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Synthesis of biotinylated glycosulfopeptides by chemoselective ligation

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Abstract—Tyrosine sulfation and *O*-glycosylation are two post-translational modifications playing an essential role in modulation of biological activity, protein folding and cellular communication. Here, a novel chemical approach for the synthesis of biotinylated glycosulfopeptides is described based on a combination of acid labile protecting groups, highly acid sensitive resins and two orthogonal chemoselective ligation reactions. © 2001 Published by Elsevier Science Ltd.

Tyrosine sulfation and *O*-glycosylation are two post-translational modifications in mature proteins playing a pivotal role in diverse biological functions, e.g. modulation of protein folding, inter- and intracellular trafficking, receptor binding and signaling.[1–3] Such protein–protein interactions strongly depend on the precise structure of the glycan moiety whereas the role of tyrosine *O*-sulfation remains elusive. Due to significant advances in synthetic and analytical methodologies, complex and structurally well-characterized glycopeptides for molecular recognition studies have become accessible.[4–6] Nevertheless, peptides containing both types of modifications may serve as more efficient tools for studying the influence of these non-peptidic moieties in biological processes. In contrast to the inherent heterogeneity of post-translationally modified recombinant proteins and the intrinsically low quantities of sulfated glycopeptides obtained in vitro by a chemoenzymatic approach using sulfo- and glycosyltransferases,[7–9] chemical synthesis is still preferred when larger amounts of material for biostructural characterization are required.

However, the chemical synthesis of glycosulfopeptides is complicated due to their chemical instability requiring complex protection/deprotection schemes. Here, we present a novel approach for the introduction of these two post-translational modifications. The strategy for the synthesis of glycosulfopeptides is based on a combination of acid labile protecting groups, highly acid sensitive resins for the assembly of the sulfated peptide sequence and chemoselective oxime bond formation[10,11] for the convenient post-synthetic attachment of a carbohydrate moiety. In addition, to facilitate biological tests, the conjugate peptide is labeled with the biotin reporter group using chemoselective thioether ligation[12] (Scheme 1). This strategy was applied for the synthesis of a peptide sequence derived from the N-terminal region of the P-selectin glycoprotein ligand 1 (PSGL-1), a major ligand of the selectin family involved in leukocyte adhesion to the vascular endothelium during inflammation.[13–15]

In the search of potential inhibitors of the early step in the leukocyte adhesion cascade, we have focused on the N-terminal segment (42–62) of PSGL-1 containing two modifications essential for efficient adhesion to P-selectin, i.e. one sulfated tyrosine (at position 46, 48 or 51) and the sialyl Lewis x (sLe^x) modified threonine 57.[16] As a target for chemical synthesis, we have chosen a nonapeptide corresponding to residues 51 to 58 of the N-terminal sequence of PSGL-1, comprising one sulfated tyrosine (position 51), one aminooxy function on the ε-NH_2 group of the lysine replacing threonine 57 for the attachment of the carbohydrate and a non-native C-terminal cysteine residue for covalent ligation of biotin via thioether bond formation.

The key peptide Ac-**Tyr(SO_3H)**-Asp-Phe-Leu-Pro-Glu-**Lys($^{\varepsilon}NHCO$-CH_2-ONH_2)**-Glu-**Cys**-NH_2 (**2** in Scheme 1) was synthesized using the highly acid sensitive Sieber resin[17] applying Fmoc strategy in combination with acid labile side chain protecting groups (*t*Bu/Trt). The building block Fmoc-Tyr(SO_3Na)-OH was obtained by

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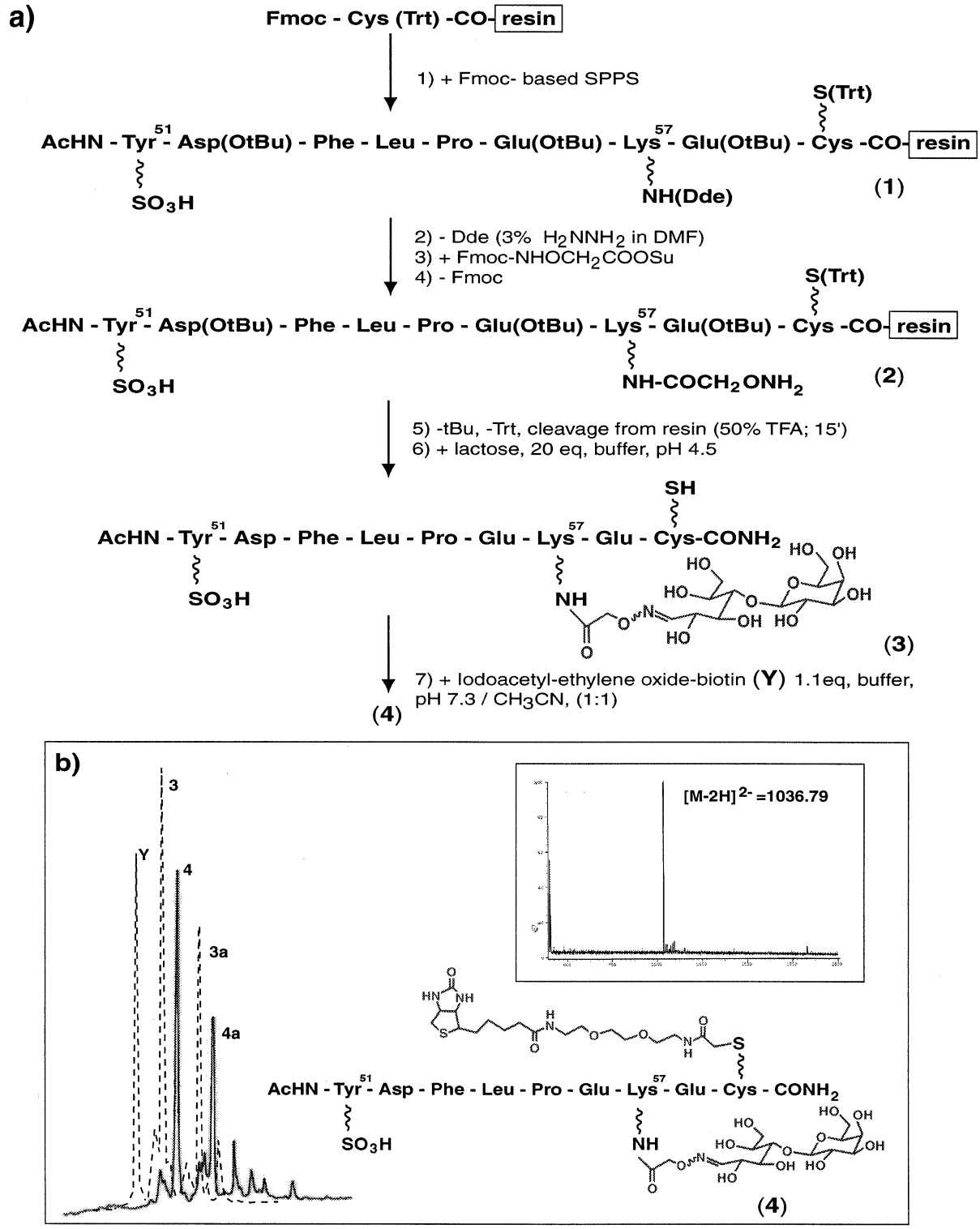

Scheme 1. (a) Fmoc-based solid-phase peptide synthesis[18] of a glycosylated sulfopeptide using highly acid-labile linker resin (Sieber amide[17]). (b) RP-HPLC profile of the reaction mixture of the biotinylation step via thioether formation (step 7) at $t=0$ (dashed lines), **Y** = biotin derivative, **3** = glycosulfopeptide, **3a** = desulfated **3**; at $t=20$ h (solid lines), **4** = biotinylated **3**, **4a** = desulfated **4**; insert: ESI-MS (negative mode): $M_{calcd}=2075.67$; $M_{found}=2075.02$.

treating N^{α}-Fmoc Tyr with sulfur trioxide dimethylformamide (SO_3 DMF) according to the procedure of Yagami et al.[19] and selectively incorporated during solid-phase peptide synthesis. After completion of the sequence, the Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) protection of the C-terminal lysine was removed with 3% hydrazine in DMF and subsequently, the aminooxy acetyl building block coupled via its hydroxysuccinimide ester (step 3). After removal of the Fmoc group (step 4), the peptide was cleaved from the

resin and the *t*Bu/Trt protecting groups removed simultaneously by short treatment (15 min, rt) with 50% TFA (Scheme 1, step 5). The subsequent condensation steps were performed without prior purification of the resulting sulfated peptide.

The second post-translational modification, the *O*-glycosylation of the monosulfopeptide (**2**, step 6) was achieved by chemoselective oxime bond formation under mild acidic conditions (pH 4.5, AcONa buffer).[20,21] As a first example, lactose was chosen as free reducing carbohydrate. The reaction proceeds to completion within less than 24 h with a 20-fold excess of lactose as monitored by HPLC. Finally, for the biotinylation of the lactosulfopeptide **3**, the free thiol of the C-terminal cysteine residue was treated with iodoacetyl functionalized biotin (step 7). As indicated by the HPLC profile of the reaction mixture (Scheme 1b) the ligation reaction via thioether formation is complete after 20 h. Despite of some loss of the highly acid labile sulfate moiety (product **4a**) that occurred already during cleavage of the peptide from the resin (step 5), biotinylated glycosulfopeptides (**4**) could be obtained in overall yields of 30–40%.[22]

In summary, we have elaborated an efficient strategy for the synthesis of biotinylated glycosulfopeptides using two orthogonal chemoselective ligation reactions in the presence of a sulfate moiety allowing for facile access to diverse glycosulfopeptide conjugates. This approach is currently used to generate a sugar library for the systematic investigation of the role of the carbohydrate moiety in PSGL-1/P-selectin mediated leukocyte adhesion and in general molecular recognition processes.

Acknowledgements

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22. Typically, for the condensation of the carbohydrate, D-(+)-lactose monohydrate (20 equiv.) was dissolved in H_2O (2 mL) and added to a solution of 0.08 mM sulfopeptide in 0.1 M sodium acetate buffer (3.2 mL), pH 4.5/CH_3CN (1:1, *v*/*v*). After completion of the reaction, the pH was adjusted to 6 and **3** isolated by SepPak® extraction in 50–60% yield ($C_{68}H_{101}N_{12}O_{32}S_2$, M_{calcd} 1662.73, M_{found} 1660.8 $[M-H]^-$, 829.8 $[M-2H]^{2-}$). For the biotinylation step, iodoacetyl-ethylene oxide-biotin (1.1 equiv.) in degassed 0.15 M phosphate buffer (4 mL), pH 7.3, was added to 75 mg (4.5×10^{-5} M) of **3** and stirred at rt under argon in the dark (20 h). HPLC-purified **4** was neutralized with 0.025 M aqueous NH_4HCO_3, lyophilized, dialyzed against H_2O at 4°C and re-lyophilized ($C_{86}H_{131}N_{16}O_{37}S_3$, M_{calcd} 2077.25, M_{found} 2075.02 $[M-H]^-$, 1036.79 $[M-2H]^{2-}$).