC (10% CH_3CN in H_2O to 70% CH_3CN in H_2O + 0.1% TFA, in 50 min) provided conjugate **39** (6 mg, 35% from HAsp(^{t}Bu)-preloaded resin **28**; 9 steps, 89%/step). R_t (10% CH_3CN in H_2O to 70% CH_3CN in H_2O + 0.1% TFA, in 50 min, analytical column) = 13.80 min. ^{1}H NMR (500 MHz, CD_3OD) δ = 8.22 (m, 2 H), 8.11 (d, J = 7.6 Hz, 1H), 8.22 (m, 2 H), 7.26 (m, 5H), 4.72 (m, 1H), 4.64 (m, 1H), 4.54 (m, 1H), 3.82 (m, 4H), 3.21 (dd, J = 14.0 and 4.7 Hz, 1H), 3.03 (dd, J = 14.0 and 5.4 Hz, 1H), 2.97 (dd, J = 14.0 and 9.6 Hz, 1H), 2.85 (m, 3H), 2.58 (qd, J = 7.4 and 1.0 Hz, 2H), 2.03 (s, 3H), 1.24 (t, J = 7.6 Hz, 3H). HRMS (ESI)$^+$ m/z calcd for $C_{24}H_{34}N_5O_9S$ [M + H]$^+$, 568.2077; found, 568.2073.

Method C: AcGlyGlyPheCys(GalNAcα1)AlaOH (45). DBU (7.2 μL, 0.05 mmol, 1 equiv) was added to a suspension of AcGlyGlyPheAzyAla-2-chlorotrityl resin **27** (99 mg, 0.05 mmol, 1 equiv) and thio 2-*N*-acetylamino-3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-galactopyranose **16** (91 mg, 0.24 mmol, 5 equiv) in DMF (3.4 mL) at 60 °C. The resulting suspension was stirred at this temperature for 22 h, and then the resin was filtered and washed with dichloromethane (25 × 1.5 mL). The ligation product was cleaved from the resin with 20% (v/v) HFIPA/CH_2Cl_2 (15 mL, 15 min, 23 °C). The residue was concentration in vacuo and dissolved in methanol (30 mL). A solution of NaOMe in MeOH (0.1 M) was added until pH 8.5, and the resulting solution was stirred for 80 min at 23 °C. Subsequently, a 1 M solution of AcOH in MeOH was added until pH 6. Concentration in vacuo and purification of the residue by preparative RP-HPLC (7% CH_3CN in H_2O to 26% CH_3CN in H_2O + 0.1% TFA, in 40 min) provided conjugate **45** (7 mg, 21% from HAla-preloaded resin **26**; 10 steps, 86%/step). R_t (7% CH_3CN in H_2O to 30% CH_3CN in H_2O + 0.1% TFA, in 40 min, analytical column) = 15.01 min. ^{1}H NMR (500 MHz, CD_3OD) δ = 8.36 (d, J = 8.6 Hz, 1H), 8.27 (t, J = 6.2 Hz, 1H), 8.22 (d, J = 6.8 Hz, 1H), 8.14 (d, J = 7.6 Hz, 1H), 8.05 (d, J = 7.6 Hz, 1H), 7.29−7.19 (m, 5 H), 5.59 (d, J = 5.5 Hz, 1H), 4.67 (m, 1H), 4.61 (m, 1H), 4.43 (m, 1H), 4.38 (pentet, J = 7.2 Hz, 1H), 4.17 (dd, J = 7.8 and 3.9 Hz, 1H), 3.87 (m, 6H), 3.72 (dd, J = 11.4 and 4.3 Hz, 1H), 3.65 (dd, J = 11.4 and 3.2 Hz, 1H), 3.23 (dd, J = 13.8 and 4.8 Hz, 1H), 3.11 (dd, J = 13.8 and 4.4 Hz, 1H), 3.00 (dd, J = 14.6 and 4.4 Hz, 1H), 2.95 (dd, J = 14.0 and 9.6 Hz, 1H), 2.02 (s, 3H), 1.98 (s, 3H), 1.41 (d, J = 7.4 Hz, 3H). HRMS (ESI)$^+$ m/z calcd for $C_{29}H_{43}N_6O_{12}S$ [M + H]$^+$, 699.2660; found, 699.2656.

AcGlu(^{t}Bu)GlyLys(Boc)Cys(Glcβ1)GlySBn (55). DBU (3.2 μL, 0.021 mmol, 1 equiv) was added to a suspension of AcGlu(^{t}Bu)GlyLys-(Boc)AzyGly-2-chlorotrityl resin **32** (51 mg, 0.021 mmol, 1 equiv) and 1-thio-β-D-glucopyranose **37** (21 mg, 0.11 mmol, 5 equiv) in DMF (1.4 mL) at 23 °C. The resulting suspension was stirred at this temperature for 3.5 days, then the resin was washed with CH_2Cl_2 (25 × 1.5 mL), and the crude product mixture was cleaved from the resin with 20% (v/v) HFIPA/CH_2Cl_2 (8 mL, 15 min, 23 °C). The residue was concentrated in vacuo to provide 16 mg of the crude product. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, 42 mg, 0.22 mmol, 12 equiv) was added to a suspension of the obtained crude AcGlu(^{t}Bu)GlyLys(Boc)Cys(β-Glc)GlyOH (14 mg), 1-hydroxybenzotriazole hydrate (HOBt, 29 mg, 0.21 mmol, 12 equiv), and benzylthiol (62 μL, 0.62 mmol, 30 equiv) in DMF (1.25 mL) at 0 °C. The resulting solution was stirred at this temperature for 24 h, and then concentrated in vacuo. Purification of the residue by preparative RP-HPLC (10% CH_3CN in H_2O to 70% CH_3CN in H_2O + 0.1% TFA, in 30 min) provided thioester **55** (8 mg, 44% from HGly-preloaded resin **21**; 10 steps, 92%/step), as a colorless oil. R_t (10% CH_3CN in H_2O to 70% CH_3CN in H_2O + 0.1% TFA, in 30 min, analytical column) = 22.8 min. ^{1}H NMR (500 MHz, DMF-d_7) δ = 8.44 (t, J = 6.1 Hz, 1H), 8.42 (t, J = 5.5 Hz, 1H), 8.28 (m, 2H), 8.16 (d, J = 8.3 Hz, 1H), 7.36−7.27 (m, 5 H), 6.69 (t, J = 5.1 Hz, 1H), 4.71 (m, 1H), 4.44 (d, J = 9.5 Hz, 1H), 4.33 (m, 2H), 4.15 (m, 4H), 3.89 (m, 3H), 3.62 (m, 1H), 3.38 (dd, J = 8.6 and 3.1 Hz, 1H), 3.32−3.23 (m, 3H), 3.02 (m, 3H), 2.92 (dd, J = 13.7 and 8.8 Hz, 1H), 2.37 (m, 2H), 2.07 (m, 1H), 1.97 (s, 3H), 1.89 (m, 1H), 1.82 (m, 1H), 1.71 (m, 1H), 1.45 (m, 4H), 1.42 (s, 9H), 1.40 (s, 9H). HRMS (ESI)$^+$ m/z calcd for $C_{42}H_{67}N_6O_{15}S_2$ [M + H]$^+$, 959.4106; found, 959.4070.

AcGluGlyLysCys(Glcβ1)GlySBn. A mixture of TFA/CH_2Cl_2/^{i}Pr$_3$SiH/HS(CH$_2$)$_2$SH = (10:10:1:1, 11 mL) was added to a flask containing AcGlu(^{t}Bu)GlyLys(Boc)Cys(β-Glc)GlySBn (**55**, 8 mg, 0.008 mmol, 1 equiv) at 23 °C. The resulting solution was stirred at this temperature for 1.5 h and then concentrated in vacuo. The residue was coevaporated with cold diethyl ether (5 mL). The resulting white precipitate was washed with cold diethyl ether (4 × 1 mL). The residue was purified by preparative RP-HPLC (5% CH_3CN in H_2O to 32% CH_3CN in H_2O + 0.1% TFA, in 35 min), providing fully deprotected thioester (6 mg, 88%) as a white solid. R_t (5% CH_3CN in H_2O to 32% CH_3CN in H_2O + 0.1% TFA, in 30 min, analytical column) = 20.0 min. ^{1}H NMR (500 MHz, D_2O) δ = 7.23−7.16 (m, 5 H), 4.52 (dd, J = 8.0 and 6.5 Hz, 1H), 4.41 (d, J = 9.9 Hz, 1H), 4.18 (dd, J = 8.8 and 5.6 Hz, 1H), 4.14 (dd, J = 8.8 and 5.6 Hz, 1H), 4.04−3.95 (m, 4H), 3.75 (m, 3H), 3.52 (dd, J = 12.4 and 6.1 Hz, 1H), 3.33 (m, 2H), 3.23 (q, J = 9.4 Hz, 1H), 3.16 (t, J = 9.5 Hz, 1H), 3.12 (dd, J = 14.3 and 6.4 Hz, 1H), 2.86 (dd, J = 14.3 and 8.4 Hz, 1H), 2.78 (m, 2H), 2.31 (m, 2H), 1.95 (m, 1H), 1.87 (s, 3H), 1.81 (m, 1H), 1.65 (m, 1H), 1.56 (m, 1H), 1.48 (m, 2H), 1.23 (m, 2H). HRMS (ESI)$^+$ m/z calcd for $C_{33}H_{51}N_6O_{13}S_2$ [M + H]$^+$, 803.2956; found, 803.2974.

AcGluGlyLysCys(Glcβ1)GlyCysAlaSerPheAspOH (56). Sodium phosphate buffer (100 mM, pH 7.5, 4.3 mL,), containing 6 M guanidine hydrochloride and PhSH (4% v/v) was added to a mixture of AcGluGlyLysCys(β-Glc)GlySBn (3.2 mg, 0.004 mmol, 1 equiv) and HCysAlaSerPheAspOH (4.5 mg, 0.008 mmol, 2.1 equiv) at 23 °C. The resulting solution was stirred for 28 h, and the reaction was monitored by analytical C18 RP-HPLC. Following reaction, the product was purified by preparative RP-HPLC (7% CH_3CN in H_2O to 16% CH_3CN in H_2O + 0.1% TFA, in 40 min) by direct injections of crude reaction mixture, and fractions containing product were lyophilized. Repurification of the product by the same method provided glycoconjugate **56** (4.1 mg, 83%), as a white solid. R_t (7% CH_3CN in H_2O to 16% CH_3CN in H_2O + 0.1% TFA, in 35 min, analytical column) = 23.0 min. ^{1}H NMR (500 MHz, D_2O) δ = 7.21−7.09 (m, 5H), 4.52 (dd, J = 7.8 and 6.3 Hz, 1H), 4.48 (m, 1H), 4.43 (m, 2H), 4.37 (t, J = 6.3 Hz, 1H), 4.24−4.16 (m, 4H), 3.80 (m, 3H), 3.61 (m, 2H), 3.53 (dd, J = 12.4 and 6.7 Hz, 1H), 3.34 (m, 2H), 3.26−3.17 (m, 2H), 3.06 (dd, J = 14.1 and 7.3 Hz, 1H), 3.02 (dd, J = 13.8 and 6.0 Hz, 1H), 2.90 (m, 2H), 2.82 (t, J = 7.6 Hz, 2H), 2.77−2.65 (m, 4H), 2.33 (t, J = 7.8 Hz, 2H), 1.95 (m, 1H), 1.88 (s, 3H), 1.83 (m, 1H), 1.68 (m, 1H), 1. 60 (m, 1H), 1.51 (m, 2H), 1.26 (m, 2H), 1.20 (d, J = 7.3 Hz, 3H). HRMS (ESI)$^+$ m/z calcd for $C_{48}H_{74}N_{11}O_{22}S_2$ [M + H]$^+$, 1220.4451; found, 1220.4435.

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Supporting Information Available: List of abbreviations, complete citation for ref 4b, and experimental details for the preparation and analytical characterization of prepared compounds. This information is available free of charge via the Internet at http://pubs.acs.org.

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