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[4] For the synthesis of $Tb_{13}Br_{18}B_3$ [$Gd_{13}Br_{18}B_3$], Ta capsules were filled with $TbBr_3$ (1000 mg) [$GdBr_3$ (750 mg)], Tb (433 mg) [Gd (323 mg)] and B (14.4 [10.7] mg; 5N, Aldrich) and sealed under Ar by arc-melting. These were heated for 30 days at 950 °C [6 days at 1000 °C]. Further details are analogous to the procedures described in reference [3g]. The compounds can be obtained as almost single-phase products according to X-ray powder diagrams. They are sensitive to moisture and need to be handled in a dry inert gas atmosphere.

[5] Single-crystal X-ray investigation (average structure): Stoe IPDS diffractometer, $Mo_{K\alpha}$ (0.71073 Å), $2\theta_{max} = 60.6°$; orthorhombic, space group *Immm*; $a = 3.9923(2)$, $b = 17.0462(12)$, $c = 28.3039(19)$ Å for $Gd_{13}Br_{18}B_3$ and $a = 3.9637(3)$, $b = 16.9063(13)$, $c = 28.1068(18)$ Å for $Tb_{13}Br_{18}B_3$; least-squares refinement on F^2 (G. M. Sheldrick, SHELXL 97 program for the refinement of crystal structures, University of Göttingen, Göttingen (Germany), **1997**); R_1 [$I \geq 2\sigma(I)$] = 0.025 (0.022), wR_2 [all data] = 0.057 (0.046) for 1668 (1627) reflections and 76 (76) parameters for $Gd_{13}Br_{18}B_3$ ($Tb_{13}Br_{18}B_3$). Further details on the crystal structure investigation may be obtained from the Fachinformationszentrum Karlsruhe, 76344 Eggenstein-Leopoldshafen, Germany (fax: (+49) 7247-808-666; e-mail: crysdata@fiz-karlsruhe.de), on quoting the depository numbers CSD-412541 ($Gd_{13}Br_{18}B_3$) and CSD-412540 ($Tb_{13}Br_{18}B_3$). A detailed description of the structural analysis as well as an analysis of symmetry relationships and additional real structure phenomena will be published elsewhere (O. Oeckler, L. Kienle, H. Mattausch, O. Jarchow, A. Simon, *Z. Kristallogr.*, submitted).

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Enzymatic Elongation of the LEC14 Antigen Generates a β-1,2 Arm on *N*-Glycans**

Ingo Prahl and Carlo Unverzagt*

This work is dedicated to Professor Hans Paulsen on the occasion of his 80th birthday

Nearly all proteins of the blood serum and on cell surfaces in higher organisms are glycosylated.[1,2] On these glycoproteins, a multitude of asparagine-linked oligosaccharides (*N*-glycans) with similar structures are found. The structural diversity of these *N*-glycans is generated biosynthetically from a common core pentasaccharide $Man_3GlcNAc_2$. In biologically relevant complex *N*-glycans, this core pentasaccharide is extended by up to five antennae and can be modified further by the addition of single sugar residues, for example, core fucose or a bisecting GlcNAc residue. Recently, mutants of Chinese hamster ovary cells (CHO cells) termed LEC14 were found to exhibit *N*-glycans with a new glycosylation motif at the core pentasaccharide.[3] The structural analysis indicated an additional GlcNAc residue β-1,2 linked to the β-mannoside of the core trisaccharide. The relevant glycosyltransferase was characterized and termed GlcNAc-TVII.[4] To confirm the proposed structure and generate derivatives for biological evaluation, we have synthesized the LEC14 nonasaccharide, functionalized with a spacer. Analysis of the NMR spectroscopic data indicates that the proposed structure is correct. Unexpectedly, the single GlcNAc residue present in the LEC14 motif of the synthetic target compound was recognized by galactosyltransferase, which resulted in a to date unknown type of branching extension. The occurrence of this novel antenna (β-1,2 arm) generates a third branch at the central β-mannoside. This arm adds a new element to the structural motifs of *N*-glycan cores, which permits even greater variability.

A protected form of the LEC14 nonasaccharide **1** was obtained by using a modular building-block approach[5] based on regio- and stereoselective glycosylations.[6] The synthesis of the LEC14 *N*-glycan was designed to allow a comparison with the isolated natural compound and to give access to neoglycoconjugates for future investigation of the biological activity of this unusual *N*-glycan.[7] Furthermore, the synthetic LEC14 *N*-glycan should be elongated using glycosyltransferases to give full length *N*-glycans with biologically relevant termini.

The conversion of the protected nonasaccharide **1** into neoglycoconjugates required several synthetic steps, which included the coupling of the carbohydrate moiety to a spacer that can be selectively activated for later attachment to the carrier.[8] Prior to the coupling of the spacer, all protecting groups except the three benzyl groups and the anomeric azido group were removed sequentially.

First, the three *p*-methoxybenzyl groups (Mpm) of the fucose residue in compound **1** were oxidatively removed using ceric ammonium nitrate (CAN) in acetonitrile/water. The phthaloyl groups of the nonasaccharide **2** were removed in a one-pot reaction comprising three steps, which was developed for use with *N*-glycans without affecting anomeric azido groups. Using ethylene diamine in *n*-butanol[9] at 80 °C yielded an intermediate pentaamino compound. After acetylation and *O*-deacetylation, the water-soluble nonasaccharide **3** was isolated by solid-phase extraction (SPE) in 96 % overall yield. Subsequent reduction of the azido group using excess propanedithiol in methanol/triethylamine[10] yielded the corresponding glycosylamine after removal of the volatile reagents. The amine was immediately acylated with excess *N*-benzyloxycarbonyl-6-aminohexanoic acid **4** using *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/hydroxybenzotriazole (TBTU/HOBt) in *N*-methyl-2-pyrrolidone (NMP). Under these conditions, *O*-acylation was observed, which could be reversed by quenching the reaction mixture with aqueous methylamine prior to workup. After purification by reverse-phase (RP)-HPLC, **5** was obtained in 31 % yield. Catalytic hydrogenation over palladium hydroxide furnished the aminohexanoyl-derivatized nonasaccharide **6** in 95 % yield after size-exclusion chromatography (Scheme 1).

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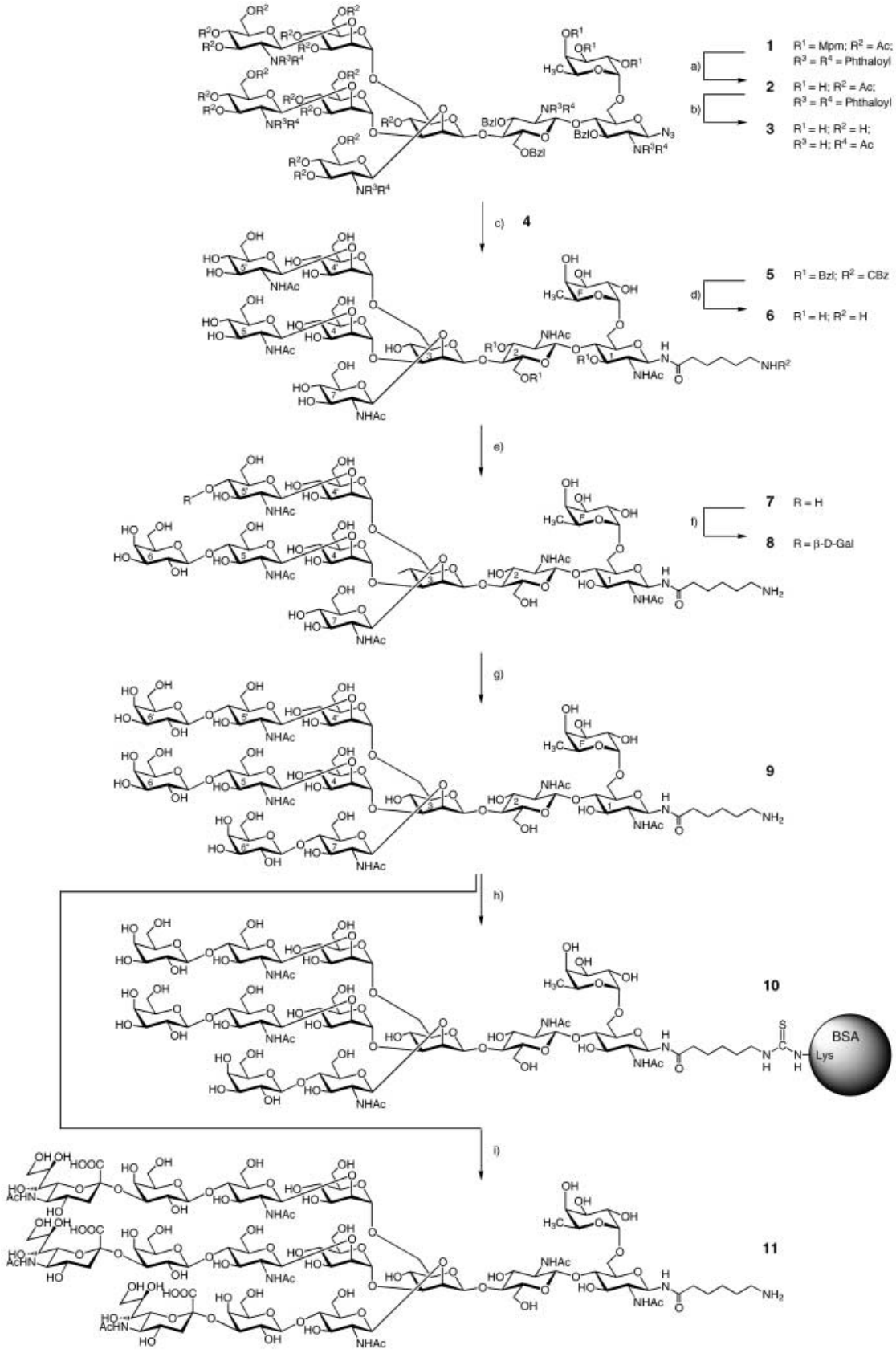


Scheme 1. Synthesis of LEC14 conjugates: a) $[(NH_4)_2Ce(NO_3)_6]$ (CAN), CH_3CN/H_2O, 80 °C (71 %); b) 1. ethylenediamine, *n*BuOH, 80 °C; 2. Ac_2O, pyridine; 3. $MeNH_2$ (41 %) in H_2O (96 % for steps 1–3); c) 1. propanedithiol, NEt_3, MeOH; 2. CBz-aminohexanoic acid **4**, TBTU, HOBt, *N*-methylpyrrolidone (31 % for steps 1 and 2 after RP-HPLC); d) $PdO/H_2O/H_2$, MeOH/AcOH (95 %); e,f,g) UDP-Gal (1 equiv), galactosyltransferase, alkaline phosphatase (80 %, 84 %, and 88 %, respectively); h) 1. thiophosgene, CH_2Cl_2/H_2O, $NaHCO_3$; 2. BSA, H_2O, $NaHCO_3$; i) CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac), α-2,3-sialyltransferase, alkaline phosphatase (yield not determined). Mpm = *p*-methoxybenzyl, Ac = acetyl, Bzl = benzyl, CBz = benzyloxycarbonyl.

The structure of **6** was confirmed by electrospray ionization (ESI) MS[11] and a series of 2D NMR experiments[12] (COSY, TOCSY, HMQC, HMQC–COSY, NOESY). Compound **6**[13] and the isolated LEC14[3] nonasaccharide differ only in their aglycon part, which is aminohexanoic acid or asparagine, respectively. Despite this difference, both compounds show nearly identical proton resonances throughout the molecule (Table 1).

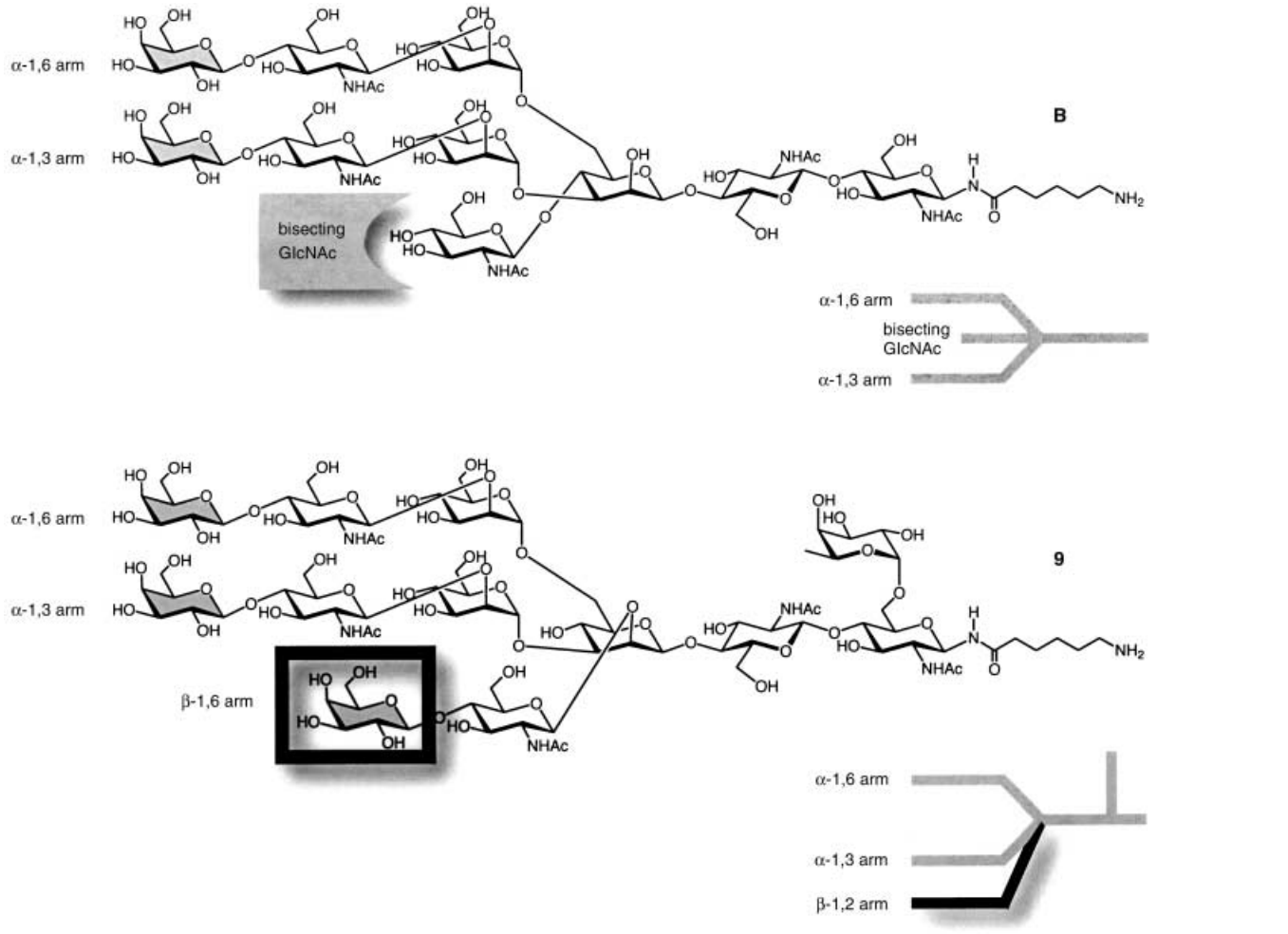
The very high level of similarity throughout the anomeric fingerprint region[14] led us to the conclusion that the structural assignments obtained from minute amounts of isolated LEC14 nonasaccharide–asparagine[3] are correct. In particular, the novel β-1,2 linkage of the GlcNAc residue attached to the central β-mannoside (LEC14 motif) was confirmed by a total synthesis of the entire *N*-glycan fragment.

Table 1. Comparison of the ^{1}H NMR spectroscopic data of **6** with that of isolated LEC14. Spectra were recorded in D_2O with DMSO (in the case of **6**) or acetone (isolated LEC14) as an internal standard. H-6^F of compound **6** has been calibrated to H-6^F of the natural LEC14 for better comparison.

Compound	δ of anomeric protons^{residue} [ppm][a]									
	1^1	1^2	1^3	1^4	$1^{4'}$	1^5	$1^{5'}$	1^7	1^F	6^F
6	5.03	4.71	4.77	5.11	4.89	4.49	4.53	4.69	4.87	1.20
LEC14[3]	5.07	4.57	(4.75)	5.11	4.88	4.54	4.54	4.68	4.86	1.20

[a] Ring description see Scheme 1.

The conversion of the spacer-bound synthetic **6** into a neoglycoprotein was then planned. A continuation of the synthesis by enzymatic methods is convenient and efficient for obtaining biologically relevant galactose termini on *N*-glycans.[15] Compound **6** carries two terminal *N*-acetylglucosamine residues (5 and 5′, see Scheme 1), which are known to be readily elongated by galactosyl transferase (EC 2.4.1.22). An initial reaction using galactosyl transferase, alkaline phosphatase,[16] and excess uridine-5′-diphospho (UDP)-galactose under conditions optimized for *N*-glycans surprisingly yielded not only the expected undecasaccharide **8**, but also the dodecasaccharide **9**. Both structures were later confirmed by ESI–MS and high-field 2D NMR spectroscopy. Compound **9** carries an additional galactose residue on the β-1,2-linked GlcNAc moiety. The occurrence of this novel enzymatic extension was highly unusual because an enzymatic galactosyl functionalization of the homologous β-1,4-linked single *N*-acetylglucosamine residue in the bisecting-type *N*-glycan (type **B**; Scheme 2) is not observed here.[17,18] On the other hand, the isolated LEC14 asparagine was obtained after a series of glycosidase



Scheme 2. Enzymatic formation of the β-1,2 arm as a novel branching motif, which extends the β-1,2-linked GlcNAc residue of the LEC14 conjugate **9**. In contrast, enzymatic extension does not occur on the bisecting GlcNAc residue of the homologous compound **B** with two antennae. BSA = bovine serum albumin.

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treatments, which makes it likely that the unusual extension was lost during the isolation process.

To investigate the regioselectivity of the unexpected enzymatic elongation of compound **6** in detail, only one equivalent of UDP-galactose was used in the galactosyltransferase reaction. After size-exclusion chromatography, the decasaccharide **7** was obtained in 80% yield. The selectivity of the galactosylation of the α-1,3 arm versus the α-1,6 arm was greater than 3.8:1. This is in good accordance with the known preference of glycosyltransferases for the α-1,3 arm of *N*-glycans.[19] Subsequent galactosylation of **7** with one equivalent of UDP-galactose yielded **8**. During this step, the formation of **9** (**8**:**9** approximately 4:1) was observed. Compounds **8** and **9** could not be separated by size-exclusion chromatography.

In a final reaction using excess UDP-galactose the undecasaccharide **8** was converted into the pergalactosyl dodecasaccharide **9** in 88% yield. The galactosyl derivatization of the different GlcNAc residues takes place in the following sequence, as shown by NMR spectroscopy: α-1,3 arm > α-1,6 arm > β-1,2-linked GlcNAc. The low accessibility of the unusual β-1,2-linked GlcNAc residue appears to coincide with the least reactivity towards galactosyltransferase.

As pergalactosyl derivatization could be readily achieved, enzymatic persialyl derivatization was also investigated. Sialylation was performed by incubation of **9** with α-2,3-sialyl transferase (E.C. 2.4.99.6), alkaline phosphatase,[16] and an excess of the donor substrate CMP-*N*-acetylneuraminic acid (CMP = cytidine-5′-monophospho-). Within two hours, ESI–MS revealed single, double, and a small amount of triple sialyl derivatization. After two days, the persialylated compound **11** was found to be the main product by ESI–MS.

To investigate the ligand properties of the novel galactosyl-derivatized LEC14-*N*-glycan **9** in comparison with core fucosyl-derivatized,[7] bisecting,[18] or unsubstituted *N*-glycans[7,20] the neoglycoconjugate **10** was synthesized. First, the free amino group of **9** was converted into an isothiocyanate,[21] which was then coupled to carbohydrate-free bovine serum albumin. On the purified neoglycoconjugate **10**, the ligand density averaged 3.2 *N*-glycans per carrier protein, as determined by a colorimetric assay[22] and supported by MALDI-TOF spectrometry. Neoglycoconjugate **10** can be used to probe the to date unknown biological properties of the novel galactosyl-derivatized LEC14-*N*-glycan in glycobiological studies.[20]

In summary, we have shown by total synthesis that the structure of the LEC14 motif is correct and that the single GlcNAc residue can be built up enzymatically to form a novel third branch attached to the central β-mannoside. The occurrence of this extended β-1,2 arm in vitro suggests that this additional branch is also generated in the cellular system, which thus expands the structural variability of *N*-glycans in general.

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