Removal of Acyl Protective Groups from Glycopeptides: Base Does Not Epimerize Peptide Stereocenters, and β-Elimination Is Slow

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Epimerization of glycopeptide stereocenters and β-elimination have been considered as important potential side reactions on deacylation of glycopeptides which have the carbohydrate moieties protected with *O*-acyl groups. Since no systematic investigation of these side reactions has been reported, a model acetylated, *O*-linked glycotripeptide and its three epimers at the α-carbon stereocenters were prepared. The model glycopeptide did not undergo any epimerization (<1%) or β-elimination, as determined by ^{1}H NMR spectroscopy, under various conditions which are in common use for deacetylation of glycopeptides. Under more severe conditions, which are required for removal of *O*-benzoyl groups, β-elimination occurred slowly and was accompanied by slight (<5%) epimerization. The surprisingly low tendency of glycopeptides to undergo base catalyzed epimerization and β-elimination is most likely due to protection of the α-carbon stereocenters by deprotonation of the adjacent amide groups.

Introduction

The carbohydrate moieties of glycoproteins have several important functions. For instance, they provide protection against proteolysis, influence transport and uptake of proteins, determine human blood groups, and regulate recruitment of leucocytes to sites of inflammation.[1] To enhance the understanding of the biological functions of glycoproteins large efforts have recently been focused on the chemical synthesis of glycopeptides.[2,3] In general, the use of glycosylated amino acids as building blocks has proved to be the most versatile approach to both *N*- and *O*-linked glycopeptides. Thus, glycopeptides can be readily prepared by solid phase synthesis,[2,3] allowing both multiple glycopeptide synthesis and approaches to combinatorial glycopeptide libraries.[4]

In solid phase glycopeptide synthesis, the 9-fluorenylmethoxycarbonyl (Fmoc) group has predominantly been used for α-amino group protection in combination with acetyl or benzoyl groups for the carbohydrate hydroxyl groups.[3] The *O*-acyl protective groups stabilize the glycosidic bonds against the acidic conditions used for cleavage from the solid phase and deprotection of amino acid side chains. A potential drawback with the Fmoc-acyl protective group combination is the need for deprotection under basic conditions. This has caused great concern since carbohydrate chains linked to serine and threonine in glycoproteins can be removed by base-catalyzed β-elimination, and amino acid residues can undergo base-catalyzed epimerization.[2,5−8] The weak base morpholine ($pK_a = 8.3$) has therefore been advocated for Fmoc-cleavage in glycopeptide synthesis instead of piperidine ($pK_a = 11.1$) which is normally used in peptide synthesis.[2] However, we and others recently showed that piperidine is preferred to morpholine, and that it causes neither β-elimination nor epimerization of *O*-linked glycopeptides.[9] In some cases such base-catalyzed side reactions have been encountered during removal of acyl protective groups from *O*-linked glycopeptides,[6−8] but the extent of the side reactions has not been studied in any detail. We have therefore initiated a systematic investigation of conditions used for deacylation of glycopeptides using the tripeptide **6** which has a β-D-galactosyl residue linked to L-threonine (Scheme 1) as a model *O*-linked glycopeptide.

Results and Discussion

The glycosylated L-threonine building block **4** (Scheme 1) was prepared in 57% yield by silver trifluoromethanesulfonate-promoted glycosylation of the pentafluorophenyl ester **2**[10] with acetobromogalactose[11] (**1**). Preparation of the D-*allo*-threonine building block **5**, from **1** and the pentafluorophenyl ester **3**, was achieved in 82% yield when silver silicate[12] was used as promoter. In contrast, under silver trifluoromethanesulfonate promotion the yields of **5** were irreproducible and **5** was often contaminated by the corresponding orthoester. The *O*-acetylated glycopeptide **6** and its three diastereoisomers **7**−**9** were prepared from **4** and **5** on solid phase, and the glycopep-

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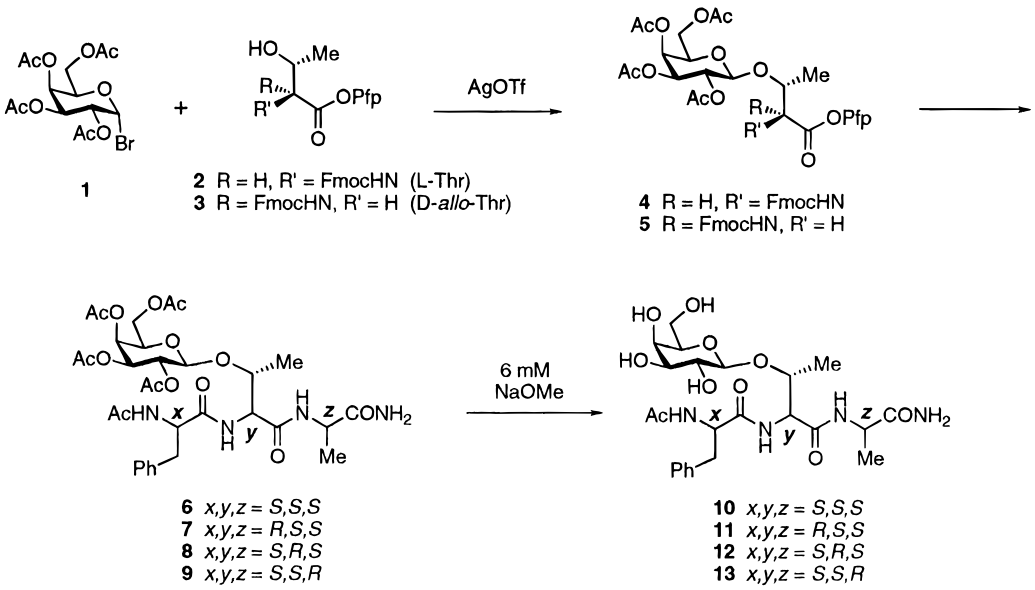
Scheme 1[a]



[a] *x*, *y* and *z* denote the stereochemistry at C-α of the amino acids

Table 1. ^{1}H NMR Data [δ (*J*, Hz)] for the *O*-Acetylated Glycopeptides 6−9 in Acetone-d_6[a]

residue	proton	6	7	8	9
Phe	α	4.63 (5.2, 7.3, 8.7)	4.53	4.47−4.55	4.66
	β	2.98 (8.7, 14.0)	3.02 (7.8, 13.6)	2.94 (9.6, 13.6)	2.97 (8.6, 14.1)
		3.19 (5.3, 14.1)	3.11 (7.6, 13.5)	3.18 (5.4, 13.8)	3.18 (5.4, 14.1)
	arom	7.15−7.30	7.20−7.35	7.10−7.35	
Thr	α	4.50 (3.8, 7.3)	4.17−4.28[b]	4.44 (5.0, 7.9)	4.54 (4.4, 7.1)
	β	4.24−4.30[b]	4.17−4.28[b]	4.10−4.25[b]	
	γ	1.13 (6.4)	0.97 (6.4)	1.21 (6.4)	1.11 (6.4)
Ala	α	4.34 (7.3)	4.34	4.30 (7.3)	4.41
	β	1.39 (7.2)	1.40 (7.3)	1.37 (7.3)	1.38 (7.2)
	amide	6.18, 6.47	6.34, 6.75	6.23, 6.84	6.19, 6.88
Gal	H-1	4.88 (7.9)	4.75 (7.9)	4.81 (7.3)	4.89 (7.9)
	H-2	5.10 (7.9, 10.5)	5.04 (7.9, 10.5)	5.05−5.18[b]	5.11 (7.8, 10.5)
	H-3	5.18 (3.5, 10.5)	5.14 (3.5, 10.5)	5.05−5.18[b]	5.18 (3.4, 10.5)
	H-4	5.40 (0.8, 3.5)	5.38 (3.5)	5.38 (2.9)	5.41 (3.4)
	H-5	4.24−4.30[b]	4.17−4.28[b]	4.10−4.25[b]	4.27−4.32[b]
	H-6	4.15 (8.5, 14.0)	4.13	4.10−4.25[b]	4.19
	H-6′	4.24−4.30[b]	4.17−4.28[b]	4.10−4.25[b]	4.27−4.32[b]

[a] Obtained at 500 MHz and 298 K with residual acetone-d_5 (δ_H 2.05 ppm) as internal standard. [b] Resonances not well resolved due to spectral overlap.

tides were cleaved from the resin, using procedures that have been described previously.[13] Purification by reversed-phase HPLC gave the glycopeptides **6**−**9** in 45−60% yields, and their stuctures were confirmed by ^{1}H (Table 1) and ^{13}C NMR spectroscopy, as well as by high resolution fast atom bombardment mass spectrometry. Importantly, ^{1}H NMR spectroscopy confirmed that no detectable epimerization of the α-carbon stereocenters had occurred during synthesis of glycopeptides **6**−**9**. Glycopeptide **6** was then deacetylated under the following conditions, each of which are in common use for deprotection of glycopeptides: (A) hydrazine hydrate in methanol[14] (1:5) for 3 h; (B) saturated methanolic ammonia[8] for 6 h; and (C) 6 mM methanolic sodium methoxide for 30 min. In recent investigations, de-*O*-acetylation of glycopeptides in methanolic solution has been performed using 10−100 mM sodium methoxide,[15] at pH values between 8 and 10.5,[15a,8] as determined using pH paper.[16] In the present investigation even milder basic conditions, *i.e.* 6 mM methanolic sodium methoxide, were employed. Using either of the three methods involving deacetylation with hydrazine, ammonia, or sodium methoxide, **6** was quantitatively converted into **10** as determined by reversed-phase HPLC and 500 MHz ^{1}H NMR spectroscopy of the *crude* reaction products. Deacetylation of the diastereoisomeric glycopeptides **7**−**9** with methanolic sodium methoxide also quantitatively gave the products **11**−**13**, which had ^{1}H NMR spectra that were distinct from each other and from that of **10** (Table 2). The region containing the threonine and alanine methyl groups (δ

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(16) In several investigations where methanolic sodium methoxide has been used for de-*O*-acetylation of glycopeptides, only the pH but not the concentration of sodium methoxide used in the reaction has been reported (cf. *e.g.* refs 6 and 8). However, the pH of 6 mM methanolic sodium methoxide was found to be 8−8.5 when determined using dry pH paper, but 10.5−11 when the paper was wet. Due to this ambiguity, the concentration of methanolic sodium methoxide should be specified, in preference to the apparent pH on wet or dry pH-paper, when the conditions employed for deacetylation are described.

Table 2. ^{1}H NMR Data [δ (J, Hz)] for the Deacetylated Glycopeptides 10−13 in MeOH-d_4[a]

residue	proton	10	11	12	13
Phe	α	4.68 (5.3, 9.4)	4.56 (7.4, 8.4)	4.61 (7.7)	4.68 (5.6, 9.3)
	β	2.92 (9.5, 14.1)	3.00 (7.4, 13.3)	2.96 (7.9, 13.6)	2.91 (9.1, 14.1)
		3.19 (5.3, 14.1)	3.04 (8.4, 13.5)	3.08 (7.5, 13.5)	3.19 (5.6, 14.3)
	arom	7.15−7.30	7.10−7.30	7.20−7.35	7.15−7.30
	Ac	1.91	1.94	1.91	1.91
Thr	α	4.61 (4.2)	4.26−4.33[b]	4.25 (3.7)	4.45 (4.7)
	β	4.35−4.42[b]	4.26−4.33[b]	3.78−3.87[b]	4.27 (4.7, 6.4)
	γ	1.21 (6.4)	0.85 (6.3)	1.27 (6.5)	1.20 (6.4)
Ala	α	4.31 (7.2, 14.5)	4.26−4.33[b]	4.31 (7.3, 14.6)	4.34−4.40
	β	1.40 (7.3)	1.42 (7.3)	1.38 (7.3)	1.37 (7.2)
Gal	H-1	4.35−4.42[b]	4.24	4.14 (7.5)	4.89 (7.9)
	H-2	3.47−3.53[b]	3.42−3.48[b]	3.43−3.48[b]	3.47−3.52[b]
	H-3	3.47−3.53[b]	3.42−3.48[b]	3.43−3.48[b]	3.47−3.52[b]
	H-4	3.83[b]	3.77−3.82[b]	5.38 (2.9)	3.80−3.85[b]
	H-5	5.59 (0.9, 4.7, 7.4)	3.50 (1.0, 4.6, 7.4)	3.78−3.87[b]	3.57 (1.0, 3.2, 5.7)
	H-6	3.73 (4.6, 11.5)	3.64−3.70	3.73 (4.8, 11.3)	3.66−3.73
	H-6′	3.82 (7.3, 11.5)	3.77−3.82	3.78−3.87[b]	3.80−3.85[b]

[a] Obtained at 500 MHz and 298 K with residual CHD_2OD (δ_H 3.31 ppm) as internal standard. [b] Resonances not well resolved due to spectral overlap.

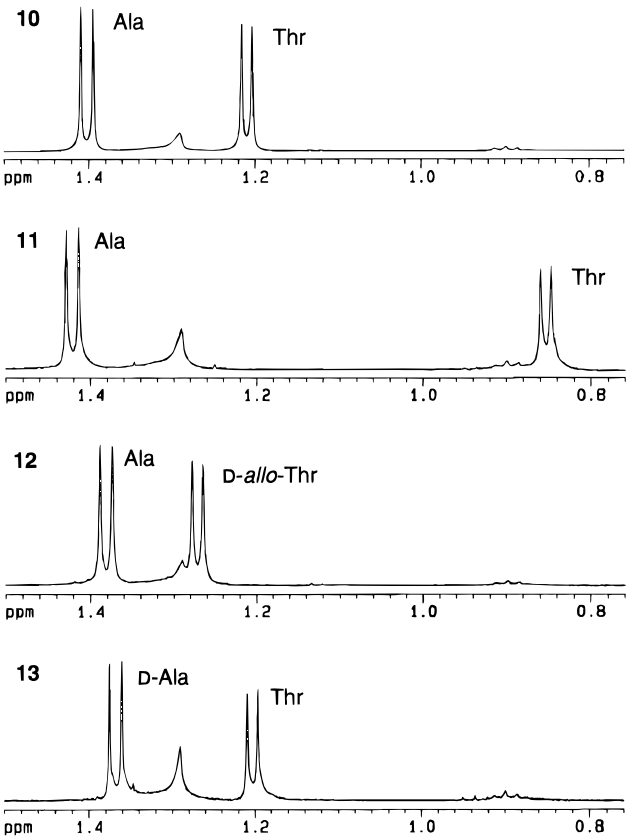


Figure 1. ^{1}H NMR spectra of the region containing the threonine and alanine methyl groups in the crude glycopeptides **10**−**13**.

= 0.8−1.5 ppm) was especially characteristic for the four diastereomeric glycopeptides as shown in Figure 1. Careful inspection of this "finger print" region did not reveal any contamination of the epimerized glycopeptides **11**−**13** in crude **10** obtained by either of the three methods used for deacetylation. Addition of portions of purified **12** to **10** established that the detection limit for epimerization was between 0.5 and 1%.

De-*O*-acetylation of glycopeptides has been performed[15b] with 100 mM methanolic sodium methoxide, and similar or even more drastic conditions are required[6,8] for removal of the more persistent *O*-benzoyl groups. Therefore, 120 mM methanolic sodium methoxide, *i.e.* a 20-fold higher concentration than above, was also used for

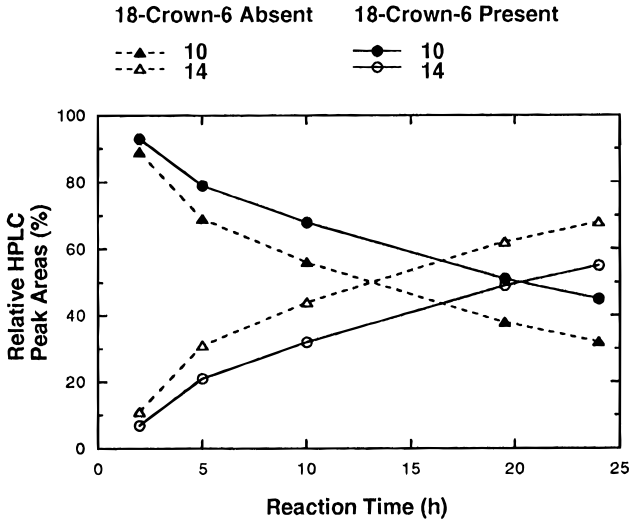


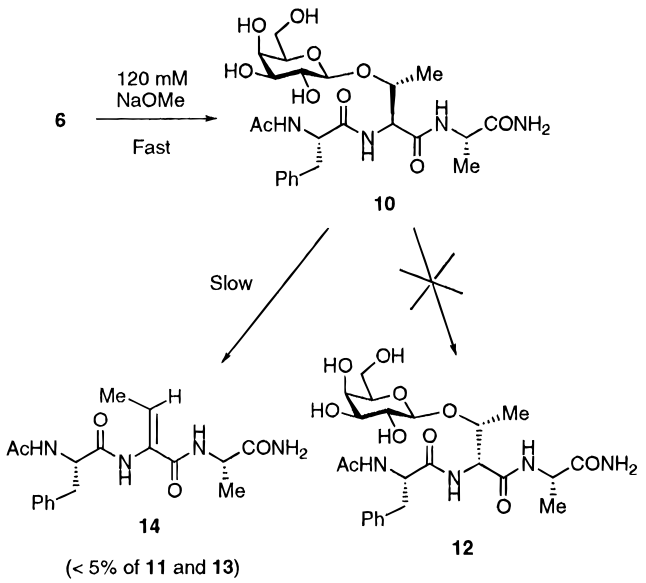
Figure 2. Transformation of glycopeptide **10** into the β-elimination product **14** with methanolic sodium methoxide in the absence and presence of 18-crown-6.

deacetylation of **6**. Analytical reversed-phase HPLC then revealed that conversion into **10** was almost instantaneous and was followed by the slow disappearance of **10** (Figure 2). Simultaneously a new compound was formed which was identified, using difference NOE spectroscopy and ^{1}H NMR chemical shifts,[17] as the product of *anti*-elimination, **14** (Scheme 2). Interruption of the β-elimination when 50% of **10** had been consumed, and separation of **10** from **14**, revealed that remaining **10** had not undergone any epimerization of the threonine residue to give **12**, as determined by ^{1}H NMR spectroscopy. However, careful inspection of the NMR spectrum showed that slight epimerization of both the phenylalanine and the alanine residue had occurred to give **11** and **13** (<1.5 and 3.5%, respectively). It should be pointed out that the epimers **11**−**13** have identical HPLC retention times as **10** (t_R = 13 min) and were therefore not removed in the purification, whereas **14** (t_R = 16 min) was easily separated from **10**.

When deacetylation of **6** instead was performed in aqueous solution with 0.1 M sodium hydroxide, *i.e.* under conditions resembling those used for cleavage of *O*-linked

(17) Srinivasan, A.; Stephenson, R. W.; Olsen, R. K. *J. Org. Chem.* **1977**, *42*, 2256−2260.

Scheme 2



carbohydrates from glycoproteins,[18] a complex mixture of products was obtained. As revealed by ^{1}H NMR spectroscopy of the crude product, both β-elimination and epimerization had occurred during the treatment with aqueous sodium hydroxide. In multiple peptide synthesis cleavage of peptides from the resin has been performed with 0.1 M aqueous sodium hydroxide.[19] In view of the above results such conditions should not be employed in glycopeptide synthesis and may also be less suitable for synthesis of peptides.

It has been proposed that complexation of cations, such as sodium ions, by the carbohydrate moieties of *O*-linked glycopeptides significantly facilitates β-elimination.[2] 18-Crown-6 efficiently complexes sodium ions in methanolic solutions (log $K_{ass.}$ = 4.27),[20] and a 20% excess of 18-crown-6 was therefore added to 100 mM methanolic sodium methoxide used for deacetylation of **6**. As shown in Figure 2 the rate of β-elimination of **10** to give **14** decreased in the presence of 18-crown-6 so that the time required to reach 50% β-elimination increased by ~50%. Complexation of sodium ions by the carbohydrate moiety was thus revealed to have a small but significant influence on the rate of base-induced β-elimination of glycopeptides.

O-Linked glycosides of serine and threonine have been suggested to undergo E1cB elimination in aqueous sodium hydroxide with an amino acid enolate ion as an intermediate.[18] Formation of the α,β-unsaturated alkene **14** (but not the isomeric β,γ-unsaturated alkene, Scheme 2) suggests that sodium methoxide-catalyzed β-eliminations of *O*-linked glycopeptides in methanolic solution are also E1cB-like to a large extent. However, since epimerization of **10** to give **12** did not occur during the β-elimination it seems unlikely that a stable enolate ion constitutes an intermediate in the elimination. Instead the elimination seems to have some E2 character. This was also supported by formation of only the *Z*-alkene **14**, but not the *E*-isomer, from **10**.

In contrast to previous concerns,[2,5,7,8] the results described here suggest that epimerization does not constitute a problem in base-catalyzed deacetylations of glycopeptides, provided that the reaction is performed in methanolic solution. Furthermore, ^{1}H NMR spectroscopy, but not reversed-phase HPLC, was found to be a sensitive tool for studies of epimerization. However, β-elimination and slight (<5%) epimerization of the *O*-linked glycopeptide **10** did occur in methanolic solution, but only slowly when higher concentrations of sodium methoxide were employed than normally used for removal of *O*-acetyl groups. Cleavage of *O*-benzoyl groups requires such drastic conditions, which need to be carefully adjusted so as to minimize β-elimination.[6,8] If possible, it may therefore be desirable to avoid *O*-benzoyl protective groups in synthesis of *O*-linked glycopeptides. Due to the loss of the carbohydrate moiety, the β-elimination product **14** displayed a different mobility on reversed-phase HPLC than the parent glycopeptide **10**. Similar differences between the mobilities of β-elimination products and the parent glycopeptides have been found in other cases by us[21] and others.[9b] As a consequence, when β-elimination has been encountered, purification by HPLC can be expected to give the desired glycopeptide which has undergone no or slight epimerization at the α-carbons.

The surprisingly low tendency of glycopeptides to undergo base-catalyzed epimerization and β-elimination is most likely due to protection of the α-carbon stereocenters by deprotonation of the adjacent amide groups, as suggested in studies directed toward alkylations of peptide enolates.[22] In cases when epimerization of a glycosylated serine or threonine residue has been reported[7,8] these residues were unable to undergo conversion into a protective aza-enolate since they were located *N*-terminally and had an underivatized α-amino group.

Further studies of epimerization and β-elimination during deacylation of glycopeptides are now being performed. These include glycopeptides which carry carbohydrates other than galactose linked to both serine and threonine, and glycopeptides that contain a larger variety of amino acid residues than in the model glycopeptide **6**.

Experimental Section

General Procedures. TLC was performed on silica gel 60 F_{254} (E. Merck) with detection by UV light and charring with H_2SO_4. Flash column chromatography was performed on dry silica gel (Matrex, 60 Å, 35−70 μm, Grace Amicon) with distilled solvents. CH_2Cl_2 was dried by distillation from CaH_2 immediately before being used as solvent in the glycosylations. Organic solutions were dried over Na_2SO_4 before being concentrated.

The ^{1}H NMR spectra were recorded at 300 or 500 MHz, and at 25 °C, for solutions in $CDCl_3$ [residual $CHCl_3$ (δ_H 7.26) as internal standard], MeOH-d_4 [residual CHD_2OD (δ_H 3.31)], or acetone-d_6 [residual acetone-d_5 (δ_H 2.05)]. First-order chemical shifts and coupling constants were obtained from one-dimensional spectra, and proton resonances were assigned from COSY experiments. Proton resonances that could not be assigned are not reported. The ^{13}C NMR spectra were recorded at 75 or 125 MHz, and at 25 °C, for solutions in $CDCl_3$

(18) Wakabayashi, K.; Pigman, W. *Carbohydr. Res.* **1974**, *35*, 3−14.
(19) Valerio, R. M.; Benstead, M.; Bray, A. M.; Campbell, R. A.; Maeji, N. J. *Anal. Biochem.* **1991**, *197*, 168−177.
(20) Buschmann, H.-J. *Chem. Ber.* **1985**, *118*, 2746−2756.

(21) Within a project dealing with the T cell response to neoglycopeptides (cf. ref 13), the decapeptide DYGISQINSR carrying per-*O*-benzoylated lactose at the central serine was prepared. Debenzoylation of this glycopeptide was accompanied by extensive β-elimination (~75%), but the deprotected glycopeptide and the product of β-elimination had significantly different retention times on reversed-phase HPLC (14.0 and 16.2 min, respectively, when analyzed as described in the Experimental Section). Sjölin, P.; Kihlberg, J. Unpublished results.
(22) Seebach, D. *Aldrichim. Acta* **1992**, *25*, 59−66.

[$CDCl_3$ (δ_C 77.0) as internal standard], MeOH-d_4 [CD_3OD (δ_C 49.15)], or acetone-d_6 [CD_3COCD_3 (δ_C 29.92)]. Ions for positive fast atom bombardment mass spectra were produced by a beam of Xe atoms (6 keV) from a matrix of glycerol and thioglycerol.

Analytical and preparative HPLC separations were performed on Kromasil C-8 columns (100 Å, 50 μm, 4.6 or 20 × 250 mm, respectively) with flow rates of 1.5 or 11 mL/min, detection at 214 nm, and the solvent systems A, aqueous 0.1% CF_3CO_2H; and B, 0.1% CF_3CO_2H in MeCN. The homogeneity of the glycopeptides was determined by analytical HPLC using a linear gradient of 0−100% B in A during 60 min.

N-(9-Fluorenylmethoxycarbonyl)-D-*allo*-threonine[23] (Fmoc-D-*allo*-Thr), 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide[11] (**1**), and *N*-(9-fluorenylmethoxycarbonyl)-L-threonine pentafluorophenyl ester[10] (**2**) were prepared according to the indicated literature methods.

***N*-(9-Fluorenylmethoxycarbonyl)-D-*allo*-threonine Pentafluorophenyl Ester (3).** 1,3-Diisopropylcarbodiimide (570 μL, 1.62 mmol) was added to Fmoc-D-*allo*-Thr (429 mg, 1.26 mmol) and pentafluorophenol (680 mg, 1.62 mmol) in ethyl acetate (15 mL) at 0 °C. After 1 h the mixture was allowed to attain room temperature during 2 h before removal of precipitated diisopropylurea by filtration. The solution was diluted with CH_2Cl_2 (85 mL) and washed with saturated aqueous $NaHCO_3$ (100 mL). The aqueous phase was extracted with CH_2Cl_2 (70 mL), and the combined organic phases were dried, filtered, and concentrated. Flash column chromatography (heptane−ethyl acetate, 1:1) of the residue gave **3** (0.48 g, 75%): $[\alpha]^{25}_D$ +20° (*c* 0.61, $CDCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.67 (bs, 1H, NH), 4.76 (bs, 1H, H-α), 4.33 (bs, 1H, H-β), 4.51 (d, J = 6.7 Hz, 2H, OCOCH_2CH), 4.26 (t, J = 6.7 Hz, 1H, OCOCH$_2$C*H*), 1.39 (bd, J = 5.4 Hz, 3H, H-γ); ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.6, 156.2, 143.6, 143.5, 141.4, 127.8, 127.1, 125.0, 120.3, 68.6, 67.7, 59.4, 47.1, 19.2. Anal. Calcd for $C_{25}H_{18}NO_5F_5$: C, 59.2; H, 3.6; N, 2.8. Found: C, 59.4; H, 3.6; N, 2.6.

***N*-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-threonine Pentafluorophenyl Ester (4).** Silver triflate (0.64 g, 2.5 mmol) was added to **1**[11] (1.10 g, 2.68 mmol), **2**[10] (0.833 g, 1.64 mmol), and molecular sieves (4 Å, 1.1 g) in dry CH_2Cl_2 (15 mL) at 0 °C. After 40 min the mixture was allowed to attain room temperature during 70 min, and it was then diluted with CH_2Cl_2 (50 mL) and filtered. The solution was washed with saturated aqueous $NaHCO_3$ (80 mL), the aqueous phase was extracted with CH_2Cl_2 (30 mL), and the combined organic phases were dried, filtered, and concentrated. Flash column chromatography (heptane−ethyl acetate, 3:1) of the residue gave **4** (0.78 g, 57%): $[\alpha]^{25}_D$ −24° (*c* 0.30, $CDCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.73 (d, J = 9.0 Hz, 1H, NH), 5.38 (d, J = 2.9 Hz, 1H, H-4), 5.18 (dd, J = 7.8, 10.3 Hz, 1H, H-2), 5.04 (dd, J = 3.4, 10.5 Hz, 1H, H-3), 4.72 (dd, J = 2.5, 9.1 Hz, 1H, H-α), 4.58 (dd, J = 2.7, 6.1 Hz, 1H, H-β), 4.52 (d, J = 7.8 Hz, 1H, H-1), 4.50 (dd, AB-type, J = 7.2, 10.8 Hz, 1H, OCOCH_2CH), 4.43 (dd, AB-type, J = 7.4, 10.5 Hz, 1H, OCOCH_2CH), 4.27 (t, J = 7.1 Hz, 1H, OCOCH$_2$C*H*), 4.03 (m, 2H, H-6,6′), 3.89 (t, J = 6.4 Hz, 1H, H-5), 2.17, 2.07, 2.01, and 2.00 (4s, each 3H, Ac), 1.31 (d, J = 6.2 Hz, 3H, H-γ); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 170.3, 170.1, 169.4, 166.3, 156.5, 143.8, 143.6, 141.3, 127.8, 127.1, 125.1, 120.0, 98.6, 73.7, 70.7, 70.6, 69.0, 67.4, 66.7, 60.8, 58.6, 47.2, 20.8, 20.6, 20.5, 20.4, 16.7; MS (FAB): calcd for $C_{39}H_{37}NO_{14}F_5$ 838 (M + H^+), found 838. Anal. Calcd for $C_{39}H_{36}NO_{14}F_5$: C, 55.9; H, 4.3; N, 1.7. Found: C, 55.9; H, 4.4; N, 1.4.

***N*-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-D-*allo*-threonine Pentafluorophenyl Ester (5).** A solution of **1**[11] (110 mg, 0.268 mmol) and **3** (80 mg, 0.158 mmol) in CH_2Cl_2 (5 mL) was stirred with molecular sieves (3 Å, 200 mg) for 1 h at 20 °C under argon. Silver silicate[12] (250 mg, activated under vacuum at 100 °C for 48 h) was then added and after a further 4 h at 20 °C the mixture was filtered and concentrated. Flash column chromatography (heptane−ethyl acetate, 2:1) of the residue gave **5** (108 mg, 82%): $[\alpha]^{25}_D$ +23° (*c* 0.26, $CDCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.25 (d, J = 8.8 Hz, 1H, NH), 5.27 (d, J = 2.8 Hz, 1H, H-4), 5.11 (dd, J = 8.0, 10.4 Hz, 1H, H-2), 4.96 (dd, J = 3.3, 10.4 Hz, 1H, H-3), 4.74 (dd, J = 5.7, 10.9 Hz, 1H, OCOCH_2CH), 4.64 (dd, J = 1.8, 8.7 Hz, 1H, H-α), 4.55 (dd, J = 5.4, 10.9 Hz, 1H, OCOCH_2CH), 4.23 (t, J = 5.5 Hz, 1H, OCOCH$_2$C*H*), 4.19 (d, J = 7.9 Hz, 1H, H-1), 4.00 (m, 1H, H-β), 3.91 (m, 2H, H-6,6′), 3.36 (bt, J = 6.4 Hz, 1H, H-5), 2.13, 2.04, 1.99, and 1.77 (4s, each 3H, Ac), 1.43 (d, J = 6.6 Hz, 3H, H-γ); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 170.2, 169.0, 164.8, 155.8, 143.8, 143.7, 141.6, 141.3, 127.9, 127.2, 127.1, 124.9, 124.6, 120.0, 101.0, 78.5, 70.7, 70.7, 68.6, 66.9, 66.2, 61.7, 58.4, 47.3, 20.6, 20.1, 17.9; MS (FAB): calcd for $C_{39}H_{37}NO_{14}F_5$ 838 (M + H^+), found 838. Anal. Calcd for $C_{39}H_{36}NO_{14}F_5$: C, 55.9; H, 4.3; N, 1.7. Found: C, 56.2; H, 4.1; N, 1.6.

***N*$^\alpha$-Acetyl-L-phenylalanyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-threonyl-L-alanine Amide (6), *N*$^\alpha$-Acetyl-D-phenylalanyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-threonyl-L-alanine Amide (7), *N*$^\alpha$-Acetyl-L-phenylalanyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-D-*allo*-threonyl-L-alanine Amide (8), and *N*$^\alpha$-Acetyl-L-phenylalanyl-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-threonyl-D-alanine Amide (9).** The acetylated glycopeptides **6**−**9** were prepared, as described previously,[13] by manual Fmoc solid phase synthesis on an aminomethylated polystyrene resin functionalized with the Rink linker. Cleavage from the resin was effected with TFA−H_2O, 9:1, and glycopeptides **6**−**9** were obtained in 45−60% yields after purification with reversed-phase HPLC. HRMS (FAB): calcd for $C_{32}H_{45}N_4O_{14}$ 709.2932 (M + H^+), found for **6**: 709.2941, **7**: 709.2916, **8**: 709.2930, and **9**: 709.2940. ^{1}H NMR data for compounds **6**−**9** are given in Table 1.

***N*$^\alpha$-Acetyl-L-phenylalanyl-3-*O*-(β-D-galactopyranosyl)-L-threonyl-L-alanine Amide (10), *N*$^\alpha$-Acetyl-D-phenylalanyl-3-*O*-(β-D-galactopyranosyl)-L-threonyl-L-alanine Amide (11), *N*$^\alpha$-Acetyl-L-phenylalanyl-3-*O*-(β-D-galactopyranosyl)-D-*allo*-threonyl-L-alanine Amide (12), and *N*$^\alpha$-Acetyl-L-phenylalanyl-3-*O*-(β-D-galactopyranosyl)-L-threonyl-D-alanine Amide (13).** Deacetylation of **6**−**9** with 6 mM methanolic sodium methoxide as described below and purification by reversed-phase HPLC gave the glycopeptides **10**−**13** in quantitative yields. HRMS (FAB): calcd for $C_{24}H_{37}N_4O_{10}$ 541.2510 (M + H^+), found for **10**: 541.2513, **11**: 541.2507, **12**: 541.2501, and **13**: 541.2507. ^{1}H NMR data for compounds **10**−**13** are given in Table 2.

***N*$^\alpha$-Acetyl-L-phenylalanyl-(2-amino-Z-but-2-enoyl)-L-alanine Amide (14).** Methanolic sodium methoxide (2 M, 1500 μL) was added to glycopeptide **6** (31 mg, 44 μmol) in MeOH (31 mL). The time-course of the reaction was monitored by reversed-phase analytical HPLC, and after 16 h the solution was neutralized with Duolite (H^+) resin. Purification by reversed-phase HPLC gave **10** (4.1 mg, 7.6 μmol) and **14** (3.0 mg, 8.3 μmol). Compound **14**: ^{1}H NMR (500 MHz, acetone-d_6) δ Phe 7.80 (bs, 1H, NH), 4.49 (q, J = 6.8 Hz, 1H, H-α), 3.18 (dd, AB-type, J = 6.9, 13.8 Hz, 1H, H-β), 3.07 (dd, AB-type, J = 8.1, 13.8 Hz, 1H, H-β′), 1.93 (s, 3H, Ac); ΔAba 8.67 (bs, 1H, NH), 6.51 (q, J = 7.0 Hz, 1H, H-β), 1.49 (d, J = 7.1 Hz, 1H, H-γ); Ala 7.52 (bd, J = 6.7 Hz, 1H, NH), 6.99 and 6.20 (2 bs, 2H, $CONH_2$), 4.33 (qv, J = 7.3 Hz, 1H, H-α), 1.34 (d, J = 7.3 Hz, 1H, H-β).

Methods Used for Deacetylation of 6. (A) Hydrazine hydrate:[14] A solution of **6** (5 mg) in N_2H_4·H_2O−MeOH (1:5, 5 mL) was stirred at 20 °C for 3 h. Acetone was then added, and the solution was concentrated. (B) Ammonia:[8] A solution of **6** (5.5 mg) in methanolic ammonia (8 mL, saturated at 0 °C) was stirred in a stoppered flask at 20 °C for 6 h and then concentrated. (C) Sodium methoxide:[8] Methanolic sodium methoxide (2 M, 15 μL) was added to a solution of **6** (5 mg) in MeOH (5 mL) so that the pH reached 10.5−11 (wet pH paper). After 30 min at 20 °C the solution was neutralized with Duolite (H^+) resin, filtered, and concentrated. (D) Sodium hydroxide: A solution of **6** (5 mg) in aqueous 0.1 M sodium hydroxide (5

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mL) was stirred at 20 °C for 2 h 30 min and then neutralized with Duolite (H^+) resin, filtered, and concentrated.

β-Elimination of **6** in the presence and absence of 18-crown-6. Methanolic sodium methoxide (2 M, 50 μL) was added simultaneously to two solutions of glycopeptide **6** (1 mg) in MeOH (950 μL). One of the solutions also contained 18-crown-6 (120 μmol). The time-course of the reactions were monitored in parallel by reversed-phase analytical HPLC.

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Supporting Information Available: 500 MHz ^{1}H NMR spectra and tabulated 125 MHz ^{13}C NMR data for glycopeptides **6**−**13** and the product of β-elimination **14** (11 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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