Peptide Synthesis

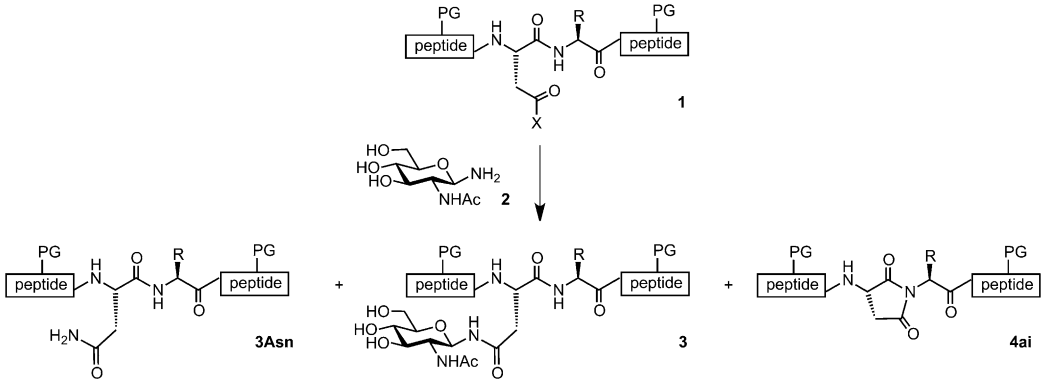
DOI: 10.1002/anie.201204272

Convergent Solid-Phase Synthesis of N-Glycopeptides Facilitated by Pseudoprolines at Consensus-Sequence Ser/Thr Residues**

*Vera Ullmann, Marisa Rädisch, Irene Boos, Jutta Freund, Claudia Pöhner, Stefan Schwarzinger, and Carlo Unverzagt**

Dedicated to Professor Hans Paulsen on the occasion of his 90th birthday

N-Glycosylation is an important posttranslational modification of proteins. A carbohydrate is transferred to an asparagine within an Asn-X-Ser/Thr consensus sequence.[1] The study of the biological aspects of N-glycosylation often requires the synthesis of N-glycopeptides,[2] which are accessible by two main approaches. In the sequential mode glycosylamino acid cassettes are used for peptide elongation. After incorporation of larger oligosaccharides the solubility and reactivity of the peptide is affected and side reactions, for example, involving free OH groups[3] complicate further elongation. In the convergent mode (Lansbury aspartylation)[4] the sugar is connected to an aspartate after complete assembly of the peptide (Scheme 1). The main drawback of the convergent mode is the formation of cyclic aspartimides during peptide elongation and sugar coupling;[5] this formation depends on the peptide sequence,[6] and the coupling conditions.[7] We found that a pseudoproline (Ψpro)[8] at the consensus-sequence Ser/Thr residue (Asn-X-Ser/Thr(Ψpro)) efficiently suppresses the formation of aspartimides in the convergent synthesis of N-glycopeptides on the solid phase.



Scheme 1. Lansbury aspartylation leads to glycopeptide **3**, Asn peptide **3Asn** and aspartimide side product **4ai**. PG = protecting group.

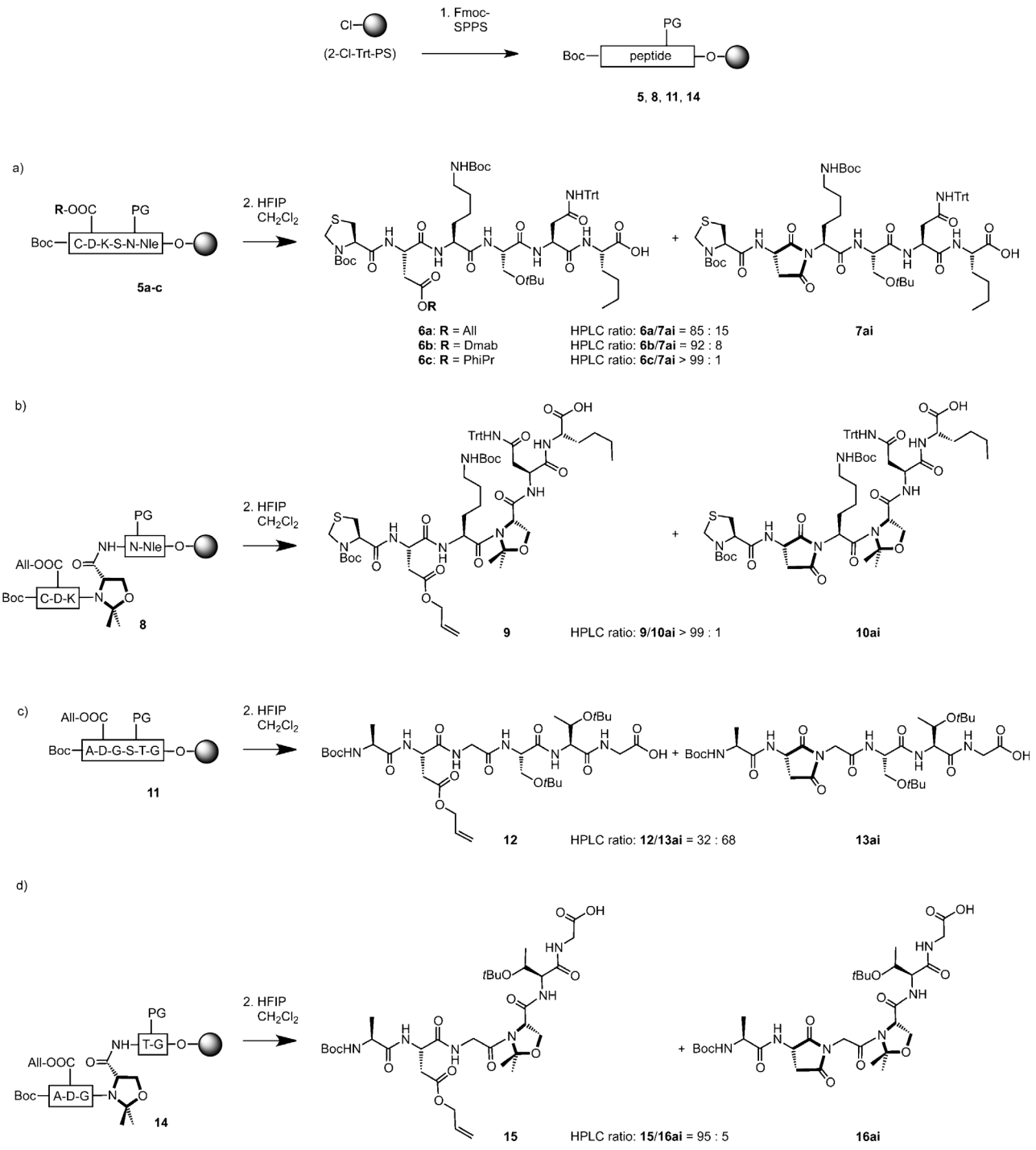
The lack of pure N-glycoproteins for biological studies has stimulated research into their synthesis; these syntheses were carried out mainly by ligation techniques.[9] The required glycopeptides and their thioesters are difficult to obtain as the sugar component interferes with the peptide synthesis. The syntheses of longer glycopeptide thioesters are particularly difficult as they require, for example, additional ligation steps[9f,10] or segment couplings.[3b] For longer N-glycopeptides the convergent approach provides advantages over the sequential approach,[11] especially as the Lansbury aspartylation[4] has been shown to be efficient also on the solid phase.[12] The undesired aspartimide formation can be avoided by peptide backbone (NH) protection,[13] however, this approach is tedious for residues other than glycine, and causes racemization when coupling backbone-protected dipeptides.[12b] Aspartimide formation during peptide elongation can be reduced by using bulky groups to protect the Asp side chain,[14] for example, the 2-phenylisopropylester (Ph*i*Pr);[12a] however, trityl anchors are also cleaved under the reaction conditions for Ph*i*Pr removal. Dmab-protected[15] Asp residues are compatible with trityl anchors, but the backbone protection of the neighboring amino acid is required.[13a,16]

To provide complex N-glycopeptide thioesters by solid-phase Lansbury aspartylation we compared three Asp-side-chain protecting groups for the Fmoc-SPPS of an interleukin-6 (IL-6) 43–48 hexapeptide. The allyl-protected peptide[17] **6a** showed the highest percentage of aspartimide **7ai** formation (15%), followed by the Dmab peptide **6b** (8%), and the Ph*i*Pr[12a] peptide **6c** (<1%; Scheme 2). As well as the protecting group many factors are known to contribute to aspartimide formation[5] for example, the steric bulk of the neighboring amino acid, the basicity of the reaction media, and also the overall conformation[18] of the peptide C-terminal relative to the Asp moiety. We reasoned that constraining the

[*] V. Ullmann, M. Rädisch, I. Boos, J. Freund, Dr. C. Pöhner, Dr. S. Schwarzinger, Prof. C. Unverzagt
Bioorganische Chemie, Gebäude NW1, Universität Bayreuth
95440 Bayreuth (Germany)
E-mail: carlo.unverzagt@uni-bayreuth.de

[**] This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201204272.



Scheme 2. a) Fmoc-SPPS of IL-6 43-48Nle **6a–c** with different Asp protection groups, b) IL-6 43-48Nle with Ser(Ψpro); c, d) synthesis of ADGSTG peptides with Ser(O*t*Bu) (**12**) or Ser(Ψpro) (**15**). All = allyl, Boc = *tert*-butyloxycarbonyl, 2-Cl-Trt-PS = 2-chlorotrityl polystyrene, Dmab = 4-(*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methyl-butyl]-amino)benzyl, Fmoc = 9-fluorenylmethoxycarbonyl, HFIP = hexafluoroisopropanol, Nle = norleucine, SPPS = solid phase peptide synthesis, Trt = trityl.

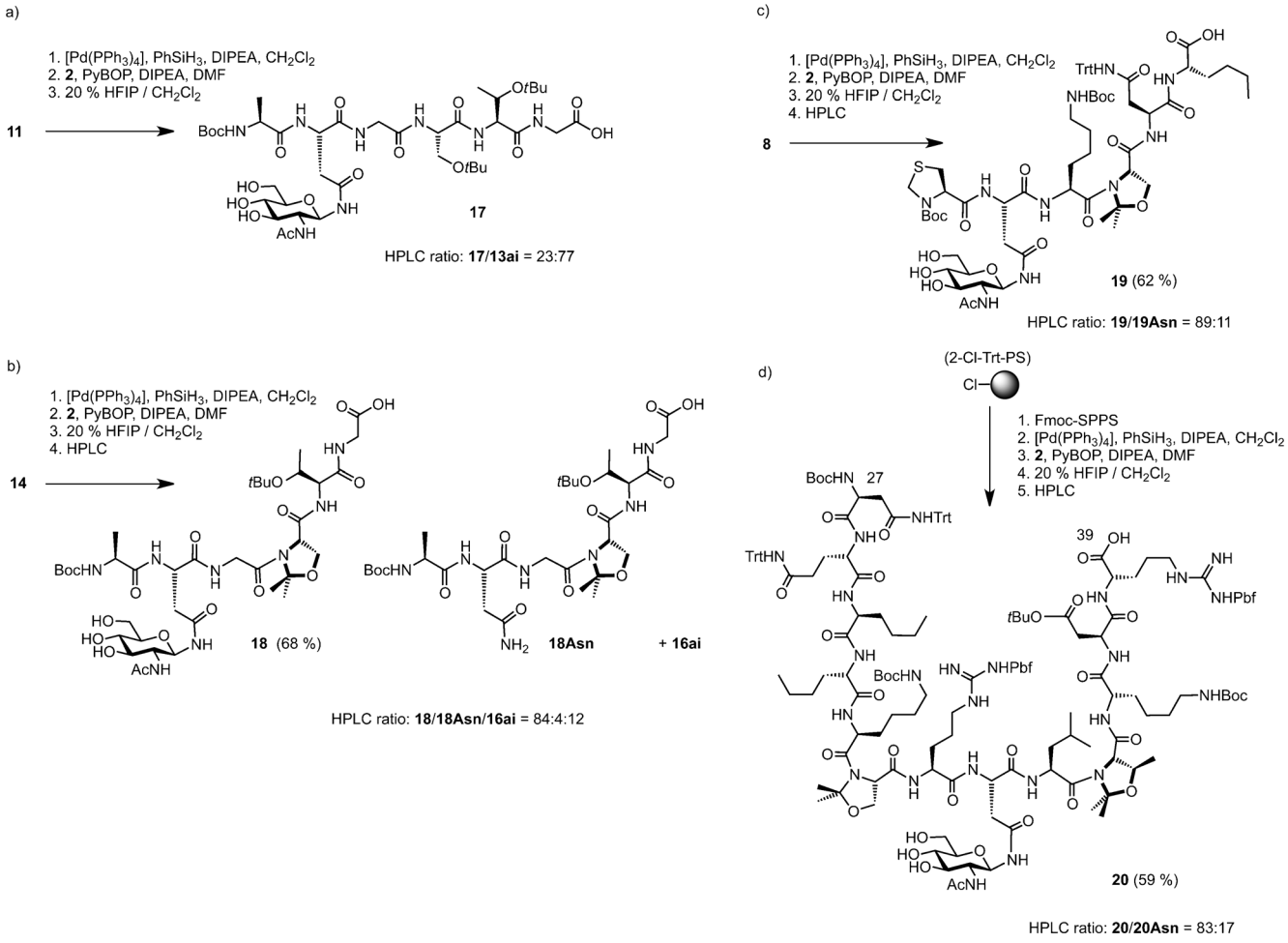
flexibility of this peptide part might influence aspartimide formation. This hypothesis was investigated by converting the *trans* conformation of the Lys-Ser amide bond into a stable *cis* conformation by using a Ser($\Psi^{Me,Me}$pro) pseudoproline dipeptide.[8] Pleasingly, in the synthesis of peptide **9** the presence of the pseudoproline almost completely eliminated the succinimide formation (product **10ai**) despite the presence of the susceptible allyl ester (Scheme 2b).[17] This hitherto unnoticed pseudoproline-based conformational effect was tested by investigating the synthesis of peptides **12** and **15**, in which a glycine, which is the residue that promotes aspartimide formation the most,[6,19] is next to the Asp allylester. In the case of Ser(O*t*Bu) peptide **12** aspartimide formation was extensive (68% **13ai**; Scheme 2c),

whereas the Ser(Ψpro) peptide **15** gave rise to only 5% of cyclized side product **16ai** (Scheme 2d). Aspartimide formation mainly occurred during Fmoc removal. The purified Asp-Gly-containing peptides **12** and **15** were stable under simulated coupling conditions (DIPEA, 3 equiv), whereas simulated cleavage conditions (20% piperidine) gave aspartimides. Thus, mild conditions including a hexamethyleneimine/*N*-methylpyrrolidine/Cl-HOBt mixture[20] were tested for the deprotection of elongated Asp-Gly-containing peptides, and only 7% of aspartimides **35ai** and **38ai** were found (Figure S3).

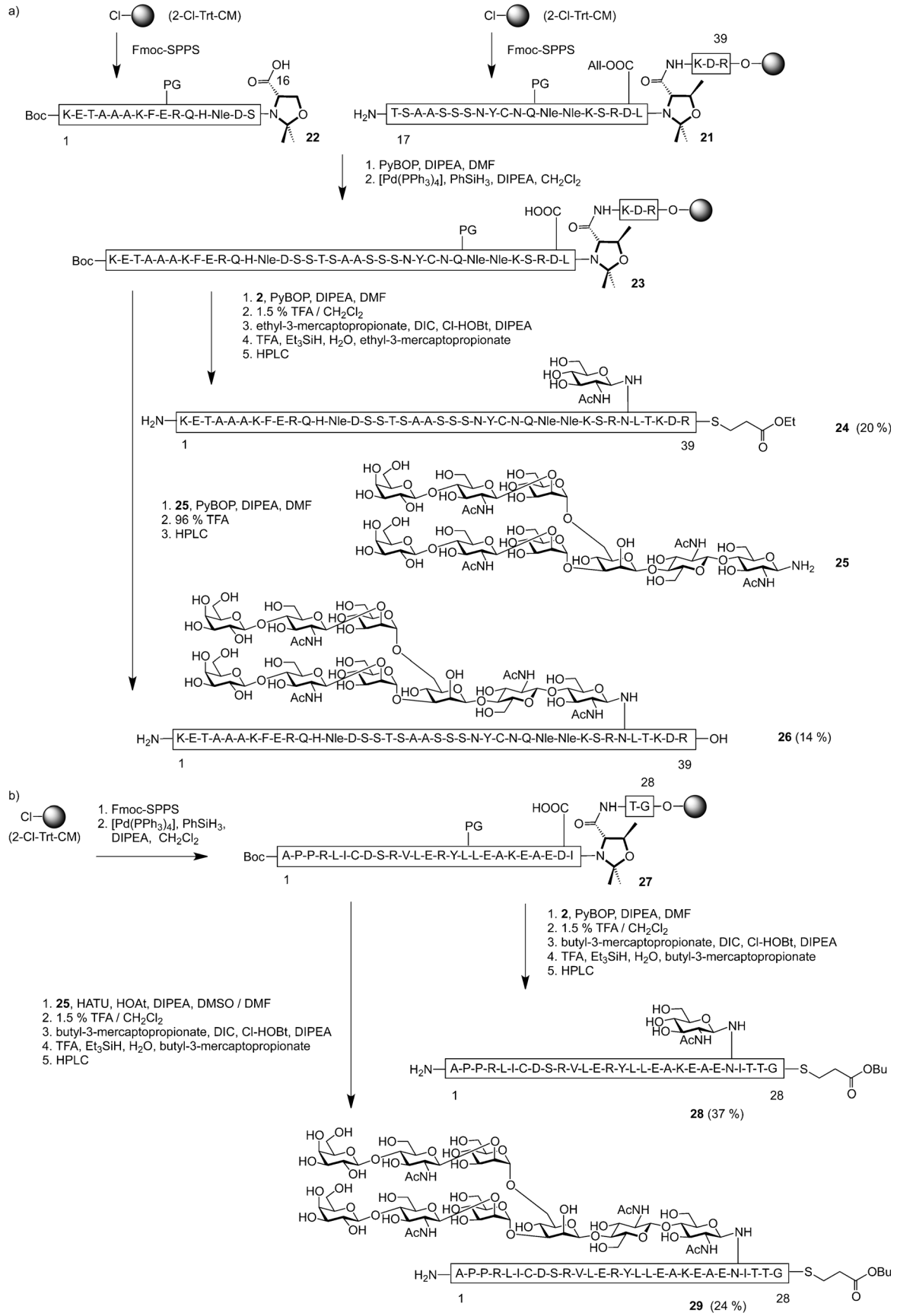
The allyl protecting groups of Asp-Gly-containing peptide resins **11** and **14** were selectively cleaved[21] in the presence of DIPEA, which prevented acidic cleavage of the peptide from the linker. Solid-phase Lansbury aspartylation was carried out by activating the peptides with PyBOP in the presence of GlcNAc-NH_2 **2** (Scheme 3). The ratio of glycopeptide **17** to aspartimide **13ai** was 23:77, thus indicating the formation of additional aspartimide during aspartylation (Scheme 3a). For the pseudoproline peptide **14** efficient conversion into glycopeptide **18** (84%) took place accompanied by formation of aspartimide **16ai** (12%) and only 4% of the Asn peptide **18Asn** (Scheme 3b). Glycopeptide **18** was obtained in 68% yield after HPLC. The allyl protecting group was removed from Asp-Lys-containing peptide **8** and then it was coupled to **2** to give glycopeptide **19** in 62% yield upon isolation (Scheme 3c).

The preparative scope of the pseudoproline-assisted convergent N-glycopeptide formation was probed with the synthesis of a ribonuclease (RNase) fragment. The RNase 27–39 peptide containing a Thr(Ψpro) was synthesized on 2-Cl-Trt-PS resin, the allyl group was cleaved at Asp 34, and then it was coupled with **2**. Glycopeptide **20** and the Asn side product **20Asn** were obtained in a 83:17 ratio with no detectable aspartimide (Scheme 3d). Cleavage from the resin and HPLC purification gave **20** (59%). The GlcNAc-β-Asn linkage was confirmed by NMR spectroscopy for glycopeptides **18**, **19**, and **20** ($J_{1,2} \approx 9$ Hz; see the Supporting Information Scheme S6).

The advantages of pseudoprolines in the convergent assembly of peptides and glycopeptides were combined in the case of difficult RNase 1–39 glycopeptides (Scheme 4a). The RNase 17–39 peptide **21** was synthesized on a 2-Cl-trityl-CM resin and coupled with the RNase 1–16 fragment **22**. Racemization during segment condensation on the solid support is avoided owing to the presence of the C-terminal



Scheme 3. Solid phase coupling of GlcNAc-NH_2 **2** to peptides. DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, GlcNAc = *N*-acetylglucosamine, PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.



Scheme 4. Convergent synthesis of glycoprotein segments: a) RNase 1–39, b) EPO 1–28). DIC = diisopropylcarbodiimide, DMSO = dimethylsulfoxide, Cl-HOBt = 6-chloro-1-hydroxybenzotriazole, 2-Cl-Trt-CM = 2-chlorotrityl ChemMatrix, HATU = 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOAt = 1-hydroxy-7-azabenzotriazole.

pseudoproline of **22**.[3b] Removal of the allyl groups of the RNase 1–39 peptide gave **23**, which was coupled with **2** and converted into thioester **24** by the in situ thioesterification method.[22] After HPLC, thioester **24** was obtained in 20% yield, which is the same yield as for a previous approach using GlcNAc-Asn.[3b] Coupling of the complex nonasaccharide amine **25** gave RNase glycopeptide **26** in 14% yield despite considerable formation of the asparagine side product. The nonasaccharide amine **25**[23] was obtained from a sialoglycopeptide[23b] by enzymatic cleavage followed by anomeric azidation,[24] and reduction (Supporting Information Scheme S7).

The pseudoproline-assisted Lansbury aspartylation was then applied to the erythropoietin (EPO) 1–28 sequence (Scheme 4 b). Resin-bound EPO 1–28 **27** was synthesized in satisfactory purity by linear Fmoc-SPPS on the polar 2-Cl-Trt-CM resin, and no accompanying cyclization of the Asp 24 allylester ocurred despite the numerous coupling and deprotection steps. Using PyBOP **27** was coupled to **2**, then the glycopeptide was cleaved, thioesterification was carried out followed by deprotection. HPLC analysis showed high conversion into the glycopeptide thioester (78%) accompanied by some asparagine formation (22%) and no detectable aspartimide. Purification by HPLC gave the EPO 1–28 GlcNAc thioester **28** in a yield of 37%. When the EPO 1–28 peptide **27** was reacted with nonasaccharide amine **25**, there was extensive asparagine formation. Thus, a series of coupling reagents was screened for **2** and the best reaction conditions were applied to **25** (Supporting Information, Table 2). In situ thioesterification of the resulting nonasaccharide glycopeptide was unexpectedly difficult because the glycopeptide acid was retained on the resin after mild acidic cleavage. An optimized thioesterification procedure, however, gave the desired glycopeptide thioester **29** in 24% yield after HPLC.

We have found a general method for improved convergent N-glycopeptide synthesis on the solid phase. By converting the consensus-sequence Ser/Thr moieties into a pseudoproline the formation of aspartimides is highly reduced, both in peptide elongation and in the subsequent aspartylation. The robust approach uses standard building blocks compatible with automated peptide synthesis, thus facilitating the availability of glycopeptide and glycoprotein libraries.

Received: June 1, 2012
Published online: ■■ ■■, ■■■■

Keywords: aspartylation · glycopeptides · protecting groups · pseudoproline · solid-phase synthesis

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Communications

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V. Ullmann, M. Rädisch, I. Boos, J. Freund, C. Pöhner, S. Schwarzinger, C. Unverzagt* _____ ■■■■–■■■■

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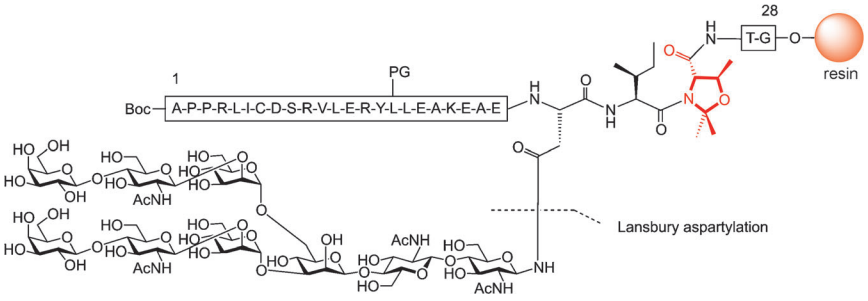

Remote control: The formation of aspartimides is highly reduced during peptide elongation and convergent sugar couplings of Asp-X-Ser/Thr peptides that contain a pseudoproline (red; see scheme). The robust approach efficiently joins complex peptides and N-glycans on the solid phase thus facilitating the availability of glycopeptides and glycoproteins.