Solution- and Solid-Phase Synthesis of N-Protected Glycopeptide Esters of the Benzyl Type as Substrates for Subtilisin-Catalyzed Glycopeptide Couplings

Krista Witte, Oliver Seitz, and Chi-Huey Wong*

Contribution from the Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

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Abstract: This paper describes the solution- and solid-phase synthesis of glycopeptides containing a GlcNAc moiety at different positions and investigates them as substrates for subtilisin-catalyzed glycopeptide condensation, with an aim to develop enzymatic synthesis of complex glycopeptides and glycoproteins. A systematic study was performed to determine the sites at which the protease subtilisin will accept a glycosylated residue in enzymatic peptide ligations. It was found that Fmoc-glycopeptide esters with unprotected side chains can be prepared by solid-phase methodology using the Rink amide resin. Both removal of the side chain protecting groups as well as release from the resin was accomplished by treatment with acid to provide glycopeptide esters of the benzyl type which were used directly as substrates. One such 12 residue peptide ester was used in a subtilisin-catalyzed glycopeptide condensation in a kinetically controlled mode to demonstrate the utility of this approach.

Introduction

Glycoproteins display their glycan structures in several different glycoforms.[1] The total synthesis of glycoproteins can provide well-defined materials serving as tools for the investigation of their structures and biological properties. Furthermore, it would allow the introduction of unnatural amino acids or carbohydrate moieties and enable the generation of a much higher glycoprotein diversity than would the recombinant expression techniques.[2] Several methods have been developed for the synthesis of small glycopeptides to achieve this goal;[3] however, there is still no general method available for the preparation of homogeneous glycoproteins.

Solid-phase peptide synthesis (SPPS) provides a rapid and efficient access to peptides in the range of 3−40 amino acid residues.[4] During the synthesis of larger fragments, uncoupled sequences, side products and epimers accumulate resulting in dramatically decreased yields and purities of the final products. The synthesis of large peptides via condensation of peptide fragments synthesized by solid-phase methods seems to be more feasible.[5] The intermediates can be purified, and the isolation of the product, which differs largely in molecular weight from the individual fragments, is carried out easily. Chemical fragment condensations, however, suffer from poor solubility of the protected peptide fragments[6] and are prone to racemization at the *C*-terminus of the acyl donor. In contrast, enzyme-catalyzed fragment condensations[7] are free of racemization and use side chain unprotected substrates, increasing the solubility of the peptide fragments. Most commonly the kinetically controlled approach is exploited using proteases together with peptide esters as acyl donors. Because of its broad substrate specificity, subtilisin and engineered variants thereof have found wide applications in peptide[8] and glycopeptide[9a] coupling reactions. The strategy has recently been used in protein synthesis[10] in conjugation with glycosyl transferase reactions to prepare glycoproteins.[9b]

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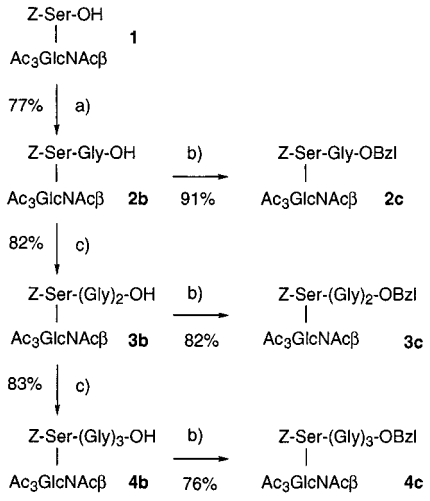
The construction of the N-protected, side chain free peptide esters is a crucial step in the synthesis of acyl donors for enzymatic coupling. Routine solid-phase synthesis furnishes unprotected peptide fragments, which are difficult to react selectively at the *C*-terminus. These free peptides can then be converted to active esters through an exopeptidase-catalyzed reaction, although the reported yields are in the 20−60% range.[11] Protected peptide fragments are suitable precursors, however, they often give only poor yields and may be racemized during the esterification.[12] Alternatively, resin-bound peptides can be converted to esters by alcoholysis of base-labile peptide linker bonds.[6] The linkers commonly used in the Fmoc strategy, however, require cleavage conditions too harsh for the synthesis of base-sensitive *O*-glycopeptides. The incorporation of peptide glycolate-phenylalanylamide esters[13] in peptides prepared by solid-phase synthesis has been employed in subtiligase-catalyzed fragment condensations[12b,14] and cyclizations.[15] The base-sensitivity of this linkage in Fmoc synthesis,[16,17] complicates its use in routine protocols.[18] A general method for the routine synthesis of N-protected, side chain free glycopeptide ester donors for fragment condensations is thus highly desirable. Presented here is the Fmoc-based solid-phase synthesis of base-stable glycopeptide PAM (2-(1′-(hydroxymethyl)phen-4′-yl)-acetamide) esters and their use in subtilisin-catalyzed peptide bond formations.

In the condensation of glycopeptide fragments the glycosylation site becomes of particular importance. The position and type of the glycosidic linkage largely affects the substrate properties of the corresponding fragments. In this study various *O*-glycopeptide esters of the benzyl type were employed for subtilisin-mediated peptide couplings in order to systematically investigate the influence of the glycosylation site on peptide bond formation.

Results and Discussion

Using Glycopeptides as Acyl Donors. Synthesis of *O*-Ac$_3$GlcNAc-dipeptide, -tripeptide, and -tetrapeptide Benzyl Esters. In the synthesis of glycopeptides and glycoproteins, glycosyltransferases can be used to construct carbohydrate structures on the assembled protein backbone.[19] Following this strategy, one carbohydrate, which acts as substrate for the transferases, has to be included in the chemical synthesis of the peptide fragments. *N*-acetylglucosamine (GlcNAc) was chosen as *O*-linked glycan, since this carbohydrate serves as substrate for a set of glycosyltransferases in the synthesis of the SLe^x-tetrasaccharide[20] and is therefore suited to demonstrate the versatility of chemo-enzymatic methods in the total synthesis of glycoproteins. The peracetylated GlcNAc residue was attached to the *N*-terminal serine of peptide benzyl esters **2c**−**4c** (Scheme 1) to probe the P2−P4-site specificity of subtilisin. Only glycine residues were incorporated, to exclude any steric interaction of the amino acids in *C*-terminal direction.

Scheme 1. Synthesis of Glycopeptide Benzyl Esters



a) *i* HCl·Gly-OtBu, DCC, HOBt, NMM, CH_2Cl_2, DMF; ;*ii* TFA, CH_2Cl_2;
b) BzlBr, CH_2Cl_2, sat. $NaHCO_3$, Bu_4NBr, 2–7d;
c) *i* HCl·Gly-OtBu, HBTU, HOBt, NMM, DMF;*ii* TFA.

The glycosyl amino acid Z-Ser(βAc$_3$GlcNAc)−OH (**1**)[21] was coupled to glycine *tert*-butyl ester (Scheme 1). Acidolytic removal of the tBu group yielded the *C*-terminal unprotected glycodipeptide **2b**. This procedure was repeated twice to furnish glycotripeptide **3b** and glycotetrapeptide **4b**. Conjugates **2b**, **3b**, and **4b** were converted to the benzyl esters **2c**, **3c**, and **4c** by nucleophilic esterification, which proceeds under phase transfer conditions.[22] The benzyl ester was used as it was found to be a good leaving group for subtilisin.

Solid-Phase Synthesis of N-Protected *O*-Ac$_3$GlcNAc-tripeptide and −tetrapeptide PAM Esters. In the development of a methodology to produce activated ester directly from the solid phase for use in enzymatic ligations, a double linker strategy was envisioned. An acid- and base-stable PAM anchor,[23] originally developed for Boc-SPPS, was inserted between the growing peptide chain and an acid-labile resin. The acid-labile Rink amide[24] resin was chosen to provide, upon acidolytic cleavage from the resin and concomitant deprotection of the side chains,[25] the peptide PAM amide. This activated ester would then act as a modified benzyl ester and serve as a leaving group in subsequent peptide ligation.

To test this proposal an initial study was performed using Fmoc-alanine (Scheme 2). For the synthesis of a conjugate of

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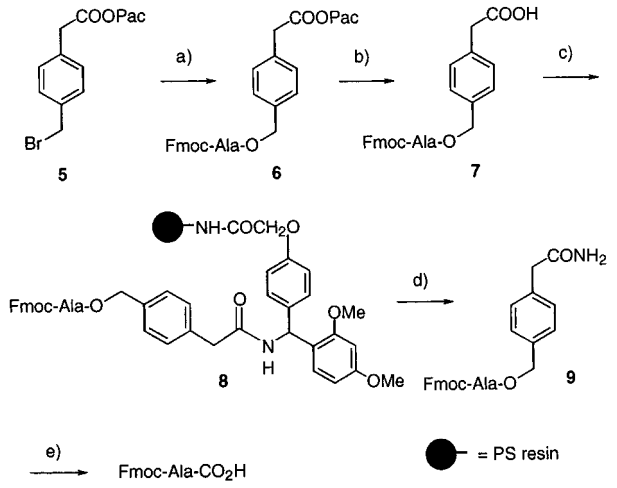
Scheme 2. Synthesis of an Fmoc-amino Acid PAM Handle and Subsequent Demonstration of Its Acceptance as a Substrate of Subtilisin



a) 1 eq Fmoc-Ala, CH_2Cl_2, sat. $NaHCO_3$, Bu_4NBr, 71%; b) Zn/AcOH, 74%; c) Rink-amide polystyrene resin, HOBt, NMM, HBTU, DMF, 0.25 mmol/g loading; d) 95% TFA, 2.5% triethylsilane, 2.5% H_2O, 89%; e) 50 mM tris, pH 8.4, 2 mU/mL of subtilsin BPN', >95%.

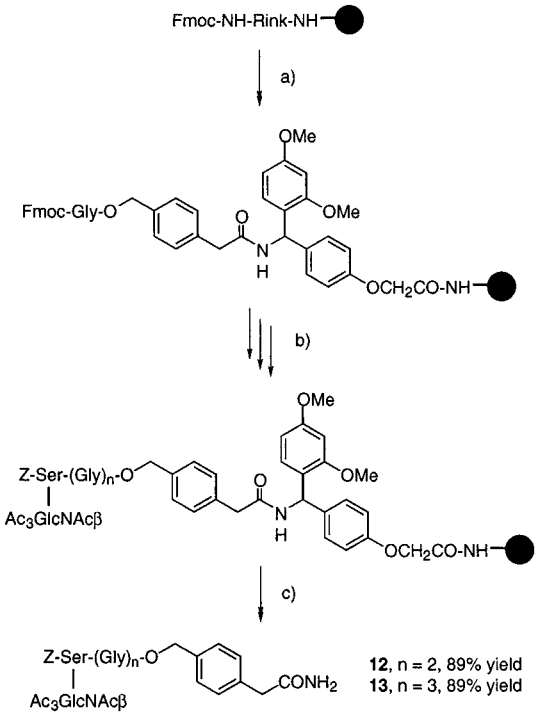
the starting amino acid and the PAM linker the Pac ester **5**[26] was reacted with the N-protected amino acid under phase transfer conditions. Reductive acidolysis released the carboxyl group yielding the amino acid ester **7**. Since the Pac ester is orthogonally stable against most of the protective groups used in Fmoc-SPPS, this scheme should provide a general access to a large variety of protected amino acid PAM esters. The Fmoc−amino acid−PAM conjugate **7** was attached to the Rink amide resin using HBTU activation.[27] Standard trifluoroacetic acid deprotection conditions released the Fmoc-Ala-PAM amide **9** in 92% yield based on initial loading. Acceptance of this ester as a substrate of subtilisin was tested by subjecting **9** to enzymatic hydrolysis conditions. Under these conditions all **9** was converted to Fmoc-alanine within 30 min.

Use of this methodology was then extended to glycopeptides (Scheme 3). Synthesis of the conjugate of the starting amino acid and the PAM linker as well as attachment to the resin was performed as described above. A standard Fmoc protocol[28] was applied for the synthesis of the glycopeptide PAM esters **12** and **13**. In each protocol the Z-Ser(Ac_3GlcNAc) building block **1** was the last amino acid to be coupled. The PAM esters **12** and **13** were released in an overall yield of 89% by treating the resin with TFA.

Interestingly, careful NMR analysis of compound **12**, pure by TLC and HPLC, showed the occurrence of two compounds in a ratio of 83:17. In the ^{1}H NMR the amide proton of serine and the anomeric proton as well as the methyl protons of the 2′-acetamido group displayed two sets of signals. Since the coupling constant of the anomeric proton was not changed, it is most likely that the second set of signals originates from a epimerized serine moiety. Further evidence was provided by the enzymatic peptide coupling which will be discussed below.

Influence of the Glycosylation Site in the Acyl Donor of Subtilisin-Catalyzed Peptide Couplings. The glycopeptide benzyl esters **2c**, **3c**, and **4c** were used to probe the influence

Scheme 3. Solid-Phase Synthesis of glycopeptide PAM Esters



a) *i* 20% piperidine/DMF; *ii* **11**, HBTU, HOBt, NMM, DMF; *iii* Ac_2O / pyr; b) *i* 20% piperidine/DMF; *ii* Fmoc-AA, HBTU, HOBt, NMM, DMF; *iii* Ac_2O/pyr; c) TFA/ethanedithiol/H_2O-mixture (95:2.5:2.5).

of the glycosylation site in the P2−P4[29] positions in subtilisin-mediated peptide couplings. Initially, the ligation was attempted between the benzyl esters **2c** and **4c** and glycine methyl ester. The glycotetrapeptide ester **4c** was treated with glycine methyl ester in a DMF/buffer (7:3) mixture at pH 8.4 in the presence of wild type subtilisin. No coupling was observed after 6 h; instead the hydrolyzed material **4b** was isolated in 32% yield. Use of the glycodipeptide ester **2c** led to 36% of hydrolysis after 5 days.

Subsequent peptide coupling reactions used glycine amide as the nucleophile. Amino acid amides are better nucleophiles than the corresponding esters,[30] which may undergo self-condensation. Scheme 4 shows the reaction of the conjugates **2c**, **3c**, and **4c** with glycine amide. The freely soluble conjugates **2c** and **3c** differed drastically in their donor properties. After 15 min **3c** was completely converted (Figure 1a) and the aminolysis product **15** was obtained in 66% yield (Table 1). The hydrolysis product **3b** was isolated in 30% yield. The glycodipeptide ester **2c** showed no reaction indicating that a *O*-glycosidic linkage at the P2 site is less favorable. The glycotetrapeptide ester **4c** was not completely soluble but dissolved during the course of the reaction. After 6 h 99% of the starting material was converted. The glycopentapeptide product **16** was isolated in a 44% yield, and the hydrolyzed product **4b**, in 54% yield.

The same tendencies were observed using the highly soluble glycopeptide PAM esters **12** and **13**. The tetrapeptide PAM ester **13** reacted similarly to the tetrapeptide benzyl ester **4c** (compare Figures 1a−c) yielding 44% of coupling product **16**

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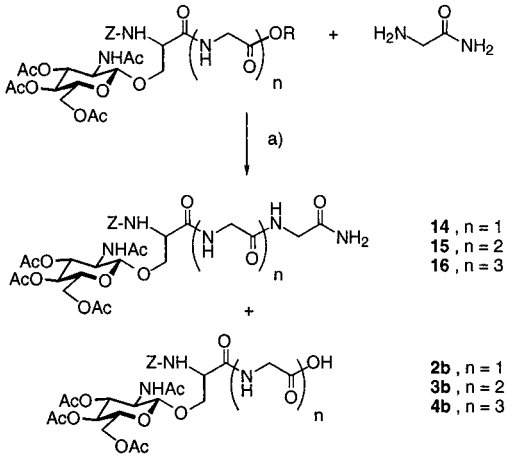
Scheme 4. Enzymatic Peptide Coupling Using Glycopeptide PAM Esters as Acyl Donors and Glycine Amide as Nucleophile



a) 20 mM substrate, O.3M Gly-NH_2, DMF/50 mM TEA (7:3), subtilisin BPN', pH 8.4, 6 h.

Table 1. Subtilisin-Catalyzed Reactions of Glycopeptide Benzyl Esters **2c**, **3c**, and **4c** and Glycopeptide PAM Esters **12** and **13** with Glycine Amide

n	R	aminolysis	hydrolysis[a]
1	Bzl	**14**, 0%[a]	**2b**, o%[a]
	PAM	nd	nd
2	Bzl	**15**, 66%[a]	**3b**, 30%[a]
	PAM	**15**, 71%[b]	**3b**, 25%[b]
3	Bzl	**16**, 44%[a]	**4b**, 54%[a]
	PAM	**16**, 44%[a]	**4b**, 56%[a]

[a] Isolated yields. [b] Yield corrected for presence of epimerized starting material which cannot act as a substrate.

and 56% of hydrolysis product **4b**. The tripeptide ester **12** was, as discussed above, an 83:17 mixture of two epimers. The enzyme catalyzed reaction on this mixture led to the formation of the coupling product **15** and hydrolysis product **3b** in 59% and 21% yield, respectively. The unreacted epimer, the minor component of the starting material, was reisolated in a 12% yield. Since the epimer was not either hydrolyzed or ligated, it was apparently not an acceptable substrate for subtilisin. To compare yields of the subtilisin reactions we have discounted the epimerized material (since it cannot act as starting material) in the data presented in Table 1. Upon correction for the 17% of nonreactive epimerized tripeptide, the yields for the aminolysis and hydrolysis of the tripeptide are 71% of **15** and 25% of **3b**.

All reactions were monitored by HPLC. Figure 1a−c shows the time course of the enzymatic coupling reactions. Both isolated yields and the qualitative kinetic data suggest that the glycotripeptide esters **3c** and **12** are better acyl donors than the glycotetrapeptide esters **4c** and **13**. They are not only converted more rapidly (Figure 1a), but also give higher aminolysis/hydrolysis ratios (Table 1, Figure 1b,c). The interpretation is complicated by the fact that **4c** is not completely soluble decreasing the effective concentration of the acyl donor. However, the freely soluble glycotetrapeptide PAM ester **13** is converted faster (Figure 1a) but shows a reactivity similar to the benzyl ester **4c** (Figure 1b,c) indicating that in fact the tripeptide esters are the best substrates.

It can be concluded that *O*-glycosylation of the P2 site leads to structures which are weak substrates (Table 1). *O*-glycosylation of the P3 site is readily accepted by subtilisin. Interestingly, subtilisin is able to differentiate between different

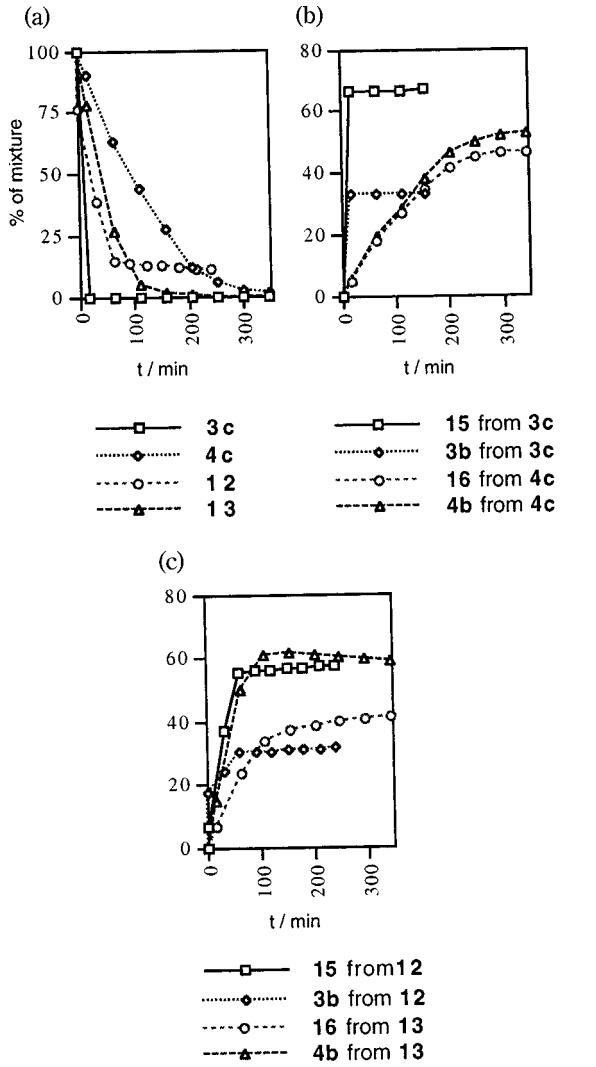


Figure 1. Time course of the subtilisin-catalyzed reactions of glycopeptide esters **3c**, **4c**, **12**, and **13** with glycine amide under the conditions described in Scheme 5. The relative amounts are based on calibrated HPLC data.

stereoisomers in the P3 site. Figure 1a shows that the glycotripeptide PAM ester **12** is rapidly turned over until after 1 h at which time 14% of the starting material remains and the conversion rate drops to almost zero. This is attributed to the presence of epimerized starting material and the poor reactivity of the D-isomer.[31,32] A shift of the glycosylation site to the P4 residue decreases the acylating potential (Figure 1c). This is difficult to explain since both of the important binding sites, the P1 and the P2 sites, are equal in the tri- and tetrapeptide substrates. We speculate that, in the absence of any productive interaction of the P1 and the P2 sites, binding pockets for the P3 and the P4 sites play a decisive role. Subtilisin possesses a deep hydrophobic pocket for the P4 site,[14,33] which in the case of the tripeptide esters **3c** and **12** might be occupied by the Z group. The tetrapeptide esters **4c** and **13** have to incorporate the bulky acylated carbohydrate into this binding site leading to less favorable substrate properties.

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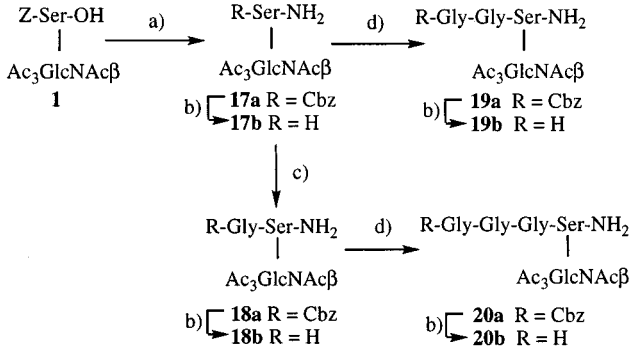
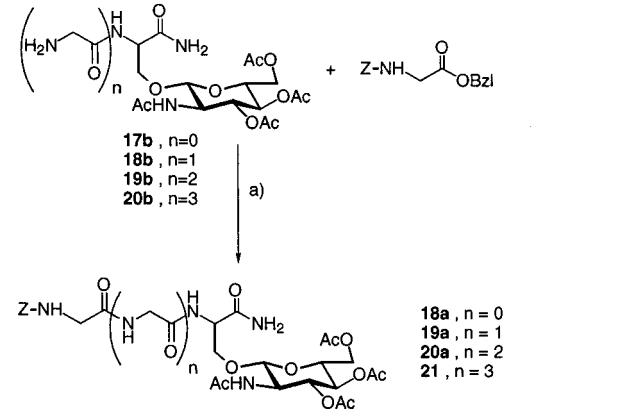
Scheme 5. Synthesis of *O*-Ac_3GlcNAc Peptide Amides



a) NH_4OH, EDC, HOBt, THF, 91%; b) $Pd(OH)_2$, H_2, >99% c) Z-Gly-OH, HBTU, HOBt, NMM, DMF, 87%; d) Z-Gly-Gly-OH, HBTU, HOBt, NMM, DMF, 72-75%.

Scheme 6. Enzymatic Peptide Coupling Using Glycopeptide Amides as Acyl Acceptors and Z-GlyOBzl as the Acyl Donor



a) 20 mM substrate, 60 mM Z-GlyOBzl, DMF/50 mM TEA (7:3), subtilisin BPN', pH 8.4, 6 h.

A comparison of the benzyl esters **3c** and **4c** with the PAM esters **12** and **13** reveals that the PAM ester is readily accepted as leaving group. PAM ester **13** reacts even faster than the corresponding benzyl ester **4c** (Figure 1a) perhaps due to the increasing solubility of the PAM group.

Using Glycopeptide Amides as Acyl Acceptors. Synthesis of *O*-Ac_3GlcNAc-dipeptide, -tripeptide, and -tetrapeptide Amides. Following the same strategy as outlined for the investigation of acyl donors, a series of glycopeptide amides each containing one GlcNAc moiety were synthesized (Scheme 5). These peptide amides were in turn used to probe the specificity of the P1′−P4′ binding subsites of subtilisin.

The glycosyl amino acid **1** was amidated to give **17a**, which was then deprotected by hydrogenation using a Degussa type catalyst to provide the N-unprotected $Ac_3GlcNAcSerNH_2$, **17b**. The di- and tripeptides were furnished by the coupling of **17b** with Z-Gly and Z-Gly-Gly to give **18a** and **19a**, respectively. The tetrapeptide was reached by the deprotection of **18a** to give free glycopeptide amide **18b** and subsequent coupling to Z-Gly-Gly to give **20a**.

Influence of Glycosylation Site in the Acyl Acceptor of Subtilisin-Catalyzed Peptide Couplings. The N-terminal unprotected glycosyl peptide amides **17b**, **18b**, **19b**, and **20b** were used to probe the substrate specificity of subtilisin in the P1′−P4′ positions (Scheme 6). Reactions were typically run for 6 h at which time no Z-GlyOBzl was detectable by TLC. Only aminolysis products were isolated and quantified due to the fact the hydrolysis product in this set of reactions was Z-Gly and of no further interest. Conditions identical to those in the previously discussed study were used with the exception of the concentrations of the acyl donor and acyl acceptors. A 3-fold excess of acceptor was used in all reactions. In contrast to the glycopeptide donors discussed earlier, all of the glycopeptide amide acceptors were soluble under the reactions conditions.

Table 2. Subtilisin-Catalyzed Ligations of Z-GlyOBzl with Glycopeptide Amides **13b**, **14b**, **15b**, and **16b**

n	acyl acceptor	aminolysis product	yield (%)
0	**17b**	**18a**	0
1	**18b**	**19a**	37
2	**19b**	**20a**	54
3	**20b**	**21**	62

In all of the following reactions Z-GlyOBzl was used as the acyl donor. No ligation was observed upon the reaction of **17b** with Z-GlyOBzl, and after 6 h all of the benzyl ester had been hydrolyzed to the free acid (Table 2). All three of the compounds **18b**, **19b**, and **20b** were substrates for subtilisin-catalyzed ligation, although to varying degrees. The ligation of **18b** gave a 37% yield of aminolysis product whereas the one amino acid increase in length of **19b** and **20b** gave 54% of **20a** and 62% of **21**, respectively.

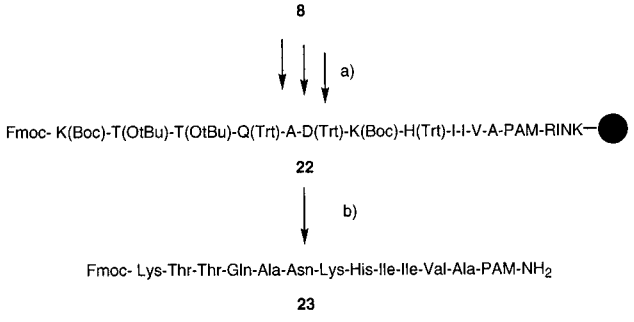
From these studies one can conclude that glycosylation is not accepted at the P1′ position. The S1′ subsite in the enzyme binding site is a narrow pocket and will often not accept branched amino acids side chains.[2a,34] It is therefore not surprising the bulky carbohydrate was not accepted. Glycosylation in the P2′ position was accepted as a substrate although with a low yield. The S2′ subsite consists of an open hydrophobic surface, and steric constraints are not as important; however the natural tendency of subtilisin toward hydrophobic residues at the P2′ position indicates that the relatively hydrophilic sugar may have unfavorable interactions.[2a,34] When the carbohydrate moiety was moved into the S3′ or S4′ subsite, the yields increased substantially. These subsites are larger and less restrictive on accepted residues.[2a,34]

This trend in which the yield increases as the peptide grows is opposite of what one would expect for a stepwise synthesis of a peptide. In a chemical peptide synthesis, as sequential steps are performed, one would expect the yield to decrease as the peptide and, thus, the synthesis grows longer. With kinetically controlled enzymatic ligation we have shown that a longer substrate will actually give a better yield and a larger glycopeptide product.

It is important to note that though the yield of enzymatic coupling is relatively low due to a significant degree of ester hydrolysis, it can be improved with the use of organic solvent stable subtilisin variants in higher concentrations of DMF or thiosubtilisin variants in aqueous solutions as demonstrated previously.[9a] It is also worth mentioning that glycine was chosen as a spacer in these studies to minimize side chain interactions. Subtilisin is fairly permissive in its substrate specificity, however, there are certain residues which, while accepted, are not optimal for enzyme action.[35] In a previous publication the flexibility of the enzyme has been summerized, and it is noted that glycine is a poor residue for the S1 subsite.[2a] This is not as limiting as it may seem as subtilisin better accepts a number of different residues. For example tyrosine, phenylalanine, tryptophan, and methionine as well as as dansylated,

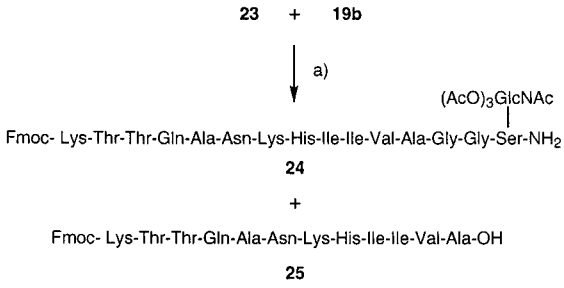
(34) Active site model based on the following: Bonneau, P. R.; Graycar, T. P.; Estell, D. A.; Jones, J. B. *J. Am. Chem. Soc.* **1991**, *113*, 1026.
(35) Philipp, M.; Bender, M. L. *Mol. Cell. Biochem.* **1983**, *51*, 5.

Scheme 7. Synthesis of Fmoc−Peptide Amide **23**



a) *i* 20% piperidine/DMF; *ii* Fmoc-AA, HBTU, HOBt, NMM, DMF; *iii* Ac_2O/pyr; b) TFA/triethylsilane/H_2O-mixture (95:2.5:2.5).

Scheme 8. Subtilisin-Catalyzed Glycopeptide Ligations



a) DMF:50 mM triethanolamine (9:1), subtilisin variant.

γ-diphenyl and polycyclic aromatic unnatural amino acids are all better accepted than glycine by the S1 subsite. As has been demonstrated in the literature as well as in the study discussed below, a sizable increase in yield can be obtained by using a more accepted substrate.

Subtilisin-Catalyzed Aminolysis of a Long-Chain Peptide Ester To Give a Glycopeptide Amide Product. In an effort to demonstrate the utility of this method on a more typical peptide sequence, the subtilisin-catalyzed fragment condensation to form a 15 residue long glycopeptide amide was undertaken (Scheme 7). It was hoped that by using a long chain peptide ester which is not optimal for subtilisin but has several of the preferred amino acids at certain sites, the acceptance of the peptide by the enzyme would be increased and a subsequent increase in yield would be obtained.

The sequence of the peptide acyl donor **23** was taken from RNase B and is near the *C*-terminal of the protein. The previously described Rink amide resin loaded with the Fmoc-Ala-Pam linker **8** was used as the initial amino acid and solid support. Standard Fmoc solid-phase peptide synthesis methodology was employed to produce the full length, fully protected, resin-bound peptide **22**. Standard TFA deprotection of the peptide side chains and concomitant release from the resin gave the N-terminal protected, activated peptide ester **23** in an excellent yield of 89% based on initial resin loading. The small amounts of impurities were removed by HPLC, and the peptide was characterized by MALDI mass spectrometry.

Subtilisin-catalyzed peptide ligation was performed as described in Scheme 8. The tripeptide **19b** from the previous study was used as the glycopeptide acyl acceptor in these ligations. All reactions were followed by MALDI mass spectrometry and quantified by calibrated data. Initial studies were performed using subtilisin in a 7:3 ratio of DMF/buffer. Although the aminolysis product **24** was formed, a significant portion of hydrolysis product **25** was also detected. To decrease the rate of the hydrolysis reaction, the portion of DMF was increased and conversely the available water was decreased. A 9:1 ratio of DMF/buffer gave a significant increase in aminolysis-to-hydrolysis ratio when the reaction was allowed to proceed at 37 °C (Table 3). Yields given represent those obtained from a reaction by employing a subtilisin variant 8397 K256Y.[36] This variant has been found to have higher stability in high temperatures and high percentages of organic solvents. A lower aminolysis-to-hydrolysis ratio was obtained (along with slightly lower overall yields) when wild type subtilisin BPN′ was used.

Table 3. Subtilisin-Catalyzed Ligations of **23** and **19b**

subtilisin variant	% **24**	% **25**	aminolysis/hydrolysis
wild type	79	20	3.95
8397 K256Y	84	16	5.25

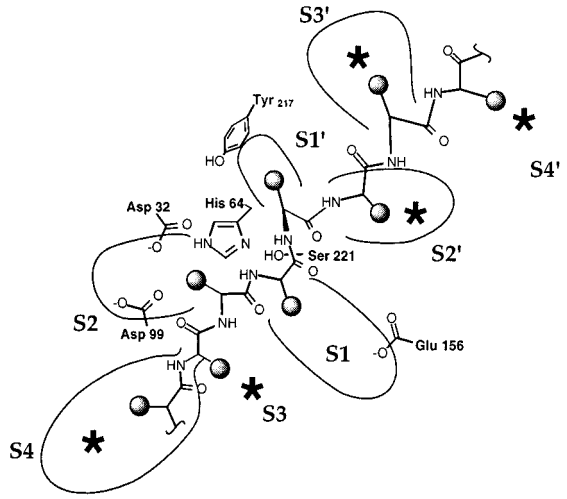


Figure 2. Schematic drawing of the general features of the subtilisin active site. Sites at which glycosylated residues are accepted are indicated by an asterisk. When a potential site is assessed, the following trends are useful. The S1 site prefers large hydrophobes but will also accept large unnatural amino acids as well as peptide isosteres. The S2 site prefers hydrophobic or basic residues. The S3 site has broad specificity as does S4. On the acyl acceptor S1′ generally needs to be a small residue such as alanine, glycine, or proline. The S2′ site prefers larger hydrophobes but will accept a range of amino acids and isosteres. S3′ and S4′ are both fairly flexible, although S3′ has a preference for large hydrophobes as well.

The significant increase in yield shown in the ligation (84%) over that of **19b** with Z-Gly benzyl ester (54%) reemphasizes the assertion that the trends shown in Figure 1a,b, and not the yields, are the important information from the study. Yields can be greatly improved by use of both better substrates and more tailored enzyme variants.

Summary

Glycopeptide esters of benzyl alcohol and 2-(1′-(hydroxymethyl)phen-4′-yl)-acetamide (PAM) were demonstrated to serve as acyl donors in subtilisin-catalyzed peptide bond formations (results summerized in Figure 2). While *O*-glycosylation of the P1 or P2 residue was prohibitive, subtilisin efficiently catalyzed the chain elongation of conjugates with the GlcNAc moiety at the P3 and P4 residues, the former being the better substrate. An epimerized glycosyl amino acid at the P3 site was not accepted. With regard to the use of *O*-

(36) (a) Sears, P.; Schuster, M.; Wang, P.; Witte, K.; Wong, C.-H. *J. Am. Chem. Soc.* **1994**, *116*, 6521. (b) Structural information: Kidd, R. D.; Yennawar, H. P.; Sears, P.; Wong, C.-H.; Faber, G. K. *J. Am. Chem. Soc.* **1996**, *118*, 1645.

glycopeptide amides as the acceptors it was found that the GlcNAc residue can be placed at P2′, P3′, or P4′. However yields increase as the glycosylation site is moved farther away from the amide bond being formed. The base- and acid-stable glycopeptide PAM esters are readily available via Fmoc-based standard procedures of solid-phase peptide synthesis, enabling a quick and efficient access to side chain unprotected glycopeptide esters useful for enzymatic glycopeptide segment condensation. The benzyl type esters used in this study are better than methyl ester as substrates for subtilisin with respect to the reation rate and yield.[9] The utility of this methodology was further demonstrated by the subtilisin-catalyzed fragment condensation to give a 15-residue-long glycosylated peptide amide.

In summary, this paper describes a systematic study to determine the sites at which subtilisin BPN′ will accept a glycosylated residue in enzymatic glycopeptide ligation and provides a useful strategy for enzymatic glysopeptide synthesis. Given the fact that a peptide with glycine at the P1 position is a poor substrate and the fact that the enzyme accepts a broad range of substrates with different side chains, it is believed larger glycopeptides or glycoproteins can be prepared when combined with the recently developed solid-phase synthesis of glycopeptides using the HYCRON linker.[21]

Experimental Section

General Methods. Subtilisin BPN′ was purchased from Sigma (protease type XXVII). Reactions were carried out at room temperature if no specifications are given. Solid-phase synthesis was performed manually using a reaction vessel similar to the Merrifield reactor. Chromatography (silica gel 60, 230−400 mesh, Mallinckrodt) was performed on open columns if no further explanations are given. Analytical HPLC was performed on a Hewlett-Packard system (Series 1100) using a RP-C18 column (Microsorb, 5 μm, 300 Å, 250 × 4 mm) and gradients of acetonitrile/water eluents containing 0.05% TFA.

Z-Ser(βAc$_3$GlcNAc)-Gly-OtBu (2a). A solution of Z-Ser(βAc$_3$-GlcNAc)−OH (**1**) (990 mg, 1.74 mmol) and HOBt (495 mg, 3.48 mmol) in a mixture of dichloromethane (20 mL), MeCN (10 mL), and DMF (1.5 mL) was made. To this a premixed solution of DCC (377 mg, 1.83 mmol) in dichloromethane (5 mL) was added. HCl·Gly-OtBu (583 mg, 3.48 mmol) was dissolved in dichloromethane (20 mL) followed by addition of NMM (0.38 mL, 3.48 mmol). The two solutions were combined and stirred for 24 h. The solvent was evaporated in vacuo and the residue dissolved in acetone. The precipitate was removed by filtration, and the filtrate was concentrated in vacuo. The residue was dissolved in EtOAc, and the solution was washed with 0.5 M HCl, saturated $NaHCO_3$, and brine. The combined organic layers were dried over $MgSO_4$. After concentration in vacuo the residue was purified by chromatography (*n*-hexane/EtOAc, 1:6) to yield 1.0 g of a white solid. Yield: 84%. R_f: 0.45 (EtOAc). ^{1}H NMR (500 MHz, DMSO-d_6): 8.20 (t, 1H, G^{NH}, J = 5.5), 7.89 (d, 1H, S^{NH}, J = 9.0), 7.36−7.26 (m, 5H, Z^{ar-H}), 5.08 (t, 1H, H-3′, $J_{3',2'} = J_{3',4'}$ = 9.5), 5.02 (s, 2H, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.5), 4.68 (d, 1H, H-1′, $J_{1',2'}$ = 8.5), 4.27−4.23 (m, 1H, S^{α}), 4.16 (dd, 1H, H-6′^{a}, $J_{6'a,6'b}$ = 12.0, $J_{6'a,5'}$ = 4.5), 4.02−3.99 (m, 1H, H-6′^{b}), 3.85−3.68 (m, 6H, G^{α}, S^{β}, H-2′, H-5′), 1.99, 1.96, 1.90, 1.72 (4 × s, 4 × 3H, 4 × Ac), 1.39 (s, 9H, tBu).

Z-Ser(βAc$_3$GlcNAc)-Gly-OH (2b). A solution of **2a** (63 mg, 92.4 μmol) in a mixture of dichloromethane (3 mL) and TFA (5 mL) is stirred for 40 min. The mixture is concentrated in vacuo and coevaporated with toluene. The residue is purified by chromatography ($CHCl_3$/MeOH/AcOH, 85:15:1) to yield 53 mg of a white solid. Yield: 92%. R_f: 0.37 ($CHCl_3$/MeOH/AcOH, 85:15:1). ^{1}H NMR (300 MHz, DMSO-d_6): 8.16 (t, 1H, G^{NH}, J = 5.5), 7.88 (d, 1H, S^{NH}, J = 9.0), 7.37−7.30 (m, 5H, Z^{ar-H}), 5.08−5.00 (m, 3H, H-3′, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.3), 4.69 (d, 1H, H-1′, $J_{1',2'}$ = 8.5), 4.31−4.17 (m, 2H, S^{α}, H-6′^{a}), 4.05−3.99 (m, 1H, H-6′^{b}), 3.83−3.60 (m, 6H, G^{α}, S^{β}, H-2′, H-5′), 1.99, 1.96, 1.89, 1.72 (4 × s, 4 × 3H, 4 × Ac). ^{13}C NMR (125 MHz, DMSO-d_6): 171.23, 170.34, 169.86, 169.82, 169.69, 169.53, 156.06, 137.02, 128.77, 128.37, 100.39, 72.74, 71.07, 70.92, 68.72, 65.89, 62.01, 54.86, 53.28, 41.05, 23.02, 20.82, 20.76, 20.39. FAB-MS (NBA, CsI): *m/e* 758.1148 (M + Cs^+, calcd 758.1173).

Z-Ser(βAc$_3$GlcNAc)-Gly-Gly-OtBu (3a). To a solution of **2b** (502 mg, 0.80 mmol) and HOBt (285 mg, 2.01 mmol) in DMF (8 mL) was added NMM (176 μL, 1.60 mmol). After addition of HBTU (304 mg, 0.80 mmol) the mixture was combined with a solution of HCl·Gly-OtBu (336 mg, 2.01 mmol) and NMM (220 μL, 2.01 mmol) in DMF (7 mL). The mixture was stirred for 17 h before the solvent was evaporated in vacuo. The residue was dissolved in dichloromethane, and the solution was washed with 0.5 M HCl, saturated $NaHCO_3$, and brine. The combined organic layers were dried over $MgSO_4$. The solvent was evaporated in vacuo and the residue purified by chromatography (*n*-hexane/EtOAc, 1:6) to furnish 513 mg of a white solid. Yield: 87%. R_f: 0.22 ($CHCl_3$/MeOH, 94:6). ^{1}H NMR (500 MHz, DMSO-d_6): 8.18 (t, 1H, G^{NH}, J = 5.5), 8.10 (t, 1H, G^{NH}, J = 5.5), 7.89 (d, 1H, S^{NH}, J = 9.0), 7.38−7.31 (m, 5H, Z^{ar-H}), 5.08 (t, 1H, H-3′, $J_{3',2'} = J_{3',4'}$ = 10.0), 5.05−5.00 (m, 2H, Z^{CH_2}), 4.84 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.5), 4.68 (d, 1H, H-1′, $J_{1',2'}$ = 8.5), 4.28−4.25 (m, 1H, S^{α}), 4.19 (dd, 1H, H-6′^{a}, $J_{6'a,6'b}$ = 12.0, $J_{6'a,5'}$ = 4.5), 4.02 (m_c, 1H, H-6′^{b}), 3.85−3.69 (m, 8H, 2 × G^{α}, S^{β}, H-2′, H-5′), 2.01, 1.97, 1.91, 1.74 (4 × s, 4 × 3H, 4 × Ac), 1.41 (s, 9H, tBu). ^{13}C NMR (125 MHz, $CDCl_3$): 170.9, 170.2, 169.2, 169.0, 156.1, 135.9, 128.5, 128.3, 128.1, 102.0, 82.2, 72.2, 70.3, 68.1, 67.2, 61.6, 54.2, 43.0, 41.7, 28.0, 23.1, 20.7, 20.6.

Z-Ser(βAc$_3$GlcNAc)-Gly-Gly-OH (3b). A solution of **3a** (513 mg, 0.69 mmol) in TFA (20 mL) was stirred for 40 min. The mixture was concentrated in vacuo and coevaporated with toluene. The residue was purified by chromatography ($CHCl_3$/MeOH/AcOH, 85:15:1) to yield 447 mg of a white solid. Yield: 94%. R_f: 0.19 ($CHCl_3$/MeOH/AcOH, 85:15:1). ^{1}H NMR (300 MHz, DMSO-d_6): 8.34 (m, 1H, G^{NH}), 8.18 (m, 1H, G^{NH}), 7.93 (d, 1H, S^{NH}, J = 9.0), 7.34−7.29 (m, 5H, Z^{ar-H}), 5.06−5.00 (m, 2H, H-3′, Z^{CH_2}), 4.81 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.7), 4.65 (d, 1H, H-1′, $J_{1',2'}$ = 8.5), 4.24−4.14 (m, 2H, S^{α}, H-6′^{a}), 4.00−3.96 (m, 1H, H-6′^{b}), 3.83−3.65 (m, 8H, 2 × G^{α}, S^{β}, H-2′, H-5′), 1.97, 1.94, 1.88, 1.71 (4 × s, 4 × 3H, 4 × Ac). ^{13}C NMR (125 MHz, DMSO-d_6): 171.31, 170.36, 169.87, 169.79, 169.72, 169.53, 169.17, 156.14, 136.98, 128.72, 128.44, 128.18, 127.91, 100.6, 72.74, 71.06, 70.96, 68.69, 65.94, 62.00, 54.93, 53.22, 42.16, 40.79, 22.97, 22.70, 20.76, 20.48. FAB-MS (nba, CsI): *m/e* 815 (M + Cs^+, calcd 815.1).

Z-Ser(βAc$_3$GlcNAc)-Gly-Gly-Gly-OtBu (4a). The reaction and the workup was performed as described in the synthesis of **3a** using **3b** (215 mg, 0.32 mmol), HOBt (112 mg, 0.63 mmol), HCl·Gly-OtBu (134 mg, 0.80 mmol), NMM (157 μL, 1.43 mmol), and HBTU (121 mg, 0.32 mmol) in 7 mL of DMF. A 221 mg amount of a white solid was obtained. Yield: 88%. R_f: 0.44 ($CHCl_3$/MeOH, 9:1). ^{1}H NMR (300 MHz, DMSO-d_6): 8.18−8.14 (m, 3H, 3 × G^{NH}), 7.90 (d, 1H, S^{NH}, J = 9.0), 7.37−7.33 (m, 5H, Z^{ar-H}), 5.08−5.03 (m, 3H, H-3′, Z^{CH_2}), 4.83 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.5), 4.68 (d, 1H, H-1′, $J_{1',2'}$ = 8.4), 4.30−4.17 (m, 2H, S^{α}, H-6′^{a}), 4.02−3.98 (m, 1H, H-6′^{b}), 3.85−3.69 (m, 10H, 3 × G^{α}, S^{β}, H-2′, H-5′), 2.00, 1.97, 1.90, 1.73 (4 × s, 4 × 3H, 4 × Ac), 1.39 (s, 9H, tBu).

Z-Ser(βAc$_3$GlcNAc)-Gly-Gly-Gly-OH (4b). A solution of **4a** (221 mg, 0.278 mmol) in 20 mL of TFA was stirred for 40 min. The mixture was concentrated in vacuo and coevaporated with toluene. The residue was purified by chromatography ($CHCl_3$/MeOH/AcOH, 80:20:1) to yield 209 mg of a white solid. Yield: 94%. R_f: 0.13 ($CHCl_3$/MeOH/AcOH, 80:20:1). ^{1}H NMR (500 MHz, DMSO-d_6): 8.17−8.11 (m, 3H, 3 × G^{NH}), 7.88 (d, 1H, S^{NH}, J = 9.1), 7.36−7.31 (m, 5H, Z^{ar-H}), 5.06 (m, 2H, H-3′, $J_{3',2'} = J_{3',4'}$ = 9.9), 5.03−4.99 (m, 1H, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.7), 4.66 (d, 1H, H-1′, $J_{1',2'}$ = 8.5), 4.27−4.23 (m, 1H, S^{α}), 4.17 (dd, 1H, H-6′^{a}, $J_{6'a,\ 6'b}$ = 12.3, $J_{6'a,\ 5'}$ = 4.6), 4.01−3.98 (m, 1H, H-6′^{b}), 3.82−3.66 (m, 10H, 3 × G^{α}, S^{β}, H-2′, H-5′), 1.99, 1.95, 1.90, 1.72 (4 × s, 4 × 3H, 4 × Ac). ^{13}C NMR (125 MHz, DMSO-d_6): 172.28, 171.34, 170.36, 169.89, 169.76, 169.53, 169.30, 169.16, 156.17, 136.96, 128.58, 128.06, 100.44, 72.75, 71.00, 68.85, 68.68, 65.97, 62.00, 54.89, 53.17, 42.44, 41.95, 40.79, 22.83, 21.28, 20.72, 20.64. FAB-MS (NBA, CsI): *m/e* 1004 (M − H^+ + 2Cs^+, calcd 1004.1), 872 (M + Cs^+, calcd 872.2).

General Procedure for Synthesis of the Glycopeptide Benzyl Esters under Phase Transfer Conditions. To a 0.04 M solution of the glycopeptide in dichloromethane are added 1 equiv of Bu_4NBr and 5 equiv of benzyl bromide are added. An equal volume of saturated $NaHCO_3$ solution is added and the mixture is stirred vigorously for 24 h before an additional 2.5 equiv of benzyl bromide together with half of the initial volume of saturated $NaHCO_3$ solution is added. After 24 h the 2-phase system is diluted with dichloromethane and saturated $NaHCO_3$. The organic layer is separated and the aqueous layer extracted with dichloromethane. The combined organic layers are dried over $MgSO_4$ and concentrated in vacuo. The residue is purified by chromatography.

Z-Ser(βAc$_3$GlcNAc)-Gly-OBzl (2c). A 50 mg (80 μmol) amount of **2a** was reacted according to the general procedure. Chromatography ($CHCl_3$/MeOH, 94:6) yields 52 mg of a white solid. Yield: 91%. R_f: 0.52 ($CHCl_3$/MeOH, 9:1). ^{1}H NMR (300 MHz, DMSO-d_6): 8.38 (t, 1H, G^{NH}, J = 5.7), 7.88 (d, 1H, S^{NH}, J = 9.0), 7.36−7.29 (m, 10H, Bzl^{ar-H}, Z^{ar-H}), 5.11−5.00 (m, 5H, H-3′, Bzl^{CH_2}, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.7), 4.68 (d, 1H, H-1′, $J_{1',2'}$ = 8.5), 4.29−4.26 (m, 1H, S^{α}), 4.18 (dd, 1H, H-6′^{a}, $J_{6'a,6'b}$ = 12.3, $J_{6'a,5'}$ = 4.6), 4.00 (dd, 1H, H-6′^{b}, $J_{6'b,6'a}$ = 12.2, $J_{6'b,5'}$ = 2.0), 3.90−3.67 (m, 6H, G^{α}, S^{β}, H-2′, H-5′), 1.98, 1.96, 1.90, 1.72 (4 × s, 4 × 3H, 4 × Ac). ^{13}C NMR (125 MHz, $CDCl_3$): 171.09, 170.75, 169.91, 169.52, 169.31, 155.95, 136.02, 135.09, 128.63, 128.54, 128.47, 128.42, 128.22, 128.06, 101.32, 72.27, 71.97, 69.00, 68.20, 67.23, 67.09, 61.86, 54.22, 53.67, 41.56, 23.13, 20.64, 20.59. FAB-MS (NBA,CsI): *m/e* 848 (M + Cs^+, calcd 848.3).

Z-Ser(βAc$_3$GlcNAc)-Gly-Gly-OBzl (3c). A 54 mg (79 μmol) amount of **3a** was reacted according to the general procedure. Chromatography ($CHCl_3$/MeOH, 94:6) yields 50 mg of a white solid. Yield: 82%. R_f: 0.46 ($CHCl_3$/MeOH, 9:1). ^{1}H NMR (300 MHz, DMSO-d_6): 8.31−8.18 (m, 2H, 2 × G^{NH}), 7.89 (d, 1H, S^{NH}, J = 9.0), 7.39−7.28 (m, 10H, Bzl^{ar-H}, Z^{ar-H}), 5.12−4.97 (m, 5H, H-3′, Bzl^{CH_2}, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.7), 4.67 (d, 1H, H-1′, $J_{1',2'}$ = 8.5), 4.29−4.22 (m, 1H, S^{α}), 4.18 (dd, 1H, H-6′^{a}, $J_{6'a,6'b}$ = 12.3, $J_{6'a,5'}$ = 4.7), 4.00 (dd, 1H, H-6′^{b}, $J_{6'b,6'a}$ = 12.3, $J_{6'b,5'}$ = 2.0), 3.91 (d, 2H, G^{α}, J = 6.0), 3.85−3.65 (m, 6H, G^{α}, S^{β}, H-2′, H-5′), 1.98, 1.95, 1.89, 1.72 (4 × s, 4 × 3H, 4 × Ac). ^{13}C NMR (125 MHz, $CDCl_3$): 170.34, 169.84, 169.72, 169.53, 169.46, 156.13, 136.99, 136.11, 128.67, 128.57, 128.30, 128.26, 128.07, 128.03, 100.49, 72.75, 71.02, 68.91, 68.71, 66.10, 65.95, 62.01, 54.90, 53.17, 42.16, 40.87, 22.84, 20.72, 20.65, 20.58. FAB-MS (NBA, CsI): *m/e* 905 (M + Cs^+, calcd 905.3), 795 (M + Na^+, calcd 795.4).

Z-Ser(βAc$_3$GlcNAc)-Gly-Gly-Gly-OBzl (4c). A 58 mg (78 μmol) amount of **4b** was reacted according to the general procedure. After 2 days benzyl bromide (150 μL) was added together with 1 N NaOH (70 μL). The mixture was stirred for further 5 days. Chromatography ($CHCl_3$/MeOH, 94:6) yielded 49 mg of a white solid. Yield: 76%. R_f: 0.40 ($CHCl_3$/MeOH, 9:1). ^{1}H NMR (500 MHz, DMSO-d_6): 8.32 (t, 1H, G^{NH}, J = 6.0), 8.16−8.13 (m, 2H, 2 × G^{NH}), 7.89 (d, 1H, S^{NH}, J = 9.0), 7.39−7.31 (m, 10H, Bzl^{ar-H}, Z^{ar-H}), 5.11 (s, 2H, Bzl^{CH_2}), 5.07 (t, 1H, H-3′, $J_{3',2'} = J_{3',4'}$ = 10.0), 5.03−4.99 (m, 2H, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.5), 4.66 (d, 1H, H-1′, $J_{1',2'}$ = 8.5), 4.28−4.23 (m, 1H, S^{α}), 4.18 (dd, 1H, H-6′^{a}, $J_{6'a,6'b}$ = 12.3, $J_{6'a,5'}$ = 4.7), 4.09−3.81 (m, 1H, H-6′^{b}), 3.90 (d, 2H, G^{α}, J = 6.0), 3.83−3.65 (m, 8H, 2 × G^{α}, S^{β}, H-2′, H-5′), 1.99, 1.96, 1.89, 1.72 (4 × s, 4 × 3H, 4 × Ac). ^{13}C NMR (125 MHz, $CDCl_3$): 170.35, 169.86, 169.73, 169.58, 169.52, 169.17, 156.16, 136.98, 136.12, 128.67, 128.58, 128.31, 128.15, 128.05, 100.46, 72.77, 71.01, 68.85, 68.71, 66.07, 65.96, 62.02, 54.90, 53.17, 42.44, 41.94, 40.88, 22.84, 20.73, 20.65, 20.58. FAB-MS (NBA, CsI): *m/e* 962 (M + Cs^+, calcd 962.3).

Fmoc-Ala-PAM-OPac (6). Fmoc-Ala (295 mg, 0.95 mmol) was combined with 91.3 mg (0.19 mmol) of **5** in 9.5 mL of saturated $NaHCO_3$ and 9.5 mL CH_2Cl_2. To this biphasic solution was added 64.8 mg (0.19 mmol) of tBu$_4$NHSO$_4$. The reaction was stirred vigorously for 5 h and then allowed to phase separate. The organic layer was washed with $NaHCO_3$ to remove excess Fmoc-Ala. After washing of the organic further with H_2O, it was dried over $MgSO_4$ and evaporated to dryness. Column chromatography afforded 94.6 mg of product, 71% yield. R_f: 0.40 (ethyl acetate/hexane, 2:1). ^{1}H NMR (400 MHz, $CDCl_3$): 7.88 (d, 2H, Pac^{ortho}, $J_{o,m}$ = 7.4), 7.76 (d, 2H, $Fmoc^{H-4,H-5}$, $J_{4,3} = J_{5,6}$ = 7.5), 7.62−7.58 (m, 4H, $Fmoc^{H-1,H-8}$, Pac^{para}), 7.47, (t, 2H, Pac^{meta}, $J_{m,o} = J_{m,p}$ = 7.7), 7.39 (t, 2H, $Fmoc^{H-3,H-6}$, $J_{3,2} = J_{3,4} = J_{6,7} = J_{6,5}$ = 7.4), 7.36−7.29 (m, 6H, $Fmoc^{H-2,H-7}$, $HMPA^{ar-H}$), 5.33 (s, 2H, Pac^{CH_2}), 5.17 (s, 2H, $HMPA^{OCH_2}$), 4.39 (m, 3H, $Fmoc^{CH_2}$, Ala^{CH}), 4.20 (t, 1H, $Fmoc^{H-9}$, J_{H-9,CH_2} = 7.1), 3.82 (s, 2H, $HMPA^{CH_2CO}$), 1.44 (d, 3H, Ala^{CH_3}, $J_{CH_3,CH}$ = 7.1). HRMS (FAB, pos): *m/e* 710.1155 (M + Cs^+, calcd 710.1182).

Fmoc-Ala-PAM (7). Zinc dust was activated by washing with 1N HCl (6×), washing with H_2O (6×), washing with ethanol (6×), and last washing with ethyl ether (6×). The activated zinc dust (565 mg, 8.7 mmol) was combined with 200 mg of **6** (0.35 mmol) in 10 mL of 85% HOAc. The reaction was stirred vigorously and monitored by TLC. Once all starting material disappeared the Zn was removed by filtration over Celite and washed with 1.5 mL 85% HOAc. The filtrate was then combined with 22 mL of Et_2O and 18 mL of H_2O, shaken and allowed to separate. The aqueous phase was titrated to pH 1−1.5 with 6 N HCl, and the solution was shaken again. The aqueous layer was washed once more with Et_2O, and the organic layers were combined and washed further with 6 aliquots of H_2O. The solution was removed under vacuum, and HOAc was azeotroped off with benzene. The resulting solid was put under vacuum in the presence of KOH for 12 h to yield 119.5 mg of product (74% yield). R_f: 0.30 (*n*-Hex/EtOAc/AcOH, 1:2:0.05). ^{1}H NMR (400 MHz, $CDCl_3$): 7.76 (d, 2H, $Fmoc^{H-4,H-5}$, $J_{4,3} = J_{5,6}$ = 8.0), 7.59 (d, 2H, $Fmoc^{H-1,H-8}$, $J_{1,2} = J_{8,7}$ = 6.0), 7.40 (t, 2H, $Fmoc^{H-3,H-6}$, $J_{3,2} = J_{3,4} = J_{6,7} = J_{6,5}$ = 7.3), 7.33−7.26 (m, 6H, $Fmoc^{H-2,H-7}$, $HMPA^{ar-H}$), 5.35 (d, 1H, Ala^{NH}, J = 6.4), 5.16 (m, 2H, $HMPA^{OCH_2}$), 4.45−4.34 (m, 3H, $Fmoc^{CH_2}$, Ala^{CH}), 4.21 (t, 1H, $Fmoc^{H-9}$, J_{H-9,CH_2} = 7.1), 3.64 (s, 2H, $HMPA^{CH_2CO}$), 1.44 (d, 3H, $J_{CH_3,CH}$ = 7.0). ^{13}C NMR (100 MHz, $CDCl_3$): 178.5, 176.6, 173.9, 156.74, 142.3, 134.5, 129.7, 128.5, 127.7, 127.0, 125.0, 120.0, 67.1, 66.8, 50.8, 47.1, 40.3. HRMS (FAB, pos): *m/e* 592.0719 (M + Cs^+, calcd 592.0736).

Immobilization of 7 on Rink-Amide Resin. Fmoc-Rink amide AM resin (0.25 mmol/g, 400 mg, 0.1 mmol) was treated with DMF/morpholine (1:1, 10 mL) for 1 h. The resin was washed with DMF and suspended with a solution of the Fmoc−amino acid−PAM conjugate **7** (60 mg, 0.131 mmol), HOBt (30 mg, 0.196 mmol), NMM (29 μL, 0.262 mmol), and HBTU (50 mg, 0.131 mmol) in DMF (11 mL). After 16 h the reactants were removed by filtration. The resin was washed with DMF before Pyr/Ac_2O (3:1, 12 mL) was added. After 10 min of shaking the resin was washed with DMF.

Release of Fmoc-Ala-PAM Amide (9). A small portion (50 mg) of the above resin was treated with 95% TFA, 2.5% triethylsilane, and 2.5% H_2O for 5 min. The solution was removed from the resin and evaporated under a stream of nitrogen, followed by vacuum to dryness to give 5.1 mg of **9**. Yield: 89% R_f: 0.36 (CH_2Cl_2/MeOH/NH_4OH, 9:1:0.05). ^{1}H NMR (400 MHz, $CDCl_3$): 7.75 (d, 2H, $Fmoc^{H-4,H-5}$, $J_{4,3} = J_{5,6}$ = 8.0), 7.57 (d, 2H, $Fmoc^{H-1,H-8}$, $J_{1,2} = J_{8,7}$ = 7.4), 7.39 (t, 2H, $Fmoc^{H-3,H-6}$, $J_{3,2} = J_{3,4} = J_{6,7} = J_{6,5}$ = 7.4), 7.33−7.26 (m, 6H, $Fmoc^{H-2,H-7}$, $HMPA^{ar-H}$), 5.33 (d, 1H, terminal^{NH_2}, J = 7.8), 5.16 (s, 2H, $HMPA^{OCH_2}$), 4.45−4.34 (m, 3H, $Fmoc^{CH_2}$, Ala^{CH}), 4.20 (t, 1H, $Fmoc^{H-9}$, J_{H-9,CH_2} = 7.0), 3.56 (s, 2H, $HMPA^{CH_2CO}$), 1.43 (d, 3H, $J_{CH_3,CH}$ = 7.1). ^{13}C NMR (100 MHz, $CDCl_3$): 180.1, 178.0, 174.1, 156.8, 142.1, 134.4, 134.0, 129.7, 128.5, 127.6, 126.9, 125.3, 119.8, 67.3, 66.8, 50.8, 47.4, 40.0. HRMS (FAB, pos): *m/e* 591.0888 (M + Cs^+, calcd 591.0896).

Enzymatic Hydrolysis of (9). Compound **9** was dissolved in 50 mM tris pH 8.4 (500 μL). To this was added 10 μL of a 0.1 U/mL solution of subtilisin BPN′. The reaction was allowed to proceed at room temperature and monitored by TLC. After 30 min all **9** was hydrolyzed to Fmoc-Ala as visualized by TLC (CH_2Cl_2/MeOH/NH_4-OH, 9:1:0.05).

Fmoc-Gly-PAM-OPac (10). Fmoc-Gly (0.86 g, 2.9 mmol), **5** (1.00 g, 2.9 mmol) and Bu_4NBr (0.93 g, 2.9 mmol) was reacted as described above. Chromatography yielded 1.37 g of a solid. Yield: 84%. R_f: 0.63 (*n*-Hex/EtOAc/AcOH, 1:2:0.05). ^{1}H NMR (500 MHz, $CDCl_3$): 7.87 (d, 2H, Pac^{ortho}, $J_{o,m}$ = 7.3), 7.76 (d, 2H, $Fmoc^{H-4,H-5}$, $J_{4,3} = J_{5,6}$ = 7.5), 7.59−7.57 (m, 4H, $Fmoc^{H-1,H-8}$, Pac^{para}), 7.45, (t, 2H, Pac^{meta}, $J_{m,o} = J_{m,p}$ = 7.8), 7.39 (t, 2H, $Fmoc^{H-3,H-6}$, $J_{3,2} = J_{3,4} = J_{6,7} = J_{6,5}$ = 7.3), 7.35−7.24 (m, 6H, $Fmoc^{H-2,H-7}$, $HMPA^{ar-H}$), 5.36 (t, 1H, G^{NH}, $J_{NH,\alpha}$ = 5.5), 5.33 (s, 2H, Pac^{CH_2}), 5.16 (s, 2H, $HMPA^{OCH_2}$), 4.38 (d, 2H, $Fmoc^{CH_2}$, $J_{CH_2,H-9}$ = 7.1), 4.21 (t, 1H, $Fmoc^{H-9}$, J_{H-9,CH_2} = 7.1),

4.01 (d, 2H, G^{α}, $J_{\alpha,NH} = 5.8$), 3.80 (s, 2H, $HMPA^{CH_2CO}$). ^{13}C NMR (125 MHz, $CDCl_3$): 191.85, 170.73, 169.82, 156.22, 143.73, 141.20, 134.03, 133.96, 133.86, 129.68, 128.80, 128.68, 127.65, 127.02, 125.03, 119.92, 67.13, 66.84, 66.32, 47.00, 42.75, 40.44. HRMS (FAB, pos): *m/e* 696.0984 ($M + Cs^+$, calcd 696.0998).

Fmoc-Gly-PAM (11). Compound **10** was deprotected as described above. The residue was dissolved in dichloromethane/EtOAc (1:1, 20 mL). Addition of *n*-hexane led to precipitation of product, which was collected by filtration yielding 0.87 g of colorless solid. Yield: 85%. R_f: 0.36 (*n*-Hex/EtOAc/AcOH, 1:2:0.05). ^1H NMR (500 MHz, $CDCl_3$): 7.76 (d, 2H, $Fmoc^{H-4,H-5}$, $J_{4,3} = J_{5,6} = 7.5$), 7.59 (d, 2H, $Fmoc^{H-1,H-8}$, $J_{1,2} = J_{8,7} = 7.5$), 7.40 (t, 2H, $Fmoc^{H-3,H-6}$, $J_{3,2} = J_{3,4} = J_{6,7} = J_{6,5} = 7.3$), 7.33−7.26 (m, 6H, $Fmoc^{H-2,H-7}$, $HMPA^{ar-H}$), 5.30 (t, 1H, G^{NH}), 5.17 (s, 2H, $HMPA^{OCH_2}$), 4.40 (d, 2H, $Fmoc^{CH_2}$, $J_{CH_2,H-9} = 7.1$), 4.22 (t, 1H, $Fmoc^{H-9}$, $J_{H-9,CH_2} = 7.1$), 4.03 (d, 2H, G^{α}, $J_{\alpha,NH} = 5.5$), 3.64 (s, 2H, $HMPA^{CH_2CO}$). ^{13}C NMR (100 MHz, $CDCl_3$): 180.23, 169.26, 152.80, 141.27, 135.01, 134.24, 129.69, 128.79, 127.72, 127.07, 125.08, 119.98, 67.25, 66.87, 47.06, 42.81, 40.46. HRMS (FAB, pos): *m/e* 446.1616 ($M + Cs^+$, calcd 446.1604).

Immobilization of 10 on Rink-Amide Resin. Fmoc-Rink amide AM resin (0.56 mmol/g, 1 g) was treated with DMF/morpholine (1:1, 10 mL) for 54 min. The resin was washed with DMF and suspended with a solution of the Fmoc−amino acid−PA conjugate **10** (0.87 g, 1.8 mmol), HOBt (0.36 g, 2.5 mmol), NMM (0.50 mL, 3.6 mmol), and HBTU (0.68 g, 1.8 mmol) in DMF (11 mL). After 16 h the reactants were removed by filtration. The resin was washed with DMF before Pyr/Ac_2O (3:1, 12 mL) was added. After 10 min of shaking the resin was again washed with DMF.

General Procedure for the Preperation of the Glycopeptide PAM Amides. Fmoc Removal. The resin was suspended in DMF/piperidine (8:2, 10 mL). After 15 min of shaking the resin was thoroughly washed with DMF.

Coupling. The resin was treated with a 3−5-fold excess of the Fmoc−amino acid as a 0.12 M solution in DMF, which contained 1.5 equiv HOBt, 2.0 equiv NMM, and 1.0 equiv HBTU per equivalent of Fmoc−amino acid. After 3−5 h of shaking the reactants were removed by filtration. The resin was washed with DMF.

Capping. The resin was suspended in Pyr/Ac_2O (3:1, 8 mL) and shaken for 6 min. Reactants were removed by filtration, and the resin was thoroughly washed with DMF.

Peptide Cleavage. The resin was shaken in a TFA/ethanedithiol/H_2O mixture (95:2.5:2.5, 10 mL) for 1.5 h unless otherwise noted. After filtration the resin was washed repeatedly with TFA. The combined filtrates were concentrated to dryness in vacuo.

Z-Ser(βAc_3GlcNAc)-Gly-Gly-OPAM (12). The synthesis was carried out by using one-half of the Fmoc−dipeptide resin following the general procedure. The crude material was purified by chromatography ($CHCl_3$/MeOH, 9:1) to yield 265 mg of a colorless solid. According to NMR analysis the product was a 83:17 mixture of two compounds, which showed no resolution in the HPLC analysis. Yield: 89% (based on the initial loading of the resin with the Rink linker). R_t: 22.03 min (Microsorb, C18, 5 μm, 250 × 4 mm, 0 min (10% B)−30 min (50% B), A = 0.05% TFA in H_2O, B = 0.05% TFA in MeCN). ^1H NMR (400 MHz, DMSO-d_6): 8.24−8.21 (m, 2H, 2 × G^{NH}), 7.87 (d, 0.83H, S^{NH}, $J = 9.0$), 7.76 (d, 0.17H, S^{NH}, $J = 8.9$), 7.46 (s, 1H, PAM^{NH}), 7.36−7.23 (m, 9H, PAM^{ar-H}, Z^{ar-H}), 6.87 (s, 1H, PAM^{NH}), 5.09−4.98 (m, 5H, PAM^{OCH_2}, H-3′, Z^{CH_2}), 4.83 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'} = 9.7$), 4.67, 4.66 (2 × d, 1H, 2 × H-1′, $J_{1',2'} = 8.5$), 4.28−4.23 (m, 1H, S^{α}), 4.17 (dd, 1H, H-6′^{a}, $J_{6'a,6'b} = 12.2$, $J_{6'a,5'} = 4.6$), 4.01−3.98 (m, 1H, H-6′^{b}), 3.94−3.61 (m, 8H, 2 × G^{α}, S^{β}, H-2′, H-5′), 3.36 (s, 1H, PAM^{CH_2CO}), 1.99, 1.95, 1.89 (3 × s, 3 × 3H, 3 × AcO), 1.73, 1.70 (2 × s, 3H, AcNH). ^{13}C NMR (100 MHz, DMSO-d_6): 172.15, 170.18, 169.71, 169.71, 169.67, 169.56, 169.36, 169.27, 155.98, 136.82, 136.55, 133.89, 129.21, 129.17, 128.41, 128.05, 127.98, 127.91, 127.86, 127.82, 100.55, 100.31, 72.58, 70.84, 68.74, 68.52, 68.43, 65.84, 65.78, 61.84, 54.97, 54.72, 52.99, 41.97, 41.93, 40.67, 22.67, 20.55, 20.46, 20.41. HRMS (FAB, pos): *m/e* 962.2095 ($M + Cs^+$, calcd 962.2012).

Z-Ser(βAc_3GlcNAc)-Gly-Gly-Gly-OPAM (13). The synthesis was carried out by using the second half of the Fmoc−dipeptide resin following the general procedure. The crude material was purified by chromatography ($CHCl_3$/MeOH, 85:15) to yield 281 mg of a colorless solid. Yield: 89% (based on the initial loading of the resin with the Rink-linker). R_t: 22.30 min (Microsorb, C18, 5 μm, 250 × 4 mm, 0 min (10% B)−30 min (50% B), A = 0.05% TFA in H_2O, B = 0.05% TFA in MeCN)). ^1H NMR (400 MHz, DMSO-d_6): 8.30 (t, 1H, G^{NH}, $J = 5.8$), 8.16−8.12 (m, 2H, 2 × G^{NH}), 7.88 (d, 1H, S^{NH}, $J = 9.0$), 7.46 (s, 1H, PAM^{NH}), 7.36−7.22 (m, 9H, PAM^{ar-H}, Z^{ar-H}), 6.87 (s, 1H, PAM^{NH}), 5.09−4.98 (m, 5H, PAM^{OCH_2}, H-3′, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'} = 9.7$), 4.66 (d, 1H, H-1′, $J_{1',2'} = 8.5$), 4.28−4.23 (m, 1H, S^{α}), 4.18 (dd, 1H, H-6′^{a}, $J_{6'a,6'b} = 12.2$, $J_{6'a,5'} = 4.6$), 4.01−3.98 (m, 1H, H-6′^{b}), 3.88 (d, 2H, G^{α}, $J = 5.9$), 3.83−3.68 (m, 8H, 2 × G^{α}, S^{β}, H-2′, H-5′), 3.36 (s, 1H, PAM^{CH_2CO}), 1.99, 1.95, 1.89, 1.72 (4 × s, 4 × 3H, 4 × Ac). ^{13}C NMR (100 MHz, DMSO-d_6): 172.15, 170.18, 169.70, 169.56, 169.39, 169.36, 169.01, 155.99, 136.80, 136.55, 133.89, 129.20, 128.41, 128.05, 127.92, 127.88, 100.28, 72.59, 70.83, 68.52, 65.80, 61.83, 54.72, 53.00, 42.27, 41.96, 41.76, 40.68, 22.67, 20.55, 20.48, 20.41. HRMS (FAB, pos): *m/e* 1019.2262 ($M + Cs^+$, calcd 1019.2287).

Studies in the Subtilisin-Catalyzed Chain Elongation of 2c, 3c, and 4c. Reaction of Z-Ser(βAc_3GlcNAc)-Gly-OBzl (2c) with Gly-OMe. Compound **2c** (17 mg, 27.2 μmol) was dissolved in a 0.3 M solution of HCl·Gly-OMe (2 mL, 600 μmol) in DMF/50 mM TEA (7:3), pH 8.4. To the mixture was added 0.5 mL of a solution of subtilisin (50 mg/mL) in DMF/50 mM TEA (7:3), pH 8.4. The mixture was stirred for 2 days. TLC control showed the presence of a new compound together with unaffected starting material. Again 0.5 mL of the subtilisin solution was added. After 2 d of stirring the pH had dropped to 6.2. To the mixture was added HCl·Gly-OMe (112 mg, 0.89 mmol). The pH was adjusted to 8.4 by addition of a 8 M NaOH solution before 0.5 mL of the subtilisin solution was added. After 1 d of stirring TLC control still showed the presence of starting material. The pH had dropped to 7. The reaction mixture was diluted with 30 mL of chloroform and 20 mL of 1 M HCl. The organic layer was separated. The aqueous layer was extracted twice with chloroform (20 mL). The combined organic layers were washed with 10 mL of brine. The brine layer was reextracted twice with chloroform. The combined organic layers were dried over $MgSO_4$. The volatiles were removed by distillation in vacuo. The residue was chromatographed ($CHCl_3$/MeOH, 9:1; $CHCl_3$/MeOH/AcOH, 85:15:1) to yield 6.5 mg of the newly formed product and 9 mg (46%) of starting material. TLC and NMR comparison with an authentic reference identified the newly formed product to be **2b**. Yield: 38%.

Reaction of Z-Ser(βAc_3GlcNAc)-Gly-Gly-Gly-OBzl (4c) with Gly-OMe. 4c (16 mg, 19.3 μmol) was dissolved in a 0.3 M solution of HCl·Gly-OMe (0.77 mL, 77 μmol) in DMF/50 mM TEA (7:3), pH 8.4. To the mixture was added 15.4 μL of a solution of subtilisin (50 mg/mL) in DMF/50 mM TEA (7:3), pH 8.4. After being stirred for 6 h at room temperature, the mixture was diluted with chloroform (20 mL) and 0.13 M HCl (16 mL). The organic layer was separated and the aqueous layer extracted 3× with chloroform. The combined organic layers were washed with brine. The aqueous layer was reextracted with chloroform. The organic layers were combined and concentrated in vacuo. The residue was purified by 2-fold GPC (Sephadex G10, 0.05 M NH_4OAC, and Biogel P2, H_2O). Lyophilization yielded 4.5 mg of the hydrolyzed material **4b**. Yield: 32%.

General Procedure for the Subtilisin-Catalyzed Reactions of the Glycopeptide Benzyl Esters 2c, 3c, and 4c and the Glycopeptide PAM Esters 12 and 13 with Gly-NH_2. The glycopeptide ester was dissolved in a 0.3 M glycinamide solution in DMF/50 mM TEA (7:3, pH 8.4) to give a 0.02 M concentration. A solution (50 mg/mL) of subtilisin (8.1 U/mg) in DMF/50 mM TEA (7:3, pH 8.4) was added until a final concentration of 12 U of subtilisin/mL resulted. After being stirred for 6 h at room temperature the mixture was directly subjected to GPC on a Sephadex LH-20 column ($CHCl_3$/MeOH, 1:1).

Reaction of Z-Ser(βAc_3GlcNAc)-Gly-OBzl with Gly-NH_2. A 0.5 mg (0.7 μmol) amount of **2c** is reacted (see the general procedure). TLC control shows no conversion of the starting material after 6 h.

Z-Ser(βAc_3GlcNAc)-Gly-Gly-Gly-NH_2 (15). Reaction of 3c. Compound **2c** (19.0 mg, 24.6 μmol) was reacted according to the general procedure. GPC furnishes a material which was further purified

by chromatography ($CHCl_3$/MeOH/AcOH, 85:15:1) yielding 12.0 mg of coupled product **15** (66%) and 5.0 mg of the hydrolysis product **2b** (30%).

Reaction of 12. Compound **12** (9.2 mg, 11.1 μmol) was reacted according to the general procedure. GPC furnishes 1.1 mg of unreacted material (12%), 4.8 mg of coupled product **15** (59%), and 1.6 mg of hydrolysis product **2b** (21%). R_f = 0.13 ($CHCl_3$/MeOH/AcOH, 85:15:1). ^{1}H NMR (500 MHz, DMSO-d_6): 8.15 (t, 1H, G^{NH}, J = 5.5), 8.11 (t, 1H, G^{NH}, J = 5.7), 8.07 (t, 1H, G^{NH}, J = 5.8), 7.88 (d, 1H, S^{α}, J = 9.0), 7.36−7.30 (m, 5H, Z^{ar-H}), 7.20 (s, 1H, amide^a), 7.06 (s, 1H, amide^b), 5.06 (t, 1H, H-3′, $J_{3',2'} = J_{3',4'}$ = 9.9), 5.03−4.99 (m, 2H, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.7), 4.67 (d, 1H, H-1′, $J_{1',2'}$ = 8.4), 4.27−4.23 (m, 1H, S^{α}), 4.17 (dd, 1H, H-6′^{a}, $J_{6'a,6'b}$ = 12.3, $J_{6'a,5'}$ = 4.6), 3.99 (m_c, 1H, H-6′^{b}), 3.83−3.69 (m, 8H, 2 × G^{α}, S^{β}, H-2′, H-5′), 3.61 (d, 2H, G^{α}, J = 5.7), 1.99, 1.96, 1.89, 1.72 (4 × s, 4 × 3H, 4 × Ac). ^{13}C NMR (125 MHz, DMSO-d_6): 171.09, 170.35, 169.92, 169.87, 169.74, 169.53, 169.37, 169.16, 156.16, 136.98, 128.58, 128.05, 100.46, 72.76, 71.01, 68.87, 68.70, 65.97, 62.01, 54.89, 53.18, 42.50, 42.36, 42.05, 22.85, 20.73, 20.66, 20.59. ESI-MS (pos): *m/e* 761 (M + Na^+, calcd 761.0), 739 (M + H^+, calcd 739.0).

Z-Ser(βAc$_3$GlcNAc)-Gly-Gly-Gly-Gly-NH$_2$ (16). Reaction of 4c. Compound **4c** (20.0 mg, 24.1 μmol) was suspended in the reaction mixture according to the general procedure. The reaction mixture became clear after 3 h. GPC furnished 8.5 mg of the coupled product **16** (44%) and 9.6 mg of the hydrolysis product **4b** (54%).

Reaction of 13. Compound **13** (19.9 mg, 22.4 μmol) was reacted according to the general procedure. GPC furnished 7.9 mg of the coupled product **16** (44%) and 9.3 mg of the hydrolysis product **4b** (56%). R_f = 0.12 ($CHCl_3$/MeOH/AcOH, 85:15:1). ^{1}H NMR (500 MHz, DMSO-d_6): 8.17−8.12 (m, 3H, 3 × G^{NH}), 8.05 (t, 1H, G^{NH}), 7.88 (d, 1H, S^{α}, J = 8.8), 7.36−7.30 (m, 5H, Z^{ar-H}), 7.20 (s, 1H, amide^a), 7.07 (s, 1H, amide^b), 5.07 (t, 1H, H-3′, $J_{3',2'} = J_{3',4'}$ = 9.9), 5.03−4.98 (m, 2H, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.7), 4.67 (d, 1H, H-1′, $J_{1',2'}$ = 8.4), 4.26−4.24 (m, 1H, S^{α}), 4.17 (dd, 1H, H-6′^{a}, $J_{6'a,6'b}$ = 12.3, $J_{6'a,5'}$ = 4.6), 3.99 (m_c, 1H, H-6′^{b}), 3.83−3.71 (m, 10H, 3 × G^{α}, S^{β}, H-2′, H-5′), 3.61 (d, 2H, G^{α}, J = 5.9), 1.99, 1.96, 1.89, 1.72 (4 × s, 4 × 3H, 4 × Ac). ^{13}C NMR (125 MHz, DMSO-d_6): 171.11, 170.35, 169.87, 169.74, 169.51, 169.30, 169.20, 156.16, 136.97, 128.20, 127.90, 100.46, 72.77, 71.05, 68.87, 68.71, 65.96, 62.01, 54.87, 53.23, 42.37, 42.18, 42.05, 41.93, 22 (broad). MALDI-MS (α-cyanocinnamic acid): *m/e* 834 (M + K^+, calcd 834.5), 818 (M + Na^+, calcd 818.4), 796 (M + H^+, calcd 796.4).

Z-Ser(βAc$_3$GlcNAc)-NH$_2$ (17a). To a solution of **1** (149.6 mg, 0.27 mmol) in THF (50 mL) was added HOBt (62 mg, 0.40 mmol) and EDC (51.7 mg, 0.27 mmol). This mixture was stirred at room temperature for 20 min. Aqueous ammonia was added slowly by syringe (180 μL, 2.7 mmol) as the reaction cleared. The reaction was stirred for 1 h after the addition was complete. Solvent was removed under vacuum, and the residue was taken up in dichloromethane and washed three times with saturated sodium bicarbonate solution. The organic layer was further washed with water and dried over $MgSO_4$. Solvent was removed by evaporation to give a white solid. Chromatography purification (90% CH_2Cl_2/10% MeOH/0.1% NH_4OH) gave 135.3 mg of white solid. Yield: 90.6%. R_f: 0.6 (90% CH_2Cl_2/10% MeOH/0.1% NH_4OH). ^{1}H NMR (500 MHz, DMSO-d_6): 7.88 (d, 1H, $GlcNAc^{NH}$, J = 9.0), 7.37−7.30 (m, 5H, Z^{ar-H}), 7.24 (s, 1H, S^{NH_2a}), 7.16 (s, 1H, s^{NH_2b}), 7.05 (d, 1H, S^{NH}, J = 8.5), 5.06 (t, 1H, H-3′, J = 9.5), 5.01 (s, 1H, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9.5), 4.66 (d, 1H, H-1′, $J_{1',2'}$ = 8.5), 4.18 (m, 1H, H-6′^{a}), 4.12 (m, 1H, S^{α}), 3.99 (m, 1H, H-6′^{b}), 3.85−3.81 (m, 2H, S^{β}), 3.70−3.67 (m, 2H, H-2′, H-5′), 1.99 (s, 3H, Ac), 1.96 (s, 3H, Ac), 1.89 (s, 3H, Ac), 1.72 (s, 3H, Ac). ^{13}C NMR (250 MHz, DMSO-d_6): 171.38, 170.27, 169.53, 169.44, 169.02, 156.31, 136.92, 133.90, 133.18, 101.09, 73.56, 71.49, 67.02, 65.80, 62.30, 54.92, 53.54, 23.06, 21.08. HRMS (FAB, pos): *m/e* 700.1142 (M + Cs^+, calcd 700.1119).

Z-Gly-Ser(βAc$_3$GlcNAc)-NH$_2$ (18a). Compound **17a** (116.6 mg, 0.206 mmol) was added to 25 mL of ethanol to give a white suspension. $Pd(OH)_2$ (Degussa type, 10 mg) was added and the flask flushed with nitrogen. The flask was then evacuated and backfilled with hydrogen from a filled balloon. The reaction was stirred under H_2 for 2 h at which time no white solid was apparent. The catalyst was filtered off over Celite and solvent removed by evaporation to give a white solid Ser(βAc$_3$GlcNAc)-NH$_2$, **17b** (89.1 mg). Yield: 99.9% Compound **17b** (64.9 mg, 0.15 mmol) was then dissolved in DMF. To this solution was added Z-Gly (94.1 mg, 0.45 mmol), HOBt (69 mg, 0.45 mmol), and NMM (125 μL, 0.9 mmol). After all material was dissolved, HBTU was added to the reaction (257 mg, 0.68 mmol), and the reaction was stirred under N_2 overnight. The solvent was removed under vacuum, and the residue was taken up in dichloromethane and washed three times with saturated sodium bicarbonate solution. The organic layer was further washed with water and dried over $MgSO_4$. Solvent was removed by evaporation to give a white solid. Chromatography purification (92% CH_2Cl_2/8% MeOH/0.1% NH_4OH) gave 81.5 mg of a white solid. Yield: 87.1%. R_f: 0.5 (92% CH_2Cl_2: 8% MeOH:0.1% NH_4OH). ^{1}H NMR (500 MHz, DMSO-d_6): 7.85 (m, 2H, $GlcNAc^{NH}$, S^{NH}), 7.51 (t, 1H, G^{NH}, J = 5), 7.37−7.34 (m, 5H, Z^{ar-H}), 7.26 (br d, 2H, S^{NH_2}, J = 6), 5.10 (t, 1H, H-3′, J = 9), 5.05 (s, 1H, Z^{CH_2}), 4.86 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9), 4.68 (d, 1H, H-1′, $J_{1',2'}$ = 8), 4.39 (m, 1H, H-6′^{a}), 4.22 (m, 1H, S^{α}), 4.03 (m, 1H, H-6′^{b}), 3.87 (m, 2H, S^{β}), 3.76−3.66 (m, 3H, G^{α}, H-2′),3.74 (s, 1H, H-5′), 2.03 (s, 3H, Ac), 1.98 (s, 3H, Ac), 1.92 (s, 3H, Ac), 1.79 (s, 3H, Ac). ^{13}C NMR (250 MHz, DMSO-d_6): 170.93, 170.19, 169.72, 169.69, 169.35, 169.14, 156.14, 137.04, 128.42, 127.88, 127.84, 100.25, 72.64, 70.85, 68.71, 68.51, 65.59, 65.48, 61.80, 53.12, 52.43, 43.58, 22.74, 20.58, 20.47, 20.41. HRMS (FAB, pos): *m/e* 757.1357 (M + Cs^+, calcd 757.1333).

Z-Gly-Gly-Ser(βAc$_3$GlcNAc)-NH$_2$ (19a). Deprotection of **17a** as stated above gave **17b**. Compound **17b** (21.65 mg, 0.05 mmol) was then dissolved in DMF, and to it was added Z-Gly-Gly (40 mg, 0.15 mmol), HOBt (22 mg, 0.15 mmol), NMM (41 μL, 0.3 mmol), and HBTU (87 mg, 0.23 mmol). The reaction was stirred under N_2 overnight, and then workup was as described above. Chromatography purification (92% CH_2Cl_2/8% MeOH/0.1% NH_4OH) gave 25.5 mg of a white solid. Yield: 75.1%. R_f: 0.4 (92% CH_2Cl_2/8% MeOH:0.1% NH_4OH). ^{1}H NMR (500 MHz, DMSO-d_6): 8.14 (m, 1H, G^{NH}), 7.85 (m, 2H, $GlcNAc^{NH}$, S^{NH}), 7.53 (m, 1H, G^{NH}), 7.35−7.32 (m, 5H, Z^{ar-H}), 7.22 (br d, 2H, S^{NH_2}, J = 7), 5.07 (t, 1H, H-3′, J = 9), 5.02 (s, 1H, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9), 4.68 (d, 1H, H-1′, $J_{1',2'}$ = 8), 4.35 (m, 1H, H-6′^{a}), 4.19 (m, 1H, S^{α}), 4.00 (m, 1H, H-6′^{b}), 3.83 (m, 2H, S^{β}), 3.76−3.64 (m, 6H, G^{α}, H-2′, H-5′), 2.01 (s, 3H, Ac), 1.95 (s, 3H, Ac), 1.89 (s, 3H, Ac), 1.76 (s, 3H, Ac). ^{13}C NMR (250 MHz, DMSO-d_6): 170.94, 170.19, 169.71, 169.67, 169.36, 130.14, 129.54, 128.83, 128.42, 127.89, 100.25, 70.81, 68.46, 65.62, 61.77, 60.00, 55.31, 54.99, 50.08, 43.60, 22.75, 20.59, 20.48, 20.42. HRMS (FAB, pos): *m/e* 704.2366 (M + Na^+, calcd 704.2391).

Z-Gly-Gly-Gly-Ser(βAc$_3$GlcNAc)-NH$_2$ (20a). Deprotection of **18a** as described above gave Gly-Gly-Ser(βAc$_3$GlcNAc)-NH$_2$, **18b**. In DMF **18b** (29.4 mg 0.06 mmol), Z-Gly-Gly (48 mg, 0.18 mmol), HOBt (41 mg, 0.27 mmol), NMM (50 μL, 0.36 mmol), and HBTU (68 mg, 0.18 mmol) were stirred under N_2 overnight. Workup was as described above. Chromatography purification (90% CH_2Cl_2/10% MeOH/0.1% NH_4OH) gave 31.9 mg of white solid. Yield: 72.5%. R_f: 0.4 (90% CH_2Cl_2/10% MeOH/0.1% NH_4OH). ^{1}H NMR (500 MHz, DMSO-d_6): 8.16 (m, 2H, G^{NH}), 7.86 (m, 2H, $GlcNAc^{NH}$, S^{NH}), 7.47 (t, 1H, G^{NH}, J = 6), 7.37−7.32 (m, 5H, Z^{ar-H}), 7.21 (br d, 2H, S^{NH_2}, J = 3), 5.06 (t, 1H, H-3′, J = 9), 5.02 (s, 1H, Z^{CH_2}), 4.82 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'}$ = 9), 4.63 (d, 1H, H-1′, $J_{1',2'}$ = 8), 4.34 (m, 1H, H-6′^{a}), 4.19 (m, 1H, S^{α}), 4.00 (m, 1H, H-6′^{b}), 3.86−3.65 (m, 10H, S^{β}, G^{α}, H-2′, H-5′), 2.00 (s, 3H, Ac), 1.95 (s, 3H, Ac), 1.89 (s, 3H, Ac), 1.76 (s, 3H, Ac). ^{13}C NMR (250 MHz, DMSO-d_6): 171.20, 170.70, 169.78, 169.62, 169.52, 157.20, 136.88, 136.58, 130.24, 129.80, 102.93, 73.10, 72.92, 70.51, 68.71, 68.23, 65.51, 60.01, 56.26, 54.02, 53.12, 52.48, 43.47, 22.71, 20.51, 20.67, 20.41. HRMS (FAB, pos): *m/e* 871.1795 (M + Cs^+, calcd 871.1762).

General Procedure for the Subtilisin-Catalyzed Reactions of Glycopeptide Amides 17a, 18a, 19a, and 20a with Z-Gly Benzyl Ester. The glycopeptide amide was deprotected as described above. The deprotected glycopeptide amides (**17b**, **18b**, **19b**, and **20b** respectively) were dissolved in a 0.06 M solution of Z-GlyOBz in DMF/50 mM TEA (7:3, pH 8.4) to give a 0.02 M concentration. To this solution a solution (50 mg/mL) of subtilisin (8.1 U/mg) in DMF/50 mM TEA (7:3, pH 8.4) was added until a final concentration of 12 U of subtilisin/mL resulted. After being stirred for 6 h at room

temperature, the mixture was directly subjected to GPC on a Sephadex LH-20 column ($CHCl_3$/MeOH, 1:1).

Deprotection and Reaction of Z-Ser(βAc$_3$GlcNAc)-NH$_2$ with Z-GlyOBzl. A 0.5 mg (0.88 μmol) amount of **17a** was reacted (see the general procedure). TLC control showed no conversion of the starting material after 6 h.

Deprotection and Enzymatic Ligation of 18a and Z-GlyOBzl To Give Z-Gly-Gly-Ser(βAc$_3$GlcNAc)-NH$_2$ (19a). Compound **18a** (10.0 mg, 16.0 μmol) was reacted according to the general procedure. GPC furnished a material which was further purified by chromatography (92% CH_2Cl_2/8% MeOH/0.1% NH_4OH) yielding 4.0 mg of coupled product **19a** (37%). Both HRMS and NMR spectra were in good agreement with those reported above.

Deprotection and Enzymatic Ligation of 19a and Z-GlyOBzl To Give Z-Gly-Gly-Gly-Ser(βAc$_3$GlcNAc)-NH$_2$ (20a). Compound **19a** (12.8 mg, 18.7 μmol) was reacted according to the general procedure. GPC furnished a material which was further purified by chromatography (90% CH_2Cl_2/10% MeOH/0.1% NH_4OH) yielding 8.1 mg of coupled product **20a** (54%). Both HRMS and NMR spectra were in good agreement with those reported above.

Deprotection and Enzymatic Ligation of 16a and Z-GlyOBzl To Give Z-Gly-Gly-Gly-Gly-Ser(βAc$_3$GlcNAc)-NH$_2$ (21). Compound **20a** (15.9 mg, 21.5 μmol) was reacted according to the general procedure. GPC furnished a material which needed no further purification yielding 10.7 mg of coupled product **21** (62%). R_f: 0.3 (92% CH_2Cl_2/8% MeOH/0.1% NH_4OH). ^{1}H NMR (500 MHz, DMSO-d_6): 8.17 (m, 3H, G^{NH}), 7.86 (m, 2H, $GlcNAc^{NH}$, S^{NH}), 7.47 (t, 1H, G^{NH}, $J = 6$), 7.35−7.32 (m, 5H, Z^{ar-H}), 7.21 (br d, 2H, S^{NH_2}, $J = 2.5$), 5.07 (t, 1H, H-3′, $J = 9$), 5.01 (s, 1H, Z^{CH_2}), 4.85 (t, 1H, H-4′, $J_{4',3'} = J_{4',5'} = 9$), 4.63 (d, 1H, H-1′, $J_{1',2'} = 8$), 4.33 (m, 1H, H-6′^{a}), 4.19 (m, 1H, S^{α}), 4.00 (m, 1H, H-6′^{b}), 3.86−3.65 (m, 12H, S^{β}, G^{α}, H-2′, H-5′), 2.00 (s, 3H, Ac), 1.95 (s, 3H, Ac), 1.89 (s, 3H, Ac), 1.76 (s, 3H, Ac). HRMS (FAB, pos): *m/e* 928.2043 (M + Cs^+, calcd 928.2009).

SPPS of Long-Chain Peptide Ester (23). Standard Fmoc-SPPS protocol was used to elaborate **8** as described in the general procedure above. Deprotection and cleavage from the resin was accomplished simultaneously by treatment with 95% TFA, 2.5% triethylsilane, and 2.5% H_2O for 20 min. The solution was drained from the vessel and the resin washed with TFA three times. The solution was evaporated to dryness to give a white solid. The solid was taken up in methanol, the insoluble material was filtered out, and the methanol solution was reduced to 5 mL volume. To this was added 100 mL of Et_2O was added to precipitate the peptide. The solid was isolated by centrifugation and taken up in H_2O. The aqueous solution was lyophilized to give the crude product. The compound was purified by HPLC. Yield: 128.4 mg, 87.3%. MALDI MS: *m/e* 1714 (M + Na^+, calcd 1714).

Enzymatic Ligation of 23 and 19b. The most successful conditions are described herein. One equivalent of **23** (1.4 mg, 0.94 μmol) and 3 equiv of **19b** (1.3 mg, 2.8 μmol) were dissolved in 90 μL of DMF. To this was added 8 μL of 50 mmol triethanolamine, pH 7.8. After the solution cooled, 2 μL of 10 mg/mL subtilisin stock was added and the reaction was kept at 37 °C. After 4 h, 2 μL of a 1 mg/mL solution of (phenylmethyl)sulfonyl fluoride in CH_3CN was added to stop the reaction. The reaction mixture was then analyzed by MALDI mass spectroscopy. MALDI of **23**: *m/e* 2096 (M + Na^+, calcd 2096). MALDI of **24**: *m/e* 1567 (M + Na^+, calcd 1567).

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