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# REVIEWS

## Difficulties Encountered During Glycopeptide Syntheses

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Methods for the efficient solid-phase synthesis of glycopeptides have developed rapidly over the past two decades. Incorporation of both *O*- and *N*-linked glycosides, as well as branched carbohydrates, into peptides is readily achieved. Synthetic glycoproteins of modest size have also been constructed. As glycopeptide synthesis protocols have progressed, so has the recognition of distinct categories of synthetic difficulties. Such categories include (a) unstable glycosidic linkages, (b) multifunctional amino acids not easily glycosylated and incorporated into peptides, and (c) glycosylated peptide sequences that are subject to side reactions. In the present overview, we describe specific examples for each category of problematic glycopeptide syntheses, as well as solutions to these problems. (J Biomol Tech 2001;12:44–68)

KEY WORDS: glycopeptide, glycosylated amino acid, aspartimide, collagen, proteoglycan, side reactions.

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There are more than 100 different posttranslational modifications of amino acid side-chains that have been described.[1,2] While many of these modifications are simply metabolic errors, others have important biological consequences and are therefore tightly controlled by specific enzymes.[3] Amino acid derivatives containing certain common posttranslational modifications, such as phosphorylation or sulfation, are now routinely prepared or even commercially available, but the formation of specific glycated residues presents a formidable challenge. This is due to the wide range of chemical properties possessed by the carbohydrates found in glycopeptides/glycoproteins and the highly specific nature of these modifications.

Most secreted and cell surface eukaryotic proteins are found glycosylated in vivo and are now thought to have three important biological roles. First, glycosylation can serve as a recognition marker for a cell, both in the context of cell–cell and cell–extracellular matrix interactions. In this same category is glycosylation expressly for the specific targeting of a protein for a subcellular compartment,[4] such as mannose-6-phosphate tagging of lysosomal enzymes.[5] A second role concerns the alteration glycosylation has on the physical properties of the protein. Frequently, glycosylation will render a protein resistant to hydrolysis, significantly increase solubility of a protein, or even drastically affect the overall folding and/or physical bulk of a protein.[2,4,6–8] A third role for glycosylation is in signal transduction, and is analogous to protein phosphorylation. Specifically, dynamic *O*-(*N*-acetyl-D-glucose) modulation occurs in response to T-cell activation, insulin signaling, and glucose metabolism, as well as control of the cell cycle.[9,10] This may occur through a "yin-yang" reciprocity between protein phosphorylation and *O*-(*N*-acetyl-D-glucose) modification.[9] In any of the above roles, the degree of glycosylation can vary from a single sugar residue to elaborately branched oligosaccharide structures or even lengthy carbohydrate polymers, all emanating from a protein core.

Topologically, cells can perform most glycosylation reactions in either the endoplasmic reticulum or Golgi compartments of the cell, although examples of cytosolic glycosyl transferases have been found. Each modification is accomplished with one of the hundreds of different, specific enzymes for carbohydrate addition.[11] Glycosylations can occur either directly to the protein being modified, such as with glycosaminoglycan biosynthesis, or assembled initially on a dolichol phosphate scaffold and later transferred to the appropriate residue in the protein of interest, such as with *N*-linked oligosaccharides.[11,12] With either route, no template mechanism or coding molecules exists for these modifications, and therefore the specific carbohydrate sequences result directly from a high degree of enzyme specificity.

To understand the biological functions of glycoproteins, methodology for site-specific incorporation of carbohydrates into peptides has developed rapidly. Three approaches have been developed to obtain a glycosylated peptide.[13] For the first approach ("direct condensation" or "convergent"), a complete or partially completed peptide is constructed that is fully protected during peptide assembly, but can be differentially deprotected to allow formation of the glycosidic linkage.[14] Because of the likelihood of secondary structure formation within the peptide and/or steric factors from neighboring residues, problems with the accessibility of the nucleophile to the activated sugar can be encountered. Nucleophile accessibility can especially be a problem during on-resin couplings, where the juxtaposition of neighboring peptide chains are defined and intramolecular steric strain can become a significant obstacle, depending on the specific peptide sequence.[15] Thus, this approach has found limited use for the preparation of *O*-linked glycopeptides, but has achieved success for complex *N*-linked glycopeptides.[16–18] For the second approach ("building block" or "pre-assembly"), preglycosylated amino acids are used in the stepwise assembly of glycopeptides. It is known that the presence of glycosyl moiety does not seriously affect the coupling reactivity of the amino acid, and thus, with the proper glycosylated amino acid at hand, the peptide synthesis can proceed more or less as usual. The mild conditions of Fmoc chemistry are more suited for glycopeptide syntheses than Boc chemistry, as repetitive acid treatments can be detrimental to sugar linkages.[19] Fmoc-Ser, -Thr, -Tyr, -Hyl, -Hyp, and -Asn have all been incorporated successfully with glycosylated side-chains. Side-chain glycosylation is performed with glycosyl bromides or glycose–$BF_3$•$Et_2O$ for Ser, Thr, Tyr, Hyl, and Hyp, and glycosylamines for Asp (to produce a glycosylated Asn). The side-chain glycosyl is usually hydroxyl protected by either Bzl or acetyl groups,[14,20–22] although some solid-phase syntheses have been successful with no protection of glycosyl hydroxyl groups.[23–25] Glycosylated residues are incorporated as preformed pentafluorophenyl (Pfp) esters or in situ with *N,N'*-dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) or *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxy-7-azabenzotriazole (HATU/HOAt).[14,20,21] These sugars are relatively stable to Fmoc removal by piperidine, morpholine, or 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), brief treatments (less than 2 h) with TFA for side-chain deprotection and peptide-resin cleavage, and palladium treatment for peptide-resin cleavage from allyl-based linkers. Deacetylation and debenzoylation may be performed with methanolic hydrazine or sodium methoxide, before or after glycopeptide-resin cleavage.[19,26] In addition, acid-labile protecting groups have been developed for the carbohydrate moiety, such as *tertiary*-butyldimethylsilyl (TBDMS), *tertiary*-butyldiphenylsilyl (TBDPS), and isopropylidene.[27,28] For the third glycosylated peptide approach, preglycosylated amino acids are used in the stepwise assembly of glycopeptides as described above, and the glycan chain is then elaborated either in solution or on the solid-phase.[13]

Several side reactions have been documented to occur during glycopeptide synthesis. DBU used in combination with silyl protection can result in decomposition of the glycopeptide.[29] Removal of acetyl groups can result in β-elimination or Cys-induced degradation of the peptide backbone.[27,30–32] Model glycopeptide studies showed that, in general, conditions used for deacetylation and debenzoylation result little or no β-elimination and epimerization.[33] In the present overview, we describe three different problems encountered in glycopeptide syntheses. These problems are (a) a glycosidic linkage that is unstable to peptide synthesis conditions, (b) a trifunctional amino acid that is difficult to glycosylate and incorporate into peptides, and (c) a peptide sequence that undergoes side reactions before glycosylation. Solutions to these problematic glycopeptide syntheses are also discussed.

## UNSTABLE GLYCOSIDIC LINKAGES

There has been significant recent interest in elucidating the regulatory mechanisms involved in determining glycosaminoglycan (GAG) identity during chain assembly. The phosphorylation of the chain-initiating xylose residue in the proteoglycan linkage structure (Fig. 1) is an early biosynthetic event and may be intimately involved in determining ultimate GAG identity

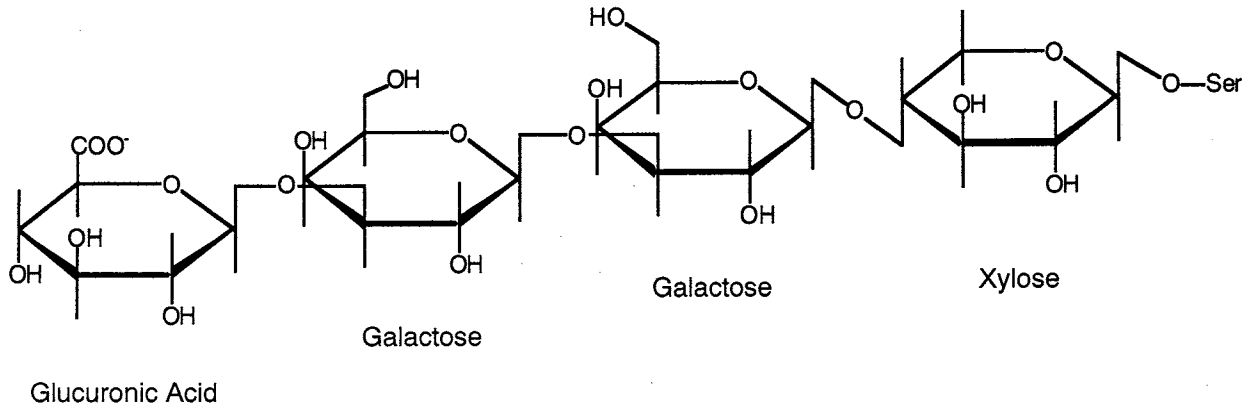



**FIGURE 1**

Proteoglycan linkage structure.

(dermatan sulfate vs. chondroitin sulfate).[34,35] To better understand this process, a specific assay for the enzyme(s) responsible for this phosphorylation needed to be developed. Thus, the ultimate goal of this synthesis was the production of sufficient quantities of glycopeptides based on the proteoglycan linkage structure to be employed in an enzymatic assay. This was accomplished in a manner that allowed for comparison of two synthetic strategies to determine the feasibility of each approach[36] (Borgia, Fields, and Oegema, manuscript in preparation). The sequence for glycopeptides that were prepared were derived from the work of Bourdon and coworkers.[37] They examined the sequence requirements of xylosyl transferase, the enzyme that primes proteoglycan core proteins for ultimate glycosaminoglycan assembly in the cell. Xylosyl transferase requires Ser in the context of Ser-Gly-X-Gly, where X is any amino acid. Furthermore, the presence of an acidic cluster of three to four residues just upstream of this motif enhances the probability of xylosylation.[37]

Solid-phase glycopeptide synthesis methodology was utilized to create novel β-glycopeptides derived from the proteoglycan linkage structure. Xylopeptides were prepared through either the "direct condensation" (Leu-Glu-Asp-Glu-Ala-**Ser**-Gly-Ile-Gly-Val-$NH_2$) or "building block" (Gly-Ser-Gly-**Ser**-Gly-Ser-Gly-$NH_2$) approaches.[38] A peracetylated, α-trichloroacetiimidate xylose residue served as the glycosylating reagent for both the resin-bound peptide (direct condensation) and with the Fmoc-Ser(OH)-OPfp residue (building block).[39,40]

The formation of glycated amino acid residues can be accomplished by a number of distinct strategies. Most glycation reactions rely on nucleophilic moieties within the peptide of interest to complete an SN2-type mechanism to an appropriate (electrophilic) anomeric center equipped with an efficient leaving group. The first efficient means of accomplishing this condensation was with the Koenigs-Knorr method of glycation (Fig. 2), in which the anomeric hydroxyl is exchanged with a halogen moiety and condensation occurs in the presence of a heavy metal promoter, frequently mislabeled as a catalyst.[40,41]

Unfortunately, this approach offers limited anomeric specificity in some instances, due to the

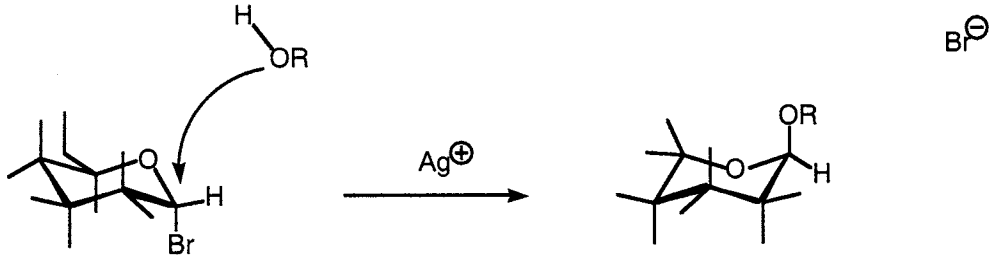



**FIGURE 2**

Koenigs-Knorr method of glycoside formation. Glycosidic linkages are formed with the nucleophilic addition of an alcohol with a bromo- or chloro-derived carbohydrate, and a heavy metal promoter.

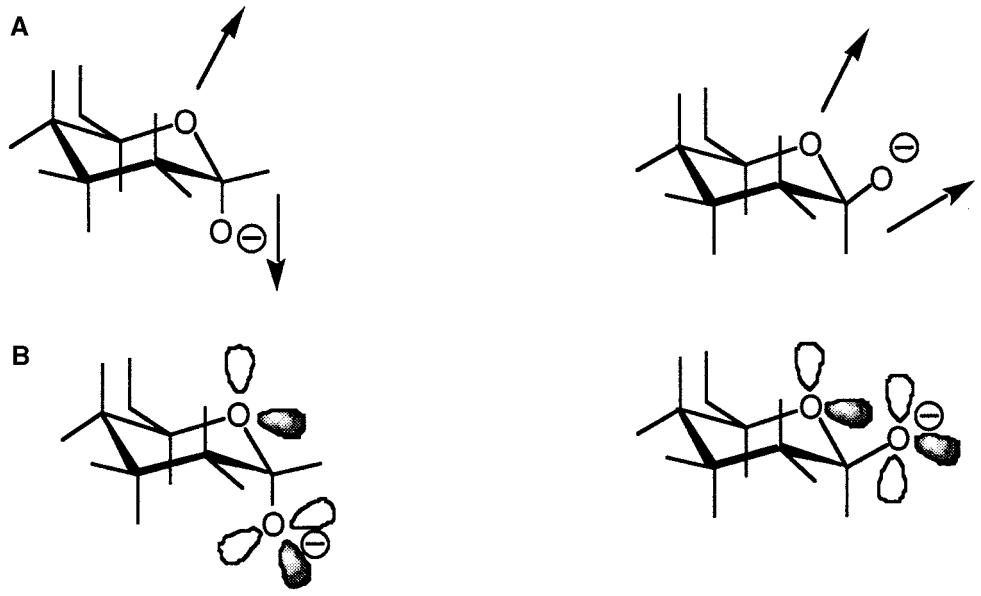



**FIGURE 3**

The "kinetic anomer" effect. Enhanced nucleophilicity of the β-oxide results from the juxtaposition of additive dipole/dipole forces. Steric interactions ultimately direct formation of the α-product. **(A)** Dipole-dipole forces. **(B)** Steric interactions.

neighboring group stabilization (from the C-2 oxygen) being the only means to direct anomeric outcome.[40,42] Depending on the specific carbohydrate being activated, this may or may not provide adequate anomeric control. Other activating moieties have been developed which offer improved specificity for a desired anomeric product. Many of these derivatives, such as the thioglycosides or the glycosyl fluorides, employ the same basic

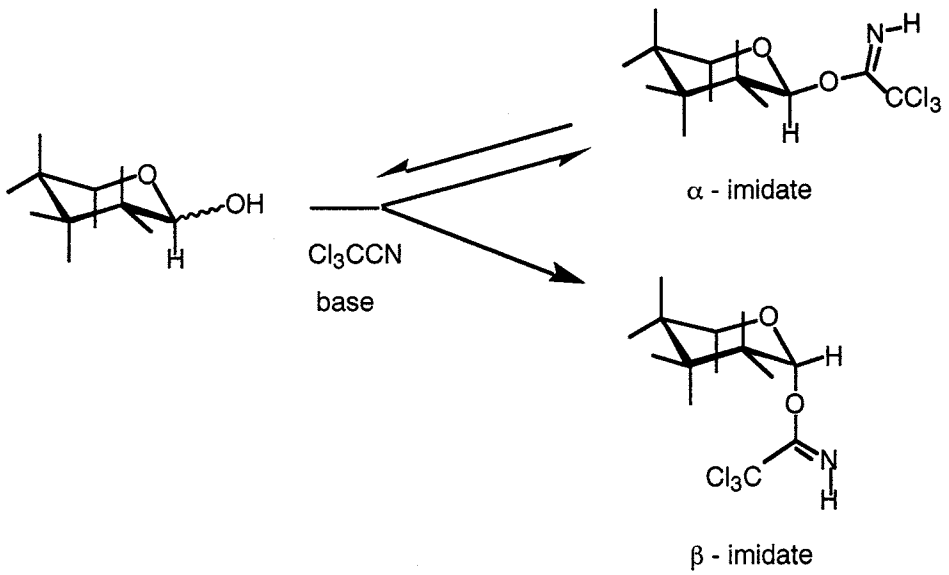



**FIGURE 4**

Base-catalyzed formation of imidates. Extraction of the anomeric proton leads to formation of the trichloroacetimidate adduct. The β-anomer is formed first in a kinetically directed mechanism, but a slow retro-reaction leads to formation of the thermodynamically favored α-derivative.

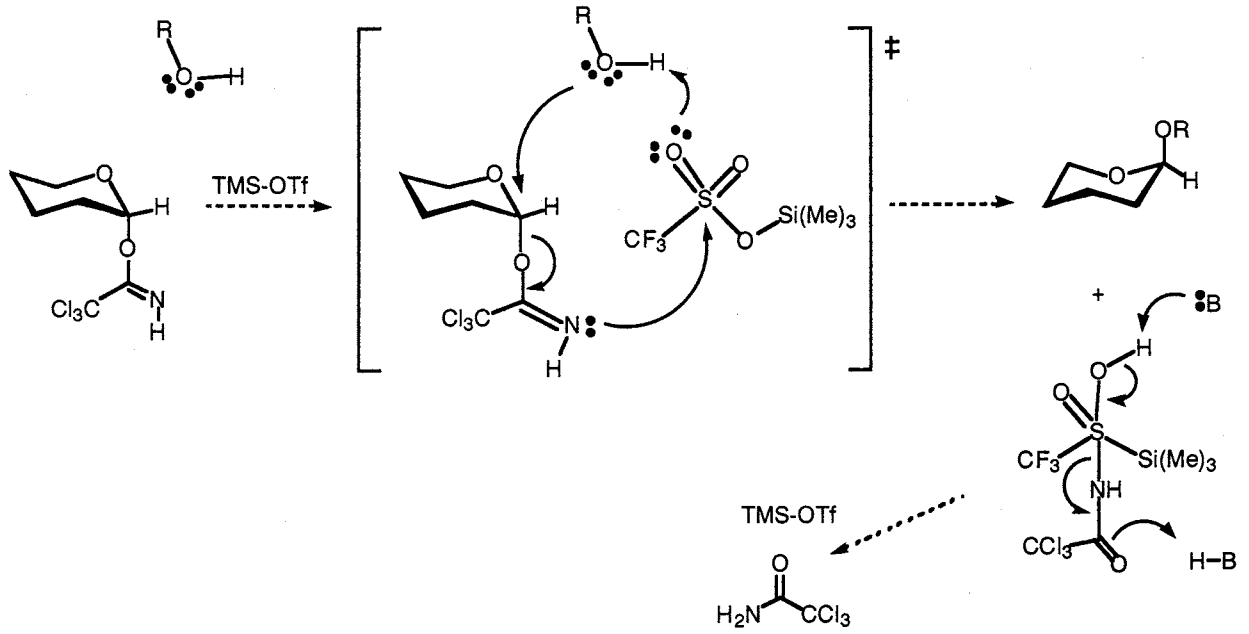



**FIGURE 5**

Mechanism of glycosylation. A cyclic, eight-membered transition state complex is formed between the Lewis acid catalyst (TMSOTf), the carbohydrate-trichloroacetimidate, and the entering nucleophile that ultimately results in formation of the glycosidic linkage.

rationale as the Koenigs-Knorr methodology.[40] More recently, the introduction of the trichloroacetimidate method has offered superb diastereoselectivity and excellent efficiency when compared with that of its predecessors. This direct activation method is distinguished from the anomeric-oxygen exchange reactions by the retention of the anomeric oxygen in the activated sugar. The precise control over the anomer production is afforded ultimately by a kinetic versus thermodynamic mechanism.[40] That is, glycosidic β-oxides possess a superior nucleophilicity when compared to α-oxides. This increased nucleophilicity results from the large dipole moment possessed only by the β-oxide, as well as the dipole moment generated by additive dipole–dipole forces (Fig. 3).

Although the β-imidate possesses the kinetic advantage, a base catalyzed retroreaction occurs to allow formation of the α-imidate product (Fig. 4), which is thermodynamically preferred due to the axial position of the electron-withdrawing moiety and absence of the negative steric forces (associated with the β-anomer).[40,43] Thus, to control the anomeric outcome, a strong base can be employed with a long reaction time to afford the α-imidate or, conversely, a weak base catalyst with a short reaction time will provide the β-anomer.

Formation of the glycosidic linkage is accomplished with the introduction of the nucleophilic residue in the presence of a Lewis acid catalyst. The mechanism for this reaction is predicted by computerized molecular orbital energy-minimization programs to occur via a (nonplanar) cyclic, eight-member transition state (Fig. 5).[44] Because this reaction proceeds through an SN2- (type) reaction, an inversion of configuration is afforded with the final product. That is, the α-imidate will yield the β-glycoside, whereas the β-imidate will yield the α-glycoside.

The activated xylose residue was first assembled in a manner similar to that described by Rio et al.[39,45] The synthetic scheme outlined previously allows for either partial or complete formation of the proteoglycan linkage structure in high (>90%) yields and in derivatives appropriate for the fabrication of the glycopeptides through the building block approach.

Synthesis of glycopeptide I (Fig. 6) via the direct condensation approach required the initial formation of a peptide with an unprotected seryl residue. Condensation of the activated sugar could then be performed with the orthogonally protected peptide in a Lewis-acid-catalyzed reaction. The sequence to be assembled was Boc-Leu-Glu(O*t*Bu)-Asp(O*t*Bu)-Glu(O*t*Bu)-Ala-Ser[O-(2,3,4-tri-O-acetyl)-β-D-xylopyranoside]-Gly-Ile-Gly-Val-Rink amide 4-methylbenzhydrylamine resin.

The accessibility of the unprotected hydroxyl moiety to the electrophilic sugar was, perhaps, the most

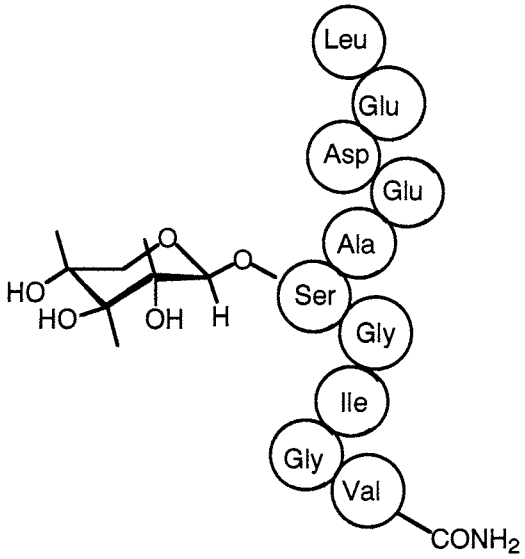



**FIGURE 6**

Sequence of glycopeptide I.

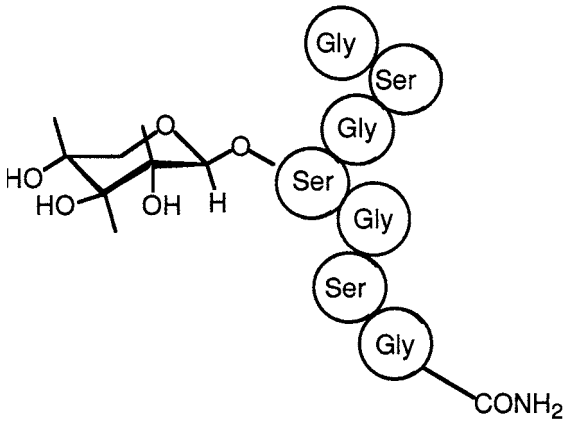



**FIGURE 7**

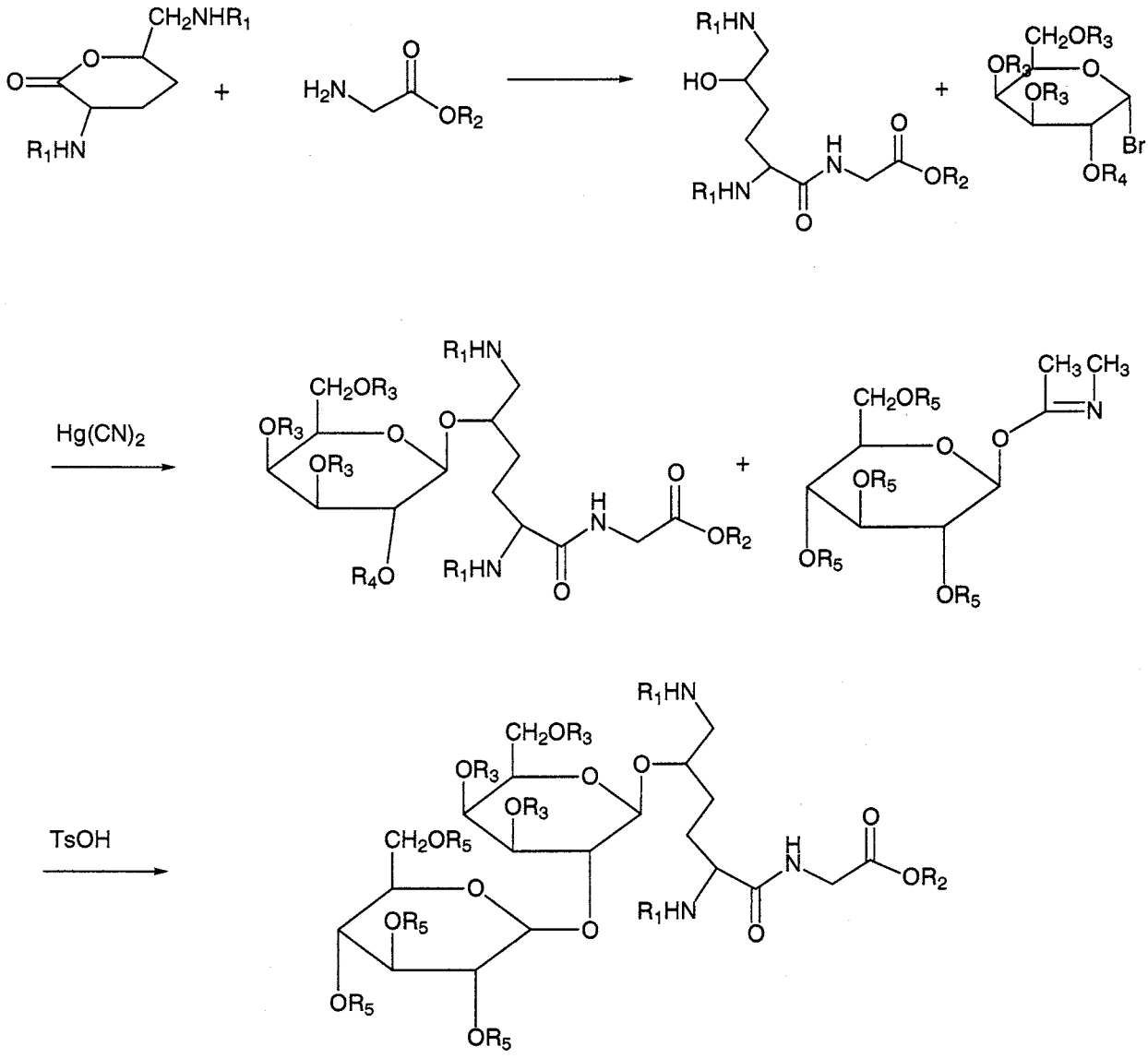
Sequence of glycopeptide II.

limiting factor for efficient glycosylation. To minimize this potential problem, peptide assembly was halted after addition of the unprotected hydroxyl group. Addition of Fmoc-Ser was accomplished with carbodiimide/HOBt, minimizing potential branching from the unprotected hydroxyl. To quantify the accessibility of the seryl hydroxyl, acetylation with small, electrophilic reagents was performed. Acetylation was achieved through either condensations with acetic anhydride or carbodiimide-activated acetic acid. Peptides were then cleaved from the resin, and analytical RP-HPLC and MALDI-TOF analyses performed. The optimal reaction time for either of these methods was found to be 3.5 h, with the acetic anhydride affording 35% conversion to the acetylated derivative while acetic acid provided 27% conversion. Both peptide products were analyzed by MALDI-TOF mass spectrometry, which confirmed formation of a novel species at m/z 716.8 Da (predicted 711.2 Da). Based on these experiments, optimal glycation should be about 30%.

Glycation of the truncated peptide was performed with conditions identical to those to be used for preparation of glycated Fmoc-Ser (see later discussion). Analysis of the crude glycosylated peptide product by analytical RP-HPLC revealed formation of a novel species (42.1% of the total material). Mass spectral analysis showed a species of m/z 779.4 Da (784.9 Da predicted), confirming the presence of truncated glycopeptide I. It appeared that the efficiency of glycosylation was even higher than that of acetylation, implying a higher reactivity for the trichloroacetimidate compared with the two methods used for the acetylation. Addition of the remaining peptide residues was achieved using the carbodiimide/HOBt. Cleavage of the mature peptide, after deprotection in 50% morpholine, was accomplished with 95% TFA, and the resulting material was purified by preparative RP-HPLC. Analysis of the product by MALDI-TOF demonstrated an m/z 1329 Da (predicted 1339 Da for acetylated glycopeptide I + 4 $Na^+$).

The direct condensation approach provided glycopeptide I with an overall yield of 3%. Although this yield is rather low, it is similar to values reported previously.[38] One of the aims of this work was to reevaluate the direct condensation approach using the trichloroacetimidate method of glycan formation, which has been reported to be more efficient and anomerically specific than the Koenigs-Knorr chemistry used previously.[40] However, no improvements appeared to be offered by the trichloroacetimidate method, suggesting that the formation of the glycosidic linkage is not a limiting factor in the success of this synthesis.

The construction of glycopeptide II (Fig. 7) by the building block method was dependent on the initial preparation of anomerically pure glycosylated Fmoc-Ser. Fmoc-Ser-OPfp was first synthesized, then condensed with the activated sugar residue. The glycation reaction was catalyzed by the Lewis acid, trimethylsilyl trifluoromethanesulfonate, which forms an eight-member cyclic transition state with both the entering nucleophile and the trichloroacetimidate moiety of the carbohydrate facilitating the condensation reaction (see earlier discussion). Fmoc-L-Ser[*O*-(2,3,4-tri-*O*-acetyl)-β-D-xylopyranoside]-OPfp was obtained in 71% yield. Interestingly, the small degree of the α-anomer found during this reaction stems from the ability of a strong Lewis acid such as TMSOTf to

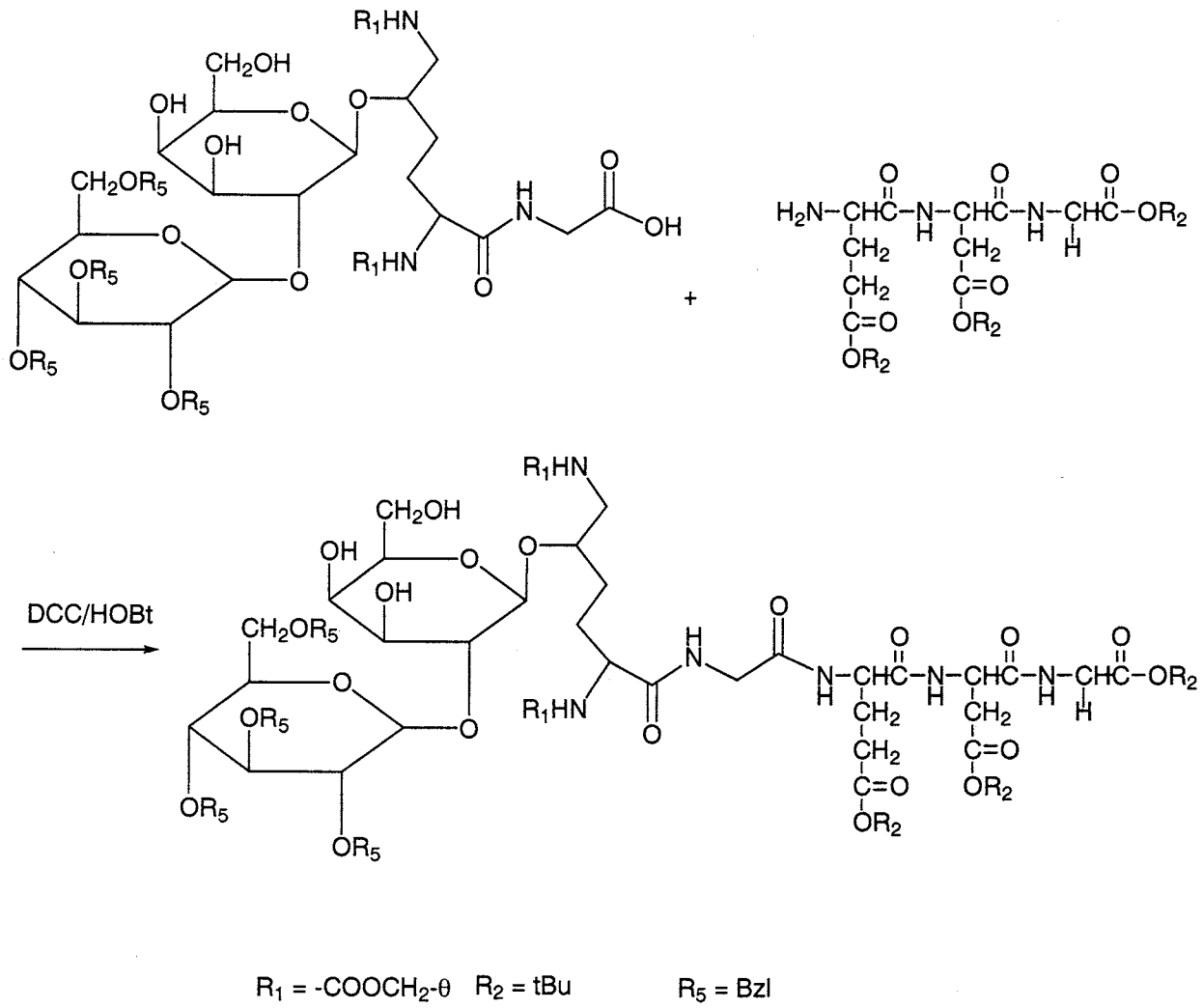



$R_1$ = -COOCH$_2$-θ  $R_2$ = tBu  $R_3$ = Ac  $R_4$ = Lev = CH$_3$-CO-(CH$_2$)$_2$-CO-  $R_5$ = Bzl

**SCHEME 1**

induce the leaving of the trichloroacetimidate moiety from an anomeric carbon prior to nucleophilic attack, thus affording a solvent-stabilized carbocation species. This anomeric carbocation is vulnerable to nucleophilic addition from either face of the sugar, and thus either anomer may result.[40,44] The alternative mechanism did not prove significant, however, as the reaction was performed in the relatively nonpolar 1,2 dichloroethane, which destabilizes the carbocation intermediate.

Incorporation of the glycosylated amino acid residue into the peptide sequence occurred with more than 99% efficiency. Addition of the remaining residues was performed as before addition of the glycated Fmoc-Ser derivative. After cleavage and purification, glycopeptide II (before deacetylation) was obtained in rather low yield (59%) compared to the synthesis of nonglycated peptides of this size. Product loss was thought to emanate from the β-elimination reaction, which has been previously demonstrated to be a significant factor affecting the success of glycopeptide synthesis.[46,47] One side product (25.5% of the total) isolated by semipreparative RP-HPLC gave an m/z 519.6 Da. This side product corresponds to

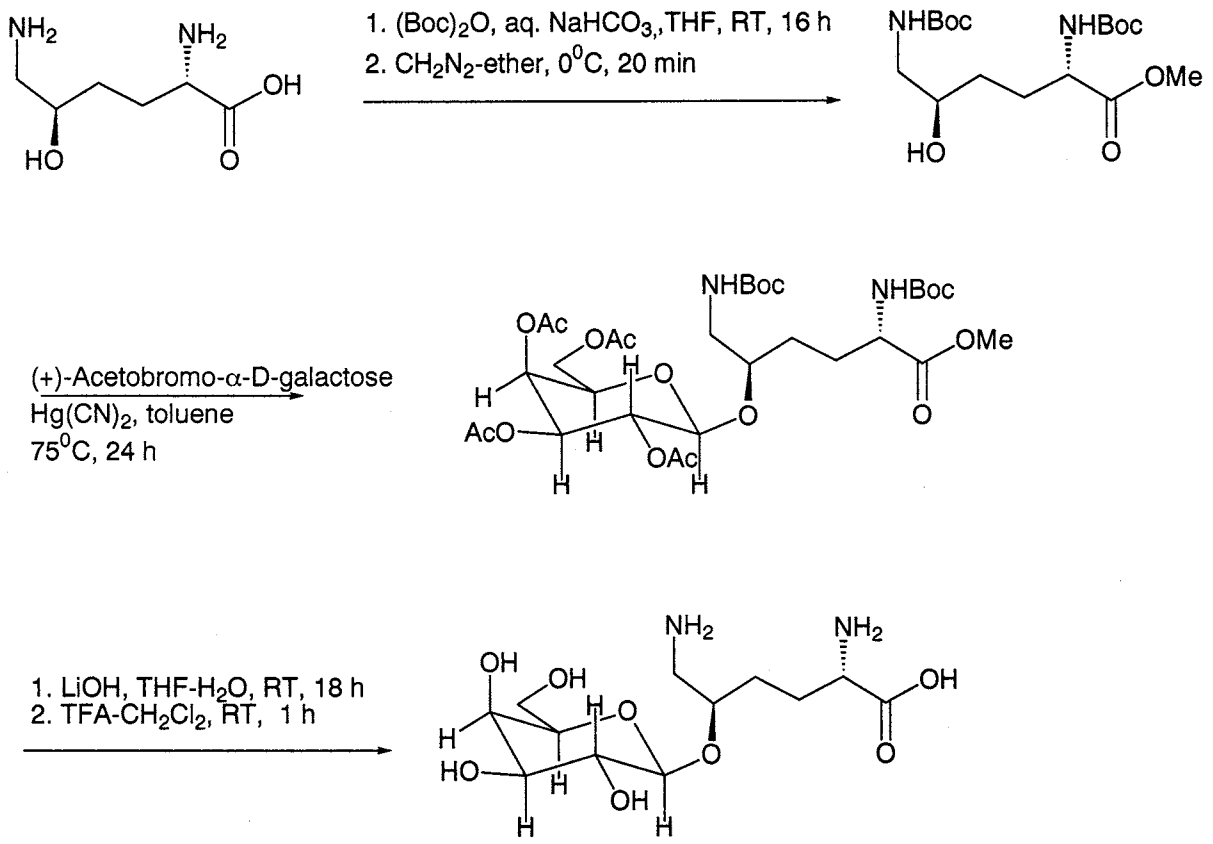



**SCHEME 2**

glycopeptide II with a dehydroalanyl residue instead of xylosylseryl (predicted 511.9 Da).

$^1$H-NMR analyses (1D and TOCSY) were performed to verify the structure of the desired product. The TOCSY spectrum confirmed the presence of three distinct Ser residues; one apparently constrained with the carbohydrate moiety and indicative of an overall static conformation for the glycopeptide. The sequence was determined to be Gly-Ser-Gly-Ser-Gly-Ser-Gly, with the second Ser predicted to contain the xylose residue. The xylose was identified as existing in primarily (>95%) the β-anomeric form. Deacetylation of glycopeptide II with sodium methoxide in $CH_3OH$ cleanly provided the desired product in 51.3% yield. Mass spectral analysis revealed a single species with m/z 637.8 Da (predicted 638.6 Da).

Mechanistically, the β-elimination reaction involves the extraction of the α-hydrogen of the Ser/Thr residue with a base, resulting in the elimination of the β-positioned sugar moiety, and ultimate formation of the α, β-double bond. Piperidine, a mild base commonly used to remove *N*-terminal Fmoc groups, may have induced β-elimination. Boc-Gly was utilized as the *N*-terminal residue, to avoid excessive exposure of the glycopeptide to basic conditions. The Boc moiety is removed by the acidic cleavage conditions and limits exposure of the peptide to base by 25%, which (it is hoped) improves the overall yield of the synthesis. Traditionally, chemists have shied away from bases such as piperidine ($pK_a$ 11.1) for Fmoc removal with glycopeptides, utilizing weaker bases, such as morpholine ($pK_a$ 8.3), instead.[19] However, there have been conflicting reports as to whether the switch from piperidine to morpholine is necessary. Several groups have attested that piperidine does not promote β-elimination reactions, under

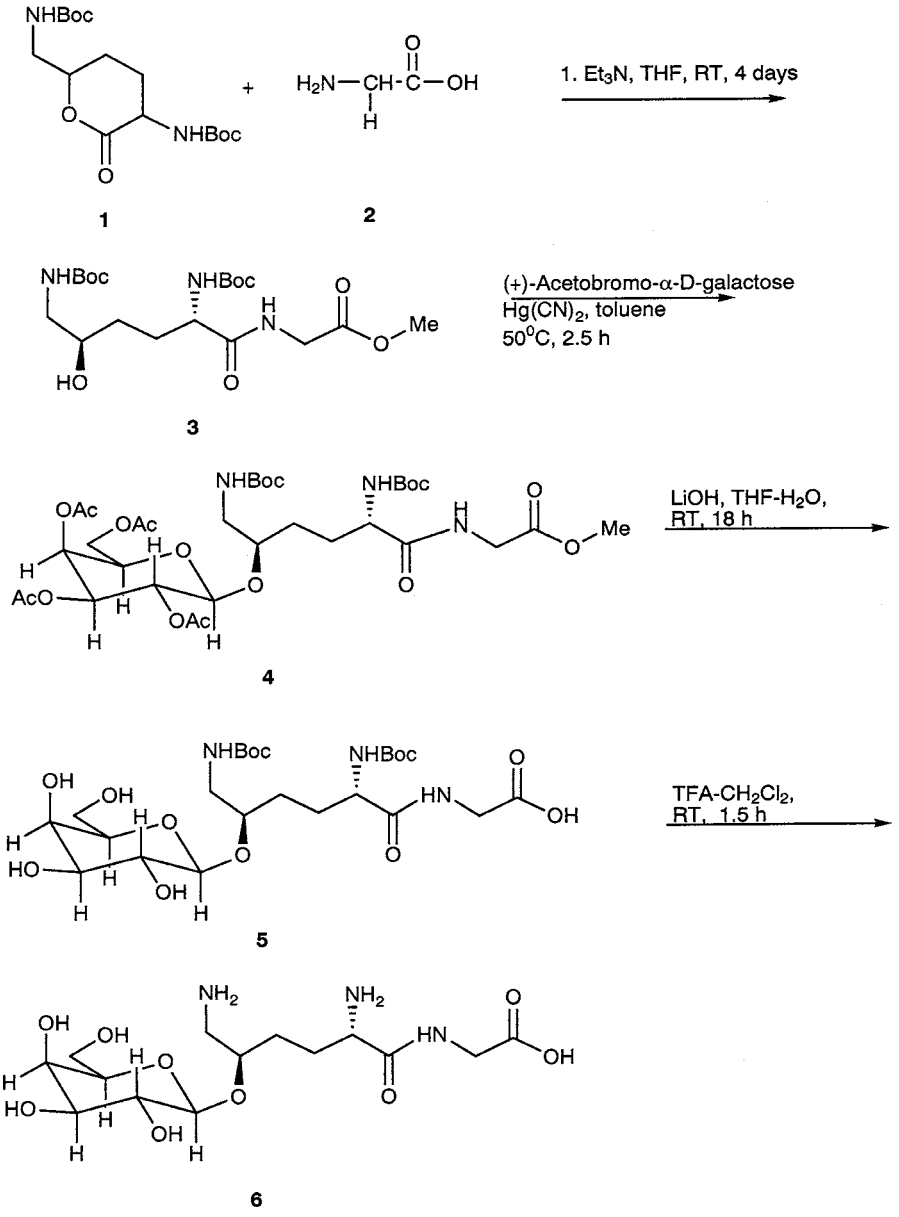



**SCHEME 3**

the conditions required to remove the Fmoc moiety with peptide sequences that were generally neutral and possessing either an *N*-acetylglucosamine or *O*-mannosyl carbohydrates.[33,46,47] In addition, when strong base (0.1 M NaOH) was required to remove acetyl groups from multiple *O*-mannosyl residues, no β-elimination was seen.[48] Unfortunately, there is no clear answer to the dilemma of what induces this side reaction. The propensity to β-eliminate may ultimately prove to be a phenomenon very specific to peptide sequence and possibly glycone identity.

## MULTIFUNCTIONAL AMINO ACIDS

Hydroxylysine (Hyl) is the major glycosylation site within collagens. The δ-hydroxyl group may be posttranslationally modified by the monosaccharide galactose (β-D-galactopyranosyl) or the disaccharide glucose-galactose [α-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl].[49,50] Interest in glycosylation of collagen stems from the recent reports of T-cell recognition of a glycosylated sequence within type II collagen,[51] the identification of melanoma and breast carcinoma binding sites within type IV collagen that contain glycosylated Hyl residues,[52–58] and the activation of specific tyrosine receptor kinases by glycosylated type I collagen.[59] Preparation of Hyl derivatives for incorporation by peptide synthesis has a long and troubled history. Initial attempts at synthesizing ε-amino protected Hyl resulted in severe solubility problems, and thus Hyl protected by the same group at both the α- and ε-amino groups was used for peptide synthesis.[60–62] In addition, Hyl readily undergoes intramolecular lactone formation, which reduces the efficiency of peptide elongation.[61,62] The first successful solution phase synthesis of a tetrapeptide-containing glycosylated Hyl did not use a Hyl building block, but rather the lactone form of the amino acid.[63,64] Enzymatic and chromatographic techniques were used to obtain the desired isomer, (5R)-5-hydroxy-L-lysine, as a benzyloxycarbonyl (Cbz) protected lactone. This derivative was then used in the solution phase synthesis of the *O*-(2-*O*-α-D-glucopyranosyl)-β-D-galactopyranoside of optically pure Hyl-Gly (Scheme 1) and the pentapeptide Hyl-Gly-Glu-Asp-Gly (Scheme 2). Overall, preparation of these two peptides required (a) enzymatic resolution of α-chloroacetyl-ε-Cbz-δ-hydroxy-DL-lysine (normal and allo forms), (b) carbobenzoylation of the obtained L-

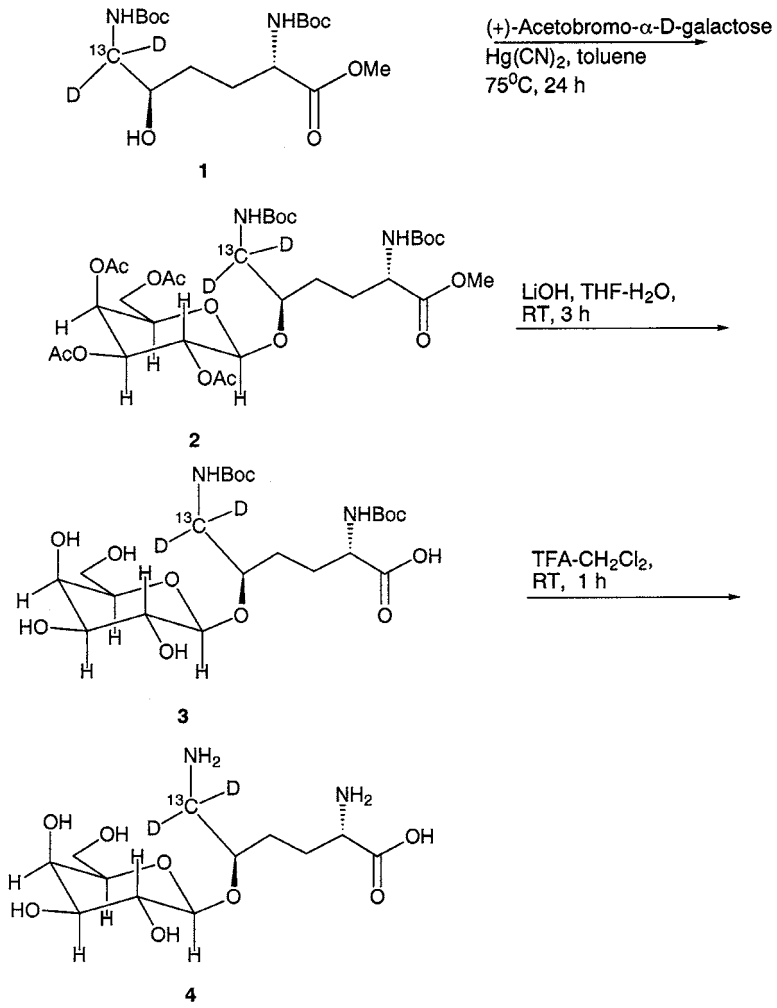



**SCHEME 4**

mixture to provide the dicarbobenzoxylated lactone (normal and allo forms), (c) separation of lactone diastereomers by flash chromatography to provide the lactone (normal L-form), and (d) aminolysis of the lactone (normal L-form) with the *tert*-butyl (*t*Bu) ester of Gly resulting in the formation of a dipeptide. Neighboring group participation from levulinyl (Lev) group ensured the 1,2-*trans*-galactosidic linkage. Mild hydrazinolysis then removed the Lev group to provide the corresponding alcohol, without affecting other esters. This alcohol was coupled repeatedly with 1-*O*-(*N*-methyl)-actimidyl-2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranose to form the final α-linked disaccharide.

The glycosylated dipeptide was coupled with the properly protected tripeptide Glu(O*t*Bu)-Asp(O*t*Bu)-Gly-OBzl in a mixture of *N,N*-dimethylformamide (DMF) and tetrahydrofuran (THF) in presence of HOBt, *N*-ethylmorpholine (NEM), and DCC. Removal of all protecting groups was achieved in 94% yield to obtain the desired product.

Adamczyk et al.[65] reported the synthesis of (−)-galactosyl-Hyl and the dipeptide (−)-galactosyl-Hyl-Gly (Scheme 3). The amino acid and dipeptide are used as calibrators, controls, and clinical reference standards in the development of immunoassays.[66] Hyl was first converted to the bis-Boc acid, then to its



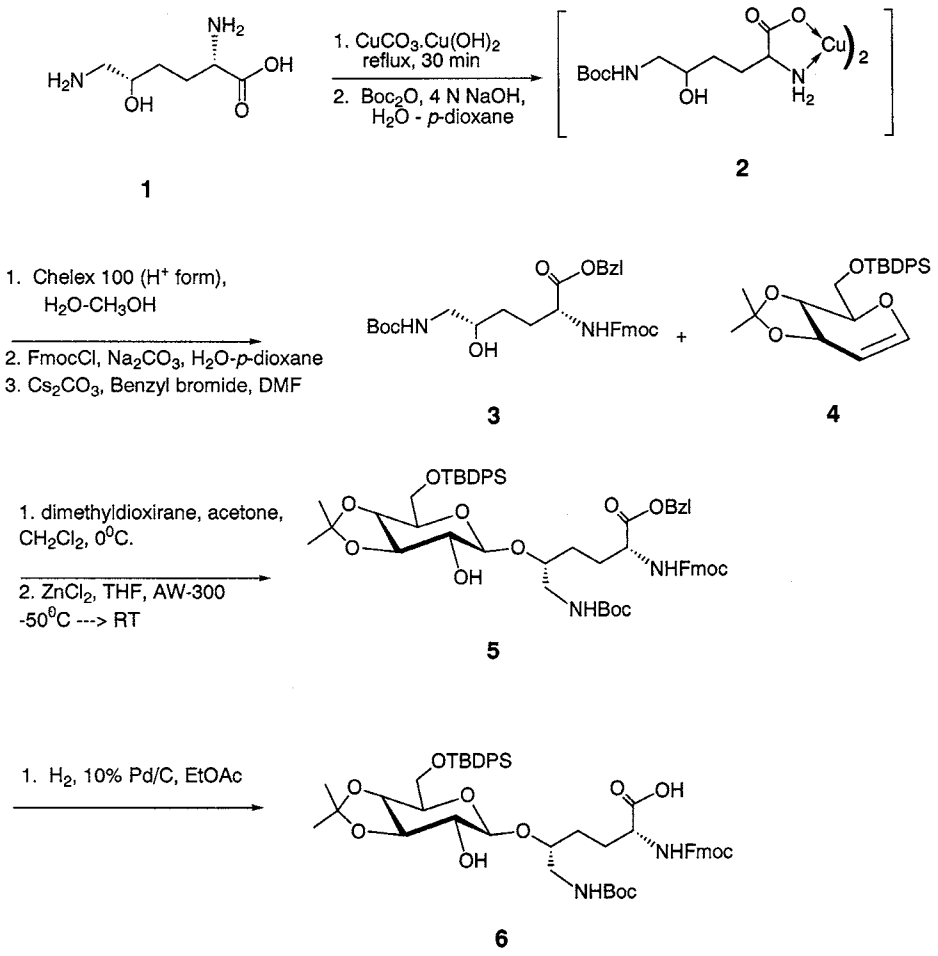

**SCHEME 5**

methyl ester by reaction with ethereal-diazomethane (95% yield). The bis-Boc methyl ester was glycosylated using 2.0 equivalent of (+)-acetobromo-α-D-galactose and 2.2 equivalent of mercury (II) cyanide in toluene at 75°C for 24 h (22% yield). Removal of the carbohydrate acetyl groups was carried out using alkaline hydrolysis (LiOH in THF-$H_2O$) in 85% yield. Removal of the Boc groups was achieved using TFA in $CH_2Cl_2$ in 69% yield.

The (−)-galactosyl-Hyl-Gly dipeptide was initiated from the bis-Boc-protected lactone of Hyl, which in turn was prepared from Hyl. The lactone was first reacted with methyl glycinate in THF to provide the protected dipeptide (Scheme 4). The protected dipeptide was then glycosylated using 2.0 equivalent of (+)-acetobromo-α-D-galactose and 2.2 equivalent of mercury (II) cyanide in toluene at 50°C for 2.5 h. Alkaline hydrolysis gave **5** in 51% yield (Scheme 4). Removal of the Boc-protecting groups using TFA-DCM (1:1) gave the dipeptide in 84% yield as a TFA salt (Scheme 4).

Adamczyk et al.[67] also reported a synthesis of an isotopically labeled glycosylated Hyl from a labeled Hyl derivative (Scheme 5). Isotopically labeled glycosylated Hyl is used as an internal standard for quantification of glycosylated Hyl by mass spectrometry. In this case, isotopically labeled Hyl derivative **1** was synthesized first and then glycosylated using 2.0 equivalent of (+)-acetobromo-α-D-galactose and 2.2 equivalent of mercury (II) cyanide in toluene at 75°C for 24 h (20% yield). Alkaline hydrolysis provided **3** in 76% yield. Removal of the Boc-protecting groups using TFA-DCM gave the isotopically labeled amino acid in 98% yield as a TFA salt.

Although practical for the synthesis of glycosylated Hyl standards, the use of bis-Boc protection of Hyl does not allow for peptide elongation via the α-amino group of Hyl. Also, the use of the Hyl lactone

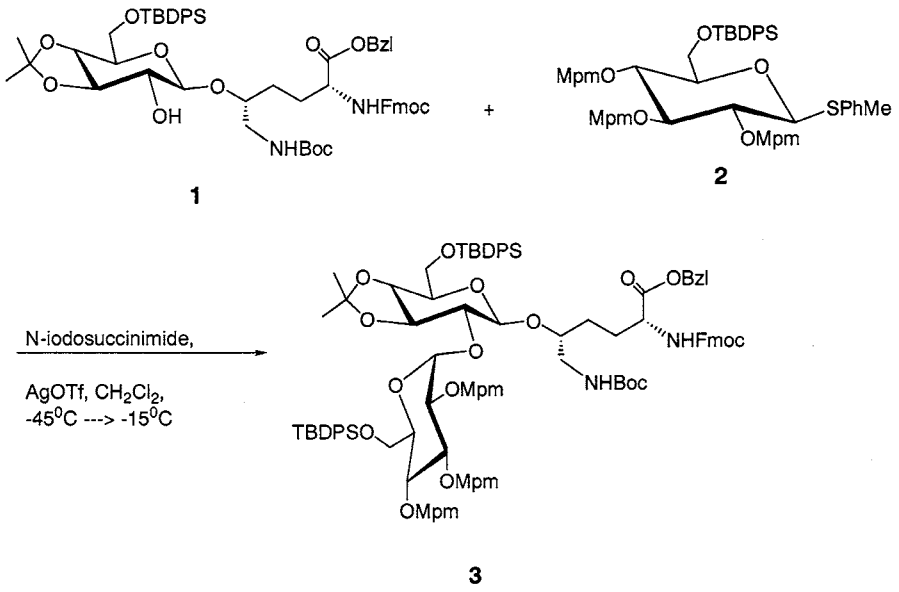



**SCHEME 6**

is detrimental for efficient solid-phase synthesis.[68] Far more desirable is the construction of glycosylated Fmoc-amino acid building blocks for solid-phase applications. The first successful synthesis for glycosylated Fmoc-Hyl(ϵ-Boc) was reported by Broddefalk et al.[28,51] (Scheme 6). Preparation of this derivative was based on the use of Lewis acid-catalyzed opening of α-1,2-anhydrosugars, prepared by epoxidation of the corresponding glycal by nucleophilies. This reaction creates not only a β-glycosidic linkage but also a free hydroxyl group at the C-2 center of sugar. Hyl was protected by converting it into its copper complex, followed by regioselective Boc protection of ϵ-amino group. After the decomposition of the copper complex, the α-amino and carboxyl groups were protected with Fmoc and benzyl groups, respectively. Epoxidation of galactal with dimethyldioxirane gave the corresponding α-1,2-anhydrosugar, which under zinc chloride promotion gave β-galactoside in 37% yield. The regioselective removal of the benzyl ester provided glycosylated Fmoc-Hyl(ϵ-Boc) (Scheme 6).

The above preparation of glycosylated Fmoc-Hyl(ϵ-Boc) required 7 steps, with the final derivative obtained in 21.4% yield. Attempts to attach an α-D-glucosyl moiety to glycosylated Hyl were unsuccessful. Diglycosylation of Hyl using glycosyl donor **2** (Scheme 7, where Mpm = 4-methoxybenzyl) promoted by various thiofilic reagents were then attempted.[69] These reactions resulted in formation of several side products, and the yield of diglycosylated Hyl never exceeded about 10%. The low yields were attributed to the lability of the ϵ-amino Boc group under the acidic conditions of glycosylations. A more stable protecting group at the ϵ-amino position of Hyl was required, but it needed to be easily removed during the acid-catalyzed cleavage of the target glycopeptide from the solid-phase support.

Broddefalk et al.[69] showed that use of the Cbz group for the protection of the ϵ-amino position of Hyl would result in the successful synthesis of a building block having the α-D-glucopyranosyl-(1 → 2)-β-D-galactopyranosyl moiety linked to Hyl. The same strat-




**SCHEME 7**

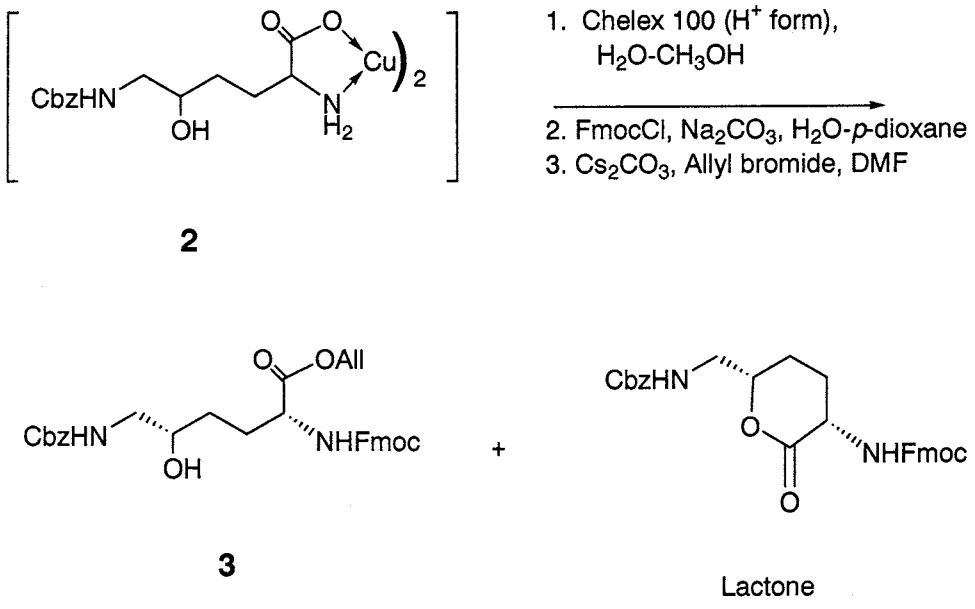
egy was used for the synthesis of Fmoc-Hyl(ϵ-Cbz)-OAll (where All is allyl) as was used for Fmoc-Hyl(ϵ-Boc)-OBzl. First, Hyl was converted to the copper complex, followed by Cbz protection of ϵ-amino group. After the decomposition of the copper complex, the α-amino was protected with Fmoc and the carboxyl group was protected by formation of an allyl ester. The overall yield was lower (37%) compared to the synthesis of Fmoc-Hyl(ϵ-Boc)-OBzl (68%), due to lactone formation (linkage between the Hyl hydroxyl and carboxyl groups) during the esterification of Fmoc-Hyl(ϵ-Boc) using allyl bromide (Scheme 8).

Epoxidation of galactal with dimethyldioxirane gave the corresponding α-1,2-anhydrosugar, which further reacted with Fmoc-Hyl(ϵ-Cbz)-OAll under zinc chloride promotion to provide an anomeric mixture (β/α = 3.8:1 in 51% yield) (Scheme 9). From this mixture, the required β-galactoside was isolated in 30% yield. During glycosylation, lactonization of Hyl was observed. Lactone formation resulted in allyl alcohol being released, which in turn reacted with the galactal epoxide to give allyl galactoside (Scheme 10) as a side product.

The α-galactosylation of glycosylated Hyl was performed with the reactive thioethyl glucoside (Scheme 11). Thioethyl glucoside was prepared from ethyl 1-thio-β-D-glucopyranoside in two steps. Treatment of ethyl 1-thio-β-D-glucopyranoside with triisopropylsilyl chloride (TIPS) and imidazole resulted in silylation of the primary hydroxyl group, followed by protection of secondary hydroxyls using 4-methoxybenzyl chloride and sodium hydride to yield **6** (Scheme 11). Activation of **6** with *N*-iodosuccinimide and silver trifluoromethane sulfonate in $CH_2Cl_2$ allowed coupling to the HO-2 of glycosylated Hyl. An anomeric mixture of glycosides (α/β = 3.3:1, 80% yield) was obtained, from which the required glycoside **7** was obtained in 47% yield. Deprotection of **7** gave a diglycosylated Hyl (**8**) suitable for solid-phase peptide synthesis.

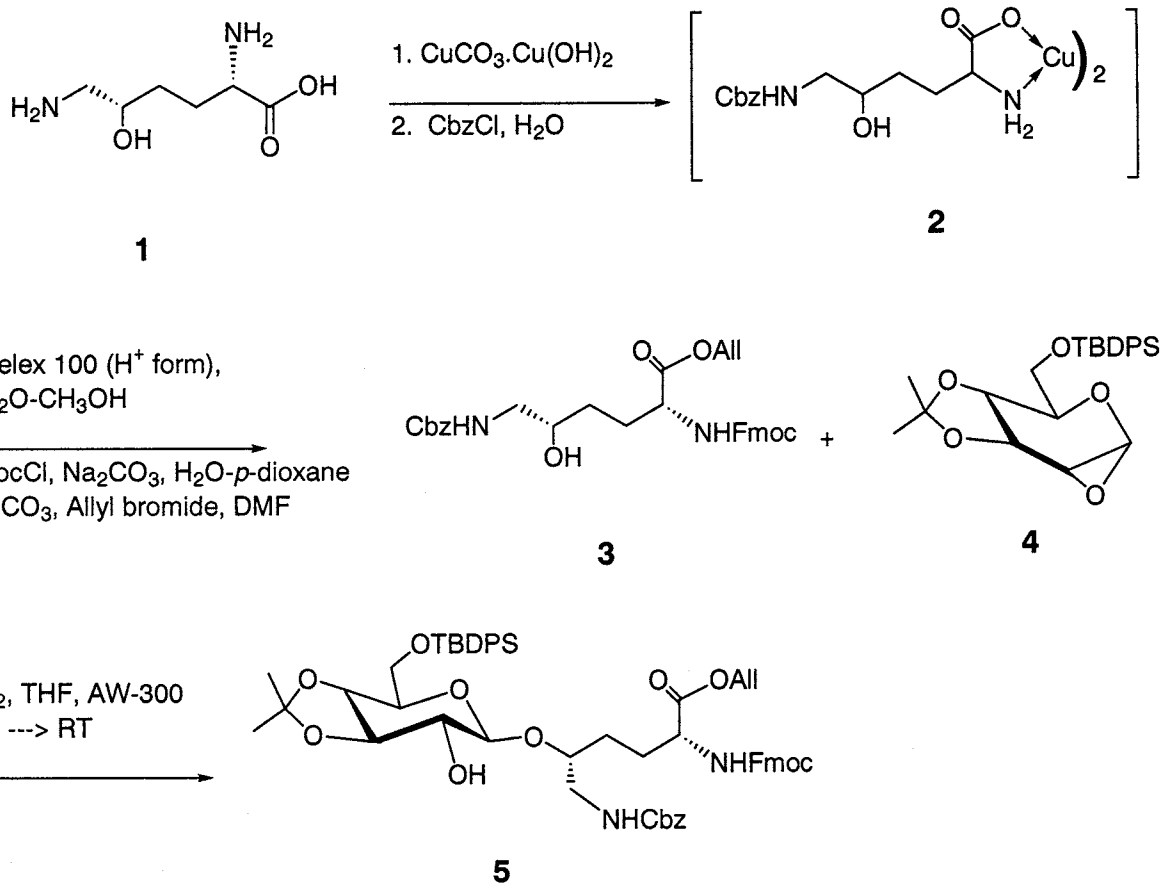
Holm et al. recently reported an improved synthesis of a galactosylated Hyl as a building block for solid-phase peptide synthesis.[70] The use of a protected 1,2-anhydrosugar was found to be more suitable for the synthesis diglycosylated Hyl, as it provides not only a 1,2-trans-glycosidic linkage but also a hydroxyl group on C-2 of the galactose residue for further attachment of an α-D-glucosyl moiety. However, this approach is less suitable for the synthesis of monoglycosylated Hyl, as the C-2 hydroxyl group on the galactose moiety can further undergo glycosylation on HO-2 of monoglycosylated Hyl. To avoid this side reaction, expensive Hyl must be used in a 2-equivalent excess. Also, the formation of an α-anomer is observed.

To improve the yield of glycosylated Hyl, glycosylation of Fmoc-Hyl(ϵ-Boc)-OBzl was carried out using peracetylated galactosyl bromide under promotion by silver silicate. This provided excellent diasteroselectivity (no α-anomer formed), but did not improve the yield (Scheme 12). The low yield was attributed to the inadequate stability of the ϵ-Boc group under the conditions of glycosylation. Thus, the Boc group was replaced by the Cbz group to protect



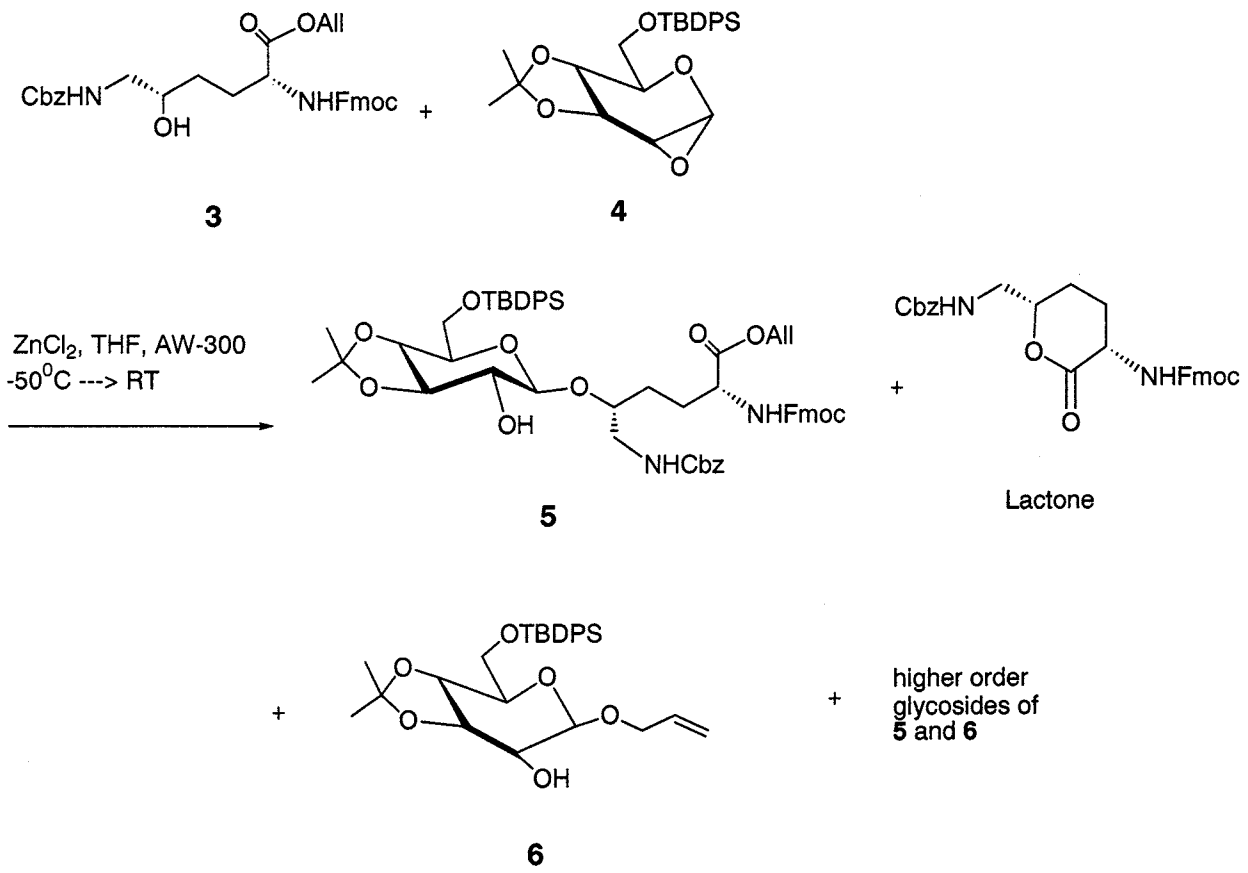

**2** **3**

**SCHEME 8**




**1** **2** **3** **4** **5**

**SCHEME 9**




**SCHEME 10**

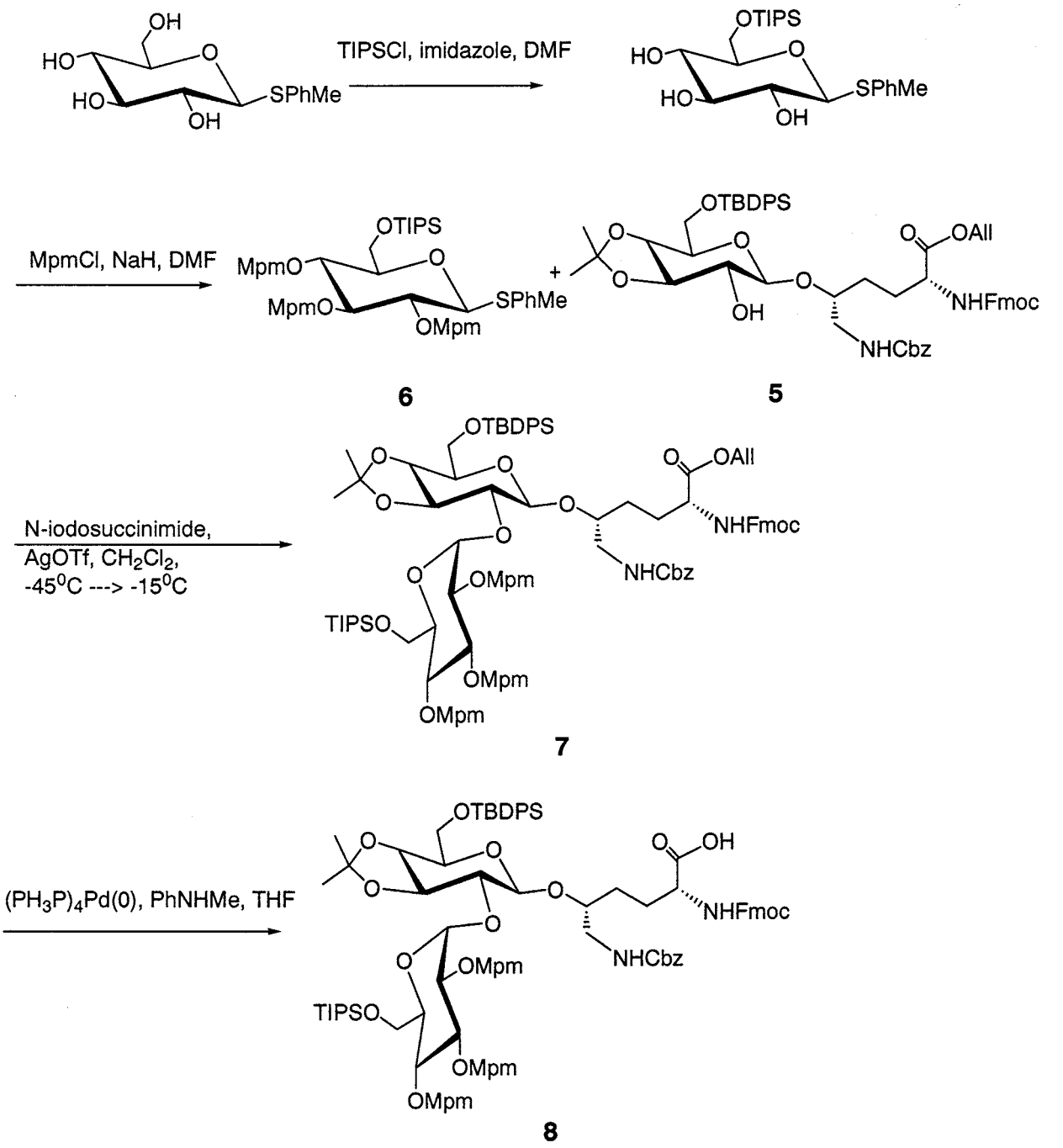
the ϵ-amino group of Hyl. Different α-carboxyl group protecting groups, such as Bzl, diphenylmethyl, and All, were also evaluated. Thus, the α-carboxyl group of Hyl was esterified by treating the cesium salt of Fmoc-Hyl(ϵ-Cbz)-OH with benzyl, diphenylmethyl, or allyl bromide in dry DMF. Lactonization was once again observed, which made isolation of Fmoc-Hyl(ϵ-Cbz)-OR (where R = Bzl, diphenylmethyl, or All) difficult and resulted in low yields.

Fmoc-Hyl(ϵ-Cbz)-OBzl was glycosylated using silver silicate promotion and galactosyl bromide. The yield was 70%, and only β-anomer was obtained. However, removal of the benzyl group using Pd/C(en) catalyst failed to provide glycosylated Hyl (Scheme 13).

The glycosylation of diphenylmethyl protected Fmoc-Hyl(ϵ-Cbz) under silver silicate promotion using galactal bromide yielded a mixture of β-glycoside and its orthoester in 4:1 ratio. The orthoester formation was reported previously by Nukada[71] during a silver silicate promoted glycosylation using glycosyl bromide. The 2-*O*-acetylated donor leads to the formation of orthoesters.[72] However, such orthoesters can be induced to give 1,2-*trans* coupling products by mild Lewis acid treatment or increasing the temperature. Products **5** and **6** (Scheme 14) were then cleaved with $H_2O$-TFA (1:9) to provide glycosylated Hyl (48% yield) suitable for solid-phase synthesis.

The allyl protected Fmoc-Hyl(ϵ-Cbz), treated under the same conditions as Fmoc-Hyl(ϵ-Cbz)-OBzl, gave the β-anomer of glycosylated Hyl in 82% yield. Formation of orthoester and lactonization was observed during glycosylation.[69] Deallylation by *N*-methylaniline in THF catalyzed by $(PPh_2)_4Pd(0)$ provided glycosylated Hyl (83% yield) suitable for solid-phase synthesis (Scheme 15). Derivative **6** was used to prepare glycopeptide fragments from the 261–278 region of type II collagen.

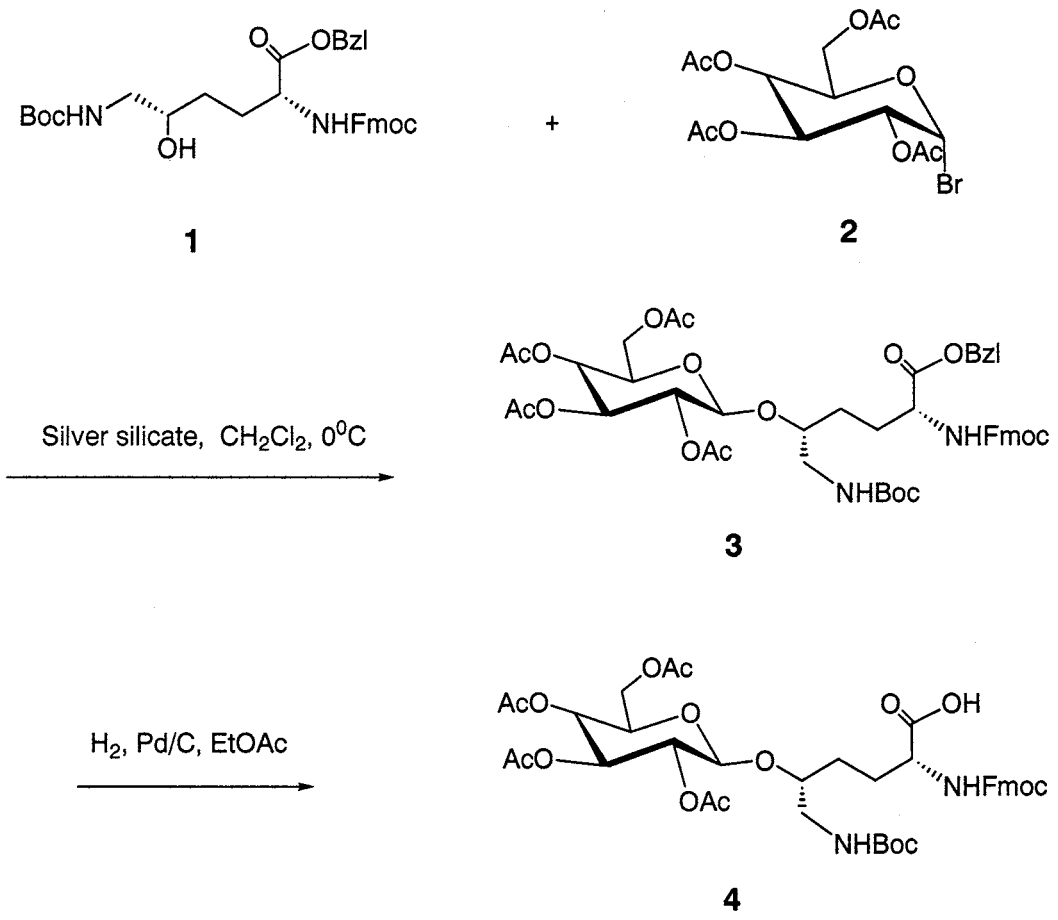
Our group sought a more direct and convenient method for the synthesis of glycosylated Fmoc-Hyl derivatives.[73] Our previous work had shown that either the Boc or allyloxycarbonyl (Aloc) group was suitable for side-chain protection of Hyl, but, due to solubility problems, Cbz was not.[68] Thus, Hyl derivatives bearing either Boc or Aloc side-chain protection were prepared (Scheme 16). First, the copper complex of Hyl was formed, which was then followed by Boc or Aloc protection of the ϵ-amino group of Hyl.




**SCHEME 11**

The copper complex of Hyl(ε-Boc) was then coupled with a 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide. Removal of copper was efficiently carried out with $Na^+$ ion exchange resin, eluting the desired Hyl(ε-Boc, *O*-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-OH with $CH_3OH$–$H_2O$ (1:1). The Fmoc group was then introduced at the α-amino group of Hyl(ε-Boc, *O*-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-OH by reacting it with 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide ester (FmocOSu) in a $H_2O$–acetone solvent system in the presence of $NaHCO_3$. Fmoc-Hyl(ε-Boc, *O*-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) was synthesized in overall 29% yield.

The above procedure is the most convenient method for the preparation of glycosylated Hyl derivatives for use in Fmoc solid-phase synthesis. The procedure requires only four distinct steps, as opposed to previous procedures of seven or more steps. This




**SCHEME 12**

procedure avoids the formation of a lactone, as the hydroxyl group of Hyl becomes unavailable prior to the decomposition of copper complex and protection of the α-amino group. Also, complete stereoselectivity was achieved. The preparation of glycosylated derivatives using amino acid copper complexes may also prove to be generally applicable for trifunctional compounds.

Derivative **4** (Scheme 16) was used for the solid-phase synthesis of the α1(IV)1263–1277 sequence from type IV collagen (Fig. 8). This sequence promotes tumor cell binding and signal transduction,[54] and contains a glycosylated Hyl residue in position 1265.[74] α1(IV)1266–1277 was assembled using Fmoc solid-phase chemistry. The $H_2N$-peptidyl-resin was then removed from the instrument, and the last three amino acids were coupled manually in an orbital shaker. Glycosylated derivative **4** was coupled using threefold molar excesses of Fmoc-amino acid and HOAt, a 2.7-fold molar excess of HATU, and a sixfold molar excess of *N,N*-diisopropylethylamine (DIEA) in DMF for 18 h. Fmoc-Val and Fmoc-Gly were coupled using fourfold molar excesses of Fmoc-amino acid and HOBt, a 3.6-fold excess of HBTU, and an eightfold molar excess of DIEA in DMF for 1 h. Fmoc groups were removed with piperidine–DMF (1:4) for 1 h. Analysis of the peptide-resin by Edman degradation chemistry revealed the desired sequence. Peptide-resin cleavage and side-chain deprotection proceeded with $H_2O$–TFA (1:19) for 1.5 h. The peptide was purified by preparative RP-HPLC and deacetylated with 2 M methanolic sodium methoxide[75] for 1 h at 20°C. The product was homogeneous by analytical RP-HPLC, and MALDI-MS analysis gave the desired mass ($[M+Na]^+$ 1638.5 Da; theoretical 1637.77 Da). Treatment of the peptide with carbazole-sulfuric acid reagent was positive for carbohydrate.

## SIDE REACTIONS OF GLYCOSYLATED SEQUENCES

The direct condensation method of glycopeptide synthesis has found considerable use for the incorporation

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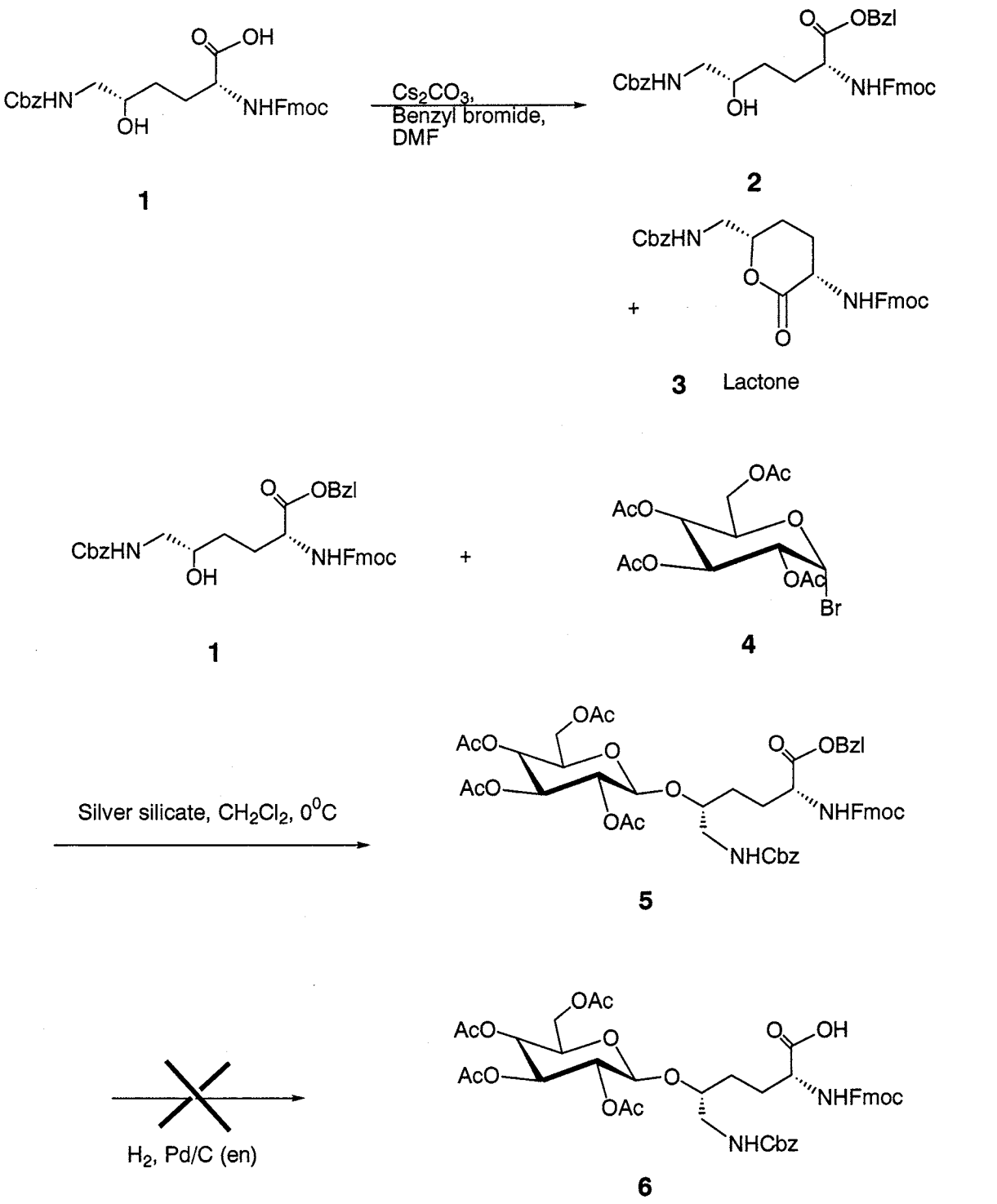
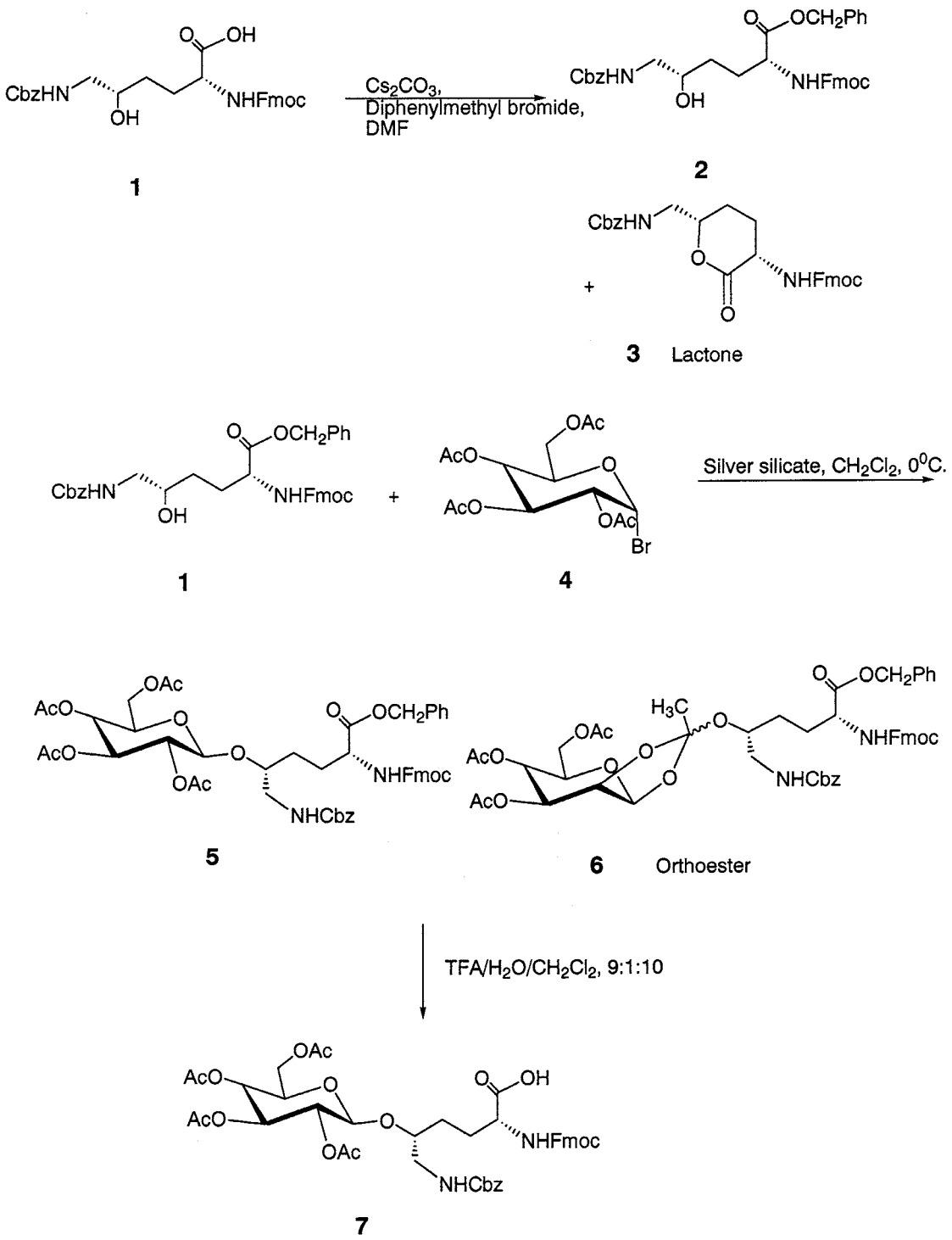
*N*-



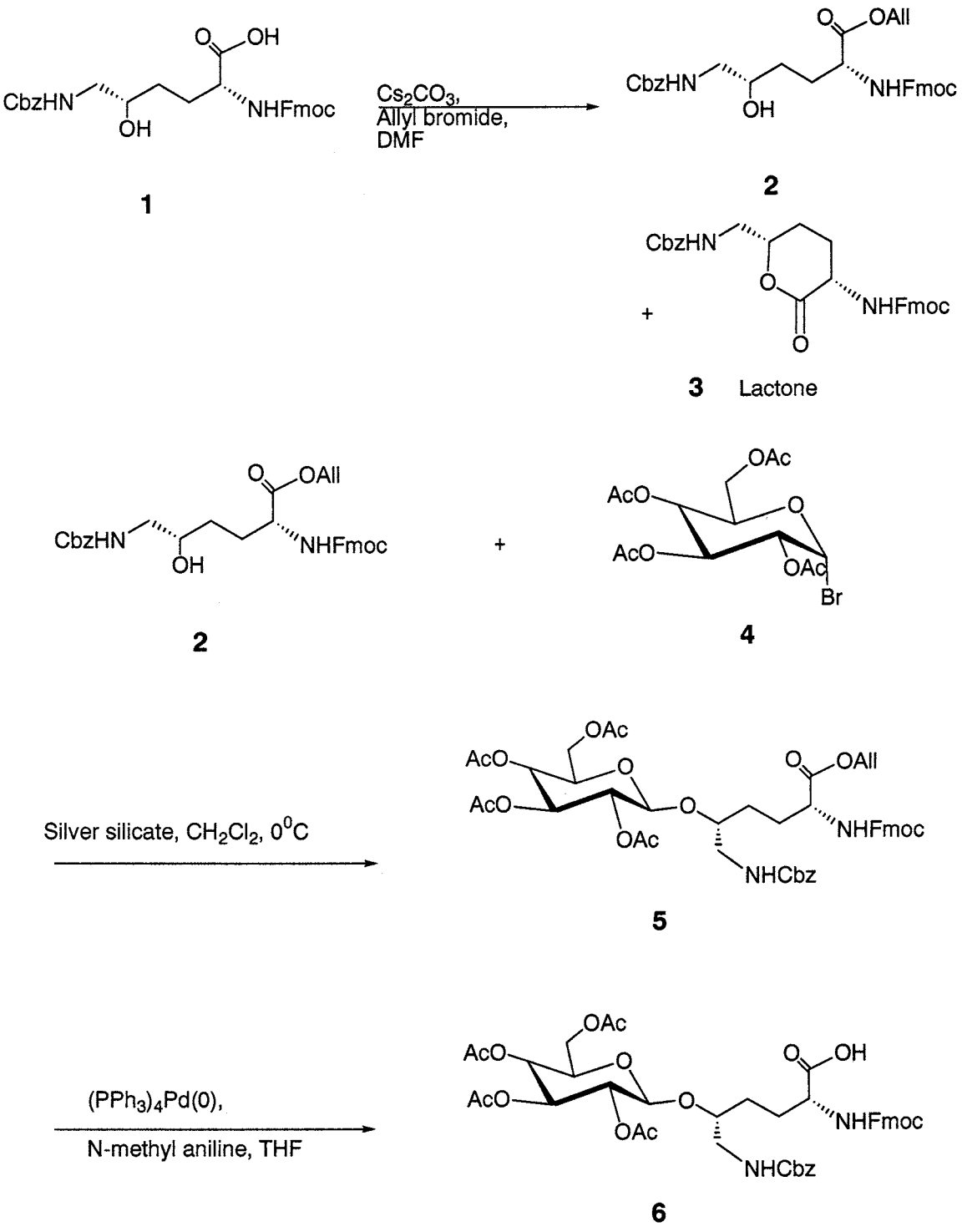

**SCHEME 13**

linked complex carbohydrates. To construct *N*-linked glycopeptides in this fashion, a peptide containing an Asp residue must first be assembled. Addition of the carbohydrate moiety will convert the Asp residue to an Asn residue. A sometimes serious side reaction with protected Asp residues involves an intramolecular condensation to form an aspartimide, which can then hydrolyze to the desired α-peptide and the undesired byproduct with the peptide chain growing from the β-carboxyl[76–78] (Fig. 9). Aspartimide formation has been




**SCHEME 14**

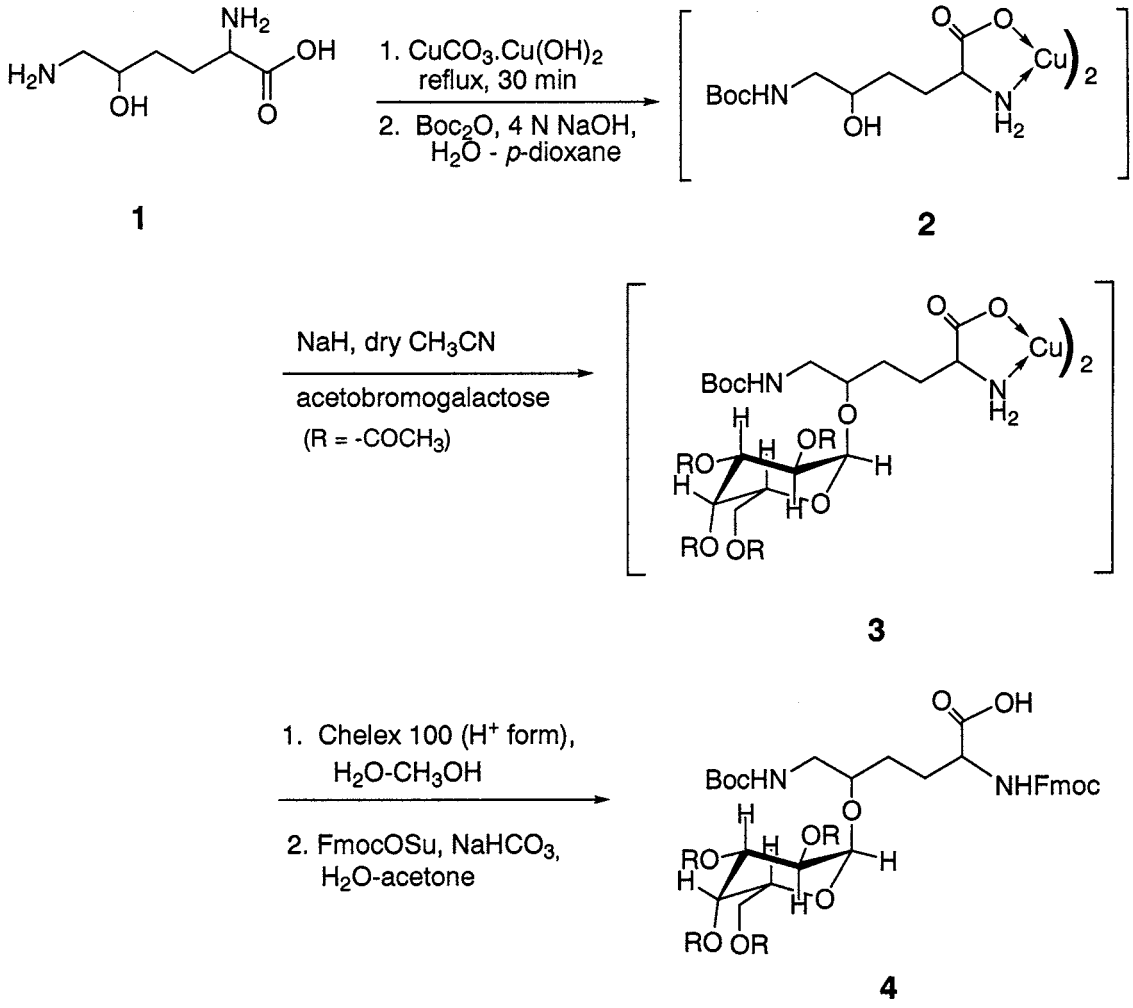
described in the synthesis of at least two Asp-containing peptides used for *N*-linked glycopeptide construction,[18,79] and has been suggested to have occurred in other glycopeptide sequences.[80,81] Aspartimide formation is sequence dependent, with Asp(OBzl)-Gly, -Ser, -Thr, -Asn, and -Gln sequences showing the greatest tendency to cyclize under basic conditions[76,82,83]; the same sequences were also found to be susceptible in strong acid.[77,78,84] For models containing Asp(OBzl)-Gly, the rate and extent of aspartimide formation was




**SCHEME 15**

substantial both in base (100% after 10-min treatment with piperidine–DMF (1:4), 50% after 1–3-h treatment with $Et_3N$ or DIEA) and in strong acid (a typical value is 36% after 1-h treatment with HF at 25°C).

The conversion of side-chain protected Asp residues to aspartimide residues can occur by repetitive base treatments used in Fmoc chemistry.[83] The cyclic aspartimide can then react with piperidine to form the α- or β-piperidide (Fig. 9). Aspartimide for-




**SCHEME 16**

mation can be rapid, and is dependent upon the Asp side-chain protecting group. Treatment of Asp(OBzl)-Gly, Asp(OcHex)-Gly, and Asp(O*t*Bu)-Gly with piperidine–DMF (1:4) for 4 h resulted in 100%, 67.5%, and 11% aspartimide formation, respectively,[83] while treatment of Asp(OBzl)-Phe with piperidine–DMF (11:9) for 1 h resulted in 16% aspartimide formation.[85] Sequence dependence studies of Asp(O*t*Bu)-X peptides revealed that piperidine could induce aspartimide formation when X = Arg(Pmc), Asn(Trt), Asp(O*t*Bu), Cys(Acm), Gly, Ser, Thr, and Thr(*t*Bu).[86,87] Aspartimide formation can also be conformation dependent.[88]

The aspartimide side reaction can be minimized or eliminated by one of several approaches. Additives can be included in the deprotection solution used to remove the Fmoc group. The most effective additives were 0.1 M HOBt or 2,4-dinitrophenol (Dnp) when used with piperidine-*N*-methylpyrrolidone (1:4) and Asp(O*t*Bu) sequences.[87] However, these additives offered no benefit when DBU was used for Fmoc removal.[87] If DBU is utilized, 1-adamantyl (1-Ada) side-chain protection of Asp is recommended to reduce aspartimide formation.[87] Because base-catalyzed aspartimide formation requires abstraction of the NH proton, no cyclization should result when the carboxyl neighbor to Asp is an *N*-alkyl amino acid. Thus, this side reaction can be eliminated by using an amide backbone protecting group (i.e., 2-hydroxy-4-methoxybenzyl) for residues in the X position of an Asp-X sequence.[79,89] However, the backbone protecting group can sterically hinder incorporation of branched or long chain carbohydrates.[79] Finally, incorporating a pseudoproline dipeptide, such as a thiazolidine ring incorporating Asp(OAll) and Cys, in place of the Asp and its neighboring residue will prevent aspartimide formation.[18,90]

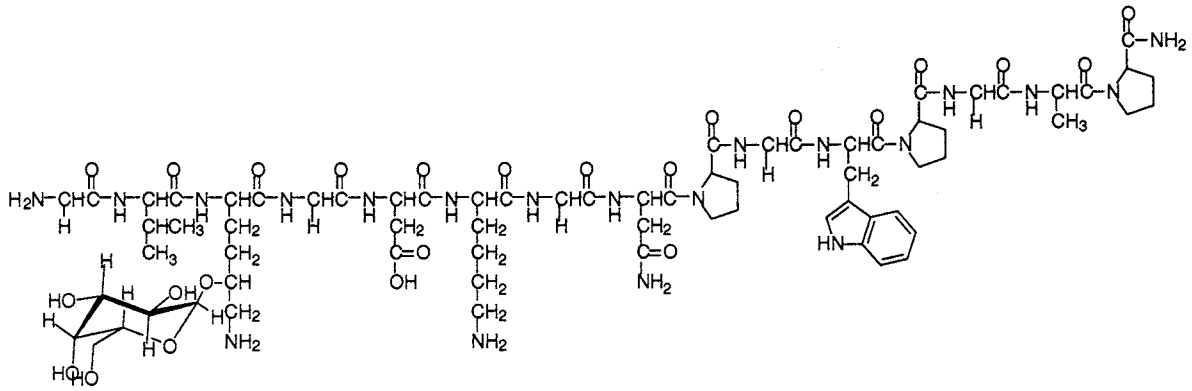


**FIGURE 8**

Sequence of glycosylated α1(IV)1263-1277.

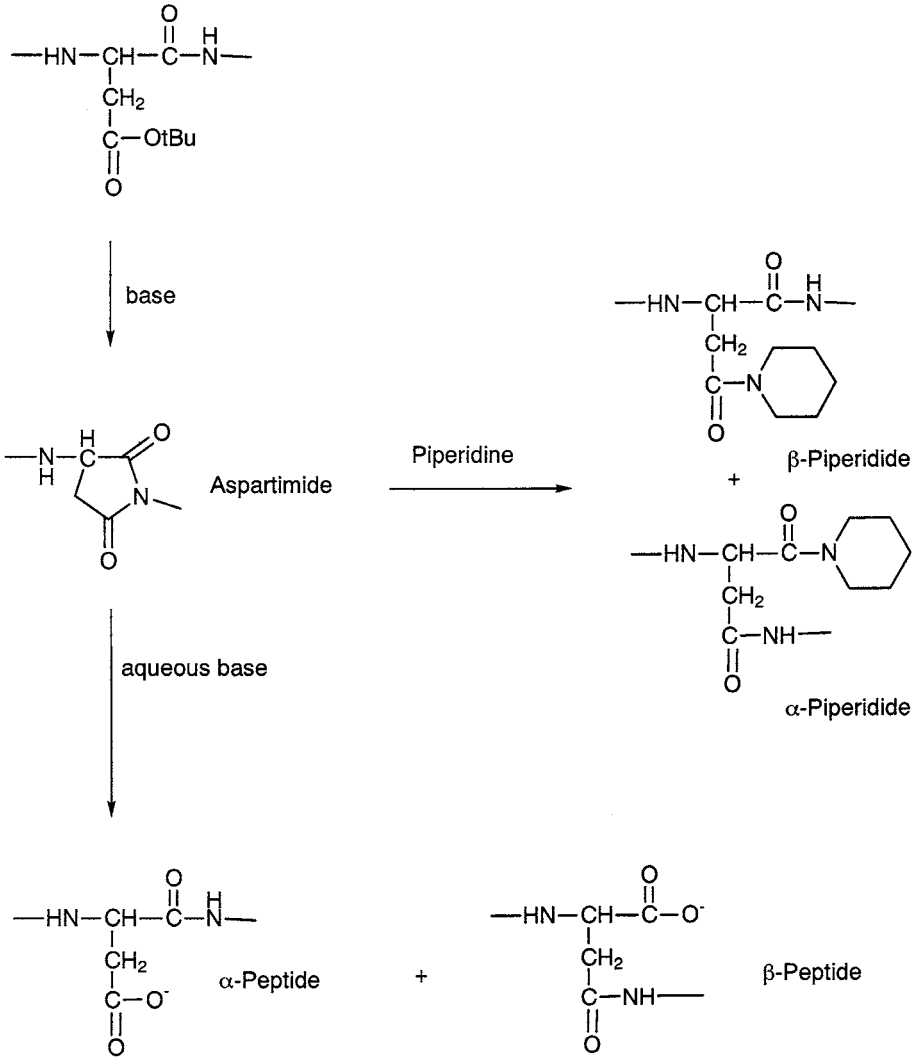



**FIGURE 9**

Formation and ring opening reactions of aspartimide.

## SUMMARY

Three different problems that can occur during glycopeptide synthesis have been described. The first problem was an unstable glycosidic linkage between xylose and Ser, subject to β-elimination by piperidine. Loss of xylose could be reduced by using a less nucleophilic base, such as morpholine, and by minimizing exposure of the glycosylated sequence to base. The second problem was a multifunctional amino acid, Hyl, which undergoes lactone formation upon activation. Glycosylated Hyl could be efficiently prepared by performing the glycosylation reaction to copper complexed Hyl, as complexed Hyl cannot undergo lactone formation. The third problem was aspartimide formation of *N*-linked glycosylated sequences. Aspartimide formation could be minimized or eliminated by several methods, including using additives in the piperidine deprotection solution or incorporating a backbone-protected amino acid prior to Asp or a pseuodproline dipeptide in place of Asp-X. Future challenges in glycopeptide synthesis will include both incorporation of a greater variety of carbohydrates and development of strategies to minimize unexpected side reactions.



## ACKNOWLEDGMENTS

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