Chemoenzymatic Synthesis of a Characteristic Phosphorylated and Glycosylated Peptide Fragment of the Large Subunit of Mammalian RNA Polymerase II

Torsten Pohl and Herbert Waldmann*

Contribution from the Department of Organic Chemistry, University of Karlsruhe, Richard-Willstätter-Allee 2, D-76128 Karlsruhe, Germany

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Abstract: The covalent modification of proteins by phosphorylation and addition of GlcNAc residues are important regulatory processes which mediate biological signal transduction. For instance, the cytosolic form of RNA polymerase II is heavily glycosylated but during its transition from an initiating to an elongating complex the carbohydrates are removed and the protein is phosphorylated. For the study of such biological phenomena, characteristic peptides which embody both types of modifications may serve as efficient tools. However, their synthesis is complicated by their pronounced acid and base lability as well as their multifunctionality. These properties make the application of protecting groups necessary which can be removed under the mildest conditions. For the construction of such peptide conjugates the enzyme labile PhAcOZ urethane blocking group was developed. This protecting group embodies (a) a functional group (a phenylacetate) that is recognized by the biocatalyst (penicillin G acylase) and that is bound by an enzyme labile linkage (an ester) to (b) a functional group (a *p*-hydroxybenzyl urethane) that undergoes a spontaneous fragmentation upon cleavage of the enzyme-sensitive bond resulting in (c) the liberation of a carbamic acid derivative which decarboxylates to give the desired peptide or peptide conjugate. When this enzymatic protecting group technique was combined with classical chemical methods, a complex phosphoglycohexapeptide was built up, which embodies two glycosylated, one phosphorylated, and one underivatized hydroxyamino acid. This peptide represents a characteristic partial structure of the repeat sequence of the large subunit of RNA polymerase II which becomes glycosylated or phosphorylated while the enzyme carries out its biological functions. The conditions under which the enzymatic deprotections proceed are so mild that no undesired side reaction is observed (i.e., no rupture or anomerization of the glycosidic bonds and no β-elimination of the phosphate or a carbohydrate occur). In addition, the specificity of the biocatalyst guarantees that the peptide bonds and the other protecting groups present are not attacked either.

Covalently modified proteins mediate the transduction of signals from the extracellular space across the plasma membrane into the interior of cells and ultimately to the cell nucleus.[1] Among the different types of covalent protein modification, in particular, the dynamic phosphorylation and dephosphorylation of hydroxyamino acids mediates the intracellular response to a wide variety of extracellular stimuli.[2] In addition, recently the β-*O*-glycosidic attachment of single *N*-acetylglucosamine (*O*-GlcNAc) residues to serine and threonine units of intracellular proteins was discovered as a second type of modification. Glycosylation with *O*-GlcNAc is analogous to phosphorylation in many ways, in particular in terms of its dynamic character and its ubiquitous occurrence.[3] *O*-GlcNAc is found in all eukaryotic cells on numerous proteins (e.g., on transcription factors,[4] oncogene products,[5] and enzymes[6] as well as on viral proteins).[7] The state of glycosylation of individual proteins is often modified during the cell cycle or in response to specific physiological stimuli. The limited data available to date suggest that the attachment and removal of *N*-acetylglucosamine is used by cells as a regulatory mechanism which is not only similar but often reciprocal to *O*-phosphorylation (i.e., phosphorylation and glycosylation by *O*-GlcNAc show a so-called "yin-yang" relationship).[3,8] Thus, *O*-GlcNAc glycoproteins often are also phosphoproteins in other sections of the cell cycle with the sites of *O*-GlcNAc addition being virtually indistinguishable from the sites of phosphorylation. An illustrative example is provided by mammalian RNA polymerase II (Scheme 1).[9]

In its cytosolic form the C-terminal domain (CTD) of its large subunit is heavily glycosylated, probably to facilitate the transport of the enzyme into the cell nucleus. During the intranuclear transition of RNA polymerase II from an initiating

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(1) (a) Hunter, T.; Karin, M. *Cell* **1992**, *70*, 375. (b) Levitzki, A. *Eur. J. Biochem.* **1994**, *226*, 1. (c) Casey, P. J. *Science* **1995**, *268*, 221. (d) Egan, S. E.; Weinberg, R. A. *Nature* **1993**, *365*, 781. (e) Boguski, M. S.; McCormick, F. *Nature* **1993**, *366*, 643.

(2) (a) Krebs, E. *Angew. Chem.* **1993**, *105*, 1173; *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1122. (b) Fischer, E. H. *Angew. Chem.* **1993**, *105*, 1181; *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1130. (c) Bridges, A. J. *Chemtracts: Org. Chem.* **1995**, *8*, 73.

(3) (a) Hart, G. W.; Haltiwanger, R. S.; Holt, G. D; Kelly, W. G. *Ann. Rev. Biochem.* **1989**, *58*, 841. (b) Hart, G. W.; Kelly, W. G.; Blomberg, M. A.; Roquemore, E. P.; Dong, L.-Y.; Kreppel, L.; Chou, T.-Y.; Snow, D.; Greis, K. *Glyco- and Cellbiology*, 44; Colloquium Mosbach 1993; Springer-Verlag: Berlin, Heidelberg, 1994.

(4) (a) Jackson, S. P.; Tjian, R. *Cell* **1988**, *55*, 125. (b) Lichtsteiner, S.; Schibler, U. *Cell* **1989**, *57*, 1179. (c) Reason, A.; Dell, A.; Morris, H. R.; Panico, M.; Treisman, R.; Marais, R.; Hart, G. W.; Haltiwanger, R. S. *Glycoconjugate J.* **1991**, *8*, 211.

(5) (a) Privalsky, M. L. *J. Virol.* **1990**, *64*, 463. (b) Chou, T.-Y.; Hart, G. W.; Dang, C. V. *J. Biol. Chem.* **1995**, *270*, 18961.

(6) (a) Datta, B.; Ray, M. K.; Chakrabarti, D.; Wylie, D. E.; Gupta, N. K. *J. Biol. Chem.* **1989**, *264*, 20620. (b) Maikrantz, W.; Smith, D. M.; Sladicka, M. M.; Schlegel, R. A. *J. Cell. Sci.* **1991**, *98*, 303.

(7) (a) Mullis, K. G.; Haltiwanger, R. S.; Hart, G. W.; Marchase, R. B.; Engler, J. A. *J. Virol.* **1990**, *64*, 5317. (b) Whitford, M.; Faulkner, P. *J. Virol.* **1992**, *66*, 3324. (c) Gonzalez, S. A.; Burrone, O. R. *Virology* **1992**, *182*, 8.

(8) Haltiwanger, R. S.; Kelly, W. G.; Roquemore, E. P.; Blomberg, M. A.; Dong, L.-Y. D.; Kreppel, L.; Chou, T.-Y.; Greis, K.; Hart, G. W. *Biochem. Soc. Trans.* **1992**, *20*, 264.

(9) Kelly, W. G.; Dahmus, M. E.; Hart, G. W. *J. Biol. Chem.* **1993**, *268*, 10416.

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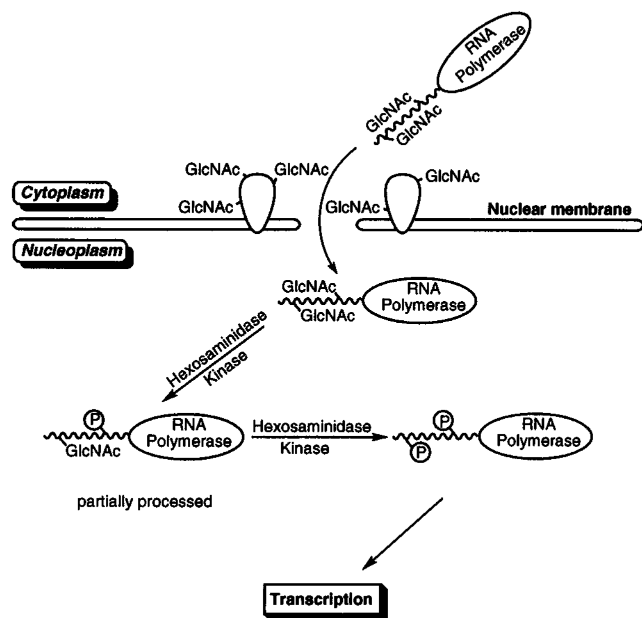
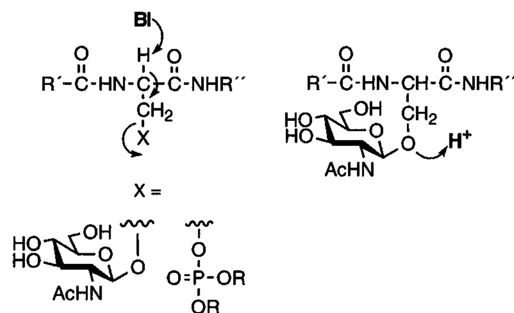
Scheme 1. Modification of the Cytosolic and the Nucleosolic Forms of RNA Polymerase II by Glycosylation with *O*-GlcNAc and by Phosphorylation



Scheme 2. Acid and Base Lability of Phospho- and Glycopeptides



to an elongating transcription complex the *O*-GlcNAc glycosides are removed and the protein is rapidly and extensively phosphorylated.

The fact that only the unphosphorylated form of RNA polymerase II contains *O*-GlcNAc suggests that phosphorylation and glycosylation are separate events and that the CTD of RNA polymerase II appears to exist in three distinct states: unmodified, phosphorylated, and glycosylated with *O*-GlcNAc. But in succession of conversion a partly glycosylated and partly phosphorylated intermediate has to appear. Thus, *O*-GlcNAc may either mask or tag phosphorylation sites. Therefore, the covalent modifications with both *O*-GlcNAc and phosphate fulfill an decisive role in the expression of genes which are transcribed under control of RNA polymerase II.

To study the biological phenomena associated with such covalently modified protein conjugates appropriate tools are required. The application of characteristic partial structures of the parent proteins which embody both types of modifications and also underivatized hydroxyamino acids may open up new opportunities for bioorganic investigations (e.g., for the generation of monoclonal antibodies (*O*-GlcNAc modified peptides are highly antigenic))[3] or for inhibition and microinjection studies. Furthermore, the precise location of the glycosylation sites may be determined by comparison of glycosylated peptides obtained from natural *O*-GlcNAc-containing glycopeptides with synthetic analogs. However, the synthesis of phospho-[10] or glycopeptides[11] alone is severely complicated by their pronounced acid and base sensitivity as well as their multifunctionality. Thus, under weakly basic conditions (pH > 8) the phosphates and the glycosides may be lost by β-elimination reactions,[10,11] and at low pH an anomerization or a rupture of the *O*-glycosides may occur[11] (Scheme 2).

These properties make necessary the application of a variety of protecting groups that can be removed selectively under the mildest and preferably neutral conditions. In the construction of phosphoglycopeptides that embody both modifications, these difficulties are potentiated. Consequently, many of the classical chemical protecting groups can not be applied in the construction of phosphoglycopeptides, and to date their synthesis has not been described. Enzymatic protecting group techniques[12] offer viable alternatives to the established classical chemical methods. Thus, on the one hand enzymatic transformations can be carried out under characteristically mild reaction conditions (pH 6−8, room temperature). In addition, enzymes often combine a high specificity for the structures they recognize and the reactions they catalyze with a broad substrate tolerance. These properties have opened up new and advantageous routes to sensitive and multifunctional lipo-,[13] glyco-,[14] phospho-,[15] and nucleopeptides.[16] In this paper, we report that by means of a combination of enzyme labile and classical protecting groups phosphoglycopeptides can be built up efficiently.[17]

Results and Discussion

As a biologically relevant target compound, the hexapeptide **2** which carries one unmodified, one phosphorylated, and two glycosylated hydroxyamino acids (Scheme 3) was chosen. Compound **2** represents a characteristic partial structure of the repeat sequence **1** of the large subunit of RNA polymerase II which becomes glycosylated or phosphorylated while the enzyme carries out its biological functions (*vide supra*).

For the synthesis of **2**, the strategy delineated in Scheme 4 was chosen. The target compound was divided into a permanently masked N- and a C-terminal amino acid (**4** and **7**) and two temporarily protected dipeptides **5** and **6**. After the amino blocking groups were removed, the peptide chain was to be completed by stepwise N-terminal chain elongation. The protecting group strategy employed the use of *O*-acetates for the GlcNAc residues of the glycopeptide fragments **6** and **7**, since *O*-acetates can be cleaved from glycosylated peptides under weakly basic conditions without β-elimination or racemization of the amino acids.[18] In addition, this allowed for the

(10) (a) Perich, J. W. *Peptides and Protein Phosphorylation*; Kemp, B. E., Ed.; CRC Press: Boca Raton, FL, 1990. (b) Shapiro, G.; Buechler, D. *Tetrahedron Lett.* **1994**, *35*, 5421.

(11) (a) Kunz, H. *Angew. Chem.* **1987**, *99*, 297; *Angew. Chem., Int. Ed. Engl.* **1987**, *26*, 294. (b) Garg, H.; Jeanloz, R. W. *Adv. Carbohydr. Chem. Biochem.* **1985**, *43*, 135.

(12) For reviews, see: (a) Pohl, T.; Nägele, E.; Waldmann, H. *Catal. Today* **1994**, *22*, 407. (b) Drauz, K.; Waldmann, H. *Enzymes in Organic Synthesis - A Comprehensive Handbook*; VCH: Weinheim, 1995. (c) Waldmann, H.; Sebastian, D. *Chem. Rev.* **1994**, *94*, 911.

(13) (a) Waldmann, H.; Nägele, E. *Angew. Chem.* **1995**, *107*, 2425; *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2259. (b) Schelhaas, M.; Glomsda, S.; Häusler, M.; Jakubke, H.-D. *Angew. Chem.* **1996**, *108*, 82; *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 106. (c) Waldmann, H.; Nägele, E.; Schelhaas, M. *Bioorg. Med. Chem.* **1996**, *5*, 75.

(14) Braun, D.; Waldmann, H.; Kunz, H. *Bioorg. Med. Chem.* **1993**, *1*, 197.

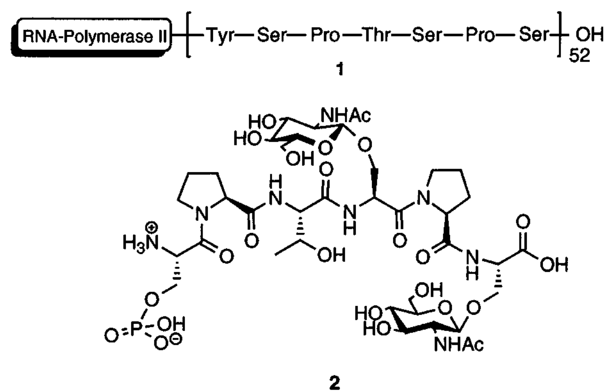
(15) (a) Waldmann, H.; Heuser, A.; Schulze, S. *Tetrahedron Lett.* **1996**, *37*, 8725. (b) Sebastian, D.; Waldmann, H. *Tetrahedron Lett.* **1997**, *38*, 2927.

(16) Gabold, S.; Waldmann, H. Submitted for publication.

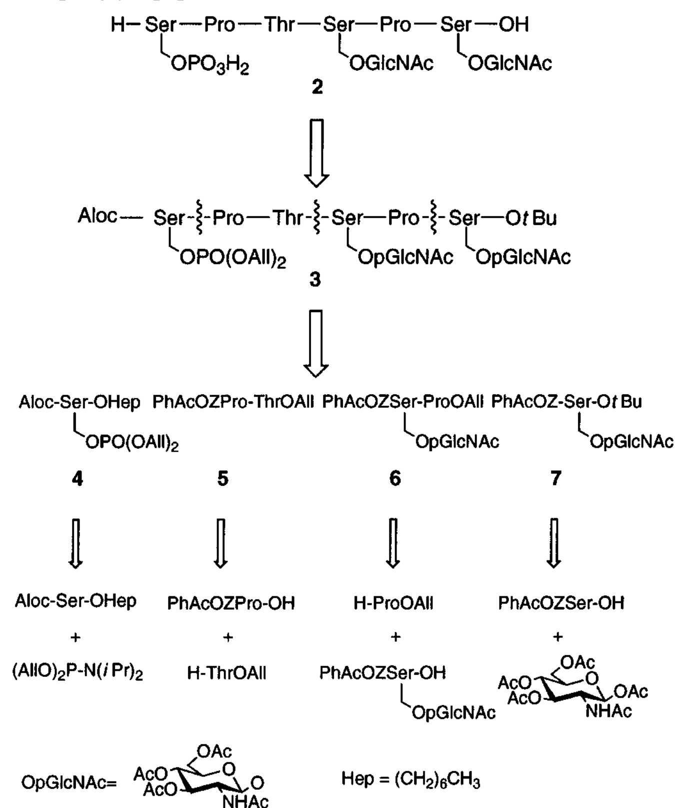
(17) Part of this work was published in preliminary form, see: Pohl, T.; Waldmann, H. *Angew. Chem.* **1996**, *108*, 1829; *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1720.

(18) Schultheiss-Reimann, P.; Kunz, H. *Angew. Chem.* **1983**, *95*, 64; *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 62.

Scheme 3. Schematic Representation of the Structure of RNA Polymerase II and the Structure of the Target Compound **2**



Scheme 4. Retrosynthetic Analysis of the Target Phosphoglycopeptide **2**



use of the *tert*-butyl ester as the permament C-terminal blocking function which had to be removed only after assembly of the entire peptide chain. Under the conditions necessary to split off this ester, acetyl-masked glycosides remain unattacked[11] and phosphopeptides[10] are not destroyed under acidic conditions either. For the masking of the phosphate and the permanent protection of the *N*-terminus in **4**, we chose allyl-based blocking groups which can be removed from phospho-[10] and glycopeptides[11] without undesired side reactions, too. Similarly, allyl esters were introduced as temporary carboxy blocking groups into the dipeptide intermediates **5** and **6**.

As a temporary N-terminal protecting group, an enzyme labile blocking function was needed. This protecting group had to meet several demands: (1) It had to be orthogonally stable to the base labile acetates, the acid labile *tert*-butyl ester, and the noble metal sensitive allyl groups. (2) It had to be removable under mild conditions to prevent β-elimination or anomerization. (3) It had to have a urethane structure to avoid racemization upon amino acid activation. (4) It had to be removable with a biocatalyst which does not attack the other functional groups present (i.e., the acetates, the allyl and the *tert*-butyl esters, the formed peptide bonds, and the phosphate and the glycosidic bonds).

In developing such a blocking group, we have drawn from our previous work on enzymatic protecting group techniques. In particular, we had devised a viable strategy for the development of enzyme labile urethane protecting groups. It consists in the use of a urethane that embodies (a) a functional group that is recognized by the biocatalyst and that is bound by an enzyme labile linkage to (b) a functional group that undergoes a spontaneous fragmentation upon cleavage of the enzyme-sensitive bond resulting in (c) the liberation of a carbamic acid derivative which decarboxylates to give the desired peptide or peptide conjugate **11** (Scheme 5).

Using this principle, the *p*-acetoxybenzyloxycarbonyl (AcOZ) group was developed whose removal could be initiated by the acetyl esterase- or lipase-catalyzed cleavage of an acetic acid ester.[13a] This urethane, however, could not be employed in the construction of **2**, since the carbohydrates were already masked as acetic acid esters which are attacked by acetyl esterase[12,19] and lipases.[12]

However, the above-mentioned principle for the development of enzyme labile urethanes is general and not restricted to a particular biocatalyst. Therefore, it could be adopted to the problem at hand by switching to a different enzyme and a different functional group recognized by it. To this end, we developed the *p*-(phenylacetoxy)benzyloxycarbonyl (PhAcOZ) group. It embodies a phenylacetic acid ester which is recognized by penicillin G acylase.[20] This biocatalyst operates under mild conditions, is highly specific for the phenylacetyl group and does not attack peptide-, phosphate- or glycosyl bonds at all. As in the case of the AcOZ group a *para*-hydroxybenzylurethane was employed as functional group that undergoes a spontaneous fragmentation reaction to a quinone methide and thereby liberates the desired amine (Scheme 5).

The PhAcOZ-protected dipeptide esters **17** were built up from amino acid esters **16** and PhAcOZ-protected amino acids **15** in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) as condensing reagents (Scheme 6). Neither by HPLC nor by NMR spectroscopy could a racemization of the activated amino acid in the formed peptides be detected. The introduction of the PhAcOZ group into the amino acids was achieved by means of *p*-(phenylacetoxy)benzyloxycarbonyl chloride **13** (PhAcOZCl). Compound **13** was obtained in high yield via acylation of *p*-hydroxybenzaldehyde, reduction of the aldehyde group in **12** to give the benzyl alcohol, and conversion to the chloroformate **13** (Scheme 6). The acylation of amino acids with **13** under the usual Schotten−Baumann conditions at pH 10−12 yielded the desired PhAcOZ amino acids only in low yield. Presumably the urethane which may be regarded as a phenyl ester (i.e., an activated ester) is cleaved under these alkaline conditions. However, upon treatment of *N,O*-bissilylated amino acids[21] **14** with the chloroformate **13**, the selectively protected amino acids **15** were obtained in satisfactory yields.

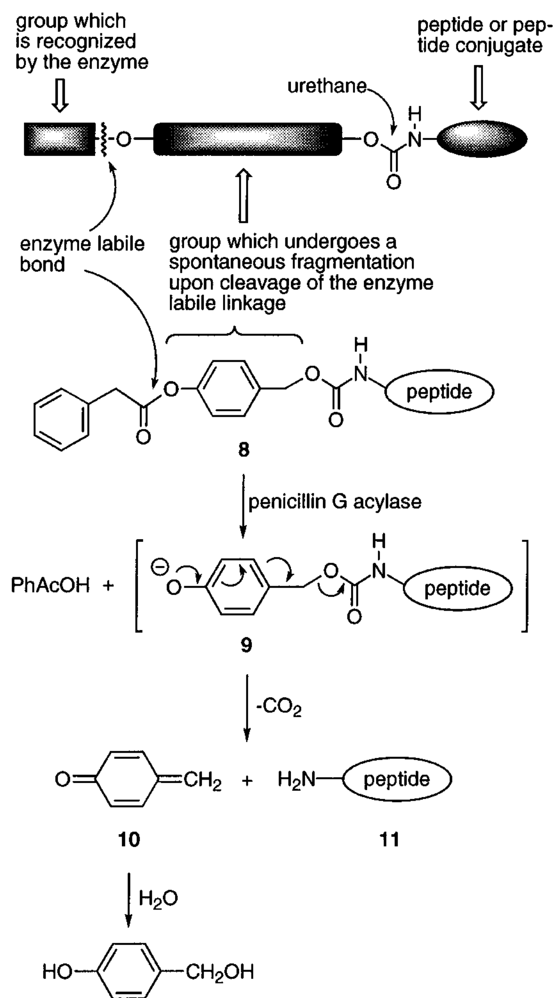
In order to determine if the PhAcOZ group can selectively be removed enzymatically, in initial experiments, the peptide allyl esters **17a** and **17b** were treated with penicillin acylase at

(19) (a) Waldmann, H.; Heuser, A.; Reidel, A. *Synlett* **1994**, 65. (b) Waldmann, H.; Heuser, A. *Bioorg. Med. Chem.* **1994**, *2*, 477.

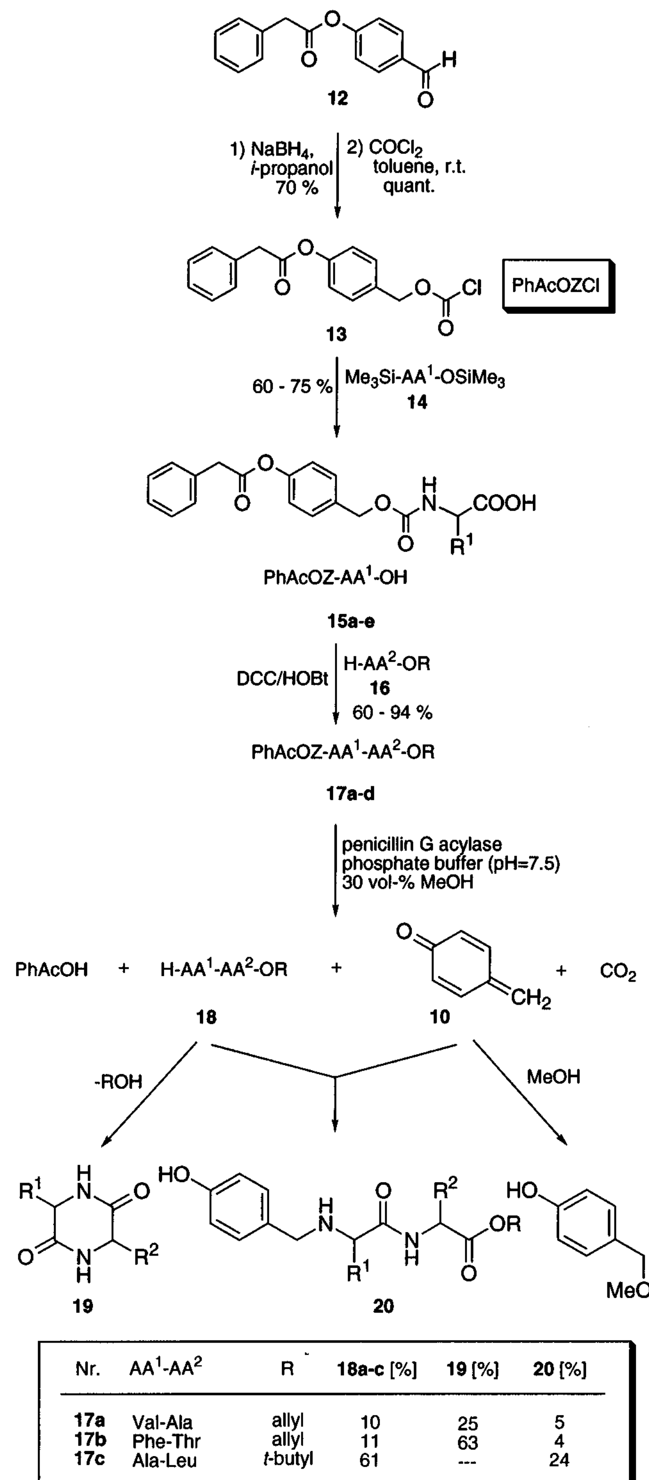
(20) (a) Pohl, T.; Waldmann, H. *Tetrahedron Lett.* **1995**, *36*, 2963 and references given therein. (b) Cole, M. *Biochem. J.* **1969**, *115*, 741, 747.

(21) Bolin, D. R.; Sytwu, I. I.; Humiec, F.; Meienhofer, J. *Int. J. Pept. Protein Res.* **1989**, *33*, 353 and references therein.

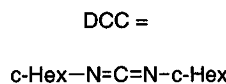
Scheme 5. Principle for the Development of Urethane Protecting Groups which Can Be Removed by an Enzyme-Initiated Fragmentation

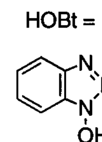


Scheme 6. Synthesis and N-Terminal Deprotection of PhAcOZ-Protected Peptides



Nr.	AA^1-AA^2	R	18a-c [%]	19 [%]	20 [%]
17a	Val-Ala	allyl	10	25	5
17b	Phe-Thr	allyl	11	63	4
17c	Ala-Leu	*t*-butyl	61	---	24



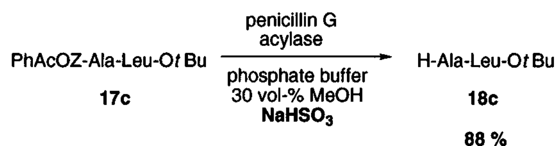


pH 7.5. In the course of the ensuing reaction, the phenylacetates were smoothly hydrolyzed to give the phenolates corresponding to **9** (Scheme 5). These intermediates underwent a spontaneous fragmentation and liberated the deprotected esters **18**, phenylacetic acid, and the quinone methide **10**. However, due to competing side reactions, the yield of **18a** and **18b** was only low. On one hand, the dipeptide esters cyclized to the diketopiperazines **19** under the reaction conditions, a well-known side reaction in peptide chemistry displayed by N-terminally unmasked dipeptide esters. On the other hand, the amino group of the peptides competed with the solvent for the electrophile **10** to give the *N*-alkylated dipeptide esters **20**. Diketopiperazine formation was overcome by using the *tert*-butyl esters which are not prone to this cyclization instead of the allyl esters. The formation of the *N*-alkylated peptides **20** was efficiently suppressed by performing the enzymatic reactions in the presence of suitable nucleophiles,[13a,22] which trap the quinone methide **11** faster than the amines **18**. Thus, if the deprotection of **17c** was carried out in the presence of an excess of $NaHSO_3$ at pH 7.5, no undesired addition product was observed and the dipeptide *tert*-butyl ester **18c** was isolated in 88% yield (Scheme 7). Alternatively, KI which was the nucleophile of choice in the enzymatic removal of the AcOZ protecting group could be employed, too, however with inferior results. To guarantee that the PhAcOZ-protected substrates are soluble under the reaction conditions and thereby accessible to the biocatalyst, the enzymatic transformations were most advantageously carried out in the presence of 30 vol % of methanol as cosolvent.

Under these optimized reaction conditions, a fast enzymatic reaction was assured and no undesired side reactions were

(22) Le Corré, G.; Guibé-Jampel, E.; Wakselman, M. *Tetrahedron* **1978**, *34*, 3105.

Scheme 7. Optimized Reaction Conditions for the Enzymatic Removal of the PhAcOZ Group



observed. The selectivity of penicillin acylase for phenylacetic acid derivatives guarantees that exclusively the PhAcOZ group is cleaved and that the C-terminal esters and the peptide bonds are left intact.

Two advantageous features of the PhAcOZ-protecting function have to be emphasized: The enzyme used recognizes the same structural element in all cases. The variable peptide part of the substrates is remote from the site of the biocatalyst's attack. Thus, possible sterically or electronically unfavorable interactions of the protein with the substrates caused by bulky amino acid side chains are guaranteed not to limit the substrate tolerance of the enzyme. Furthermore, this enzymatic protecting group technique can be applied for the construction of peptides and analogs thereof containing, for instance, unnatural amino acids including D-configurated amino acids.

For the selective deprotection of the intermediates **5** and **6**, orthogonal stability of the PhAcOZ group to the allyl ester was required (Scheme 4). This was demonstrated by selective removal of the allyl ester from the dipeptide PhAcOZ-Pro-ThrOAll **17d** by treatment with $(PPh_3)_4Pd(0)$ in the presence of morpholine as accepting nucleophile.[23] Thereby, the desired peptide carboxylic acid PhAcOZ-Pro-ThrOH was obtained without attack on the N-terminal urethane group in 93% yield (see the Experimental Section).

The PhAcOZ group thus proved to be an enzyme labile urethane blocking group which is orthogonally stable to the other protecting groups chosen and which ensures deprotection under the mildest conditions. With this synthetic tool in hand, the construction of the acid and base labile complex phosphoglycopeptide **2** following the strategy detailed above (Scheme 4) was approached.[24] The fully protected C-terminal tripeptide **27** was synthesized via the building blocks **6** and **7**. To this end, the PhAcOZ-protected serine glycoside **22** was built up in 54% yield by glycosylation of PhAcOZ serine **15e** with the oxazoline **21**[25] (Scheme 8). The C-terminally unmasked serine derivative **22** was then condensed with proline allyl ester **23** in high yield to give **6**. In addition, it was converted into the *tert*-butyl ester **7** by employing the *tert*-butyl isourea **24**.[26] The glycosylated dipeptide allyl ester **6** was then selectively deprotected at the C-terminus by palladium(0)-mediated allyl transfer to morpholine as the accepting nucleophile.[27] From the PhAcOZ-masked glycosylated amino acid ester **7**, the N-terminal protecting group was removed by treatment with penicillin G acylase under the reaction conditions described above to give the N-terminally deprotected serine derivative **26** in high yield. In the course of the enzymatic transformation, no undesired side reaction occurred. Neither an anomerization or a rupture of the glycosidic bond nor an attack on the *O*-acetates could be observed. The selectively deprotected fragments **25** and **26** were then condensed to give the fully protected diglycotripeptide **27**.

(23) Friedrich-Bochnitschek, S.; Waldmann, H.; Kunz, H. *J. Org. Chem.* **1989**, *54*, 751.

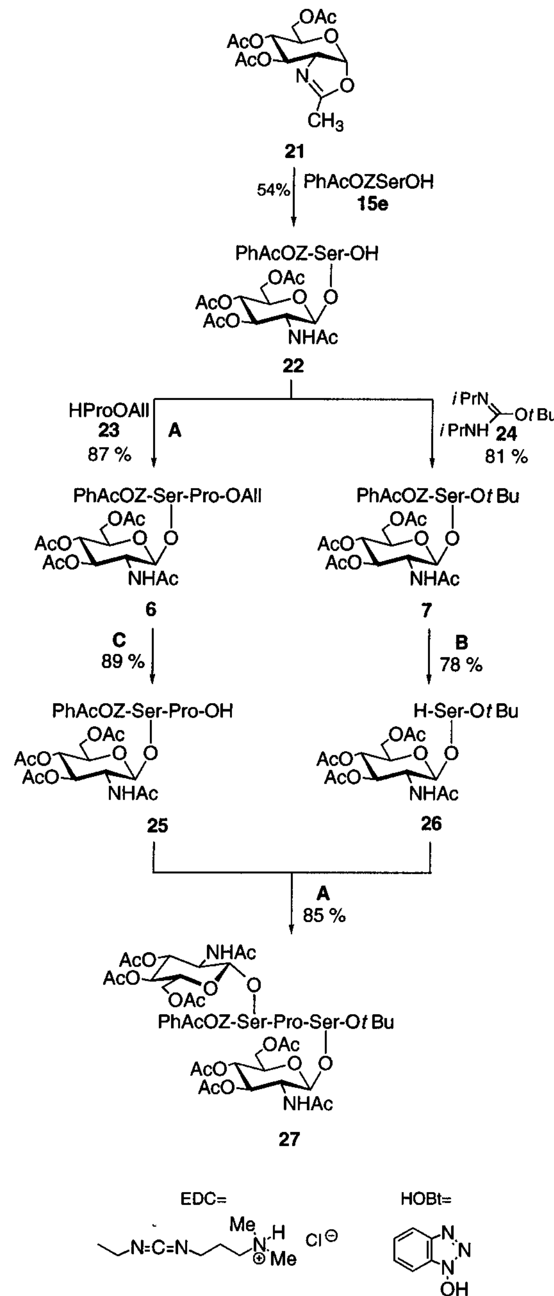
(24) For a synthesis of a glycosylated analog to **2**, see: Meinjohanns, E.; Vargas-Berenguel, A.; Meldal, M.; Paulsen, H.; Bock, K. *J. Chem. Soc., Perkin Trans. 1* **1995**, 2165.

(25) Arsequell, G.; Krippner, L.; Dwek, R. A.; Wong, S. Y. C. *J. Chem. Soc., Chem. Commun.* **1994**, 2383.

(26) Mathias, L. *Synthesis* **1979**, 561.

(27) Waldmann, H. *Liebigs Ann. Chem.* **1988**, 1179 and references given therein.

Scheme 8. Synthesis of the Diglycotripeptide **27**[a]

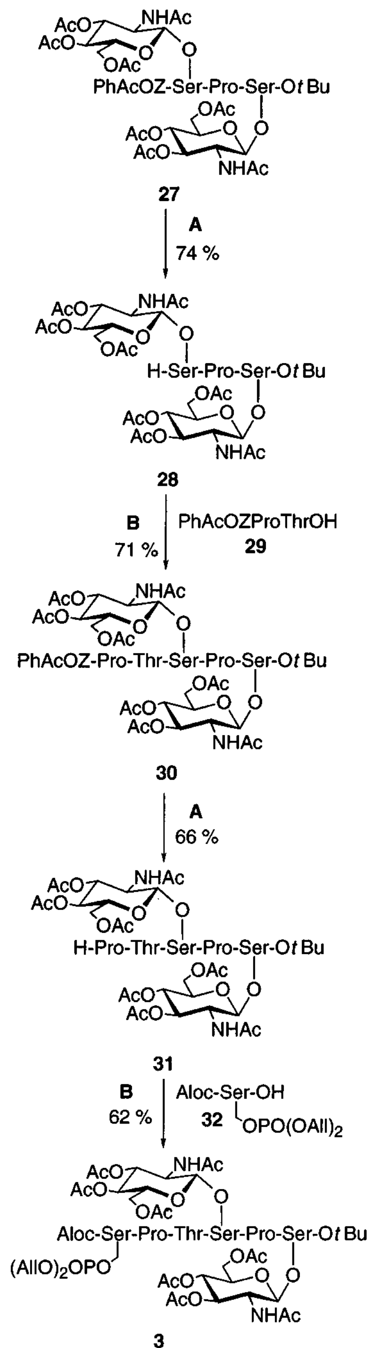


[a] Conditions: (A) EDC/HOBt (2 equiv/2 equiv); (B) penicillin G acylase, 0.07 M phosphate buffer [30 vol % methanol, $NaHSO_3$ (500 equiv), pH 7.5, 25 °C]; (C) $[(C_6H_5)_3P]_4Pd$, morpholine (1.1 equiv).

In order to elongate the peptide chain in the N-terminal direction, **27** was deprotected by means of the enzyme-initiated removal of the PhAcOZ-protected urethane under the mildest conditions (Scheme 9). Thereby, the selectively unmasked diglycotripeptide **28** was obtained in high yield and again without disturbing side reactions. It was then condensed in high yield with the dipeptide carboxylic acid **29** to give the diglycopeptide **30**, which contains two glycosylated and one unmodified hydroxyamino acid. Compound **29** was obtained by cleavage of the respective allyl ester (*vide supra*; see the Experimental Section).

From the glycosylated pentapeptide, the N-terminal PhAcOZ urethane was cleaved off by employing once more penicillin G acylase. The enzyme-initiated fragmentation proceeded smoothly, and the desired N-terminally unmasked glycopeptide **31** was formed in high yield. We stress that the acylase is capable of removing the PhAcOZ group from an N-terminal proline. If

Scheme 9. Synthesis of the Protected Phosphoglycohexapeptide **3**[a]

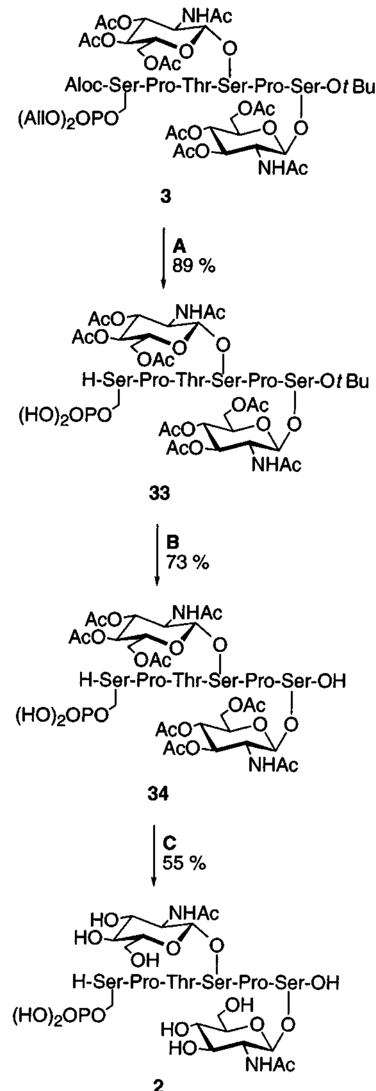


[a] Conditions: (A) penicillin G acylase, 0.07 M phosphate buffer (30 vol % methanol), $NaHSO_3$ (500 equiv), pH 7.5, 25 °C; (B) EDC/HOBt (2 equiv/2 equiv).

the phenylacetamide is employed instead, the respective peptides are not attacked by penicillin acylase.[20,27] This finding highlights a particularly important advantage of the 4-acyloxybenzyloxy-type blocking functions. The biocatalyst always has to recognize and attack the same functional group (here a phenylacetate), which is distant to the variable part of the substrates to be deprotected (here the peptide chain). Therefore, the introduction of sterically demanding or otherwise unfavorable structures (here a cyclic *N*-alkylamino acid) only has a subordinate (if any) influence on the desired enzymatic transformation.

The assembly of the peptide chain was completed by coupling of the selectively deprotected diglycopentapeptide **31** with the phosphorylated serine derivative **32** to give the complex phosphoglycopeptide **3**. The serine phosphate **32** was obtained from the respective heptyl ester **5** (Scheme 4) by lipase-mediated saponification of the ester group.[15b] Thereby, a second enzymatic protecting group technique could advantageously be employed for the construction of the RNA polymerase derived target compound.

Scheme 10. Synthesis of the Phosphoglycohexapeptide **2**[a]



[a] Conditions: (A) $[(C_6H_5)_3P]_4Pd$, formic acid/*n*-butylamine (10 equiv/6 equiv), room temperature (rt); (B) TFA, rt; (C) hydrazine hydrate (3000 equiv), methanol, rt.

Finally, all blocking functions were removed from the fully masked phosphoglycohexapeptide **3** (Scheme 10). To this end, first the N-terminal Aloc group and the allyl phosphate protecting functions were cleaved by treatment with a palladium(0) catalyst and formic acid/*n*-butylamine as recommended by Noyori et al.[28] These weakly acidic conditions were chosen to avoid any base-mediated β-elimination, in particular, of the phosphate but also of the carbohydrates. Thus, the partially deprotected hexapeptide **33** was obtained in high yield without undesired side reaction. Next, the C-terminal *tert*-butyl ester was cleaved selectively by treatment of **33** with trifluoroacetic acid. Under these conditions an anomerization or rupture of the glycosides did not occur. Finally, all *O*-acetates were removed from **34** by means of hydrazine-mediated transesterification of the acetic acid esters with methanol.[18]

(28) Hayakawa, Y.; Kato, H.; Uchiyama, M.; Kajino, H.; Noyori, R. *J. Org. Chem.* **1986**, *51*, 2402.

In this final deprotection sequence, the order in which the different blocking groups were removed had to be chosen carefully. Under the basic conditions necessary to split off the carbohydrate ester protecting groups, the diallyl phosphate would most probably have been lost by β-elimination. Therefore, the N-terminal Aloc group and the allyl phosphates were cleaved first. The resulting phosphoric acid monoester is deprotonated under basic conditions and, therefore, no longer prone to base-induced elimination. The second step had to be the cleavage of the *tert*-butyl ester under acidic conditions in the presence of peracetylated GlcNAc residues. Glycosidic bonds to acetylated carbohydrates are significantly more acid stable than, e.g., bonds to benzylated or deprotected sugars.[11] Thus, by performing the C-terminal deprotection as the second step a possible anomerization or cleavage of the *O*-glycosides was prevented.

Conclusion

We have developed an efficient chemoenzymatic strategy for the synthesis of differently functionalized complex peptide conjugates. Its usefulness was demonstrated by the first construction of the sensitive phosphoglycohexapeptide **2** which represents a characteristic partial structure of the C-terminal repeating unit of the large subunit of mammalian RNA polymerase II. In all enzymatic and nonenzymatic transformations described above, no undesired side reactions were observed. Thus, the peptide backbone and the glycosidic bonds of the carbohydrates as well as the phosphate remained intact. In addition, an anomerization of the glycosidic bonds which may occur under acidic conditions was not observed. Also a base-mediated β-elimination of the phosphate or the carbohydrates could not be detected. When enzymatic and classical chemical protecting group techniques are combined in the way described above or in similar ways, further sensitive *O*-phosphorylated and *O*-glycosylated peptide conjugates will be accessible. The use of these compounds may open up new avenues of research in bioorganic chemistry and biology.

Experimental Section

General Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 250, AM 400, and DRX-500 spectrometer. Mass spectra were measured on a Finnigan MAT MS 70 spectrometer. Analytical chromatography was performed on E. Merck silica gel 60 F_{254} plates. Flash chromatography was performed on Baker silica gel (40–64 μm). Tetrahydrofuran was distilled from potassium metal, dichloromethane was from lithium aluminium hydride, and toluene was from sodium. Penicillin G acylase was obtained in immobilized form on Eupergit C from Boehringer Mannheim.

4-(Phenylacetoxy)benzyl Chloroformate (13). A solution of 4-(phenylacetoxy)benzaldehyde (**12**) (4.3 g, 18 mmol) in 75 mL of 2-propanol was treated with sodium borohydride (0.225 g, 6 mmol) suspended in 25 mL of 2-propanol. The reaction mixture was periodically monitored by TLC. After the conversion was complete, the reaction was quenched by addition of HCl (0.5 N), the pH was adjusted to 7 with Na_2CO_3 solution, and the 2-propanol was removed under reduced pressure. After extraction with ethyl acetate, the organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. Purification of the residue by flash chromatography (silica gel; ethyl acetate/hexane 1:1) yielded 3 g (70%) of 4-(phenylacetoxy)benzyl alcohol as a white solid: mp 46 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.42–7.23 (m, 7H), 7.03 (d, J = 8.5 Hz, 2H), 4.68 (s, 2H), 3.89 (s, 2H), 1.81 (br, 1H); ^{13}C NMR (62.8 MHz, $CDCl_3$) δ 169.9, 149.9, 138.3, 133.2, 129.1, 128.5, 127.8, 127.2, 121.4, 64.5, 41.4. Anal. Calcd: C, 74.35; H, 5.83. Found: C, 74.34; H, 5.69.

A solution of 4-(phenylacetoxy)benzyl alcohol (1.24 g, 5 mmol) in 10 mL of toluene was added at 0 °C to a solution of phosgene in 5.3 mL of toluene (1.93 M). After 2 h at 0 °C, the mixture was stirred for 1 h at room temperature and the toluene was removed under reduced pressure to yield 1.5 g (99%) of **13** as a colorless oil: ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.43–7.22 (m, 7H), 7.11 (d, J = 8.5 Hz, 2H), 5.28 (s, 2H), 3.88 (s, 2H); ^{13}C NMR (62.8 MHz, $CDCl_3$) δ 169.8, 151.4, 150.7, 133.2, 130.9, 130.3, 129.3, 128.8, 127.5, 122.0, 72.6, 41.4; MS *m/e* calcd for (M^+) $C_{16}H_{13}O_4Cl$ 304.050, found 304.051.

Synthesis of PhAcOZ-Protected Amino Acids 15. To a vigorously stirred suspension of the amino acid (7.5 mmol) in 25 mL of CH_2Cl_2 was added trimethylsilyl chloride (TMS-Cl) (1.63 g, 15 mmol) in one portion. The mixture was then refluxed for 1 h and cooled to 0 °C, and diisopropylethylamine (1.68 g, 13 mmol) and chloroformate **13** (1.5 g, 5 mmol) were added. The reaction mixture was stirred at 0 °C for 30 min and at room temperature for 2 h. The solvent was removed *in vacuo*, and the residue was taken up in 25 mL of Et_2O and 50 mL of a $NaHCO_3$ solution (0.5 N). The layers were separated, and the aqueous layer was extracted with 10 mL of Et_2O. The combined organic layers were washed with 10 mL of H_2O. The pH of the combined aqueous layers was adjusted to pH 2 with HCl (0.5 N), and the aqueous solution was extracted with ethyl acetate. The combined organic layers were then dried with $MgSO_4$ and concentrated to give the desired amino acids.

***N*-(4-(Phenylacetoxy)benzyloxycarbonyl)-L-alanine (15a).** Data for **15a**: yield 75%; colorless solid, mp 91 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 10.27 (br, 1H), 7.41–7.21 (m, 7H), 7.04 (d, J = 8.5 Hz, 2H), 5.43 (d, J = 7 Hz, 1H), 5.08 (s, 2H), 4.40 (m, 1H), 3.83 (s, 2H), 1.41 (d, J = 7 Hz, 3H); ^{13}C NMR (62.8 MHz, $CDCl_3$) δ 177.5, 170.1, 155.7, 150.5, 133.8, 133.3, 129.3, 129.2, 128.7, 127.4, 121.6, 66.4, 49.5, 41.0, 18.3; $[\alpha]_D^{20}$ 6.7 (c = 1, CH_2Cl_2); MS *m/e* calcd for (M^+) $C_{19}H_{19}NO_6$ 357.121, found 357.118. Anal. Calcd: C, 63.84; H, 5.36; N, 3.92. Found: C, 63.82; H, 5.34; N, 3.88.

***N*-(4-(Phenylacetoxy)benzyloxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-L-serine (22).** To a mixture of peracetylated GlcNAc[24] (480 mg, 1.23 mmol), 4A molecular sieves, and CH_2Cl_2 (8 mL) was added $BF_3{\cdot}Et_2O$ (0.4 mL, 3.8 mmol) dropwise at 0 °C (freshly distilled under argon). After 24 h of stirring at room temperature, the formation of the oxazoline **21** was complete as monitored by TLC ($CHCl_3$/MeOH 10:1). Then, Et_3N (0.16 mL, 1.15 mmol) was added at 0 °C, the reaction mixture was stirred for 10 min, and a solution of PhAcOZ-Ser (**15e**, 465 mg, 1.25 mmol) in 12 mL of CH_2Cl_2/CH_3CN (2:1) was added. After 4–5 days at room temperature, the reaction was complete (periodic monitoring by TLC (CH_3Cl/MeOH/AcOH 80:10:1)). The mixture was neutralized at 0 °C with Et_3N, diluted with CH_2Cl_2, and filtered through Celite. The filtrate was concentrated *in vacuo*, and the residue was purified by column chromatography (silica gel, $CHCl_3$/MeOH 20:1 to 5:1) to yield 467 mg (54%) of **22** as a colorless solid: mp 87 °C; ^{1}H NMR (250 MHz, CD_3OD) δ 7.43–7.23 (m, 7H), 7.06 (d, J = 8.6 Hz, 2H), 5.21 (dd, $J_{2,3}$ = $J_{3,4}$ = 10 Hz, 1H), 5.07 (s, 2H), 4.94 (dd, $J_{3,4}$ = $J_{4,5}$ = 10 Hz, 1H), 4.64 (d, $J_{1,2}$ = 8 Hz, 1H), 4.24 (m, 2H), 4.11 (m, 2H), 3.89 (s, 2H), 3.88–3.69 (m, 3H), 2.03 (s, 3H), 2.00 (s, 3H), 1.96 (s, 3H), 1.83 (s, 3H); ^{13}C NMR (62.8 MHz, CD_3OD) δ 174.6, 172.9, 171.4, 170.9, 170.8, 170.3, 157.0, 151.0, 134.9, 134.2, 129.5, 129.3, 128.7, 127.3, 121.7, 101.0, 72.9, 71.9, 69.1, 70.0, 66.0, 62.2, 55.8, 54.3, 40.8, 21.9, 19.7, 19.7, 19.6; $[\alpha]_D^{20}$ −17.7 (c = 2, CH_3OH); FAB MS *m/e* calcd for (M^+ + 1) $C_{33}H_{38}N_2O_{15}$ 703.235, found 703.237.

Peptide Couplings to Give PhAcOZ Protected Peptides. (A) *N*-(4-(Phenylacetoxy)benzyloxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-L-serine, *tert*-Butyl Ester (7). A solution of peptide **22** (71 mg, 0.1 mmol) and *N,N′*-diisopropyl-*O*-*tert*-butylisourea (**24**) (100 mg, 0.5 mmol) in 20 mL of CH_2Cl_2 was stirred for 3 days at room temperature. After filtration to remove precipitated urea, the CH_2Cl_2 solution was extracted with phosphate buffer (pH 5), the organic layer was dried ($MgSO_4$), and CH_2Cl_2 was removed under reduced pressure. Purification of the crude residue by flash chromatography (silica gel; ethyl acetate/hexane 4:1) yielded 43 mg (56%) of **7** as a colorless solid: mp 93 °C; ^{1}H NMR (250 MHz, CD_3OD) δ 7.43–7.24 (m, 7H), 7.06 (d, J = 8.5 Hz, 2H), 5.19 (dd, $J_{2,3}$ = $J_{3,4}$ = 10 Hz, 1H), 5.06 (s, 2H), 4.94 (dd, $J_{3,4}$ = $J_{4,5}$ = 10 Hz, 1H), 4.66 (d, $J_{1,2}$ = 8 Hz, 1H), 4.39–4.07 (m, 4H), 3.89 (s, 2H), 3.88–3.67 (m, 3H), 2.03 (s, 3H), 2.01 (s, 3H), 1.96 (s, 3H), 1.85 (s, 3H), 1.47 (s, 9H); ^{13}C NMR (62.8 MHz, CD_3OD) δ 171.8, 170.9, 170.5, 170.2, 169.6, 168.5, 156.2, 150.2, 133.8, 133.0, 128.9, 128.4, 128.2, 127.0, 121.2, 99.9, 82.2, 72.2, 71.3, 68.4, 68.6, 65.8, 61.8, 54.5, 53.8, 40.8, 27.3,

22.3, 20.5, 19.9, 19.9; $[\alpha]_D^{20}$ −13.6 (c = 1, CH_3OH). Anal. Calcd: C, 58.55; H, 6.11; N, 3.69. Found: C, 58.37; H, 6.12; N, 3.44.

(B) Peptides 17a−d, 6, 27, 30, and 3. To a solution of PhAcOZ-protected amino acid, dipeptide, or serine derivative **32** (0.25 mmol) and the relevant amino acid ester or peptide *tert*-butyl ester in 5 mL of CH_2Cl_2 was added *N*-hydroxybenzotriazole (67.5 mg, 0.5 mmol) and at 0 °C the carbodiimide DCC or *N*-ethyl-*N′*-((dimethylamino)propyl)-carbodiimide hydrochloride (EDC) (0.5 mmol). After 2 h of stirring at this temperature, the mixture was left overnight at room temperature, the urea was filtered, and the solution was extracted with 3 mL of H_2O (pH 4). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude residue was purified by flash chromatography (silica gel; $CHCl_3/CH_3OH$). Data for *N*-(4-(phenylacetoxy)-benzyloxycarbonyl)-L-valyl-L-alanine, allyl ester (**17a**): yield 88%, colorless solid, mp 119 °C; ^1H NMR (250 MHz, $CDCl_3$) δ 7.41−7.27 (m, 7H), 7.04 (d, J = 8 Hz, 2H), 6.52 (d, J = 7 Hz, 1H), 5.99−5.83 (m, 1H), 5.46 (d, J = 8 Hz, 1H), 5.31 (dd, J_{trans} = 17 Hz, J_{gem} = 1.5 Hz, 1H), 5.24 (dd, J_{cis} = 10 Hz, J_{gem} = 1.5 Hz, 1H), 5.07 (s, 2H), 4.68−4.54 (m, 3H), 4.05 (m, 1H), 3.87 (s, 2H), 2.11 (m, 1H), 1.39 (d, J = 6 Hz), 0.96 (d, J = 7 Hz, 3H), 0.89 (d, J = 7 Hz, 3H); ^{13}C NMR (62.8 MHz, $CDCl_3$) δ 172.3, 170.7, 169.9, 156.2, 150.5, 133.9, 133.3, 131.4, 129.3, 129.2, 128.7, 127.3, 121.5, 118.8, 66.3, 66.0, 60.1, 48.0, 41.3, 31.3, 19.1, 18.2, 17.7; $[\alpha]_D^{20}$ −3.5 (c = 1, CH_2Cl_2). Anal. Calcd: C, 65.29; H, 6.50; N, 5.64. Found: C, 65.19; H, 6.66; N, 5.82. Data for *N*-(4-(phenylacetoxy)benzyloxycarbonyl)-L-prolyl-L-threonyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-L-seryl-L-prolyl-*O*-(2′-acetamido-3′,4′,6′-tri-*O*-acetyl-2′-deoxy-β-D-glucopyranosyl)-L-serine, *tert*-butyl ester (**30**): yield 71%, colorless solid, mp 112−116 °C; ^1H NMR (500 MHz, CD_3OD) δ 7.45−7.26 (m, 7H), 7.06 (d, J = 8.5 Hz, 2H), 5.19 (dd, $J_{2,3} = J_{3,4} = J_{2',3'} = J_{3',4'}$ = 9 Hz, 2H), 5.09 (s, 2H), 5.01 (dd, $J_{3,4} = J_{4,5} = J_{3',4'} = J_{4',5'}$ = 9 Hz, 2H), 4.73 (d, $J_{1,2}$ = 8.5 Hz, 1H), 4.69 (d, $J_{1',2'}$ = 8.5 Hz, 1H), 4.66 (t, J = 6.5 Hz, 1H), 4.57 (d, J = 6.5 Hz, 1H), 4.56−4.44 (m, 2H), 4.38−4.20 (m, 5H), 4.17−4.04 (m, 2H), 4.01−3.48 (m, 13H), 2.33−2.22 (m, 2H), 2.16−1.87 (m, 30H), 1.48 (s, 9H), 1.19, 1.06 (d, J = 6 Hz, 3H, 2 rotamers); ^{13}C NMR (125.7 MHz, CD_3OD) δ 173.8, 173.7, 173.6, 173.3, 172.9, 172.3, 172.2, 172.0, 171.8, 171.7, 171.2, 171.1, 170.5, 169.7, 157.2, 152.0, 135.6, 135.0, 130.4, 130.0, 129.6, 128.3, 122.7, 101.4, 101.1, 83.5, 74.2, 74.1, 73.1, 72.9, 70.0, 69.6, 71.7, 71.5, 68.8, 68.6, 63.1, 62.8, 61.7, 61.6, 59.6, 55.3, 55.1, 54.4, 54.2, 48.2, 47.7, 41.8, 30.6, 30.5, 28.3, 26.0, 25.5, 23.1, 23.0, 20.8, 20.8, 20.7, 20.7, 20.6, 20.6, 20.2; $[\alpha]_D^{20}$ −54.7 (c = 1, CH_3OH). Anal. Calcd: C, 55.53; H, 6.24; N, 6.67. Found: C, 55.34; H, 6.20; N, 6.77. Data for *N*-allyloxycarbonyl-*O*-(diallylphosphato)-L-seryl-L-prolyl-L-threonyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-L-seryl-L-prolyl-*O*-(2′-acetamido-3′,4′,6′-tri-*O*-acetyl-2′-deoxy-β-L-glucopyranosyl)-L-serine, *tert*-butyl ester (**3**): yield 62%, colorless amorphous solid; ^1H NMR (400 MHz, CD_3OD) δ 6.06−5.88 (m, 3H), 5.43−5.08 (m, 8H), 5.01 (dd, $J_{3,4} = J_{4,5} = J_{3',4'} = J_{4',5'}$ = 9 Hz, 2H), 4.78 (d, $J_{1,2}$ = 8.5 Hz, 1H), 4.73 (d, $J_{1',2'}$ = 8.5 Hz, 1H), 4.71 (t, J = 6 Hz, 1H), 4.64−4.43 (m, 10H), 4.41−4.26 (m, 5H), 4.23−4.07 (m, 5H), 4.02−3.77 (m, 8H), 3.72−3.61 (m, 2H), 2.40−2.19 (m, 2H), 2.16−1.85 (m, 30H), 1.46 (s, 9H), 1.19, 1.14 (d, J = 6 Hz, 3H, 2 rotamers); ^{13}C NMR (125.7 MHz, CD_3OD) δ 173.8, 173.6, 173.3, 172.3, 172.2, 171.9, 171.8, 171.6, 171.5, 171.1, 171.1, 170.0, 169.8, 169.6, 155.7, 133.2, 132.8, 132.7, 118.1, 117.9, 117.6, 100.7, 100.2, 83.7, 74.7, 74.5, 73.1, 72.7, 71.0, 70.9, 69.9, 69.8, 68.9, 68.1, 66.6, 66.5, 66.3, 62.7, 62.6, 61.0, 60.6, 60.1, 54.4, 54.3, 54.0, 53.9, 52.1, 47.9, 47.8, 30.1, 29.9, 28.0, 25.2, 25.0, 23.1, 23.0, 20.5, 20.5, 20.5, 20.4, 20.4, 20.4, 20.2; $[\alpha]_D^{20}$ −65.4 (c = 1, CH_2OH); FAB MS *m/e* calcd for (M^+ + 1) $C_{65}H_{97}N_8O_{32}P$ 1533.6025, found 1533.768.

Enzymatic Removal of the PhAcOZ Group from Peptides and Glycopeptides. Peptides 18c, 26, 28, and 31. A solution of the respective PhAcOZ-protected peptide (0.05 mmol) in a mixture of 33 mL of CH_3OH, 13 mL of a $NaHSO_3$ solution (40%), and 68 mL of a Na_2HPO_4 buffer (0.07 M) was treated with penicillin G acylase (300 units) at pH 7.5 for 24 h at room temperature. The immobilized enzyme was filtered, and the CH_3OH was removed under reduced pressure. The reaction mixture was extracted three times with $CHCl_3$, and the combined organic layers were dried ($MgSO_4$) and concentrated to dryness. The crude residue was purified by flash chromatography (silica gel, $CHCl_3/CH_3OH$ and 1 vol % Et_3N). Data for *O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-L-serine, *tert*-butyl ester (**26**): yield 78%, colorless oil; ^1H NMR (250 MHz, $CDCl_3$) δ 6.46 (d, J = 8 Hz, 1H), 5.28 (dd, $J_{2,3} = J_{3,4}$ = 10 Hz, 1H), 5.08 (dd, $J_{3,4} = J_{4,5}$ = 10 Hz, 1H), 4.78 (d, $J_{1,2}$ = 8 Hz, 1H), 4.31−4.06 (m, 3H), 3.93−3.68 (m, 3H), 3.58 (t, J = 4 Hz, 1H) 2.08 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.97 (s, 3H), 1.46 (s, 9H); ^{13}C NMR (62.8 MHz, $CDCl_3$) δ 171.7, 170.9, 170.7, 170.6, 169.4, 100.8, 81.9, 72.6, 71.8, 68.6, 71.3, 62.1, 55.0, 54.6, 27.9, 23.5, 23.3, 20.7, 20.6; $[\alpha]_D^{20}$ −29.8 (c = 1, CH_2OH); FAB MS *m/e* calcd for (M^+ + 1) $C_{21}H_{34}N_2O_{11}$ 491.224, found 491.232.

Pd(0)-Mediated Cleavage of the Allyl Esters. Peptides 25 and 29. To a solution of the dipeptide allyl ester (0.23 mmol) and 2 mol% $(Ph_3P)_4Pd$ in 20 mL of THF under an argon atmosphere was added dropwise a solution of morpholine (21 mg, 0.24 mmol) in 5 mL of THF. The mixture was stirred for 30 min, the solvent was removed under reduced pressure and the residue purified by flash chromatography (silica gel; ethyl acetate/hexane 3:1 then $CHCl_3/CH_3OH$ 10:1 to 5:1). Data for *N*-(4-(phenylacetoxy)benzyloxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-L-seryl-L-proline (**25**): yield 89%, colorless solid, mp 138 °C; ^1H NMR (250 MHz, CD_3OD) δ 7.42−7.22 (m, 7H), 7.03 (d, J = 8.5 Hz, 2H), 5.22 (dd, $J_{2,3} = J_{3,4}$ = 10 Hz, 1H), 5.08 (s, 2H), 5.04 (dd, $J_{3,4} = J_{4,5}$ = 10 Hz, 1H), 4.78−4.45 (m, 2H, 1H), 4.29 (m, 2H), 4.06 (m, 1H) 4.04−3.38 (m, 8H), 2.20 (m, 1H), 2.13−1.92 (m, 3H), 2.01 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.87 (s, 3H); ^{13}C NMR (62.8 MHz, CD_3OD) δ 173.7, 172.4, 171.9, 171.3, 170.6, 170.0, 169.5, 158.1, 152.1, 135.9, 135.2, 130.5, 130.2, 129.7, 128.3, 122.7, 101.7, 74.3, 73.0, 70.1, 69.5, 67.2, 63.2, 62.2, 55.4, 53.9, 49.3, 41.9, 30.5, 25.8, 23.0, 20.7, 20.7, 20.6; $[\alpha]_D^{20}$ −65.8 (c = 1, CH_3OH); FAB MS *m/e* calcd for (M^+ + 1) $C_{38}H_{45}N_3O_{16}$ 800.287, found 800.281.

Final Removal of All Protecting Groups. (A) *O*-Phosphato-L-seryl-L-prolyl-L-threonyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-L-seryl-L-prolyl-*O*-(2′-acetamido-3′,4′,6′-tri-*O*-acetyl-2′-deoxy-β-D-glucopyranosyl)-L-serine, *tert*-Butyl Ester (33). To a solution of peptide **3** (5 mg, 3.26 μmol) and 2 mol % $Pd(Ph_3P)_4$ in 2.5 mL of THF were added under argon atmosphere a solution of formic acid (1.5 mg, 33 μmol) in 0.5 mL of THF and a solution of *n*-butylamine (1.4 mg, 20 μmol) in 0.5 mL of THF. After 30 min of stirring at room temperature, at which time no traces of **3** could be detected by TLC, the solvent was removed under reduced pressure and the crude residue was purified by flash chromatography (silica gel; ethyl acetate/hexane 4:1 followed by acetone/hexane 3:1 and $CHCl_3/CH_3OH$ 5:1), yielding 4 mg (89%) of **33** as a colorless amorphous solid: ^1H NMR (500 MHz, CD_3OD) δ 5.21 (dd, $J_{2,3} = J_{3,4}$ = 9 Hz, 1H), 5.19 (dd, $J_{2',3'} = J_{3',4'}$ = 9 Hz, 1H), 5.01 (dd, $J_{3,4} = J_{4,5} = J_{3',4'} = J_{4',5'}$ = 9 Hz, 2H), 4.78 (d, $J_{1,2}$ = 8.5 Hz, 1H), 4.72 (d, $J_{1',2'}$ = 8.5 Hz, 1H), 4.70 (m, 1H), 4.59−4.47 (m, 3H), 4.40−4.28 (m, 4H), 4.27−4.10 (m, 5H), 4.02−3.78 (m, 8H), 3.72−3.61 (m, 4H), 2.40−2.19 (m, 2H), 2.16−1.88 (m, 30H), 1.48 (s, 9H), 1.21, 1.16 (d, J = 6 Hz, 3H, 2 rotamers); $[\alpha]_D^{20}$ −14.5 (c = 0.4, CH_3OH); FAB MS *m/e* calcd for (M^+) $C_{55}H_{85}N_8O_{30}P$ (1369.5), found 1328 (M−Ac), 1273 (M + H − H_2PO_4), 1231 (1273 − Ac), 1202 (M − serylphosphate), 1160 (1202 − Ac).

(B) *O*-Phosphato-L-seryl-L-prolyl-L-threonyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-L-seryl-L-prolyl-*O*-(2′-acetamido-3′,4′,6′-tri-*O*-acetyl-2′-deoxy-β-D-glucopyranosyl)-L-serine (34). A solution of peptide **33** (4 mg, 3 μmol) in 0.8 mL (10 mmol) of trifluoracetic acid was stirred for 4 h at room temperature. At this time, 3 mL of toluene were added and the solvent was removed under reduced pressure. For further purification, the residue was washed with hexane and then with CH_2Cl_2, yielding 2.8 mg (73%) of **34** as a colorless amorphous solid: ^1H NMR (500 MHz, CD_3OD) δ 5.23 (dd, $J_{2,3} = J_{3,4} = J_{2',3'} = J_{3',4'}$ = 9 Hz, 2H), 5.00 (dd, $J_{3,4} = J_{4,5} = J_{3',4'} = J_{4',5'}$ = 9 Hz, 2H), 4.78 (d, $J_{1,2}$ = 8.5 Hz, 1H), 4.75 (d, $J_{1',2'}$ = 8.5 Hz, 1H), 4.73−4.64 (m, 2H), 4.61−4.53 (m, 1H), 4.47 (t, J = 6 Hz, 1H), 4.43−4.27 (m, 5H), 4.27−4.10 (m, 3H), 4.08−3.77 (m, 9H), 3.75−3.61 (m, 4H), 2.41−2.18 (m, 2H), 2.16−1.87 (m, 30H), 1.19, 1.14 (d, J = 6 Hz, 3H, 2 rotamers); $[\alpha]_D^{20}$ − 10.8 (c = 0.28, CH_3OH); FAB MS *m/e* calcd for (M^+) $C_{51}H_{77}N_8O_{30}P$ (1313.4), found 1238 (M − H_2PO_4 + Na), 1215 (M − H_2PO_4), 1196 (1238 − Ac), 1145 (M − serylphosphate).

(C) *O*-Phosphato-L-seryl-L-prolyl-L-threonyl-*O*-(2-acetamido-2-deoxy-*β*-D-glucopyranosyl)-L-seryl-L-prolyl-*O*-(2′-acetamido-2′-deoxy-*β*-D-glucopyranosyl)-L-serine (2). A solution of peptide **34** (2.8 mg, 2.13 μmol) in 1.5 mL of CH_3OH was treated with hydrazine hydrate (6 mmol) for 3 h at room temperature. After cooling the reaction mixture to 0 °C, 3 mL of acetone was added, and after 1 h, the solvent was removed under reduced pressure. This procedure was repeated 10 times; then, the residue was dissolved in CH_3OH, acetone was added, and the mixture was evaporated to dryness, yielding 1.2 mg (55%) of **2** as a colorless amorphous solid: ^{1}H NMR (500 MHz, CD_3OD) δ 4.88−4.79 (m, 2H), 4.50 (d, $J_{1,2}$ = 8.4 Hz, 1H), 4.48−4.18 (m, 5H), 4.17−3.98 (m, 3H), 3.97−3.81 (m, 6H), 3.79−3.62 (m, 10H), 3.57 (t, J = 4.7 Hz, 2H), 3.53−3.41 (m, 8H), 2.41−2.19 (m, 2H), 2.17−1.89 (m, 12H), 1.19 (d, J = 6 Hz, 3H); $[\alpha]_D^{20}$ −5.5 (c = 0.1, CH_3OH); FAB MS *m/e* calcd for (M^+) $C_{39}H_{65}N_8O_{24}P$ (1060.5), found 1061.5 (M + H), 1040 (M − Ac + Na), 921 (M + H − Ac − H_2PO_4).

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Supporting Information Available: ^{1}H and ^{13}C NMR data for compounds **12**, **15b−e**, **17b−d**, **6**, **27**, **18c**, **28**, and **29** as well as copies of the ^{1}H and ^{13}C NMR spectra of compounds **12**, **13**, **15b−d**, **18c**, **22**, **25**, **26**, **28**, **29**, **31**, **3**, **33**, **34,** and **2** (33 pages). See any current masthead page for ordering and Internet access instructions.

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