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Solid-Phase Synthesis of Phosphoramidate-Linked Glycopeptides

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Dedicated to Prof. Helmut Vorbrüggen on the occasion of his 80th birthday

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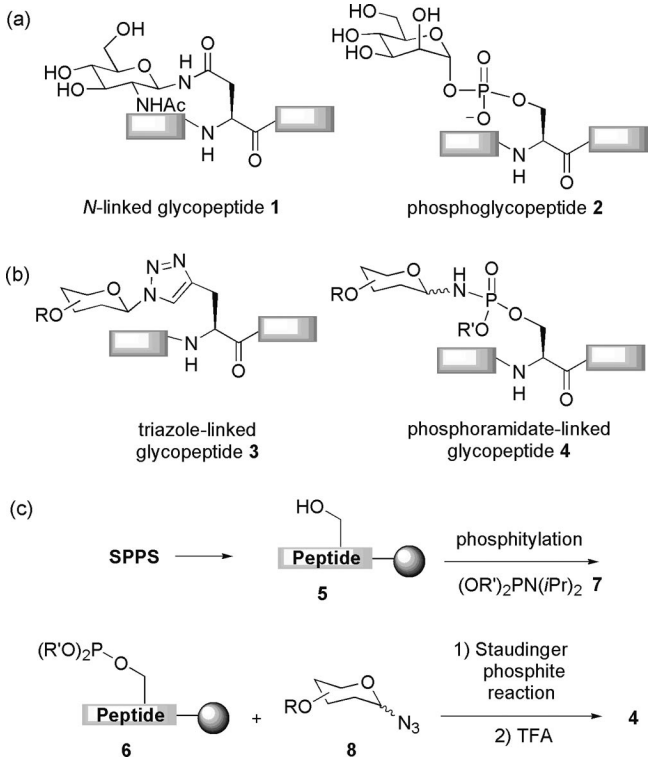
The synthesis of stable glycopeptide mimetics is of particular interest in bioorganic chemistry to allow access of glycoconjugates for biological investigations. In this paper, a straightforward solid-phase synthesis of a novel glycopeptide mimetic by a Staudinger phosphite reaction is presented. Thereby, a dimethyl phosphite containing peptide, which is obtained by standard phosphitylation of a Ser residue, is treated on the solid support with glycosyl azides, delivering phosphoramidate-linked glycoconjugates. These artificial glycopeptides show excellent stability under acidic and physiological conditions.

Introduction

Glycoconjugates consist of carbohydrates that are glycosidically linked to other biologically relevant compound classes such as peptides and proteins, lipids or phospholipids in GPI anchored-proteins.[1] They are of great importance for the life sciences, as the glycan moiety is implicated in mediating an increasing number of important biological processes, such as intra- and intercellular trafficking[2] and receptor binding and signaling.[3] Furthermore, glycosylation has a large impact on structure formation and folding and can be a vital protein modification for the normal growth and development of organisms.[4] In addition to O- and N-linked glycoproteins, which either possess an O-glycosidic linkage to the hydroxy side chain of Ser or Thr or an N-glycosidic linkage to the side chain of Asn in **1** (Scheme 1a),[5] other types of glycosylations have been identified as well. Among these oligosaccharides linked to Thr or Ser through a phosphodiester in **2** have been referred to as rare examples for phosphoglycosylation (Scheme 1a),[6] which was found in the parasite *Leishmania mexicana* with a predominant α-mannosidic phosphoglycosylation pattern.[6,7]

In general, the biological importance of glycoconjugates requires access to pure material for functional investigations.[8] Because it is difficult to isolate them from natural sources in homogeneous forms,[9] glycoconjugate synthesis has been the focus of various activities in bioorganic laboratories.[8] In addition to the synthesis of naturally occurring glycopeptides and glycoproteins, the development of mimetics such as triazole–glycoconjugates **3** has become an attractive approach in glycoscience,[5,10,11] as it can provide a simple modular synthetic access to artificial glyco-



Scheme 1. (a) Naturally occurring glycopeptides. (b) Glycopeptide mimetics. (c) Synthetic route to phosphoramidate-linked glycopeptide **4**. SPPS = solid phase peptide synthesis, TFA = trifluoroacetic acid.

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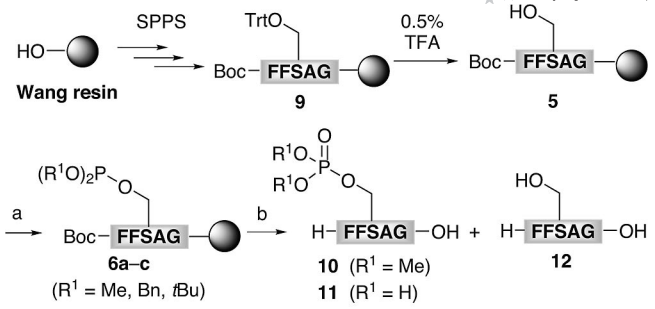
conjugates (Scheme 1b). Improved stability, resistance to hydrolysis by glycosidases, or increased biological activity can be additional advantageous properties.[12] In this paper we report the straightforward solid-phase synthesis of novel glycopeptide mimetic **4** (with R′ = Me) containing a phosphoramidate linkage that is stable under acidic and neutral conditions (Scheme 1b).[13]

Results and Discussion

The synthetic route presented here benefits from a Staudinger phosphite reaction that we recently used as a chemoselective reaction for the functionalization of proteins.[14] A particular aim in this study was to conduct conjugation of readily available carbohydrates with peptides on the solid support and combine it with standard Fmoc-based solid-phase peptide chemistry (SPPS). Our synthetic strategy involves a two-step approach in which first Ser-containing peptide **5** is converted into phosphite-peptide **6** by global phosphitylation with phosphoramidites **7** on the resin. Afterwards, **6** is treated with glycosyl azides **8**, which renders phosphoramidate-linked glycopeptide **4** after TFA cleavage from the solid support (Scheme 1c).

To apply the Staudinger phosphite reaction to the glycosylation of peptides we first investigated the synthesis of different phosphite-containing peptides **6** on solid support that differ in the phosphite alkyl substituents.[15] SPPS was performed by standard Fmoc-couplings on a Wang resin, in which the serine at the latter glycosylation site was trityl protected in **9**. After trityl removal with 0.5% TFA in CH_2Cl_2 the free hydroxy group was phosphitylated with either dimethyl-, dibenzyl-, or di-*tert*-butyl-*N*,*N*-diisopropylphosphoramidite.[16] This reaction was performed in dry DMF in the presence of 1*H*-tetrazole (Figure 1).[17] The conversion to the corresponding dimethyl-, dibenzyl-, and di-*tert*-butyl peptidyl phosphites **6a**–**c** was determined by oxidation of the nascent phosphitylated peptide with *t*BuOOH, subsequent cleavage from the resin with 95% TFA, and final HPLC-MS analysis, in which phosphate **10** or **11** reflected the phosphite conversion of **6a**–**c**. In all reactions, high conversions to the P^V compounds were observed (89–96%), in which the benzyl and *tert*-butyl phosphites **6b** and **6c** led to the formation of phosphate monoester **11** due to the acid lability of the preceding phosphate triester (Figure 1).

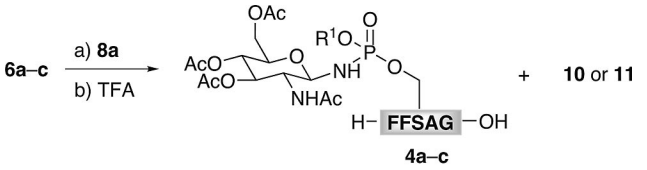
We next probed a solid-supported Staudinger phosphite reaction, in which immobilized phosphite-containing peptides **6** were treated in dry CH_2Cl_2 with acetylated β-GlcNAc azide **8a**[18,19] to afford different phosphoramidate esters **4** (Figure 2). Afterwards, water was added to ensure phosphorimidate hydrolysis, and the peptides were cleaved with 95% TFA from the solid support. It was found that dimethylphosphite-containing peptide **6a** showed 69% conversion to glycosyl-phosphoramidate methyl ester **4a**. Analogous benzyl ester **4b** was only formed in moderate rates, whereas *tert*-butyl ester **4c** was not obtained at all, and in both transformations phosphate monoester **11** was identified as the major reaction product (Figure 2). This difference in the formation of **4** can be rationalized by the acid lability of the P–N bond in saponified phosphoramidates **4b** and **4c**, in which the benzyl and *tert*-butyl esters are cleaved upon TFA exposure. In contrast, phosphoramidate **4a** showed excellent stability under neutral and acidic conditions when **4a** was treated with TFA for 2 h and no P–N bond cleavage was observed. Further stability tests under basic conditions revealed that β-elimination of the phosphorylated Ser derivative occurs when treated with Na_2CO_3 in MeOH (Scheme 2, see also the Supporting Information).



Phosphite[a]	6a (R^1 = Me)	6b (R^1 = Bn)	6c (R^1 = *t*Bu)
10	96	–	–
11	–	89	94

[a] Conversions are determined by LC–MS analysis (see Supporting Information).

Figure 1. Phosphitylation of immobilized peptides. Reagents and conditions: (a) $(MeO)_2PN(iPr)_2$, $(BnO)_2PN(iPr)_2$ or $(tBuO)_2PN(iPr)_2$ (20 equiv.), 1*H*-tetrazole (50 equiv.), 25 °C, 1.5 h, dry DMF; (b) 1. *t*BuOOH, 30 min, 25 °C, DMF; 2. 95% TFA, 3 h. DMF = dimethylformamide, Trt = trityl, TFA = trifluoroacetic acid.

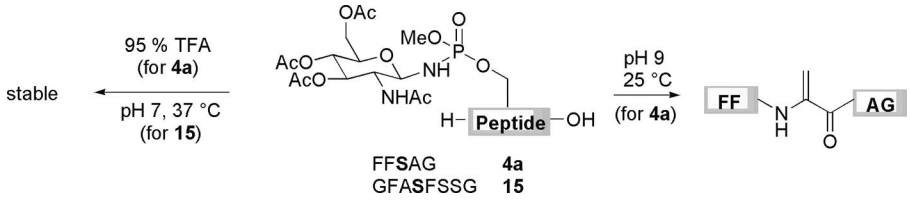


Glycopeptide[a]	6a (R^1 = Me)	6b (R^1 = Bn)	6c (R^1 = *t*Bu)
4	69	<25	–

[a] Conversions are determined by LC–MS analysis (see Supporting Information).

Figure 2. Staudinger phosphite reaction to **4**. Reagents and conditions: (a) 1. **8a** (7 equiv.), dry CH_2Cl_2, 25 °C, 45 h; 2. H_2O (110 equiv.), 15 h; (b) 95% TFA, 3 h.

Consequently, these initial investigations pointed towards the use of dimethyl-*N*,*N*-diisopropylphosphoramidite as phosphitylating agent in further optimization and stability studies. In these, we used a more challenging octapeptide, in which the Ser at the desired glycosylation site was orthogonally protected in contrast to other Ser residues in the sequence (Scheme 3). The Staudinger phosphite reaction of dimethylphosphite-containing peptide **13** with **8a** was per-

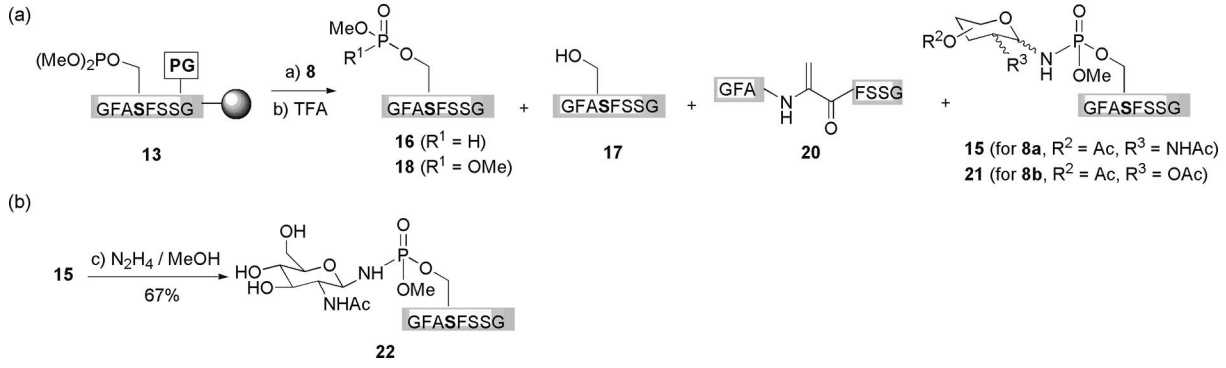


Scheme 2. Stability tests of glycopeptides **4a** and **15** (see Supporting Information).

formed at room temperature as well as at 40 °C in different dry or wet solvents (DMF, DMSO, CH_2Cl_2) and monitored by HPLC–MS (Table 1, Entries 1–9). The best reaction conditions were 45 h in dry CH_2Cl_2 at 40 °C, providing glycopeptide **15** in 57% conversion after hydrolysis (based on HPLC analysis) and in 49% overall isolated yield after semipreparative HPLC purification (see Supporting Information). Afterwards, the stability of isolated glycopeptide **15** under physiological conditions (pH 7.4, 37 °C) was analyzed by LC–MS, in which no signs of decomposition were observed after 24 h. Finally, to remove the acetyl protecting groups, glycopeptide **15** was treated with hydrazine hydrate in methanol for 1 h to deliver deprotected glycopeptide **22** at a conversion of 73% and a yield of 67%.[20]

In addition to desired glycopeptide **15**, H-phosphonate **16**, alcohol **17**, and phosphotriester **18** were identified as byproducts, which can be rationalized either by different hydrolysis pathways of phosphorimidate **14** or by oxidation or hydrolysis of nonconverted **13**, as illustrated in Scheme 3a. These pathways were further analyzed by $H_2{}^{18}O$-labeling experiments and conversion studies, which indicated that only hydrolysis routes A and B occur, as exclusively ^{18}O-labeled products **15** and **19** could be detected (Scheme 4a; Supporting Information, Scheme S1).[21] In addition, because **18** did not contain the heavy oxygen isotope, it is likely to be formed by oxidation of **13** under the reaction conditions. Thus, it can be concluded that once phosphorimidate **14** is formed, no P–N bond cleavage occurs during hydrolysis by route C.

Because the phosphitylation to peptidyl phosphite **13** was quantitative as verified by *t*BuOOH oxidation and HPLC–MS analysis, we assume that peptide alcohol **17** is formed by unselective hydrolysis of **14** through hydrolysis route B and/or by acidic hydrolysis of nonconverted **13**. To

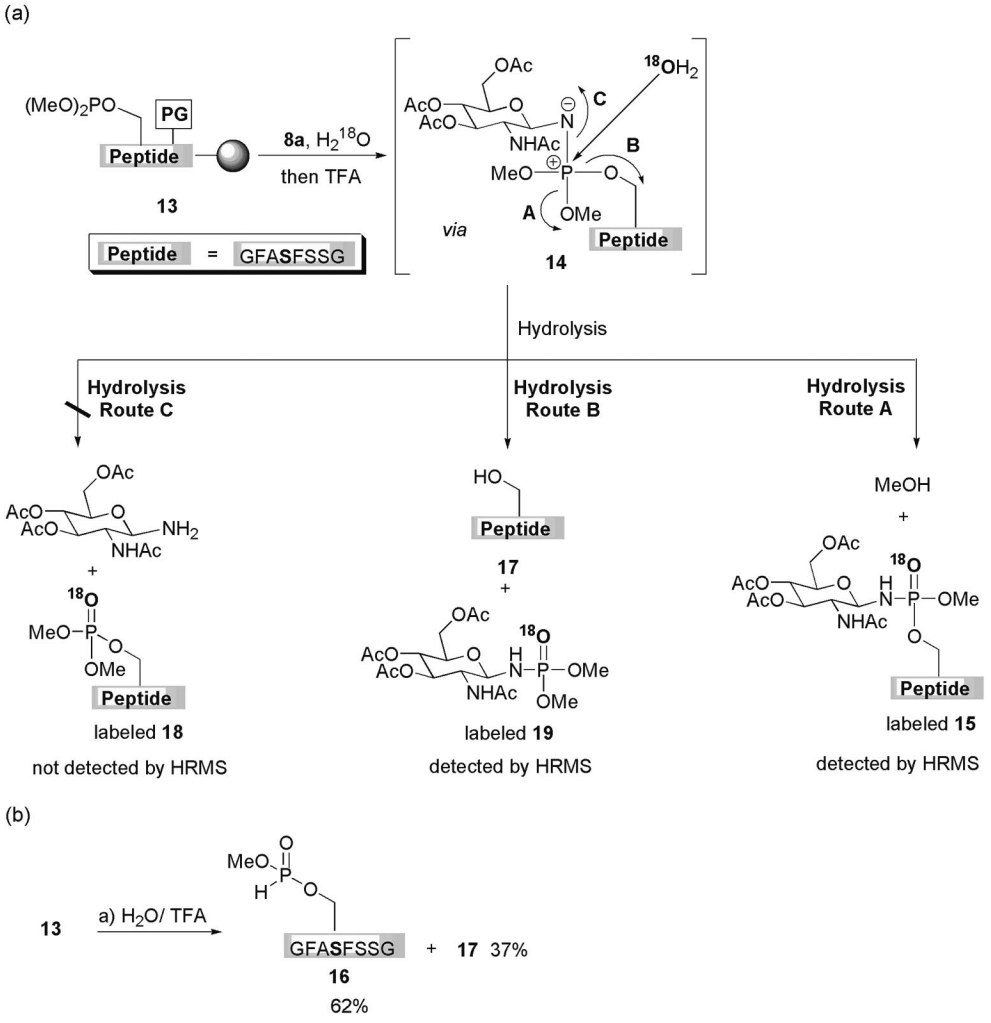


Scheme 3. (a) Staudinger phosphite reaction to **15** and **21**. (b) Deprotection of **15** to **22**. Reagents and conditions: (a) 1. **8a** (7 equiv.) or **8b** (7 equiv.), dry solvent, 25 or 40 °C, 45 h; 2. H_2O (110 equiv.), 15 h; (b) 95% TFA, 3 h; (c) NH_2NH_2, MeOH, 20 °C, 1 h.

Table 1. Conversion rates for the Staudinger phosphite reaction of peptide **13** with glycosyl azides **8a** and **8b**.

Entry[a]	Glycosyl azide	**15** or **21**	**16**	**17**	**18**	**20**	Solvent	Water added	*T* [°C]
1	**8a**	20	30	39	11	–	DMF	–	25
2	**8a**	37	2	37	8	16	DMF	–	40
3	**8a**	40	1	28	19	12	DMF	+	40
4	**8a**	53	27	9	11	–	DMSO	–	25
5	**8a**	31	–	14	37	18	DMSO	–	40
6	**8a**	44	–	22	21	13	DMSO	+	40
7	**8a**	38	7	49	6	–	CH_2Cl_2	–	25
8	**8a**	54	8	34	4	–	CH_2Cl_2	+	40
9	**8a**	57	14	25	4	–	CH_2Cl_2	–	40
10	**8b**	77	10	4	9	–	DMSO	–	25
11	**8b**	77	2	14	7	–	DMSO	–	40
12	**8b**	6	82	6	6	–	CH_2Cl_2	–	25
13	**8b**	26	48	22	4	–	CH_2Cl_2	–	40

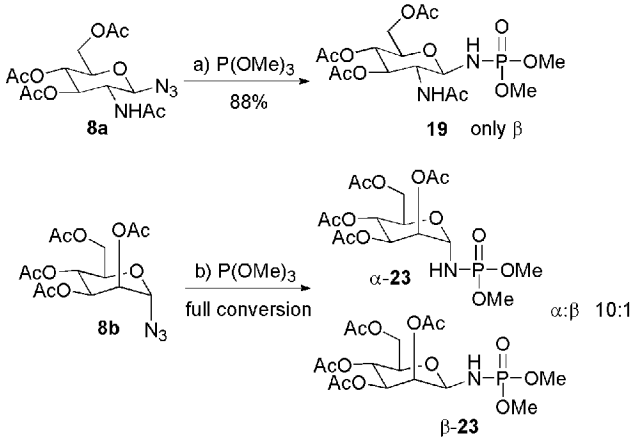
[a] Conversions are determined by LC–MS analysis (see Supporting Information).



Scheme 4. (a) Hydrolysis pathways of phosphorimidate **14** by nucleophilic attack of $H_2{}^{18}O$ at phosphorus (see Supporting Information). (b) TFA-induced hydrolysis of nonconverted **13** to **16** and **17**. Reagents and conditions: (a) H_2O (110 equiv.), 15 h, then 95% TFA, 3 h.

validate the acidic hydrolysis pathway, **13** was treated in a separate experiment with H_2O for 15 h followed by cleavage from the resin with TFA for 3 h, which furnished **17** and H-phosphonate **16** at conversions of 37 and 62%, respectively, with less than 1% of **18** present (Scheme 4b). Another byproduct in the reaction mixture included dehydroalanine **20**, which was observed as an elimination product at 40 °C in DMSO and DMF (Table 1, Entries 2, 3, 5, and 6).

In addition to the solid-phase glycosylation of **13** with β-GlcNAc azide **8a** we also intended to access a mannosylated phosphoramidate-linked peptide as an analogue of natural phosphodiester-linked glycopeptide **2**, in which mannose contains an α-linkage to the phosphorylated peptide.[22] Upon reaction of synthetically accessible acetyl α-mannosyl azide **8b**[19,23] with **13**, the conversion to glycopeptide **21** was found to be optimal in DMSO at 40 °C (Table 1, Entries 10 and 11), whereas the reaction in CH_2Cl_2 proceeded with significantly lower conversion. Under the optimal conditions, **21** was formed in 77% conversion and in an isolated yield of 64% (Table 1, Entry 11); however, partial epimerization to the β-anomer was observed (see Supporting Information). To further investigate the anomerization of glycosyl azides in Staudinger phosphite reactions with small molecules, we converted α-mann-



Scheme 5. Synthesis of glycosyl phosphoramidates **19** and **23**. Reagents and conditions: (a) $P(OMe)_3$ (5 equiv.), CH_2Cl_2, 25 °C, 6 h; (b) $P(OMe)_3$ (5 equiv.), DMSO, 40 °C, 15 h.

osyl azide **8b** in DMSO with trimethylphosphite into the corresponding phosphoramidate **23** (Scheme 5). After full conversion to **23**, a ratio of 10:1 for the α-/β-anomer was determined by NMR measurements (see Supporting Information).[24] Analogously, for β-GlcNAc azide **8a** the integrity at the anomeric centre was retained, providing β-glycosyl phosphoramidate **19** exclusively at 25 °C in CH_2Cl_2 in 88% isolated yield.[13]

Conclusions

In summary, we have developed a convergent method for the synthesis of phosphoramidate-linked glycopeptides by a Staudinger phosphite reaction on solid support. This synthetic route utilizes standard Fmoc-based solid-phase peptide synthesis and yields a novel type of glycopeptide mimetic, which shows excellent stability under physiological conditions as well as acidic deprotection conditions required for Fmoc-based peptide synthesis. The key steps in the Staudinger phosphite reaction sequence are a global phosphitylation of an unprotected Ser residue to a dimethylphosphite-containing peptide, which is followed by glycosylation with a glycosyl azide in high yields and under high retention of the anomeric linkage before the resulting phosphoramidate-linked glycopeptide is deprotected and cleaved from the resin by TFA treatment. Although not all amino acids have been screened in this study so far, this protocol is expected to tolerate also problematic amino acid functionalities, as the phosphitylation and the glycosylation steps are carried out in the presence of protected amino acids. Current investigations in our laboratory focus on the synthesis of glycoconjugate mimetics with larger carbohydrate moieties, the development of a chemoselective protocol with unprotected substrates, as well as biological recognition studies for different di- and multivalent glycoconjugates of biological relevance.

Experimental Section

General: All reagents, amino acids, and solvents were purchased from commercial suppliers and used without further purification. Glycosyl azides **8a** and **8b** were obtained by published protocols.[18a,23] Dry solvents were purchased from Acros Organics. Tetrazole solution in acetonitrile was purchased from Sigma Aldrich, acetonitrile was removed under reduced pressure at room temperature, and the solid tetrazole was dissolved immediately in dry DMF. ***CAUTION! Solid tetrazole may explode under heating or high pressure.*** Analytical HPLC and HRMS spectra were recorded with an Agilent 6210 TOF LC–MS system, Agilent Technologies, Santa Clara, CA, USA, by using Agilent Eclipse XDB-C_{18} column (5 μm, 4.6 × 150 mm) with a flow rate of 0.5 mL min^{-1}. Spray voltage and drying gas flow rate were set to 4 kV and 25 psi, respectively. Purification of glycopeptides **21**, **15**, and **22** by semipreparative HPLC was performed with a JASCO LC-2000 Plus system using a C_{18} column (5 μm, 25 × 250 mm with a flow rate of 16 mL min^{-1}). Specific gradients are given in the synthetic procedures.

Peptide Synthesis: Peptides were synthesized with an ABI 433A peptide synthesizer by using standard amide coupling conditions HBTU/HOBt (Fast-moc protocol) utilizing Fmoc-Gly-Wang resin (substitution: 0.79 mmol g^{-1}), which was purchased from Novabiochem.

General Procedure for Solid-Phase Staudinger Phosphite Reaction of Phosphitylated Peptides with Glycosyl Azides (GP I): Immobilized phosphitylated peptides **6** and **13** were prepared by using dimethyl-*N*,*N*-diisopropylphosphoramidite as a phosphitylating agent. Then, glycosyl azide **8a** or **8b** (0.07 mmol) was dissolved in dry solvent (1 mL; DMF, DMSO, or CH_2Cl_2) and added to the peptide resin, and the reaction vessel was gently agitated at either 25 or 40 °C. After 45 h, the reaction mixture was quenched by the addition of water (20 μL, 1.1 mmol) and again kept for 15 h at 25 °C. The reagents were removed by filtration; the resin was washed with DMF (3 × 3 mL) and CH_2Cl_2 (3 × 3 mL) and dried. Finally, the glycopeptide was cleaved from the resin by using TFA/TIS/water (95:2.5:2.5) over 3 h and then precipitated in cold ether and further analyzed or purified by HPLC.

General Procedure for the Global Peptide Phosphitylation on a Solid Support (GP II): The Ser residue at the latter phosphitylation site was incorporated into the peptide with a trityl side chain protecting group, which was removed on the resin immediately before the phosphitylation reaction with 0.5% TFA in dry CH_2Cl_2. The peptidyl resin (0.01 mmol) was placed in a manual peptide synthesis vessel and dried overnight under reduced pressure at 40 °C. A solution of tetrazole (0.5 mmol) and dimethyl-, dibenzyl-, or di-*tert*-butyl-*N*,*N*-diisopropylphosphoramidite (0.2 mmol) in dry DMF (0.6 mL) was added to the peptidyl resin, and the mixture was agitated gently for 1.5 h. The reagents were removed by filtration, and the peptidyl resin was washed with dry DMF (3 × 3 mL) and finally with either dry DMSO (3 × 3 mL) or CH_2Cl_2 (3 × 3 mL). Specific dry solvents are given in the synthetic procedures.

General Procedure for the Oxidation of Phosphitylated Peptides on the Solid Support (GP III): The phosphitylated peptide was prepared according to GP II with dry DMF washings at the end. Then, dry DMF was added to cover the peptidyl resin and 6 M *tert*-butyl hydroperoxide in octane (1 mL) was added, and the reaction vessel was gently agitated for 30 min. *tert*-Butyl hydroperoxide was removed by filtration, and the peptidyl resin was washed with DMF and CH_2Cl_2 and dried. Finally, the phosphorylated peptide was cleaved from the resin with TFA/TIS/water (95:2.5:2.5) over 3 h and precipitated in cold ether.

Supporting Information (see footnote on the first page of this article): Specified synthetic procedures; NMR spectroscopic data, mass spectrometry data, and HPLC traces for the peptide phosphitylation; synthesis of glycopeptide **15**, **21**, and **22**; glycosyl phosphoramidates **19** and **23**; and stability tests of **4a** and **15**.

Acknowledgments

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