Enzymatic Synthesis of Neoglycopeptide Building Blocks**

Delphine Ramos, Patrick Rollin, and Werner Klaffke*

Cellular interactions triggered by glycostructures are imperative for the smooth functioning of an organism. On the other hand, and far less beneficial, such structures serve as anchors for pathogenic organisms harmful to the host. Both these functions imply uses for such structures in biomedical applications and therefore, the need for unlimited and facile access to a library of glycopeptide structures is evident. Ideally, saccharide and peptide should be prepared in parallel, to a certain degree.

Transglutaminase (glutaminyl-peptide γ-glutamyl transferase, TGase, EC 2.3.2.13) is an acyl transferase that catalyzes the amidation reaction of primary amines with the γ-carboxamide residue of glutamine. Previous research in this area is illustrated by a few key papers and patents: the enzyme has been used to synthesize high molecular weight emulsifiers,[1] for protein immobilization,[2] to cross-link trypsin/α-amylase mixtures after preabsorption on DEAE-type ion-exchange resins (DEAE = diethylaminoethyl),[3] to prepare hapten-protein conjugates for ELISA (enzyme-linked immunosorbent assay) assays,[4] and to synthesize high molecular weight peptidoglucans.[5]

We report here on a regioselective procedure for the synthesis of N-linked-neoglycopeptides, leaving the core sugar conformation intact. Amino functionalization is achieved on the saccharide, after N-glycosylation using allylamine, by photoinduced coupling of cysteamine (2-aminoethanthiol) to the double bond (Scheme 1).

Whereas in most biotechnical applications cross-linking has been brought about by transaminase acting on ε-amino groups of lysine, the question here arose whether the amino group could also be attached through a spacer linkage to the anomeric center of a glycoside. Such a construction would mimic the Asn–glycoside linkage of N-linked glycoproteins, and it would require the least modification of the saccharide part (Asn = asparagine).

To qualify as a versatile chemo-enzymatic procedure the reaction should: 1) be performed in aqueous media, 2) allow for a wide range of substrates, 3) not depend on cofactors, like the in vitro route which uses dolichyl phosphates in peptide glycosylation does, and 4) be feasible on a preparative scale.

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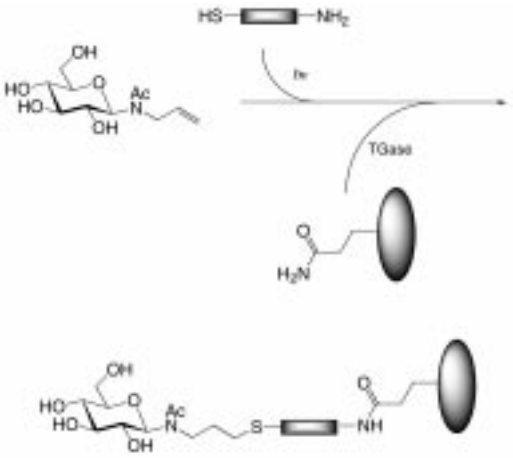
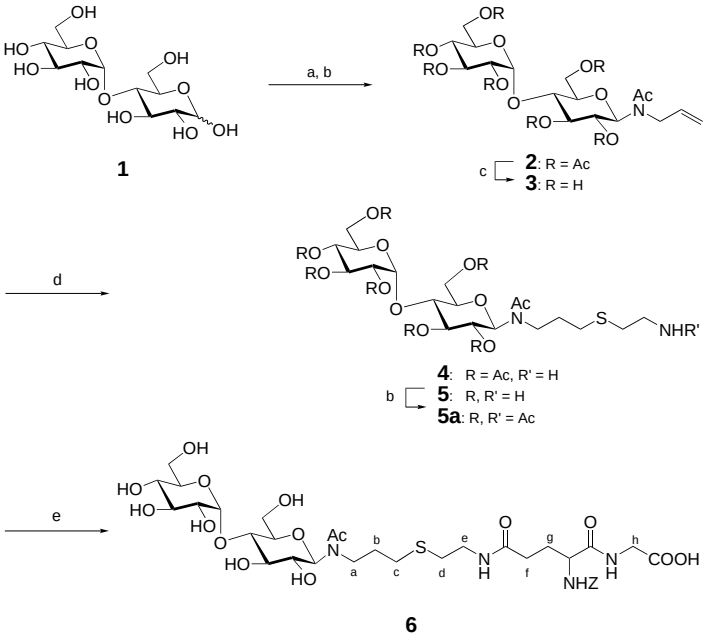
[**] This work has enjoyed generous financial support from Unilever Research Vlaardingen, which is gratefully acknowledged. Microbial transglutaminase (EC 2.3.2.13) was a kind gift from Ajinomoto Co. Europe, Hamburg.



Scheme 1. Rationale of the spacer-transglutaminase approach.

The chosen model compound maltose (**1**), was allowed to react with allylamine[6] and subsequently acetylated to yield the stable peracetylated β-configured anomer **2** (Scheme 2). Upon irradiation (254 nm) of a mixture of **2** and cysteamine



Scheme 2. Chemoenzymatic synthesis of the neoglycopeptide **6**: a) $CH_2CHCH_2NH_2$, room temperature, 72 h; b) Ac_2O/pyridine (1/2), DMAP (85% yield from maltose); c) MeONa/MeOH, pH 10 (90%); d) cysteamine hydrochloride (5 equiv), MeOH, *hν* (254 nm), argon, 8 h (80%); e) compound **5** (30 mM), Z-Gln-Gly (30 mM), TGase (20 $g l^{-1}$), phosphate buffer (Na_2HPO_4/NaH_2PO_4 0.2 M, pH 6), 50 °C, shaken for several hours (60%). DMAP = 4-dimethylaminopyridine, Z-Gln-Gly = benzyloxycarbonyl-glutamine-glycine, TGase = transglutaminase.

hydrochloride in methanol,[7–10] the peracetylated thioether **4** was obtained in 80% yield. This compound was prone to acyl migration, and therefore allylglycosamine **2** was deacetylated prior to the photoaddition reaction to yield a stable compound **3**. After the photochemical step, **3** furnished the unprotected spacer modified disaccharide **5**.

The spacer requirements were evaluated by estimating the relative activities of compounds **4**, **5**, **7**, **8**, **9**, **10**, and **11**

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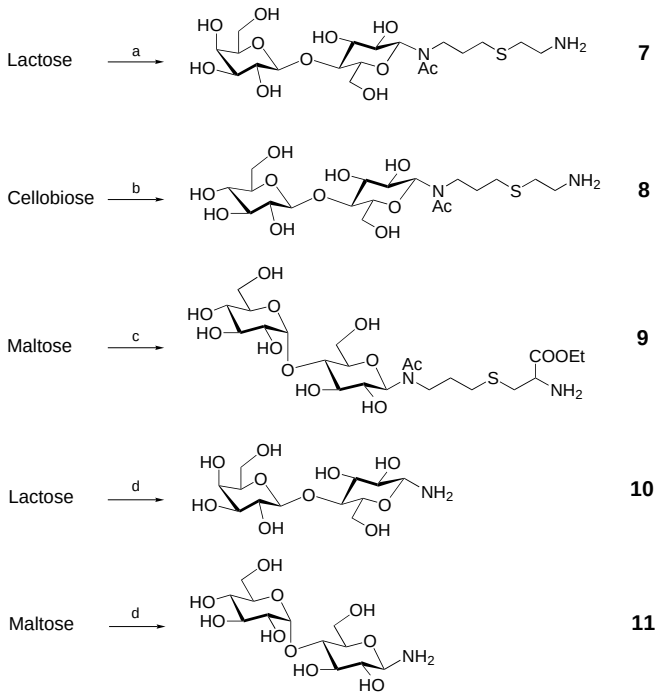
(Scheme 3) relative to hydroxylamine (100%). *N*-Acetyl-[4-*O*-α-D-glucopyranosyl)-β-D-glucopyranosyl]-3-thio(2-aminoethyl)aminopropane (**5**) was accepted as a substrate by the



Scheme 3. Synthesis of additional artificial substrates for TGase. a) 4 steps, 79% from lactose; b) 4 steps, 78% from cellobiose; c) 4 steps, 65% from maltose; d) NH_4HCO_3 (saturated solution), 40 °C, 4 d.

enzyme with a relative activity of 21% (Table 1) and, as expected, the per-O-acetylated counterpart **4** was almost inactive. Compounds **7** and **8**, prepared from lactose and cellobiose following the same procedure as for the synthesis of **5**, also proved to be good substrates for the TGase with relative activities comparable to that found for compound **5** (19 and 18%). The lack of activity for **9** may be attributed to the hindrance and the electronic situation of the amine, whereas either the instability at pH 6 or the lack of spacer yielded both lactosamine **10** and maltosamine **11**[12, 13] inactive. This research demonstrates that the primary amino spacer arm, rather than the nature of the sugar backbone, determines the recognition by the enzyme, allowing this method to be applied to a variety of saccharides.

Table 1. Evaluation of the spacer requirements.

Compound	Relative activity[a] [%]
NH_2OH	100
4	1
5	21
7	19
8	18
9	0
10	0
11	0

[a] Relative activities [$V_0^{Obs}/V_0^{NH_2OH}$] of compounds **4**, **5**, **7**, **8**, **9**, **10** and **11** were estimated against hydroxylamine (standard, 100%). Liberated NH_3 was detected by the glutamate dehydrogenase/reduced nicotinamide adenine dinucleotide phosphate (GLDH/NADPH) assay.[11]

For the preparative scale transacylation, compound **5** was allowed to react with the dipeptide benzyloxycarbonyl-glutamine-glycine (*Z*-Gln-Gly) and Tgase[14] in a buffered solution (pH 6), at 50 °C.[15, 16] The characterization of compound **6** (Table 2), after work-up, proved the covalent cross-linking between the saccharide and the peptide.

Table 2. Selected physical data for compounds **2**, **5**, **5a**, and **6**.

2: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 5.86$ (d, $J_{1,2} = 9.5$ Hz; H-1), 5.74 (dddd; C*H*=CH_2), 5.36 (d, $J_{1',2'} = 4$ Hz; H-1′), 5.34 (dd ~ t, $J_{2,3} = 9$, $J_{3,4} = 9$ Hz; H-3), 5.34 (dd, $J_{2',3'} = 10.4$, $J_{3',4'} = 9.6$ Hz; H-3′), 5.10 (d; CH=CH_2), 5.01 (dd ~ t, $J_{4',5'} = 10$, $J_{3',4'} = 9.6$ Hz; H-4′), 4.87 (dd ~ t, $J_{1,2} = 9.5$, $J_{2,3} = 9$ Hz; H-2), 4.82 (dd, $J_{2',3'} = 10.4$, $J_{1',2'} = 4$ Hz; H-2′), 4.40 (dd, $J_{6'a,6'b} = 12.3$, $J_{5',6'a} = 2.5$ Hz; H-6′a), 4.20 (dd, $J_{6'a,6'b} = 12.3$, $J_{5',6'b} = 4$ Hz; H-6′b), 4.15 (dd, $J_{6a,6b} = 12.4$, $J_{5,6a} = 4$ Hz; H-6a), 4.03 (dd, $J_{6a,6b} = 12.4$, $J_{5,6b} = 2.5$ Hz; H-6b), 3.92 (m_c, $J_{4',5'} = 10$, $J_{3,4} = 9$, $J_{5',6'b} = 4$, $J_{5',6'a} = 2.5$ Hz; H-4, H-5′), 3.75 (m_c, $J_{5,6a} = 4$, $J_{5,6b} = 2.5$ Hz; H-5, CH_2-CH=CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta =$ 171.00 – 170.00 (8 × *C*=O), 135.17 (*C*H=CH_2), 117.43 (CH=*C*H_2), 96.00 (C-1′), 80.34 (C-1), 76.27 (C-3), 74.98 (C-5), 73.04 (C-4), 71.32 (C-2), 70.49 (C-2′), 69.80 (C-3′), 69.03 (C-4′), 68.55 (C-5′), 62.99 (C-6), 61.95 (C-6′), 24.87 (*C*H_2–CH=CH_2), 22.00 – 21.00 (8 × CO*C*H_3); MS (ESI): *m*/*z*: 740 $[M+Na]^+$

5: White powder (80% yield); MS (ESI): *m*/*z*: 523 $[M+Na]^+$, 501 $[M+H]^+$. For analytical purposes, **5** was peracetylated to form **5a**

5a: ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 5.39$ (d, $J_{1',2'} = 4$ Hz; H-1′), 5.30 (m_c, $J_{2',3'} = 10.5$, $J_{1,2} = 9.7$, $J_{3',4'} = 9.7$, $J_{3,4} = 9.1$ Hz; H-2, H-3, H-3′), 5.00 (t, $J_{1,2} =$ 9.7, $J_{3',4'} = 9.7$, $J_{4',5'} = 9.7$ Hz; H-1, H-4′), 4.80 (dd, $J_{2',3'} = 10.5$, $J_{1',2'} = 4$ Hz; H-2′), 4.48 (dd, $J_{6a,6b} = 12.5$ Hz; H-6b), 4.19 (dd, $J_{6'a,6'b} = 12.1$, $J_{5',6'b} = 4.1$ Hz; H-6′b), 4.16 (dd, $J_{6a,6b} = 12.5$, $J_{5,6a} = 4$ Hz; H-6a), 4.02 (dd, $J_{6'a,6'b} = 12.1$, $J_{5',6'a} = 2.5$ Hz; H-6′a), 3.98 (dd, $J_{3,4} = 9.1$, $J_{4,5} = 9.1$ Hz; H-4), 3.90 (m_c, $J_{4',5'} =$ 9.7, $J_{5',6'b} = 4.1$, $J_{5',6'a} = 2.5$ Hz; H-5′), 3.72 (m_c, $J_{4,5} = 9.1$, $J_{5,6a} = 4$ Hz; H-5), 3.35 (m_c; CH_2(e)), 2.59 (m_c; CH_2(d)), 2.42 (m_c; CH_2(a)), 2.15 – 1.88 (m; 9 × CO*C*H_3), 1.83 (m_c, 1H; CH_2(c)), 1.71 (m_c, 3H; CH_2(c), CH_2(b)); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 171.60 – 169.30$ (9 × *C*OCH$_3$), 95.49 (C-1′), 85.26 (C-1), 75.91, 75.67 (C-2 + C-3), 74.63 (C-5), 69.95 (C-2′), 69.37 (C-4′), 69.14 (C-3′), 68.54 (C-5′), 62.45 (C-4), 62.40 (C-6), 61.33 (C-6′), 38.43 (*C*H_2(e)), 31.72 (*C*H_2(d)), 30.31 (*C*H_2(c)), 29.17 (*C*H_2(a)), 28.43 (*C*H_2(b)), 23.10 – 20.43 (9 × CO*C*H_3); MS (ESI): *m*/*z*: 859 $[M+Na]^+$, 837 $[M+H]^+$

6: ^{1}H NMR (600 MHz, D_2O): $\delta = 7.41$ (m_c; aromatic-Hs), 5.38 (d; H-1′), 5.15, 5.07 (each d, each 1H; CH_2–Ph), 4.98 (d, $J_{1,2} = 8.5$ Hz; H-1), 4.12 (m_c; C*H* (Gln)), 3.90 – 3.45 (m, $J_{6a,6b}$ or $J_{6'a,6'b} = 12.0$, $J_{3,4}$ or $J_{3',4'} = 9.5$, $J_{4,5} =$ 9.5, $J_{1,2} = 8.5$ Hz; CH_2(f), CH_2(g), CH_2(h), H-2, H-2′, H-3, H-3′, H-4′, H-5, H-5′, H-6, H-6′), 3.40 (dd, $J_{3,4} = 9.5$, $J_{4,5} =$ 9.5 Hz; H-4), 3.31 (m_c; CH_2(e)), 2.64 (m_c; CH_2(d)), 2.52 (m_c; CH_2(a)), 2.34 (m_c; CH_2(c)), 1.87 (m_c; CH_2(b)); ^{13}C NMR (150 MHz, D_2O): $\delta = 175.16 – 175.53$ (5 × *C*=O), 136.12 (aromatic-C), 128.70 – 127.69 (5 × aromatic-*C*H), 99.57 (C-1′), 86.73 (C-1), 77.19 – 69.20 (C-2, C-2′, C-3, C-3′, C-4, C-4′, C-5, C-5′, 2 × CH_2), 67.17 (*C*H_2-Ph), 60.73, 60.36 (C-6 + C-6′), 43.33 (*C*H_2) 38.42 (*C*H_2(e)), 31.87 (*C*H_2(c)), 29.10 (*C*H_2(d)), 27.85 (*C*H_2(a)), 27.12 (*C*H_2(b)); MS (ESI): *m*/*z*: 865 $[M - H+2Na]^+$ (daughter 757 $[(M - OCH_2Ph)+2Na]^+$), 859 $[M+K]^+$ (daughter 751 $[(M-OCH_2Ph)+K]^+$), 843 $[M+Na]^+$ (daughter 735 $[(M - OCH_2Ph)+Na]^+$), 819 $[M - H]^-$ (daughter 711 $[(M - OCH_2Ph) - H]^-$).

In conclusion, a practical combined approach towards new glycoprotein structures has been worked out, which requires minor modifications of the saccharide and thereby conserves the spatial arrangement of the sugar functionality. By combining both photochemical linking and enzymatic acyl transfer, a double regiochemical control can be exerted on the glycoside as well as on the peptide. Finally, this procedure should be applicable to the synthesis of neoglycopeptide building blocks and the synthesis of multivalent glycoside ligands on a suitably arranged protein scaffold.

Received: August 9, 1999 [Z13846]

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Aliphatic Hydroxylation by a Bis(μ-oxo)dicopper(III) Complex**

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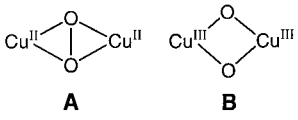
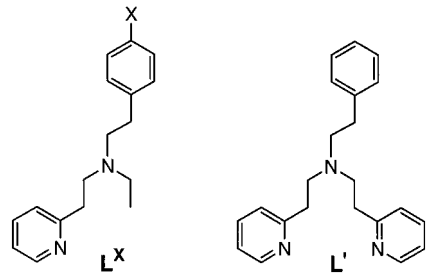
Understanding how metal – dioxygen adducts react in biochemical and synthetic transformations of organic substrates is an important research objective.[1] Significant progress toward this goal has been made through the detailed characterization of complexes derived from the reaction of dioxygen with Cu^I precursors.[2] Of the adducts characterized by X-ray crystallography to date[3–5] the (μ-η^2:η^2-peroxo)- and bis(μ-oxo)dicopper units **A** and **B**, respectively, have drawn particular attention as a consequence of their known or potential relevance to intermediates in hydroxylation reactions performed by copper oxygenases such as tyrosinase and particularly methane monooxygenase (pMMO).[6] Although **B** has yet to be observed in an enzyme system, the possibility that **A** (identified as an intermediate in tyrosinase and catechol oxidase) may convert into **B** prior to activation of the substrate C–H bond is supported by the observed reactivity of synthetic examples of these cores.[5, 7, 8] Evidence in support of the ability of core **B** to hydroxylate arene rings,[9] to abstract H atoms from the weak C–H bonds of dihydroanthracene,[10] and to oxidatively N-dealkylate ligand substituents has been uncovered.[5, 11] Mechanistic studies of the latter reaction implicate an initial hydroxylation at the activated position α to the N donor by a rate-controlling C–H bond scission to yield a presumed carbinolamine intermediate, which then decays to the product aldehyde and secondary amine.[11] However, direct observation of the hydroxylation of aliphatic C–H bonds by **B**, a reaction relevant to the function of monooxygenase (for example, pMMO), has remained elusive.[12]



Herein we describe a new set of bis(μ-oxo)dicopper complexes with ligand $\mathbf{L^X}$ ($\mathbf{L^X}$ = *p*-substituted *N*-ethyl-*N*-[2-(2-pyridyl)ethyl]-2-phenylethylamine; X = OMe, Me, H, Cl, NO_2) that decompose to a product in which the ligand is hydroxylated at its benzylic position. Detailed characterization of this newly discovered aliphatic C–H bond activation reaction by core **B** reveals important information on the fundamental chemistry underlying copper monooxygenase reactivity.



(X = OMe, Me, H, Cl, NO_2)

Figure 1 shows the spectral changes observed upon introduction of O_2 into a solution of $[Cu^I(\mathbf{L^H})(CH_3CN)]PF_6$ in acetone at $-90\,^\circ C$.[13] An absorption band at 402 nm (ε = 17700 $M^{-1}cm^{-1}$)[14] similar to those of the bis(μ-oxo)dicopper(III) complexes reported previously[5] appears gradually. Also similar to other complexes with core **B** is that the solution is ESR silent. Furthermore, the resonance Raman spectrum (λ_{ex} = 457.9 nm) of a frozen [D_6]acetone solution of the intermediate generated using $[Cu^I([D_4]\mathbf{L^H})(CH_3CN)]PF_6$ ([D_4]$\mathbf{L^H}$: *N*-ethyl-*N*-[2-(2-pyridyl)ethyl]-1,1,2,2-tetradeuterio-2-phenylethylamine) has an intense peak at 607 cm^{-1} that shifts to 578 cm^{-1} upon isotopic substitution with $^{18}O_2$ (see inset of Figure 1). This frequency and isotopic shift ($\Delta\tilde{\nu}$ = 29 cm^{-1}) are very close to those reported for bis(μ-oxo)di-

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[**] The present study was financially supported in part by a Grant-in-Aid for Scientific Research on Priority Area (Molecular Biometallics: 10129218; Electrochemistry of Ordered Interface: 10131242) from the Ministry of Education, Science, Culture, and Sports of Japan and the National Institutes of Health of the USA (postdoctoral fellowship to P.L.H., grant GM47365 for W.B.T., and grant GM33162 to L.Q.).

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