Pergamon

Tetrahedron Letters, Vol. 38, No. 32, pp. 5627-5630, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)01278-1

Building Blocks for Glycoproteins: Synthesis of the Ribonuclease B Fragment 21-25 containing an Undecasaccharide N-Glycan

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Abstract: A glycosylated fragment of the bovine glycoprotein ribonuclease B (**1**) was synthesized corresponding to amino acids 21-25 with a complex type biantennary N-glycan linked to Asn^{24}. The total synthesis of the undecasaccharide-glycopentapeptide **1** was accomplished by modern solution phase glycopeptide chemistry combined with enzymatic techniques for the removal of protective groups and a regio- and stereoselective transfer of the terminal carbohydrates using glycosyltransferases.

In the human organism glycoproteins play important roles in key biological events. It is widely recognized that the properties of glycoproteins[1] are influenced by their carbohydrate part, however the basis of these interactions remains to be elucidated. The structure-activity relationships of glycoproteins are difficult to evaluate because of the microheterogeneity present in the natural material and the tedious isolation procedures. To study the interplay between natural asparagine-linked oligosaccharides (N-glycans) and their peptide backbone an access to defined model compounds was developed. For the first time a glycoprotein fragment containing a sialylated biantennary *N*-glycan (**1**) was obtained by total synthesis (Fig. 1).

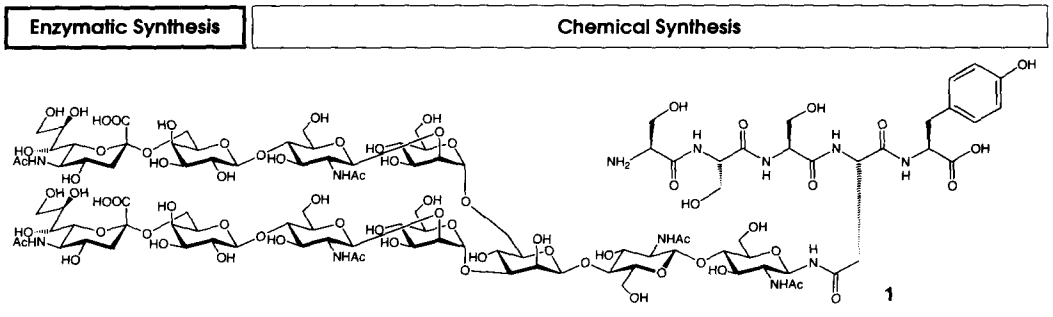

Figure 1: Amino acids 21-25 of bovine ribonuclease B (Ser-Ser-Ser-Asn-Tyr) linked to a full length biantennary N-glycan

Glycopeptide synthesis[2] has been remarkably stimulated in the last decade by an extended repertoire, mainly by solid phase techniques and enzymatic reactions. In priciple, two general strategies can be distinguished: the classical route to glycopeptides via glycosyl amino acids or the coupling of a complete peptide sequence to the carbohydrate. In the latter case the occurrence of side reactions[3] and low reactivity is critical prompting us to choose the classical strategy for the synthesis of **1**. An access to the complex glycosyl amino acid **2** as a building block for glycoprotein partial structures has been described previously.[4] Elongation of the carbohydrate chains of **2** using glycosyltransferases gave a sialylated undecasaccharide-asparagine.[4] This compound contains two additional carboxyl groups at the sialic acid moieties requiring a sophisticated protective group strategy to selectively activate the C-terminus. Thus peptide couplings were performed on the heptasaccharide level using glycosyl asparagine **2** followed by enzymatic completion of the oligosaccharide chains to give the undecasaccharide-pentapeptide **1**.

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The reactivity of the glycosyl amino acid **2** in peptide couplings could only be estimated from a few comparable reference data.[5] To examine these properties solution phase syntheses were performed on the C- and the N-terminus of the heptasaccharide asparagine **2**. First, glycosyl amino acid **2** was converted to the Fmoc derivative **3** with Fmoc-*N*-hydroxysuccinimide ester.[6] The protected heptasaccharide asparagine **3** was readily isolated by solid phase extraction and elongated with tyrosine methylester **4** in *N*-methylpyrrolidone (NMP) and TBTU[7] ((1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) as a coupling reagent. Within less than 10 minutes the reaction was completed giving the glycodipeptide **5** in 74 % yield after HPLC-purification. Presumably, the steric hindrance at the C-terminus of **3** is low despite the bulky Fmoc moiety and the complex oligosaccharide part suggesting that **3** may also be an attractive building block for solid phase synthesis. Selective liberation of the amino group in the glycopeptide **5** was affected by 10 % piperidine in NMP without hydrolysis of the methylester. However, the deprotection required prolonged reaction times (30 minutes) indicating a steric hindrance of the Fmoc group by the neighboring substituents. To remove the reagents completely the resulting amino compound **6** was purified by HPLC. Elongation of the glycodipeptide amine **6** with Fmoc-triserine 7[8] promoted by the highly activating HATU[9] (O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) proceeded rapidly (5-10 min) and gave the complex heptasaccharide glycopentapeptide **8** (70 % yield after HPLC). Despite the presence of the numerous hydroxyl functions[10] in the heptasaccharide part the elongation with the Fmoc-tripeptide **7** occurred chemoselectively at the amino group of **6**.

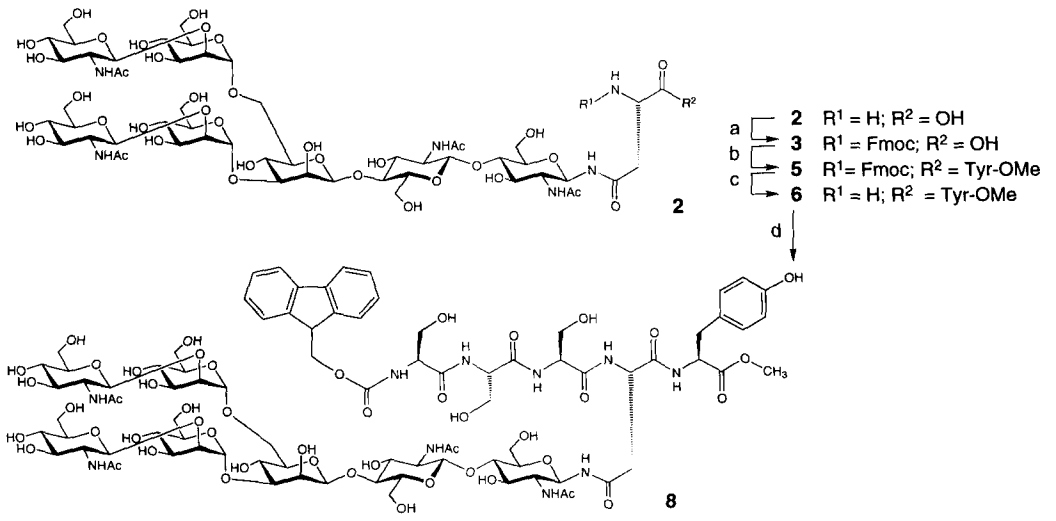

Figure 2: a) Fmoc-OSu, dioxane-H_2O (88%); b) H-Tyr-OMe **4**, TBTU, HOBt, NMP (74%); c) piperidine, NMP (87%) d) Fmoc-Ser-Ser-Ser-OH **7**, HATU, NMP (70%).

The remaining protective groups of the heptasaccharide glycopentapeptide **8** are labile under the conditions used for enzymatic elongation of the oligosaccharide part and were thus removed in a stepwise procedure. First the Fmoc group was cleaved with piperidine in NMP followed by hydrolysis of the methylester. Alkaline hydrolysis of methylesters is prone to racemization[11] suggesting a mild enzymatic[12] step instead (figure 3). Chymotrypsin cleaves peptides and peptide esters after aromatic amino acids and leucine,[13] other amino acids are not affected. This allowed a facile removal of the methylester in **9** by chymotrypsin in aqueous solution without side reactions. Size exclusion chromatography afforded the free glycopeptide **10** in 96 % yield.

A stepwise elongation to the biantennary undecasaccharide **1** was performed by an enzymatic transfer[14] of the terminal sugars galactose and N-acetylneuraminic acid to the N-acetylglucosamine residues of

10. The reaction sequence (figure 3: c-d) was conducted in a one-pot reaction. Thin layer chromatography showed that alkaline phosphatase[14i] accelerated the galactosyltransferase reaction which was complete after a few hours. The following sialylation required considerably longer reaction times and good results could only be obtained when the α2,6-sialyltransferase was added in two portions. Separation of the reaction mixture by size exclusion chromatography showed that the double galactosylation proceeded nearly quantitatively and less than three percent of the monosialylated compound could be removed. The target molecule an undecasaccharide glycopentapeptide **1**[15] was isolated in 91 % yield. The average glycosylation yield calculated for the enzymatic transfer of the four terminal residues amounts to 98 % per sugar moiety. This value demonstrates the high efficiency of alkaline phosphatase in glycosyltransferase reactions, a method that has already been adopted by numerous research groups.[14l-t]

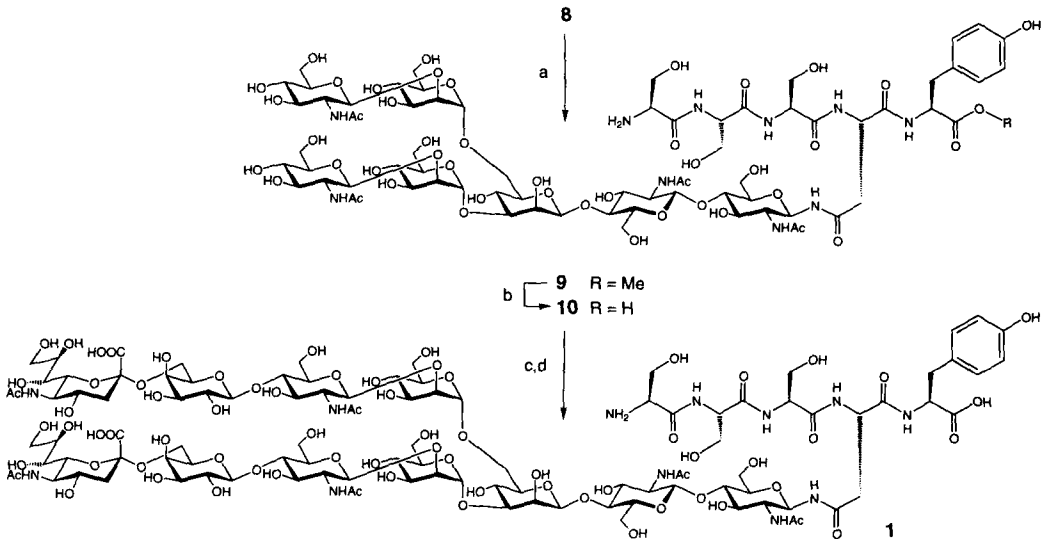

Figure 3: a) piperidine, NMP; b) chymotrypsine, H_2O (a.-b.: 96%); c) UDP-Gal, galactosyltransferase, alkaline phosphatase; d) CMP-NeuAc, α2,6-sialyltransferase, alkaline phosphatase (c-d.: 91 %).

The peptide sequence of the undecasaccharide-glycopentapeptide **1** represents the amino acids 21-25 of bovine ribonuclease B. Instead of the natural glycosylation site at Asn^{34} the synthetically less critical sequence at Asn^{24} was chosen. The undecasaccharide moiety of **1** is identical with the complex biantennary N-glycans found in human secretory ribonuclease[16a] whereas bovine RNAse B contains high mannose N-glycans.[16b] A combination of chemical and enzymatic methods allowed the first synthesis of a glycoprotein fragment carrying a full length asparagine-linked oligosaccharide. With the availability of the complex glycopeptide **1** the total synthesis of entire glycoproteins[17] is within reach.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft and the Leonhard-Lorenz-Stiftung. Gratitude is expressed to Prof. Horst Kessler for his generous support.

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b) **1**: 6.24 mg yield (91% calculated from **12**), $[\alpha]_D^{23}$ = -9.5° (0.2, H_2O) $C_{108}H_{168}N_{12}O_{72}$ MALDI-TOF-MS (negative mode; 2,5-dihydroxybenzoic acid in H_2O:EtOH= 9+1): calculated = 2760.98, found = 2757 (M-1);

NMR assigments were based on TOCSY, NOESY, HMQC, HMQC-COSY, HMQC-DEPT, HMQC-TOCSY experiments.

^{1}H-NMR (500 MHz, D_2O): δ = 7.16 (d, J_{vic} =8.4Hz, 2H, H-o Tyr), 6.90 (d, 2H, H-m Tyr), 5.19 (d, $J_{1,2}$ < 1.0Hz, 1H, H-1^4), 5.07 (d, $J_{1,2}$ = 9.6Hz, 1H, H-1^1), 5.00 (d, $J_{1,2}$ < 1.0Hz, 1H, H-1$^{4'}$), 4.83 (d, $J_{1,2}$ < 1.0Hz, 1H, H-1^3), 4.76 (t, J_{vic} =6.4Hz, 1H, αCH Asn), 4.66 (m, 3H, H-1^2, H-1^5, H-1$^{5'}$), 4.63 (t, J_{vic} =5.5Hz, 1H, αCH SerA), 4.51 (t, J_{vic} =5.4Hz, 1H, αCH SerB), 4. 50 (2d, $J_{1,2}$ = 6.5Hz, 1H, H-1^6, H-1$^{6'}$), 4.45 (dd, J_{vic} =7.2, J_{vic} =5.5Hz, 1H, αCH Tyr), 4.31 (m, 1H, H-2^3), 4.29 (t, J_{vic} =5.0Hz, 1H, αCH SerC), 4.25 (dd, $J_{2,3}$ = 2.7Hz, 1H, 1H, H-2^4), 4.17 (dd, $J_{2,3}$ = 2.7Hz, 1H, H-2$^{4'}$), 3.12 (dd, J_{gem} = 14.0Hz, J_{vic} = 5.1Hz, 1H, βCHa Tyr), 2.96 (dd, J_{vic} = 7.5Hz, 1H, βCHb Tyr), 2.81 (dd, J_{gem} = 16.1Hz, J_{vic} = 5.4Hz, 1H, βCHa Asn), 2.75-2.69 (m, 3H, βCHb Asn, H-3eq^N, H-3eq$^{N'}$), 2.14, 2.12, 2.08, 2.05 (4s, 18H, NAc), 1.77 (dd, J_{vic} = J_{gem} = 12.2Hz, 2H, H-3ax^N, H-3ax$^{N'}$).

^{13}C-NMR (125 MHz, D_2O, CD_3CN als internal standard): δ = 175.9, 173.9, 173.7, 173.6, 173.5, 172.4, 171.6, 170.3, 170.0, 169.7, 167.1 C=O, 153.2 C-p Tyr, 129.7 C-o Tyr, 128.2 C-i Tyr, 114.3 C-m Tyr, 102.6 C-1^6, C-1$^{6'}$, 100.3 C-1^2, 99.5 C-1^3, 99.2 C-2^N, C-2$^{N'}$, 98.6 C-1^4, 98.4, 98.3 C-1^5, C-1$^{5'}$, 96.0 C-1$^{4'}$, 79.7, 79.6 C-4^5, C-4$^{5'}$, 79.5 C-3^3, 78.7 C-4^2, 77.9 C-4^1, 77.3 C-1^1, 75.5 C-2^4, 75.3 C-2$^{4'}$, 75.2 C-5^1, 73.5, 73.4 C-5^2, C-5^3, C-5^5, C-5$^{5'}$, 72.7 C-5^6, C-5$^{6'}$, 72.6 C-5^4, 71.8 C-3^1, C-5$^{4'}$, 71.6 C-6^N, C-6$^{N'}$, 71.5 C-3^6, C-3$^{6'}$, 71.2, 71.1 C-3^2, C-3^5, C-3$^{5'}$, 70.8 C-8^N, C-8$^{N'}$, 69.8 C-2^6, C-2$^{6'}$,69.2 C-2^3, 68.5 C-3^4, C-3$^{4'}$, 67.5 C-7^N, C-7$^{N'}$, 67.4 C-4^6, C-4$^{6'}$, 67.2 C-4^N, C-4$^{N'}$, 66.3 C-4^4, C-4$^{4'}$, 64.9 C-6^3, 64.7 C-4^3, 62.3 C-6^6, C-6$^{6'}$, 61.8 C-9^N, C-9$^{N'}$, 60.8, 60.7 C-6^4, C-6$^{4'}$,60.2 βC SerB, 60.1 βC SerA, 59.3 C-6^5, C-6$^{5'}$, 59.2 βC SerC, 59.1 C-6^2, 59.0 C-6^1, 55.1 αC Tyr, 54.7 αC SerA, 54.4 αC SerB, 54.0 C-2^2, 53.7 C-2^5, C-2$^{5'}$, 53.5 αC SerC, 52.7 C-2^1, 51.0 C-5^N, C-5$^{N'}$, 49.2 αC Asn, 35.6 βC Tyr, βC Asn, 21.5, 21.3, 21.2, 21.1 NAc.

Ser A, B, C were not assigned to the peptide sequence.

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(*Received in Germany* 22 *May* 1997; *accepted* 19 *June* 1997)