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(71) Applicant(s)
CSL Behring Lengnau AG

(72) Inventor(s)
Schulte, Stefan; Beltz, Hans-Wilhelm; Pestel, Sabine; Weimer, Thomas; Pelzing, Matthias

(74) Agent / Attorney
FB Rice Pty Ltd, Level 23 44 Market Street, Sydney, NSW, 2000, AU

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- (71) Applicant: CSL BEHRING RECOMBINANT FACILITY AG [CH/CH]; Wankdorfstrasse 10, 3014 Bern (CH).
- (72) Inventors: SCHULTE, Stefan; Bauerbacher Strasse 46, 35043 Marburg (DE). BELTZ, Hans-Wilhelm; Im Rotenbach 6a, 35216 Biedenkopf (DE). PESTEL, Sabine; Stuempelstal 19, 35041 Marburg (DE). WEIMER, Thomas; Richard-Wagner-Strasse 8, 35075 Gladenbach (DE). PELZING, Matthias; 75 Mount View Road, Thomastown, Victoria 3074 (AU).
- (74) Agents: HAUSER, Hans-Peter et al.; CSL Behring GmbH, Emil-von-Behring-Strasse 76, 35041 Marburg (DE).

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(54) Title: METHODS FOR PREPARING MODIFIED VON WILLEBRAND FACTOR

(57) Abstract: The present invention provides modified von Willebrand Factor molecules, methods for their preparation and uses thereof. The invention further provides pharmaceutical compositions for treating coagulation disorders.



5 **Methods for preparing modified von Willebrand Factor**

FIELD OF THE INVENTION

10 The present invention relates to products and methods for improving treatment of blood coagulation disorders.

BACKGROUND OF THE INVENTION

15 There are various bleeding disorders caused by deficiencies of blood coagulation factors. The most common disorders are hemophilia A and B, resulting from deficiencies of blood coagulation Factor VIII (FVIII) and IX, respectively. Another known bleeding disorder is von Willebrand's disease (VWD).

20 In plasma FVIII exists mostly as a noncovalent complex with von Willebrand Factor (VWF), and its coagulant function is to accelerate Factor IXa dependent conversion of Factor X to Xa.

25 Classic hemophilia or hemophilia A is an inherited bleeding disorder. It results from a chromosome X-linked deficiency of blood coagulation FVIII, and affects almost exclusively males with an incidence of between one and two individuals per 10,000. The X-chromosome defect is transmitted by female carriers who are not themselves hemophiliacs. The clinical manifestation of hemophilia A is an increased bleeding tendency.

30

35 In severe hemophilia A patients undergoing prophylactic treatment FVIII has to be administered intravenously (i.v.) about 3 times per week due to the short plasma half-life of FVIII of about 12 to 14 hours. Each i.v. administration is cumbersome, associated with pain and entails the risk of an infection especially as this is mostly done at home by the patients themselves or by the parents of children having been diagnosed for hemophilia A.

It would thus be highly desirable to increase the half-life of FVIII so that pharmaceutical compositions containing such FVIII would have to be administered less frequently.

Several attempts have been made to prolong the half-life of non-activated FVIII either by reducing its interaction with cellular receptors (WO 03/093313 A2, WO 02/060951 A2), by covalently attaching polymers to FVIII (WO 94/15625, WO 97/11957 and US 4970300), by encapsulation of FVIII (WO 99/55306), by introduction of novel metal binding sites (WO 97/03193), by covalently attaching the A2 domain to the A3 domain either by peptidic (WO 97/40145 and WO 03/087355) or disulfide linkage (WO 02/103024A2) or by covalently attaching the A1 domain to the A2 domain (WO2006/108590).

Another approach to enhance the functional half-life of FVIII or VWF is by PEGylation of FVIII (WO 2007/126808, WO 2006/053299, WO 2004/075923) or by PEGylation of VWF (WO 2006/071801). The increased half-life of pegylated VWF would indirectly also enhance the half-life of FVIII present in plasma. Also fusion proteins of FVIII have been described (WO 2004/101740, WO2008/077616 and WO 2009/156137).

VWF, which is missing, functionally defect or only available in reduced quantity in different forms of von Willebrand disease (VWD), is a multimeric adhesive glycoprotein present in the plasma of mammals, which has multiple physiological functions. During primary hemostasis VWF acts as a mediator between specific receptors on the platelet surface and components of the extracellular matrix such as collagen. Moreover, VWF serves as a carrier and stabilizing protein for procoagulant FVIII. VWF is synthesized in endothelial cells and megakaryocytes as a 2813 amino acid precursor molecule. The amino acid sequence and the cDNA sequence of wild-type VWF are disclosed in Collins et al. 1987, Proc. Natl. Acad. Sci. USA 84:4393–4397. The precursor polypeptide, pre-pro-VWF, consists of an N-terminal 22-residue signal peptide, followed by a 741-residue pro-peptide and the 2050-residue polypeptide found in mature plasma VWF (Fischer et al., FEBS Lett. 351: 345-348, 1994). After cleavage of the signal peptide in the endoplasmatic reticulum a C-terminal disulfide bridge is formed between two monomers of VWF. During further transport through the secretory pathway 12 N-linked and 10 O-linked carbohydrate side chains are added. More important, VWF dimers are multimerized via N-terminal disulfide bridges and the propeptide of 741 amino acids length is cleaved off by the enzyme PACE/furin in the late Golgi apparatus.

Once secreted into plasma the protease ADAMTS13 can cleave high-molecular weight VWF multimers within the A1 domain of VWF. Plasma VWF therefore consists of a whole range of multimers ranging from single dimers of 500 kDa to multimers consisting of up to more than 20 dimers of a molecular weight of over 10,000 kDa. The VWF-HMWM hereby having the strongest hemostatic activity, which can be measured in ristocetin cofactor activity (VWF:RCO). The higher the ratio of VWF:RCO/VWF antigen, the higher the relative amount of high molecular weight multimers.

In plasma FVIII binds with high affinity to VWF, which protects it from premature elimination and thus, plays in addition to its role in primary hemostasis a crucial role to stabilize FVIII, regulate plasma levels of FVIII and as a consequence is also a central factor to control secondary hemostasis. The half-life of non-activated FVIII bound to VWF is about 12 to 14 hours in plasma. In von Willebrand disease type 3, where no or almost no VWF is present, the half-life of FVIII is only about 2 to 6 hours, leading to symptoms of mild to moderate hemophilia A in such patients due to decreased concentrations of FVIII. The stabilizing effect of VWF on FVIII has also been used to aid recombinant expression of FVIII in CHO cells (Kaufman et al. 1989, Mol Cell Biol 9:1233-1242).

VWF-derived polypeptides, in particular VWF fragments, have been described to stabilize FVIII in vitro and in vivo. WO 2013/106787 A1 is directed at chimeric proteins comprising certain VWF fragments and a FVIII protein. WO 2014/198699 A2 and WO 2013/083858 A2 describe VWF fragments and their use in the treatment of hemophilia. WO 2011/060242 A2 discloses fusion polypeptides comprising certain VWF fragments and an antibody Fc region. WO2013/093760 A2 describes a method for preparing a protein, comprising co-expressing FVIII or VWF polypeptides, including truncated forms of VWF, with a recombinant α -2,3-sialyltransferase. Yee et al. (2014) Blood 124(3):445-452 found that a VWF fragment containing the D'D3 domains is sufficient to stabilize Factor VIII in mice. However, although a VWF D'D3-Fc fusion protein exhibited markedly prolonged survival when transfused into FVIII-deficient mice, the VWF D'D3-Fc fusion protein did not prolong the survival of co-transfused FVIII.

The effect of the fermentation temperature on the sialylation level of a glycoprotein was investigated by Trummer et al (Biotech. Bioeng. (2006) Vol. 94, No. 6, p. 1033-1044) who found for erythropoietin at 30°C a decrease in sialylation by 40% and at 33°C a decrease by 20%.

Ahn et al. also investigated the effect of fermentation temperature on the sialylation level of a glycoprotein and published for erythropoietin (Biotech. Bioeng.(2008) Vol. 101, No. 6, p. 1234-1244) a percentage of asialo glycoprotein at 37°C of 2.4 %, and at 32° C of 2.1 %.

- 5 There is an ongoing need for methods increasing the half-life of FVIII and FVIII products with reduced administration frequency.

SUMMARY OF THE INVENTION

10

It has been found by the inventors that the sialylation of N-glycans of VWF fragments can be significantly increased if mammalian cells transfected with recombinant DNA encoding a VWF fragment are cultured at a lowered temperature, e.g. below 36°C. The products obtained in this way exhibit improved pharmacokinetics and a prolonged mean residence
15 time (MRT) and can be used to also improve pharmacokinetics and prolong MRT of a co-administered FVIII. It has been found by the inventors that the clearance of FVIII can be significantly reduced by co-administration of a half-life extended VWF-derived polypeptide which is characterized by a high degree of sialylation of its N-glycans. They are therefore particularly suitable for treating blood coagulation disorders. Especially VWF fragments
20 capable of binding to FVIII which comprise N-glycans wherein more than 75% of all N-glycans on average have at least one sialic acid have been shown to be particularly useful.

Another advantage of the method of the present invention as described above is that the VWF fragments obtained have a higher proportion of dimers than VWF fragments
25 produced in a conventional manner. The inventors found that the dimers have a higher affinity to FVIII than the monomers.

In particular preferred embodiments of the invention the VWF-derived polypeptide of the invention may be connected to a half-life extending moiety and is characterized by a high degree of sialylation of its N-glycans and has a particular low amount of N-glycans with
30 multivalent terminal and non-sialylated galactose residues, including a particular low amount of N-glycans with two or more terminal and non-sialylated galactose residues, and even more preferred a particular low amount of N-glycans with three or more terminal and non-sialylated galactose residues.

The present invention therefore relates to the following embodiments [1] to [53]:

[1] A method of producing a glycoprotein comprising N-glycans with increased sialylation, which comprises (i) providing cells comprising a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF), and (ii) culturing said cells at a temperature of less than 36.0°C, wherein said polypeptide comprising a truncated VWF preferably has a circulatory mean residence time (MRT) greater than that of full-length VWF.

[2] A method of producing a dimer of a glycoprotein comprising a truncated von Willebrand Factor (VWF), which comprises (i) providing cells comprising a nucleic acid encoding the amino acid sequence of the glycoprotein, and (ii) culturing said cells at a temperature of less than 36.0°C.

[3] A method of increasing the dimerization of a glycoprotein comprising a truncated von Willebrand Factor (VWF), which comprises (i) providing cells comprising a nucleic acid encoding amino acid sequence of the glycoprotein, and (ii) culturing said cells at a temperature of less than 36.0°C.

[4] The method of any one of the preceding items, wherein the cells further comprise a recombinant nucleic acid encoding a sialyltransferase, preferably an α -2,6-sialyltransferase and/or an α -2,3-sialyltransferase.

[5] The method of any one of the preceding items, wherein prior to step (ii) the cells are cultured at a temperature of 37.0°C $\pm$ 1.0°C, and step (ii) comprises culturing the cells at a temperature of 34.0°C $\pm$ 2.0°C.

[6] A method of producing a glycoprotein comprising N-glycans with increased sialylation, which comprises (i) providing cells comprising a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF) and a recombinant nucleic acid encoding an α -2,6-sialyltransferase, and (ii) culturing the cells under conditions that allow expression of the glycoprotein.

[7] The method of any one of the preceding items, wherein the cells are transfected mammalian cells, and step (i) comprises introducing into mammalian cells the nucleic acid encoding a polypeptide comprising the truncated VWF, and optionally the recombinant nucleic acid encoding a sialyltransferase.

[8] The method of any one of the preceding items, further comprising the step of recovering the glycoprotein from the culture medium.

[9] The method of any one of the preceding items, further comprising subjecting the glycoprotein obtained in any one of the preceding items to ion exchange chromatography, whereby glycoprotein with high sialylation is separated from glycoprotein with low sialylation; and collecting the fractions eluted from the ion exchange column having high sialylation.

[10] The method of any one of the preceding items, further comprising contacting the glycoprotein obtained in any one of the preceding items with a sialyltransferase and a sialic acid donor *in vitro*.

[11] The method of item [10], wherein the sialyltransferase is an α -2,6-sialyltransferase, an α -2,3-sialyltransferase, or a combination thereof.

[12] The method of any one of the preceding items, wherein the glycoprotein further comprises a half-life extending heterologous polypeptide fused to the truncated VWF.

[13] The method of item [12], wherein the half-life extending heterologous polypeptide comprises or consists of a polypeptide selected from the group consisting of albumin or a fragment thereof having a length of at least 100 amino acids, immunoglobulin constant regions and portions thereof, e.g. the Fc fragment, transferrin and fragments thereof, the C-terminal peptide of human chorionic gonadotropin, solvated random chains with large hydrodynamic volume known as XTEN, homo-amino acid repeats (HAP), proline-alanine-serine repeats (PAS), albumin, afamin, alpha-fetoprotein, Vitamin D binding protein, polypeptides capable of binding under physiological conditions to albumin or immunoglobulin constant regions, and combinations thereof.

[14] The method of any one of items [1] to [11], comprising conjugating the glycoprotein obtained in any one of the preceding items with the half-life-extending moiety.

[15] The method of item [14], wherein the half-life-extending moiety is selected from the group consisting of hydroxyethyl starch (HES), polyethylene glycol (PEG), polysialic acids (PSAs) and albumin binding ligands, e.g. fatty acid chains.

[16] The method of any one of the preceding items, wherein, on average, at least 75% of the N-glycans of the obtained glycoprotein comprise at least one sialic acid moiety.

[17] The method of any one of the preceding items, wherein, on average, at least 80% of the N-glycans of the obtained glycoprotein comprise at least one sialic acid moiety.

[18] The method of any one of the preceding items, wherein, on average, at least 85% of the N-glycans of the obtained glycoprotein comprise at least one sialic acid moiety.

5 [19] The method of any one of the preceding items, wherein, on average, at least 50% of the obtained glycoprotein is present as dimer.

[20] A glycoprotein obtainable by a method of any one of the preceding items.

[21] A glycoprotein comprising a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said glycoprotein
10 comprises N-glycans, and at least 75%, preferably at least 85%, more preferably at least 90%, preferably at least 95%, preferably at least 96%, preferably at least 97%, preferably at least 98%, more preferably at least 99% of said N-glycans comprise, on average, at least one sialic acid moiety.

[22] A glycoprotein comprising a truncated von Willebrand Factor (VWF), wherein said
15 truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said glycoprotein comprises N-glycans, wherein less than 35%, preferably less than 34%, preferably less than 33%, preferably less than 32%, preferably less than 31%, preferably less than 30%, preferably less than 29%, preferably less than 28%, preferably less than 27% preferably less than 26%, preferably less than 25%, preferably less than 24%, preferably less than
20 23%, preferably less than 22%, preferably less than 21%, preferably less than 20%, preferably less than 19%, preferably less than 18%, preferably less than 17%, preferably less than 16%, preferably less than 15%, preferably less than 14%, preferably less than 13%, preferably less than 12%, preferably less than 11%, preferably less than 10%, preferably less than 9%, preferably less than 8%, preferably less than 7%, preferably less
25 than 6% and preferably less than 5% of said N-glycans comprise, on average, two or more terminal and non-sialylated galactose residues.

[23] A glycoprotein according to items [21] and [22].

[24] A glycoprotein comprising a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said glycoprotein
30 comprises N-glycans, wherein less than 6%, preferably less than 5%, preferably less than 4%, preferably less than 3%, preferably less than 2%, and preferably less than 1% of said

N-glycans comprise, on average, three or more terminal and non-sialylated galactose residues.

[25] A glycoprotein according to items [21] and [24] or to items [22] and [24] or to items [23] and [24].

- 5 [26] The glycoprotein of items [21] to [25], wherein at least 70% of said N-glycans comprise, on average, at least one α -2,6-sialic acid moiety or one α -2,3-sialic acid moiety.

[27] The glycoprotein of any one of items [20] to [26], wherein the truncated VWF comprises (a) amino acids 776 to 805 of SEQ ID NO:9 or (b) an amino acid sequence having a sequence identity of at least 90% to amino acids 776 to 805 of SEQ ID NO:9.

- 10 [28] The glycoprotein of any one of items [20] to [27], wherein the truncated VWF comprises (a) amino acids 766 to 864 of SEQ ID NO:9 or (b) an amino acid sequence having a sequence identity of at least 90% to amino acids 766 to 864 of SEQ ID NO:9.

- [29] The glycoprotein of any one of items [20] to [28], wherein the truncated VWF consists of (a) amino acids 764 to 1242 of SEQ ID NO:9, (b) an amino acid sequence
15 having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:9, or (c) a fragment of (a) or (b).

[30] The glycoprotein of any one of items [20] to [29], further comprising a half-life extending heterologous polypeptide fused to the truncated VWF, and/or a half-life-extending moiety conjugated to the glycoprotein.

- 20 [31] The glycoprotein of item [30], wherein said half-life extending heterologous polypeptide comprises or consists of human serum albumin or a fragment thereof, wherein the length of said fragment is at least 100 amino acids.

- [32] The glycoprotein of item [30], wherein said heterologous polypeptide fused to the glycoprotein comprises or consists of a polypeptide selected from the group consisting of
25 immunoglobulin constant regions and portions thereof, e.g. the Fc fragment, transferrin and fragments thereof, the C-terminal peptide of human chorionic gonadotropin, solvated random chains with large hydrodynamic volume known as XTEN, homo-amino acid repeats (HAP), proline-alanine-serine repeats (PAS), albumin, afamin, alpha-fetoprotein, Vitamin D binding protein, polypeptides capable of binding under physiological conditions
30 to albumin or immunoglobulin constant regions, and combinations thereof.

[33] The glycoprotein of item [30], wherein said half-life-extending moiety conjugated to the glycoprotein is selected from the group consisting of hydroxyethyl starch (HES), polyethylene glycol (PEG), polysialic acids (PSAs), elastin-like polypeptides, heparosan polymers, hyaluronic acid and albumin binding ligands, e.g. fatty acid chains.

5 [34] The glycoprotein of any one of items [20] to [33], wherein the glycoprotein is a dimer.

[35] The dimeric glycoprotein of item [34], wherein the affinity of said dimeric glycoprotein to the FVIII is greater than the affinity of a monomeric glycoprotein to said FVIII, wherein said monomeric glycoprotein has the same amino acid sequence as the
10 dimeric glycoprotein.

[36] The glycoprotein of any one of items [20] to [35], wherein said truncated VWF has one or more amino acid substitution(s) relative to the amino acid sequence shown in SEQ ID NO:9, wherein the truncated VWF having said one or more amino acid substitutions has a greater affinity to FVIII than a truncated VWF consisting of the same amino acid
15 sequence except for said one or more amino acid substitutions relative to SEQ ID NO:9.

[37] The glycoprotein of item [36], wherein the affinity of said glycoprotein to the FVIII is greater than the affinity of a reference polypeptide, wherein the amino acid sequence of said reference polypeptide is identical to the amino acid sequence of said glycoprotein except that the amino acid sequence of the truncated VWF of the reference polypeptide
20 does not have said one or more substitutions relative to the amino acids sequence shown in SEQ ID NO:9.

[38] A composition comprising a population of glycoproteins as defined in any one of items [20] to [37], wherein the ratio of dimeric glycoprotein to monomeric glycoprotein in the composition is greater than 1.0, preferably greater than 1.5, more preferably greater than 2.0, most preferably greater than 2.5.
25

[39] A pharmaceutical composition comprising a glycoprotein of any one of items [20] to [37] and a pharmaceutically acceptable excipient.

[40] The pharmaceutical composition of item [39], wherein at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the glycoproteins in the composition are
30 present as dimers.

[41] A glycoprotein as defined in any one of items [20] to [37] for use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject an effective amount of said glycoprotein.

5 [42] The glycoprotein for use according to item [41], wherein said treatment further comprises administering to the subject an effective amount of a FVIII.

[43] The glycoprotein for use according to item [42], wherein the plasma MRT of the FVIII is increased, and/or the clearance of the FVIII is reduced, by the co-administration of the glycoprotein, as compared to a treatment with the FVIII alone.

10 [44] The glycoprotein for use according to item [42] or [43], wherein the frequency of administration of the FVIII is reduced as compared to a treatment with the FVIII alone.

[45] The glycoprotein for use according to any one of items [42] to [44], wherein the glycoprotein and/or the FVIII is/are administered intravenously.

[46] The glycoprotein for use according to any one of items [42] to [44], wherein the glycoprotein and/or the FVIII is/are administered subcutaneously.

15 [47] The glycoprotein for use according to any one of items [42] to [46], wherein the glycoprotein and the FVIII are administered separately.

[48] The use of a glycoprotein as defined in any one of items [20] to [37] for increasing the plasma MRT of Factor VIII.

20 [49] The use of a glycoprotein as defined in any one of items [20] to [37] for reducing the clearance of administered FVIII from the circulation.

[50] The use of item [48] or [49], wherein said Factor VIII is exogenously administered to a subject having hemophilia A.

25 [51] A pharmaceutical kit comprising (i) a FVIII and (ii) a glycoprotein as defined in any one of items [20] to [37] for simultaneous, separate or sequential use in the treatment of a blood coagulation disorder.

[52] A method of treating a blood coagulation disorder, comprising administering to a patient in need thereof an effective amount of a glycoprotein as defined in any one of items [20] to [37].

[53] A method of extending the circulatory half-life of an exogenously administered FVIII, which comprises co-administering the glycoprotein of any one of items [20] to [37].

5 DESCRIPTION OF THE DRAWINGS

Fig. 1: Mean residence time and clearance (mean) of D'D3-FP dimer quantified as albumin in rats, as determined in Example 8.1.

Fig. 2: Mean residence time and clearance (mean) of rVIII-SC quantified as FVIII antigen
10 in rats, as determined in Example 8.1.

Fig. 3: Mean residence time and clearance (mean) of D'D3-FP dimer quantified as albumin in rats, as determined in Example 8.2.

Fig. 4: Mean residence time and clearance (mean) of full length Factor VIII quantified as FVIII antigen in rats, as determined in Example 8.2.

15 Fig. 5: Legend to the glycostructures shown in figures 6 to 25

Fig. 6: Profil of lot B-140526 showing the neutral N-glycans

Fig. 7: Profil of lot B-140526 showing the mono-sialo N-glycans

Fig. 8: Profil of lot B-140526 showing the di-sialo N-glycans

Fig. 9: Profil of lot B-140526 showing the tri-sialo N-glycans

20 Fig. 10: Profil of lot B-140526 showing the tetra-sialo N-glycans

Fig. 11: Profil of lot B-140616KS showing the neutral N-glycans

Fig. 12: Profil of lot B-140616KS showing the mono-sialo N-glycans

Fig. 13: Profil of lot B-140616KS showing the di-sialo N-glycans

Fig. 14: Profil of lot B-140616KS showing the tri-sialo N-glycans

25 Fig. 15: Profil of lot B-140616KS showing the tetra-sialo N-glycans

Fig. 16: Profil of lot B-140825 showing the neutral N-glycans

Fig. 17: Profil of lot B-140825 showing the mono-sialo N-glycans

Fig. 18: Profil of lot B-140825 showing the di-sialo N-glycans

Fig. 19: Profil of lot B-140825 showing the tri-sialo N-glycans

Fig. 20: Profil of lot B-140825 showing the tetra-sialo N-glycans

Fig. 21: Profil of lot B-140623KS showing the neutral N-glycans

5 Fig. 22: Profil of lot B-140623KS showing the mono-sialo N-glycans

Fig. 23: Profil of lot B-140623KS showing the di-sialo N-glycans

Fig. 24: Profil of lot B-140623KS showing the tri-sialo N-glycans

Fig. 25: Profil of lot B-140623KS showing the tetra-sialo N-glycans

10 Fig. 26: Quantitative determination of N-glycans with two or more terminal and non-sialylated galactose residues of the comparative sample B140526. The first column shows the quantitative distribution of all N-glycans for neutral, mono-sialo, di-sialo, tri-sialo and tetra-sialo N-glycans adding up to 100%. The second column shows the percentage (relating to the 100% of all N-glycans) of N-glycans with two or more terminal and non-sialylated galactose residues. In the present sample only neutral, mono-sialo and di-sialo

15 N-glycans having two or more terminal and non-sialylated galactose residues were detected. The third column shows the percentage (relating to the 100% of all N-glycans) of N-glycans with three or more terminal and non-sialylated galactose residues. In the present sample only neutral N-glycans having three or more terminal and non-sialylated galactose residues were detected.

20 Fig. 27: Quantitative determination of N-glycans with two or more terminal and non-sialylated galactose residues of sample B140616KS according to the invention. The first column shows the quantitative distribution of all N-glycans for neutral, mono-sialo, di-sialo, tri-sialo and tetra-sialo N-glycans adding up to 100%. The second column shows the percentage (relating to the 100% of all N-glycans) of N-glycans with two or more terminal

25 and non-sialylated galactose residues. In the present sample only neutral, mono-sialo and di-sialo N-glycans having two or more terminal and non-sialylated galactose residues were detected. The third column shows the percentage (relating to the 100% of all N-glycans) of N-glycans with three or more terminal and non-sialylated galactose residues. In the present sample only neutral N-glycans having three or more terminal and non-sialylated

30 galactose residues were detected.

Fig. 28: Quantitative determination of N-glycans with two or more terminal and non-sialylated galactose residues of sample B140825 according to the invention. The first column shows the quantitative distribution of all N-glycans for neutral, mono-sialo, di-sialo, tri-sialo and tetra-sialo N-glycans adding up to 100%. The second column shows the percentage (relating to the 100% of all N-glycans) of N-glycans with two or more terminal and non-sialylated galactose residues. In the present sample only neutral, mono-sialo and di-sialo N-glycans having two or more terminal and non-sialylated galactose residues were detected. The third column shows the percentage (relating to the 100% of all N-glycans) of N-glycans with three or more terminal and non-sialylated galactose residues. In the present sample only neutral N-glycans having three or more terminal and non-sialylated galactose residues were detected.

Fig. 29: Quantitative determination of N-glycans with two or more terminal and non-sialylated galactose residues of sample B140623KS according to the invention. The first column shows the quantitative distribution of all N-glycans for neutral, mono-sialo, di-sialo, tri-sialo and tetra-sialo N-glycans adding up to 100%. The second column shows the percentage (relating to the 100% of all N-glycans) of N-glycans with two or more terminal and non-sialylated galactose residues. In the present sample only neutral, mono-sialo and di-sialo N-glycans having two or more terminal and non-sialylated galactose residues were detected. The third column shows the percentage (relating to the 100% of all N-glycans) of N-glycans with three or more terminal and non-sialylated galactose residues. In the present sample only neutral N-glycans having three or more terminal and non-sialylated galactose residues were detected.

DETAILED DESCRIPTION

In a first aspect, the present invention pertains to a method of producing a glycoprotein comprising sialylated N-glycans, which comprises (i) providing cells comprising a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF), and (ii) culturing said cells at a temperature of less than 36.0°C. Preferably, the N-glycans of the produced glycoprotein have an increased sialylation, and/or a high degree of sialylation.

In a second aspect, the present invention pertains to a method of producing a glycoprotein comprising sialylated N-glycans, which comprises (i) providing cells comprising a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF) and a recombinant nucleic acid encoding an α -2,3-sialyltransferase and/or an α -2,6-

sialyltransferase, and (ii) culturing the cells under conditions that allow expression of the glycoprotein and of the sialyltransferase(s). Preferably, the N-glycans of the produced glycoprotein have an increased sialylation, and/or a high sialylation.

The term “glycoprotein”, as used herein, refers to a protein or polypeptide that comprises one or more covalently linked oligosaccharide chains. The oligosaccharide chains may be composed of a single unbranched chain of sugar residues or may be composed of a chain of sugar residues that branches one or more times.

“N-linked glycans” are oligosaccharides that are covalently linked to asparagine residues of a polypeptide. Terminal galactoses on such N-linked glycans may be modified by the attachment of an α -2,3- or an α -2,6-linked sialic acid (as shown in Figures 5 to 25). Preferably the terminal galactoses are D-galactoses. N-glycans are usually branched and can be, for example, of a bi-, tri- or tetra-antennary type, so that there could be two, three or four terminal galactose residues in one N-glycan, which may be sialylated to varying degrees or be all non-sialylated. “Terminal” refers to the most distant position in a given branch of an N-glycan from the attachment point of the N-glycan to the peptidic chain of the glycoprotein of the invention.

The term “sialic acid” refers to the N- or O-substituted derivatives of neuraminic acid usually found as terminal monosaccharides of animal oligosaccharides (for review, see Varkis (1992) Glycobiology vol. 2 no. 1 pp. 25-40). The most common sialic acid is N-acetyl neuraminic acid. An “increased sialylation” means that at least 75% of the N-glycans of the glycoprotein comprise, on average, at least one sialic acid moiety. By way of non-limiting example an “increased sialylation of at least 75%” is determined as in Example 6 of the present invention, i.e. by enzymatically cleaving all N-glycans from a given glycoprotein of interest and then determining the amount of cleaved N-glycans with no sialic acids (“asialo N-glycans”) and the total amount of all cleaved N-glycans. A “sialylation of at least 75%” corresponds then to an amount of 25% of asialo N-glycans or less of the total amount of all cleaved N-glycans.

Increased sialylation is of importance for maintaining a given therapeutic glycoprotein longer in the circulation since glycoproteins with a reduced sialylation bind to the asialoglycoprotein receptor (ASGP-R) and are then – after receptor mediated endocytosis – finally degraded.

The ASGP-R is expressed exclusively by parenchymal hepatocytes, which contain 100,000 – 500,000 binding sites per cell. These receptors are randomly distributed over

the sinusoidal plasma membrane facing the blood capillaries. Their main function is to maintain plasma glycoprotein homeostasis by recognition, binding and endocytosis of asialoglycoproteins (Stokmaier et al (2009) Bioorganic & Medicinal Chemistry, 7254-7264).

- 5 The human ASGP-R consists of two homologous subunits, designated H1 and H2, which form a non-covalent heterologous complex with an estimated ratio of 2-5:1, respectively. This ASGP-R complex binds to glycoproteins exposing glycostructures with non-sialylated terminal D-galactose and N-acetyl-D-galactoseamine residues. It has been found that the binding affinity of glycostructures to the ASGP-R strongly depends on the
- 10 valency of the ligand. Whereas the affinity of a single D-galactose residue is only in the millimolar range, bi-, tri- and tetraantennary desialylated glycans bind with dissociation constants of 10^{-6} , 5×10^{-9} and 10^{-9} M, respectively.

Therefore in particular preferred embodiments of the invention the glycoprotein of the invention which is characterized by a high degree of sialylation of its N-glycans has a

15 particular low amount of N-glycans with multivalent terminal and non-sialylated galactose residues, including a particular low amount of N-glycans with two or more terminal and non-sialylated galactose residues, and even more preferred a particular low amount of N-glycans with three or more terminal and non-sialylated galactose residues.

In a first step, the methods of the invention comprise the step of providing cells comprising

20 a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF).

The truncated VWF

The term "von Willebrand Factor" (VWF) as used herein includes naturally occurring (native) VWF, but also variants thereof retaining at least the FVIII binding activity of

25 naturally occurring VWF, e.g. sequence variants where one or more residues have been inserted, deleted or substituted. The FVIII binding activity is determined by a FVIII-VWF binding assay as described in Example 11.

The preferred VWF in accordance with this invention is human VWF represented by the amino acid sequence shown in SEQ ID NO:9. The cDNA encoding SEQ ID NO:9 is shown

30 in SEQ ID NO:8.

The gene encoding human native VWF is transcribed into a 9 kb mRNA which is translated into a pre-propolypeptide of 2813 amino acids with an estimated molecular

weight of 310,000 Da. The pre-propolypeptide contains an N-terminal 22 amino acids signal peptide, followed by a 741 amino acid pro-polypeptide (amino acids 23-763 of SEQ ID NO:9) and the mature subunit (amino acids 764-2813 of SEQ ID NO:9). Cleavage of the 741 amino acids propolypeptide from the N-terminus results in mature VWF consisting of 2050 amino acids. The amino acid sequence of the human native VWF pre-propolypeptide is shown in SEQ ID NO:9. Unless indicated otherwise, the amino acid numbering of VWF residues in this application refers to SEQ ID NO:9, even if the VWF molecule does not comprise all residues of SEQ ID NO:9.

The propolypeptide of native VWF comprises multiple domains. Different domain annotations can be found in the literature (see, e.g. Zhou et al. (2012) Blood 120(2): 449-458). The following domain annotation of native pre-propolypeptide of VWF is applied in this application:

D1-D2-D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK

With reference to SEQ ID NO:9, the D' domain consists of amino acids 764-865; and the D3 domain consists of amino acids 866-1242.

The feature "truncated" means that the polypeptide does not comprise the entire amino acid sequence of mature VWF (amino acids 764-2813 of SEQ ID NO:9). Typically, the truncated VWF does not comprise all amino acids 764-2813 of SEQ ID NO:9 but only a fragment thereof. A truncated VWF may also be referred to as a VWF fragment, or in the plural as VWF fragments.

Typically, the truncated VWF is capable of binding to a Factor VIII. Preferably, the truncated VWF is capable of binding to the mature form of human native Factor VIII. In another embodiment, the truncated VWF is capable of binding to the single-chain Factor VIII consisting of the amino acid sequence SEQ ID NO:10. Binding of the truncated VWF to Factor VIII can be determined by a FVIII-VWF binding assay as described in Example 11.

The truncated VWF of the present invention preferably comprises or consists of an amino acid sequence having a sequence identity of at least 90% to amino acids 776 to 805 of SEQ ID NO:9 and is capable of binding to FVIII. In preferred embodiments the truncated VWF comprises or consists of an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 776 to 805 of SEQ ID NO:9 and is capable of binding to FVIII. Most preferably, the truncated

VWF comprises or consists of amino acids 776 to 805 of SEQ ID NO:9. Unless indicated otherwise herein, sequence identities are determined over the entire length of the reference sequence (e.g. amino acids 776 to 805 of SEQ ID NO:9).

5 The truncated VWF of the present invention preferably comprises or consists of an amino acid sequence having a sequence identity of at least 90% to amino acids 766 to 864 of SEQ ID NO:9 and is capable of binding to FVIII. In preferred embodiments the truncated VWF comprises or consists of an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 766 to 864 of SEQ ID NO:9 and is capable of binding to FVIII. Most preferably, the truncated
10 VWF comprises or consists of amino acids 766 to 864 of SEQ ID NO:9.

In another preferred embodiment, the truncated VWF consists of (a) an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:9, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. More preferably, the truncated VWF consists of (a) an amino acid
15 sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 764 to 1242 of SEQ ID NO:9, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Most preferably, the truncated VWF consists of (a) amino acids 764 to 1242 of SEQ ID NO:9, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII.

20

As described in more detail below, the method of the invention comprises providing cells comprising a nucleic acid encoding the polypeptide comprising the truncated VWF. The nucleic acid is introduced into suitable host cells by techniques that are known *per se*.

25 In a preferred embodiment, the nucleic acid in the host cell encodes (a) an amino acid sequence having a sequence identity of at least 90% to amino acids 1 to 1242 of SEQ ID NO:9, or (b) a fragment thereof, provided that the truncated mature VWF is still capable of binding to FVIII. More preferably, the nucleic acid encodes (a) an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at
30 least 99%, to amino acids 1 to 1242 of SEQ ID NO:9, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Most preferably, the nucleic acid encodes (a) amino acids 1 to 1242 of SEQ ID NO:9, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Especially if the glycoprotein eventually produced is a dimer, the nucleic acid will comprise a sequence encoding amino

acids 1 to 763 of VWF (e.g. SEQ ID NO:9), even if the truncated VWF in the glycoprotein does not comprise amino acids 1 to 763 of VWF (e.g. SEQ ID NO:9).

In other embodiments the truncated VWF comprises or consists of one of the following amino acid sequences, each referring to SEQ ID NO:9:

- 5 776-805; 766-805; 764-805; 776-810; 766-810; 764-810; 776-815; 766-815; 764-815;
776-820; 766-820; 764-820; 776-825; 766-825; 764-825; 776-830; 766-830; 764-830;
776-835; 766-835; 764-835; 776-840; 766-840; 764-840; 776-845; 766-845; 764-845;
776-850; 766-850; 764-850; 776-855; 766-855; 764-855; 776-860; 766-860; 764-860;
10 776-864; 766-864; 764-864; 776-865; 766-865; 764-865; 776-870; 766-870; 764-870;
776-875; 766-875; 764-875; 776-880; 766-880; 764-880; 776-885; 766-885; 764-885;
776-890; 766-890; 764-890; 776-895; 766-895; 764-895; 776-900; 766-900; 764-900;
776-905; 766-905; 764-905; 776-910; 766-910; 764-910; 776-915; 766-915; 764-915;
776-920; 766-920; 764-920; 776-925; 766-925; 764-925; 776-930; 766-930; 764-930;
15 776-935; 766-935; 764-935; 776-940; 766-940; 764-940; 776-945; 766-945; 764-945;
776-950; 766-950; 764-950; 776-955; 766-955; 764-955; 776-960; 766-960; 764-960;
776-965; 766-965; 764-965; 776-970; 766-970; 764-970; 776-975; 766-975; 764-975;
776-980; 766-980; 764-980; 776-985; 766-985; 764-985; 776-990; 766-990; 764-990;
776-995; 766-995; 764-995; 776-1000; 766-1000; 764-1000; 776-1005; 766-1005; 764-1005;
20 776-1010; 766-1010; 764-1010; 776-1015; 766-1015; 764-1015; 776-1020; 766-1020; 764-1020;
776-1025; 766-1025; 764-1025; 776-1030; 766-1030; 764-1030; 776-1035; 766-1035; 764-1035;
776-1040; 766-1040; 764-1040; 776-1045; 766-1045; 764-1045; 776-1050; 766-1050; 764-1050;
776-1055; 766-1055; 764-1055; 776-1060; 766-1060; 764-1060; 776-1065; 766-1065; 764-1065;
776-1070; 766-1070; 764-1070; 776-1075; 766-1075; 764-1075; 776-1080; 766-1080; 764-1080;
25 776-1085; 766-1085; 764-1085; 776-1090; 766-1090; 764-1090; 776-1095; 766-1095; 764-1095;
776-1100; 766-1100; 764-1100; 776-1105; 766-1105; 764-1105; 776-1110; 766-1110; 764-1110;
776-1115; 766-1115; 764-1115; 776-1120; 766-1120; 764-1120; 776-1125; 766-1125; 764-1125;
776-1130; 766-1130; 764-1130; 776-1135; 766-1135; 764-1135; 776-1140; 766-1140; 764-1140;
776-1145; 766-1145; 764-1145; 776-1150; 766-1150; 764-1150; 776-1155; 766-1155; 764-1155;
30 776-1160; 766-1160; 764-1160; 776-1165; 766-1165; 764-1165; 776-1170; 766-1170; 764-1170;
776-1175; 766-1175; 764-1175; 776-1180; 766-1180; 764-1180; 776-1185; 766-1185; 764-1185;
776-1190; 766-1190; 764-1190; 776-1195; 766-1195; 764-1195; 776-1200; 766-1200; 764-1200;
776-1205; 766-1205; 764-1205; 776-1210; 766-1210; 764-1210; 776-1215; 766-1215; 764-1215;
776-1220; 766-1220; 764-1220; 776-1225; 766-1225; 764-1225; 776-1230; 766-1230; 764-1230;
35 776-1235; 766-1235; 764-1235; 776-1240; 766-1240; 764-1240; 776-1242; 766-1242; 764-1242;
764-1464; 764-1250; 764-1041; 764-828; 764-865; 764-1045; 764-1035; 764-1128; 764-1198;
764-1268; 764-1261; 764-1264; 764-1459; 764-1463; 764-1464; 764-1683; 764-1873; 764-1482;
764-1479; 764-1672; and 764-1874.

In certain embodiments the truncated VWF has an internal deletion relative to mature wild type VWF. For example, the A1, A2, A3, D4, C1, C2, C3, C4, C5, C6 domains or combinations thereof may be deleted, and the D' domain, the D3 domain and the CK domain is retained. In further embodiments the truncated VWF does not comprise the binding sites for platelet glycoprotein Iba (GPIba), collagen and/or integrin α IIB β III (RGDS sequence within the C1 domain). In other embodiments, the truncated VWF does not comprise the cleavage site (Tyr1605-Met1606) for ADAMTS13 which is located at the central A2 domain of VWF. In yet another embodiment, the truncated VWF does not comprise the binding sites for GPIba, and/or does not comprise the binding site for collagen, and/or does not comprise the binding site for integrin α IIB β III, and/or it does not comprise the cleavage site (Tyr1605-Met1606) for ADAMTS13 which is located at the central A2 domain of VWF.

In other embodiments the truncated VWF comprises or consists of an amino acid sequence that has a sequence identity of at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, to one of the amino acid sequences recited in the preceding paragraph, provided that the truncated VWF is capable of binding to FVIII.

A glycoprotein is termed a "dimer" in the present invention if two monomers of the glycoprotein are linked covalently. Preferably the two monomeric subunits are covalently linked via at least one disulfide bridge, e.g. by one, two, three or four disulfide bridges. The cysteine residues forming the at least one disulfide bridge are preferably located within the truncated VWF portion of the glycoprotein. In one embodiment, these cysteine residues are Cys-1142, Cys-1222, Cys-1225, Cys-1227 and combinations thereof.

If the glycoprotein of the invention is a dimer, the truncated VWF preferably comprises or consists of two polypeptides each with an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, or amino acids 764 to 1227 of SEQ ID NO:9 and is capable of binding to FVIII. In preferred embodiments the truncated VWF comprises or consists of an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, or amino acids 764 to 1227 of SEQ ID NO:9 and is capable of binding to FVIII. Most preferably, the truncated

VWF comprises or consists of amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, or amino acids 764 to 1227 of SEQ ID NO:9.

The truncated VWF may be any one of the VWF fragments disclosed in WO 2013/106787 A1, WO 2014/198699 A2, WO 2011/060242 A2 or WO 2013/093760 A2, the disclosure of which is incorporated herein by reference.

Further components of the polypeptide

In addition to the truncated VWF, the glycoprotein may further comprises a half-life extending moiety. The half-life-extending moiety may be a heterologous amino acid sequence fused to the truncated VWF. Alternatively, the half-life-extending moiety may be chemically conjugated to the polypeptide comprising the truncated VWF by a covalent bond different from a peptide bond.

In certain embodiments of the invention, the half-life of the glycoprotein is extended by chemical modification, e.g. attachment of a half-life extending moiety such as polyethylene glycol (PEGylation), glycosylated PEG, hydroxyl ethyl starch (HESylation), polysialic acids, elastin-like polypeptides, heparosan polymers or hyaluronic acid. In another embodiment, the glycoprotein is conjugated to a HLEP such as albumin via a chemical linker. The principle of this conjugation technology has been described in an exemplary manner by Conjuchem LLC (see, e.g., US patent No. 7,256,253).

In other embodiments, the half-life-extending moiety is a half-life enhancing protein (HLEP). Preferably, the HLEP is an albumin or a fragment thereof. The N-terminus of the albumin may be fused to the C-terminus of the truncated VWF. Alternatively, the C-terminus of the albumin may be fused to the N-terminus of the truncated VWF. One or more HLEPs may be fused to the N- or C-terminal part of VWF provided that they do not to interfere with or abolish the binding capability of the truncated VWF to FVIII.

In one embodiment the polypeptide has the following structure:

tVWF - L1 - H, [formula 1]

Wherein tVWF is the truncated VWF, L1 is a chemical bond or a linker sequence, and H is a HLEP.

L1 may be a chemical bond or a linker sequence consisting of one or more amino acids, e.g. of 1 to 50, 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5 or 1 to 3 (e.g. 1, 2 or 3) amino acids and which may be equal or different from each other. Usually, the linker sequences are not present at the corresponding position in the wild-type VWF. Examples of suitable amino acids present in L1 include Gly and Ser. The linker should be non-immunogenic and may be a non-cleavable or cleavable linker. Non-cleavable linkers may be comprised of alternating glycine and serine residues as exemplified in WO2007/090584. In another embodiment of the invention the peptidic linker between the truncated VWF moiety and the albumin moiety consists of peptide sequences, which serve as natural interdomain linkers in human proteins. Preferably such peptide sequences in their natural environment are located close to the protein surface and are accessible to the immune system so that one can assume a natural tolerance against this sequence. Examples are given in WO2007/090584. Cleavable linker sequences are described, e.g., in WO 2013/120939 A1.

Preferred HLEP sequences are described infra. Likewise encompassed by the invention are fusions to the exact "N-terminal amino acid" of the respective HLEP, or fusions to the "N-terminal part" of the respective HLEP, which includes N-terminal deletions of one or more amino acids of the HLEP. The polypeptide may comprise more than one HLEP sequence, e.g. two or three HLEP sequences. These multiple HLEP sequences may be fused to the C-terminal part of VWF in tandem, e.g. as successive repeats.

In another embodiment of the invention, the half-life of the complex of the invention is extended by chemical modification, e.g. attachment of a half-life extending moiety such as polyethylene glycol (PEGylation), glycosylated PEG, hydroxyl ethyl starch (HESylation), polysialic acids, elastin-like polypeptides, heparosan polymers or hyaluronic acid. In another embodiment, the glycoprotein of the invention is conjugated to a HLEP such as albumin via a chemical linker. The principle of this conjugation technology has been described in an exemplary manner by Conjuchem LLC (see, e.g., US patent No. 7,256,253).

Half-life enhancing polypeptides (HLEPs)

Preferably, the half-life extending moiety is a half-life extending polypeptide (HLEP), more preferably HLEP is selected from albumin or fragments thereof, immunoglobulin constant region and portions thereof, e.g. the Fc fragment, solvated random chains with large hydrodynamic volume (e.g. XTEN (Schellenberger et al. 2009; Nature Biotechnol. 27:1186-1190), homo-amino acid repeats (HAP) or proline-alanine-serine repeats (PAS)), afamin, alpha-fetoprotein, Vitamin D binding protein, transferrin or variants thereof, carboxyl-terminal peptide (CTP) of human chorionic gonadotropin- β subunit, polypeptides or lipids capable of binding under physiological conditions to albumin or immunoglobulin constant region.

A "half-life enhancing polypeptide" as used herein is preferably selected from the group consisting of albumin, a member of the albumin-family, the constant region of immunoglobulin G and fragments thereof, region and polypeptides capable of binding under physiological conditions to albumin, to members of the albumin family as well as to portions of an immunoglobulin constant region. It may be a full-length half-life-enhancing protein described herein (e.g. albumin, a member of the albumin-family or the constant region of immunoglobulin G) or one or more fragments thereof that are capable of stabilizing or prolonging the therapeutic activity or the biological activity of the coagulation factor. Such fragments may be of 10 or more amino acids in length or may include at least about 15, at least about 20, at least about 25, at least about 30, at least about 50, at least about 100, or more contiguous amino acids from the HLEP sequence or may include part or all of specific domains of the respective HLEP, as long as the HLEP fragment provides a functional half-life extension of at least 25% compared to the respective polypeptide without the HLEP.

The HLEP portion of the glycoprotein may be a variant of a wild type HLEP. The term "variants" includes insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the FVIII-binding activity of the truncated VWF.

In particular, the proposed truncated VWF HLEP fusion constructs of the invention may include naturally occurring polymorphic variants of HLEPs and fragments of HLEPs. The HLEP may be derived from any vertebrate, especially any mammal, for example human,

monkey, cow, sheep, or pig. Non-mammalian HLEPs include, but are not limited to, hen and salmon.

Albumin as HLEP

The terms, "human serum albumin" (HSA) and "human albumin" (HA) and "albumin" (ALB) are used interchangeably in this application. The terms "albumin" and "serum albumin" are broader, and encompass human serum albumin (and fragments and variants thereof) as well as albumin from other species (and fragments and variants thereof).

As used herein, "albumin" refers collectively to albumin polypeptide or amino acid sequence, or an albumin fragment or variant, having one or more functional activities (e.g., biological activities) of albumin. In particular, "albumin" refers to human albumin or fragments thereof, especially the mature form of human albumin as shown in SEQ ID NO:11 herein or albumin from other vertebrates or fragments thereof, or analogs or variants of these molecules or fragments thereof.

In particular, the proposed truncated VWF fusion constructs of the invention may include naturally occurring polymorphic variants of human albumin and fragments of human albumin. Generally speaking, an albumin fragment or variant will be at least 10, preferably at least 40, most preferably more than 70 amino acids long.

Preferred embodiments of the invention include albumin variants with enhanced binding to the FcRn receptor. Such albumin variants may lead to a longer plasma half-life of a truncated VWF albumin variant fusion protein as compared to a truncated VWF fusion with a wild-type albumin.

The albumin portion of the proposed VWF fusion constructs of the invention may comprise at least one subdomain or domain of HA or conservative modifications thereof.

Immunoglobulins as HLEPs

Immunoglobulin G (IgG) constant regions (Fc) are known in the art to increase the half-life of therapeutic proteins (Dumont J A et al. 2006. BioDrugs 20:151-160). The IgG constant region of the heavy chain consists of 3 domains (CH1-CH3) and a hinge region. The immunoglobulin sequence may be derived from any mammal, or from subclasses IgG1,

IgG2, IgG3 or IgG4, respectively. IgG and IgG fragments without an antigen-binding domain may also be used as HLEPs. The therapeutic polypeptide portion is connected to the IgG or the IgG fragments preferably via the hinge region of the antibody or a peptidic linker, which may even be cleavable. Several patents and patent applications describe the fusion of therapeutic proteins to immunoglobulin constant regions to enhance the therapeutic protein's in vivo half-lives. US 2004/0087778 and WO 2005/001025 describe fusion proteins of Fc domains or at least portions of immunoglobulin constant regions with biologically active peptides that increase the half-life of the peptide, which otherwise would be quickly eliminated in vivo. Fc-IFN- β fusion proteins were described that achieved enhanced biological activity, prolonged circulating half-life and greater solubility (WO 2006/000448). Fc-EPO proteins with a prolonged serum half-life and increased in vivo potency were disclosed (WO 2005/063808) as well as Fc fusions with G-CSF (WO 2003/076567), glucagon-like peptide-1 (WO 2005/000892), clotting factors (WO 2004/101740) and interleukin-10 (U.S. Pat. No. 6,403,077), all with half-life enhancing properties.

Various HLEPs which can be used in accordance with this invention are described in detail in WO 2013/120939 A1.

Nucleic Acid

The nucleic acid encoding the polypeptide to be expressed can be prepared according to methods known in the art. Based on the cDNA sequence of VWF (SEQ ID NO:8), recombinant DNA encoding the above-mentioned truncated VWF constructs can be designed and generated.

Even if the glycoprotein which is secreted by the host cells does not comprise amino acids 1 to 763 of VWF, it is preferred that the nucleic acid (e.g. the DNA) encoding the intracellular precursor of the glycoprotein comprises a nucleotide sequence encoding an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 23 to 763 or preferably to amino acids 1 to 763 of SEQ ID NO:9. Most preferably, the nucleic acid (e.g. the DNA) encoding the intracellular precursor of the glycoprotein comprises a nucleotide sequence encoding amino acids 23 to 763 of SEQ ID NO:9, or amino acids 1 to 763 of SEQ ID NO:9.

Constructs in which the DNA contains the entire open reading frame inserted in the correct orientation into an expression plasmid may be used for protein expression. Typical expression vectors contain promoters that direct the synthesis of large amounts of mRNA corresponding to the inserted nucleic acid in the plasmid-bearing cells. They may also include an origin of replication sequence allowing for their autonomous replication within the host organism, and sequences that increase the efficiency with which the synthesized mRNA is translated. Stable long-term vectors may be maintained as freely replicating entities by using regulatory elements of, for example, viruses (e.g., the OriP sequences from the Epstein Barr Virus genome). Cell lines may also be produced that have integrated the vector into the genomic DNA, and in this manner the gene product is produced on a continuous basis.

Typically, the cells to be provided are obtained by introducing the nucleic acid encoding a polypeptide comprising the truncated VWF into mammalian host cells.

15 *Host cells*

Any host cell susceptible to cell culture, and to expression of glycoproteins, may be utilized in accordance with the present invention. In certain embodiments, a host cell is mammalian. Non-limiting examples of mammalian cells that may be used in accordance with the present invention include BALB/c mouse myeloma line (NSO/ 1, ECACC No: 85110503); human retinoblasts (PER.C6 (CruCell, Leiden, The Netherlands)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59, 1977); baby hamster kidney cells (BHK, ATCC CCL10); Chinese hamster ovary cells +/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216, 1980); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243 251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1 587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (HepG2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals NY. Acad. Sci., 383:44-68, 1982); MRC 5 cells; PS4 cells; human amniocyte cells (CAP); and a human hepatoma line (Hep G2). Preferably, the cell line is a rodent cell line, especially a hamster cell line such as CHO or BHK.

Methods suitable for introducing nucleic acids sufficient to achieve expression of a glycoprotein of interest into mammalian host cells are known in the art. See, for example, Gething et al., Nature, 293:620-625, 1981; Mantei et al., Nature, 281:40-46, 1979; Levinson et al. EP 117,060; and EP 117,058. For mammalian cells, common methods of introducing genetic material into mammalian cells include the calcium phosphate precipitation method of Graham and van der Erb (Virology, 52:456-457, 1978) or the lipofectamineTM (Gibco BRL) Method of Hawley-Nelson (Focus 15:73, 1993). General aspects of mammalian cell host system transformations have been described by Axel in US. Pat. No. 4,399,216. For various techniques for introducing genetic material into mammalian cells, see Keown et al., Methods in Enzymology, 1989, Keown et al., Methods in Enzymology, 185:527-537, 1990, and Mansour et al., Nature, 336:348-352, 1988.

Culturing the Cells

In a second step, the method of the first aspect of the invention comprises culturing the cells at a temperature of less than 36.0°C. In the method of the second aspect, the method comprises culturing the cells under conditions that allow expression of the glycoprotein.

The basal medium chosen for culturing the host cell line is not critical to the present invention and may be any one of, or combination of, those known to the art which are suitable for culturing mammalian cells. Media such as Dulbecco's Modified Eagle Medium, Ham's F-12 Medium, Eagle's Minimal Essential Medium and RPMI-1640 Medium and the like are commercially available. The addition of growth factors such as recombinant insulin is optional. In one embodiment, the medium is "protein-free" in the sense that it is either completely free of any protein or at least are free of any protein that is not recombinantly produced. Human serum albumin may be used as a serum-free culture supplement for the production of the glycoprotein. Preferably, the medium contains a protease inhibitor, such as a serine protease inhibitor, which is suitable for tissue culture and which is of synthetic or vegetable origin.

Generally, the present invention may be used with any cell culture method that is amenable to the expression of glycoproteins. For example, cells may be grown in batch or fed-batch cultures, where the culture is terminated after sufficient expression of the glycoprotein, after which the expressed glycoprotein is harvested. Alternatively, cells may be grown in continuous cultures (e.g. perfusion cultures), where the culture is not

terminated and new nutrients and other components are periodically or continuously added to the culture, during which the expressed glycoprotein is harvested periodically or continuously. The latter embodiment is preferred if the method comprises a temperature shift as described hereinbelow. The culture can be any conventional type of culture, such as batch, fed-batch or continuous, but is preferably continuous. Suitable continuous cultures include perfusion culture.

Cells may be grown in any convenient volume chosen by the practitioner. For example, cells may be grown in small scale reaction vessels ranging in volume from a few milliliters to several liters. Alternatively, cells may be grown in large scale commercial bioreactors ranging in volume from approximately at least 1 liter to 10, 100, 250, 500, 1000, 2500, 5000, 8000, 10,000, 12,000 liters or more, or any volume in between. The culture is typically carried out in a bioreactor, which is usually a stainless steel, glass or plastic vessel of 1 (one) to 10000 (ten thousand) litres capacity, for example 5, 10, 50, 100, 1000, 2500, 5000 or 8000 litres. The vessel is usually rigid but flexible plastic bags can be used, particularly for smaller volumes. These are generally of the 'single use' type.

Mammalian cells such as CHO and BHK cells are generally cultured as suspension cultures. That is to say, the cells are suspended in the medium, rather than adhering to a solid support. The cells may alternatively be immobilized on a carrier, in particular on a microcarrier. Porous carriers, such as Cytoline®, Cytopore® or Cytodex®, may be particularly suitable.

To obtain a high sialylation, the cells (e.g. CHO cells) are preferably cultured at a decreased temperature, e.g. at less than 36.0°C. "Decreased temperature" refers to a temperature that is lower than the optimum temperature or normal temperature for growth of the respective cell line. For most mammalian cells the normal temperature is 37°C. It is therefore preferred according to the invention that the cells (e.g. CHO cells) are cultured at a decreased temperature of 30.0 to 36.0°C, 30.5 to 35.5°C, 31.0 to 35.0°C, 31.5 to 34.5°C, 32.0 to 34.0°C, or 32.5 to 33.5°C. Preferably, the cells are cultured at a decreased temperature of 30.0°C±1.0°C, 31.0°C±1.0°C, 32.0°C±1.0°C, 33.0°C±1.0°C, 34.0°C±1.0°C, or 35.0°C±1.0°C.

The decreased temperature is maintained for a time period that is sufficient to increase the sialylation of the glycoprotein to be expressed. Preferably, the decreased temperature is maintained for at least 1 hour, at least 6 hours, at least 12 hours, at least 18 hours, at least 24 hours, at least 48 hours, at least 72 hours, at least 96 hours, at least 120 hours, or at least 144 hours. In other embodiments, the decreased temperature is maintained for

1 hour to 8 weeks, 6 hours to 6 weeks, 12 hours to 5 weeks, 18 hours to 4 weeks, 24 hours to 3 weeks, 48 hours to 14 days, 72 hours to 10 days, or 3 to 7 days.

To accomplish this, a culture may be subjected to one or more temperature shifts during the course of the culture. When shifting the temperature of a culture, the temperature shift may be relatively gradual. For example, it may take several hours or days to complete the temperature change. Alternatively, the temperature shift may be relatively abrupt. The temperature may be steadily increased or decreased during the culture process. Alternatively, the temperature may be increased or decreased by discrete amounts at various times during the culture process. The subsequent temperature(s) or temperature range(s) may be lower than or higher than the initial or previous temperature(s) or temperature range(s). One of ordinary skill in the art will understand that multiple discrete temperature shifts are encompassed in this embodiment. For example, the temperature may be shifted once (either to a higher or lower temperature or temperature range), the cells maintained at this temperature or temperature range for a certain period of time, after which the temperature may be shifted again to a new temperature or temperature range, which may be either higher or lower than the temperature or temperature range of the previous temperature or temperature range. The temperature of the culture after each discrete shift may be constant or may be maintained within a certain range of temperatures.

Typically, the cells (e.g. CHO cells) will initially be cultured at a "normal" temperature of $37.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ until the target cell density is achieved. The initial culture period is then followed by a temperature shift to the decreased temperature. After a period of culturing at the decreased temperature, a temperature shift to the normal temperature may or may not follow. Preferably, the cells (e.g. CHO cells) will initially be cultured at $37.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ for several days, followed by manufacturing at a decreased temperature of $31.0 - 35.0^{\circ}\text{C}$.

Based on the present disclosure, those of ordinary skill in the art will be able to select temperatures in which to grow cells, depending on the particular needs of the respective cell line and the particular production requirements of the practitioner.

In certain embodiments, batch and fed-batch bioreactors are terminated once the expressed glycoprotein reaches a sufficiently high titer. Additionally or alternatively, batch and fed-batch bioreactors may be terminated once the cells reach a sufficiently high density, as determined by the needs of the practitioner. For example, the culture may be terminated once the cells reach 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 percent of maximal viable cell density. Additionally or alternatively,

batch and fed-batch bioreactors may be terminated prior to excessive accumulation of metabolic waste products such as lactate and ammonium.

In certain cases, it may be beneficial to supplement a cell culture during the subsequent production phase with nutrients or other medium components that have been depleted or metabolized by the cells. As non-limiting examples, it may be beneficial to supplement a cell culture with hormones and/or other growth factors, inorganic ions (such as, for example, sodium, chloride, calcium, magnesium, and phosphate), buffers, vitamins, nucleosides or nucleotides, trace elements (inorganic compounds usually present at very low final concentrations), amino acids, lipids, or glucose or other energy source. Such supplementary components may all be added to the cell culture at one time, or they may be provided to the cell culture in a series of additions or they may be provided together with fresh medium during a perfusion culture.

Alternatively to batch and fed-batch bioreactors the invention can also be practiced when cells expressing a glycoprotein of the invention are cultured in continuous perfusion bioreactors.

One of ordinary skill in the art will be able to tailor specific cell culture conditions in order to optimize certain characteristics of the cell culture including but not limited to growth rate, cell viability, final cell density of the cell culture, final concentration of detrimental metabolic byproducts such as lactate and ammonium, titer of the expressed glycoprotein, extent and composition of the oligosaccharide side chains or any combination of these or other conditions deemed important by the practitioner.

Isolation of the Expressed Glycoprotein

In general, it will typically be desirable to isolate and/or purify glycoproteins expressed according to the present invention. In certain embodiments, the expressed glycoprotein is secreted into the medium and thus cells and other solids may be removed, as by centrifugation or filtering for example, as a first step in the purification process.

The expressed glycoprotein may be isolated and purified by standard methods including, but not limited to, chromatography (e.g., ion exchange, affinity, size exclusion, and hydroxyapatite chromatography), gel filtration, centrifugation, or differential solubility, ethanol precipitation and/or by any other available technique for the purification of proteins (See, e.g., Scopes, Protein Purification Principles and Practice 2nd Edition, Springer-

Verlag, New York, 1987; Higgins, S. J. and Hames, B. D. (eds.), Protein Expression: A Practical Approach, Oxford Univ Press, 1999; and Deutscher, M. P., Simon, M. I., Abelson, J. N. (eds.), Guide to Protein Purification: Methods in Enzymology (Methods in Enzymology Series, Vol. 182), Academic Press, 1997, each of which is incorporated
5 herein by reference). For immunoaffinity chromatography in particular, the glycoprotein may be isolated by binding it to an affinity column comprising antibodies that were raised against that glycoprotein and were affixed to a stationary support. Alternatively, affinity tags such as an influenza coat sequence, poly-histidine, or glutathione-S-transferase can be attached to the glycoprotein by standard recombinant techniques to allow for easy
10 purification by passage over the appropriate affinity column. Protease inhibitors such as phenyl methyl sulfonyl fluoride (PMSF), leupeptin, pepstatin or aprotinin may be added at any or all stages in order to reduce or eliminate degradation of the glycoprotein during the purification process. Protease inhibitors are particularly advantageous when cells must be lysed in order to isolate and purify the expressed glycoprotein. Additionally or alternatively,
15 glycosidase inhibitors may be added at any or all stages in order to reduce or eliminate enzymatic trimming of the covalently attached oligosaccharide chains. A preferred purification method is described in Example 5 of this application.

Glycoproteins expressed according to the present invention have more extensive sialylation than they would if grown under traditional cell culture conditions. Thus, one
20 practical benefit of the present invention that may be exploited at the purification step is that the additional and/or altered sialic acid residues on a glycoprotein grown in accordance with certain of the present inventive methods may confer on it distinct biochemical properties that may be used by the practitioner to purify that glycoprotein more easily, or to a greater purity, than would be possible for a glycoprotein grown in
25 accordance with more traditional methods. For example, the glycoprotein can be purified or greatly enriched by anion exchange chromatography, making use of the negative charge of the sialic acid residues. Thereby a further enrichment of glycoprotein with high sialylation can be achieved.

In a further embodiment, the sialylation of the glycoprotein obtained by a method of the
30 invention can be further increased by contacting the glycoprotein with a sialyltransferase in vitro. The sialyltransferase typically is a mammalian sialyltransferase, preferably it is a human sialyltransferase. The sialyltransferase may be an α -2,3-sialyltransferase and/or an α -2,6-sialyltransferase. Preferably, the sialyltransferase is a human α -2,3-sialyltransferase (Genbank NP_775479-ST3GAL 1) and/or a human α -2,6-sialyltransferase. Most
35 preferably, the sialyltransferase is human α -2,6-sialyltransferase identified by Genbank

NP_003023-ST6GAL 1). Further present in the *in vitro* reaction is a sialyl group donor, or sialic acid donor. Suitable donors include, e.g., Cytidine-5'-monophospho-N-acetylneuraminic acid (CMP-NANA), Roche Catalog No. 05 974 003 103. A suitable kit for *in vitro* sialylation is available from Roche (Catalog Number 07 012 250 103).

- 5 One of ordinary skill in the art will appreciate that the exact purification technique will vary depending on the character of the glycoprotein to be purified, the character of the cells from which the glycoprotein is expressed, and/or the composition of the medium in which the cells were grown.

As mentioned above, the invention, in a second aspect, relates to a method of producing
10 a glycoprotein comprising N-glycans with increased sialylation, which comprises (i) providing cells comprising a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF) and a recombinant nucleic acid encoding an α -2,3-sialyltransferase and/or an α -2,6-sialyltransferase, preferably an α -2,6-sialyltransferase, and (ii) culturing the cells under conditions that allow expression of the glycoprotein.

- 15 The α -2,3-sialyltransferase preferably is a human α -2,3-sialyltransferase. The cDNA sequence encoding human α -2,3-sialyltransferase is shown in SEQ ID NO:12, and based thereon the skilled artisan can design suitable expression vectors containing a coding sequence of α -2,3-sialyltransferase.

The α -2,6-sialyltransferase preferably is a human α -2,6-sialyltransferase. The cDNA
20 sequence encoding human α -2,6-sialyltransferase is shown in SEQ ID NO:7, and based thereon the skilled artisan can design suitable expression vectors containing a coding sequence of α -2,6-sialyltransferase.

The transfected cells can be cultured under conditions allowing expression of the glycoprotein according to known culturing methods.

- 25 The glycoprotein can be recovered and/or isolated using established purification techniques.

The embodiments described hereinabove in connection with the method of the first aspect of the invention apply to the method of the second aspect *mutatis mutandis*.

Glycoprotein of the invention

In a third aspect the invention relates to a glycoprotein obtainable by a method described herein.

In a fourth aspect, the invention relates to a glycoprotein comprising a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said glycoprotein comprises N-glycans, and at least 75%, preferably at least 80%, more preferably at least 85% of said N-glycans comprise, on average, at least one sialic acid moiety. In preferred embodiments, at least 87%, at least 90%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of said N-glycans comprise, on average, at least one sialic acid moiety. The inventors demonstrated that polypeptides comprising highly sialylated VWF fragments not only have a prolonged half-life themselves, but are also capable to extend the half-life of co-administered FVIII. In other words, administration of the glycoprotein of the invention leads to an extended half-life and/or to a reduced clearance of co-administered FVIII.

In a fifth aspect, the invention relates to a glycoprotein comprising a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said glycoprotein comprises N-glycans, wherein at least 50% of the sialyl groups of the N-glycans of the glycoproteins are α -2,6-linked sialyl groups. In general, terminal sialyl groups can be attached to the galactose groups via a α -2,3- or via a α -2,6-linkage. In one embodiment, N-glycans of the glycoprotein of the invention comprise more α -2,6-linked sialyl groups than α -2,3-linked sialyl groups. Preferably, at least 60%, or at least 70%, or at least 80%, or at least 90% of the sialyl groups of the N-glycans are α -2,6-linked sialyl groups. These embodiments can be obtained by, e.g., co-expressing human α -2,6-sialyltransferase in mammalian cells.

In a sixth aspect, the invention relates to a glycoprotein comprising a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said glycoprotein comprises N-glycans, wherein at least 50% of the sialyl groups of the N-glycans of the glycoproteins are α -2,3-linked sialyl groups. In general, terminal sialyl groups can be attached to the galactose groups via a α -2,3- or via a α -2,6-linkage. In one embodiment, N-glycans of the glycoprotein of the invention comprise more α -2,3-linked sialyl groups than α -2,6-linked sialyl groups. Preferably, at least 60%, or at least 70%, or at least 80%, or at least 90% of the sialyl groups of the N-

glycans are α -2,3-linked sialyl groups. These embodiments can be obtained by, e.g., co-expressing human α -2,3-sialyltransferase in mammalian cells.

The preferred amino acid sequences of the glycoprotein of the invention have already been described hereinabove. The embodiments described above in connection with the first aspect of the invention apply to the third, fourth, fifth and sixth aspects *mutatis mutandis*.

The "glycoprotein of the invention" as used herein refers to a glycoprotein according to the third, fourth, fifth or sixth aspect. The glycoprotein of the invention has an increased sialylation of N-glycans, and in particular an increased α -2,6-sialylation or an increased α -2,3-sialylation.

In one embodiment, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the N-glycans of the glycoprotein of the invention comprise at least one sialic acid group. In another embodiment, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% of the N-glycans of the truncated VWF within the glycoprotein of the invention comprise at least one sialic acid group.

In another embodiment, less than 25%, less than 20%, less than 15%, or less than 12%, or even less than 10%, or less than 8%, or less than 6%, or less than 5%, or less than 4%, or less than 3%, or less than 2% or even less than 1% of the N-glycans of the glycoprotein of the invention are asialo-N-glycans, i.e. they are N-glycans lacking a sialic acid group. In another embodiment, less than 25%, less than 20%, less than 15%, or less than 12%, or less than 10%, or less than 8%, or less than 6%, or less than 5%, or less than 4%, or less than 3%, or less than 2% or even less than 1% of the N-glycans of the truncated VWF within the glycoprotein of the invention are asialo-N-glycans, i.e. they do not have a sialic acid group.

In another embodiment, at least 30%, or at least 35%, or at least 40% of the N-glycans of the glycoprotein of the invention are monosialo-N-glycans, i.e. they are N-glycans with one sialic acid group. In another embodiment, at least 30%, or at least 35%, or at least 40% of the N-glycans of the truncated VWF within the glycoprotein of the invention are monosialo-N-glycans. By way of non-limiting example the amount of monosialylated N-glycans can be determined as detailed in Example 6 and in Example 12.

In yet another embodiment, at least 15%, or at least 25%, or at least 30% of the N-glycans of the glycoprotein of the invention are disialo-N-glycans, i.e. they are N-glycans with 2

sialic acid groups. In yet another embodiment, at least 15%, or at least 25%, or at least 30% of the N-glycans of the truncated VWF within the glycoprotein of the invention are disialo-N-glycans. By way of non-limiting example the amount of disialylated N-glycans can be determined as detailed in Example 6 and in Example 12.

- 5 In yet another embodiment, at least 5%, or at least 10%, of the N-glycans of the glycoprotein of the invention are trisialo-N-glycans, i.e. they are N-glycans with 3 sialic acid groups. In yet another embodiment, at least 5%, or at least 10%, of the N-glycans of the truncated VWF within the glycoprotein of the invention are trisialo-N-glycans. By way of non-limiting example the amount of trisialylated N-glycans can be determined as
10 detailed in Example 6 and in Example 12.

- In another embodiment, at least 20%, or at least 30%, or at least 40%, of the N-glycans of the glycoprotein of the invention comprise two or more sialic acid groups. In another embodiment, at least 20%, or at least 30%, or at least 40%, of the N-glycans of the truncated VWF within the glycoprotein of the invention comprise two or more sialic acid
15 groups.

- Other preferred embodiments of the invention comprise a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said glycoprotein comprises N-glycans, wherein less than 35%, preferably less than 34%, preferably less than 33%, preferably less than 32%, preferably less than 31%,
20 preferably less than 30%, preferably less than 29%, preferably less than 28%, preferably less than 27% preferably less than 26%, preferably less than 25%, preferably less than 24%, preferably less than 23%, preferably less than 22%, preferably less than 21%, preferably less than 20%, preferably less than 19%, preferably less than 18%, preferably less than 17%, preferably less than 16%, preferably less than 15%, preferably less than
25 14%, preferably less than 13%, preferably less than 12%, preferably less than 11%, preferably less than 10%, preferably less than 9%, preferably less than 8%, preferably less than 7%, preferably less than 6% and preferably less than 5% of said N-glycans comprise, on average, two or more terminal and non-sialylated galactose residues.

- Still other even more preferred embodiments of the invention comprise a truncated von
30 Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said truncated VWF comprises N-glycans, wherein less than 6%, preferably less than 5%, preferably less than 4%, preferably less than 3%, preferably less than 2%, and preferably less than 1% of said N-glycans comprise, on average, three or more terminal and non-sialylated galactose residues.

The above-described embodiments can be combined with each other. Any percentages of N-glycans mentioned above, or any indications of the degree of sialylation, are to be understood as average percentages or degrees, i.e. they refer to a population of molecules, not to a single molecule. It is clear that the glycosylation or sialylation of the individual glycoprotein molecules within a population of glycoproteins will show some heterogeneity.

It has further been found that the glycoproteins obtained in accordance with this invention have a high proportion of dimers. The glycoprotein of the invention is therefore preferably present as dimer. In one embodiment, at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% or about 100% of the glycoproteins are present as dimers. In another embodiment, the ratio dimer:monomer of the glycoprotein of the invention is at least 1.5, preferably at least 2, more preferably at least 2.5 or at least 3. The dimer formation obtained by the methods of the invention is favorable, as the dimer has an improved affinity to Factor VIII. The dimer content, and the ratio of dimer to monomer of the glycoprotein of the invention can be determined as described in Example 5.

In another preferred embodiment, the glycoprotein of the invention comprises a heterologous polypeptide, e.g. a HLEP as defined above. Most preferably, the HLEP is human serum albumin (see SEQ ID NO:11). The embodiments described supra apply here *mutatis mutandis*.

The glycoprotein of the invention is preferably capable of binding to Factor VIII (see above). In one embodiment, the affinity of the glycoprotein of the invention to Factor VIII is greater than that of human native VWF to the same Factor VIII. The factor VIII affinity may refer to human native Factor VIII, or to the Factor VIII characterized by SEQ ID NO:10.

It has been found that preparations of the glycoprotein in accordance with this invention with a high proportion of dimers do have an increased affinity to Factor VIII. Such increased affinity to Factor VIII does lead to an enhanced stabilization of Factor VIII by the glycoproteins of the present invention. Alternatively to or in combination with an increased dimer proportion also glycoproteins in accordance with the invention with mutations within the Factor VIII binding domain which do increase the affinity to Factor VIII are preferred embodiments of the invention. Suitable mutations are disclosed, e.g., in WO 2013/120939 A1.

Another aspect of the invention is a glycoprotein as defined herein for use in the treatment of a blood coagulation disorder. The treatment comprises administering to a patient an effective amount of the glycoprotein. The treatment may further comprise administering a FVIII.

- 5 Another aspect of the invention is a pharmaceutical composition comprising a glycoprotein of the invention, and a pharmaceutically acceptable excipient or carrier.

Another aspect of the present invention is a pharmaceutical kit comprising (i) a glycoprotein as defined hereinabove and (ii) a Factor VIII. Preferably, the glycoprotein and the FVIII are contained in separate compositions.

- 10 Another aspect of the present invention is a pharmaceutical kit comprising (i) a glycoprotein as defined hereinabove and (ii) a Factor VIII, for simultaneous, separate or sequential use in the treatment of a blood coagulation disorder.

- Another aspect of the invention is the use of a polypeptide as defined hereinabove for increasing the terminal half-life or mean residence time (MRT) or reducing the clearance of Factor VIII. For evaluation of the pharmacokinetic data a linear pharmacokinetics model (compound elimination via the central compartment) was applied. Accordingly, any pharmacokinetic parameters used herein are based on a linear pharmacokinetics model (compound elimination via the central compartment), unless indicated otherwise.
- 15

- The "half-life" $T_{1/2}(t)$ at a certain time t is the time it takes to halve the plasma concentration $C(t)$ that is present at time t , i.e. $C[t + T_{1/2}(t)] = C(t)/2$. The "terminal half-life" is the limit of $T_{1/2}(t)$ when t tends to infinity.. The area under the curve (AUC) can be determined to assess clearance effects. A reduction in clearance leads to higher AUC values, and to an increase in half-life.
- 20

- The term "MRT", as used herein, means the average time a drug molecule resides in the body. In a linear pharmacokinetic system with constant clearance MRT can be calculated as area under the first moment curve (AUMC) divided by AUC. The first moment curve is time multiplied by plasma concentration at that time.
- 25

- The MRT of administered FVIII is increased by at least 25%, preferably by at least 50%, more preferably by at least 75%, more preferably by at least 100%, most preferably by at least 125%, if an effective amount of the glycoprotein of the present invention is co-administered, i) relative to administration of the FVIII alone or ii) relative to administration of a reference protein which has the same protein sequence as the glycoprotein of the
- 30

invention but a completely desialylated N-glycan structure or iii) relative to administration of a reference protein which has the same protein sequence as the glycoprotein of the invention but more than 35 % of its N-glycans comprise two or more terminal and non-sialylated N-glycans and more than 6 % of its N-glycans comprise three or more terminal
5 and non-sialylated galactose residue.

The MRT of the glycoprotein prepared according to the method of the present invention comprising culturing at a reduced temperature is greater than that of a reference glycoprotein having the same amino acid sequence which was cultured at 37°C. The increase in MRT of the glycoprotein prepared according to the method of the present
10 invention (or of any glycoprotein of the present invention) relative to the reference glycoprotein is preferably at least 25%, more preferably at least 50%, most preferably at least 100%.

The term "clearance", as used herein, refers to the rate at which plasma is cleared of drug. Specifically, it is the current elimination rate of a drug divided by its current plasma
15 concentration. In a linear pharmacokinetic system after a single intravenous administration the clearance can be calculated as the ratio of dose over the area under the plasma concentration-time curve (AUC), provided the clearance is constant. The lower the clearance the longer it takes until the plasma is cleared of the drug.

20 The clearance of administered FVIII is reduced by at least 10%, preferably by at least 25%, more preferably by at least 40%, more preferably by at least 50%, most preferably by at least 60%, if an effective amount of the glycoprotein of the present invention is co-administered, i) relative to administration of the FVIII alone or ii) relative to administration of a reference protein which has the same protein sequence as the glycoprotein of the
25 invention but a completely desialylated N-glycan structure or iii) relative to administration of a reference protein which has the same protein sequence as the glycoprotein of the invention but more than 35 % of its N-glycans comprise two or more terminal and non-sialylated N-glycans and more than 6 % of its N-glycans comprise three or more terminal and non-sialylated galactose residue.

30 The clearance of the glycoprotein prepared according to the method of the present invention comprising culturing at a reduced temperature is lower than that of a reference glycoprotein having the same amino acid sequence which was cultured at 37°C. The reduction in clearance of the glycoprotein prepared according to the method of the present invention (or of any glycoprotein of the present invention) relative to the reference

glycoprotein is preferably at least 40%, more preferably at least 50%, most preferably at least 60%.

The invention further relates to a method of increasing the MRT or half-life, or to a method of reducing the clearance of Factor VIII *in vivo*, comprising administering to a subject an effective amount of a glycoprotein as defined hereinabove.

A further aspect of this invention is a method of treating a blood coagulation disorder, comprising administering to a patient in need thereof an effective amount of a glycoprotein as defined hereinabove.

A further aspect is the use of a glycoprotein as defined hereinabove for reducing the frequency of administration of FVIII in a treatment of hemophilia A. The frequency of intravenous or subcutaneous administration of FVIII may be reduced to twice per week. Alternatively, the frequency of intravenous or subcutaneous administration of FVIII may be reduced to once per week, or even lower, e.g. to once per 10 days or once per 14 days. The FVIII may be administered twice weekly, every 5 days, once weekly, every 10 days, every two weeks, every three weeks, every four weeks or once a month, or in any range between any two of the foregoing values, for example from every four days to every month, from every 10 days to every two weeks, or from two to three times a week, etc.

Another aspect is the use of a glycoprotein as defined hereinabove for reducing the dose of FVIII to be administered in a treatment of hemophilia A.

Treatment of coagulation disorder

The glycoproteins of the invention are useful for treating coagulation disorders including hemophilia A. The term "hemophilia A" refers to a deficiency in functional coagulation FVIII, which is usually inherited.

Treatment of a disease encompasses the treatment of patients already diagnosed as having any form of the disease at any clinical stage or manifestation; the delay of the onset or evolution or aggravation or deterioration of the symptoms or signs of the disease; and/or preventing and/or reducing the severity of the disease.

A "subject" or "patient" to whom a glycoprotein of the invention is administered preferably is a human. In certain aspects, the human is a pediatric patient. In other aspects, the human is an adult patient.

Compositions comprising a glycoprotein of the invention and, optionally one or more additional therapeutic agents, such as the second therapeutic agents described below, are described herein. The compositions typically are supplied as part of a sterile, pharmaceutical composition that includes a pharmaceutically acceptable carrier. This composition can be in any suitable form (depending upon the desired method of administering it to a patient).

The glycoproteins of the invention can be administered to a patient by a variety of routes such as orally, transdermally, subcutaneously, intranasally, intravenously, intraperitoneally, intramuscularly, intrathecally, topically or locally. The most suitable route for administration in any given case will depend on the particular glycoprotein, the subject, and the nature and severity of the disease and the physical condition of the subject. Typically, a glycoprotein of the invention will be administered intravenously.

The glycoprotein and the FVIII are preferably administered intravenously or subcutaneously.

In a first embodiment, both the glycoprotein and the FVIII are administered intravenously. In a second embodiment, both the glycoprotein and the FVIII are administered subcutaneously.

In another embodiment, the FVIII is administered intravenously, and the glycoprotein is administered via a different route. In further embodiments, the glycoprotein is administered subcutaneously, and the FVIII is administered via a different route. For example, the glycoprotein may be administered subcutaneously, and the FVIII may be administered intravenously.

In further embodiments, the FVIII is administered subcutaneously, and the glycoprotein is administered via a different route. In further embodiments, the glycoprotein is administered intravenously, and the FVIII is administered via a different route. For example, the glycoprotein may be administered intravenously, and the FVIII may be administered subcutaneously.

The term "Factor VIII" and "FVIII" are used interchangeably herein and encompass both plasma derived FVIII and recombinant FVIII. Recombinant FVIII encompasses without limitation full-length FVIII as well as two-chain B-domain deleted or truncated variants as well as single-chain B-domain deleted or truncated variants for example those described

in WO 2004/067566 and other FVIII variants with mutations outside the B-domain but having the biological activity of FVIII.

- 5 Determination of the effective dosage, total number of doses, and length of treatment with a glycoprotein of the invention is well within the capabilities of those skilled in the art, and can be determined using a standard dose escalation study.

Pharmaceutical compositions

- 10 Therapeutic formulations of the glycoproteins of the invention suitable in the methods described herein can be prepared for storage as lyophilized formulations or aqueous solutions by mixing the glycoprotein having the desired degree of purity with optional pharmaceutically-acceptable carriers, excipients or stabilizers typically employed in the art (all of which are referred to herein as "carriers"), i.e., buffering agents, stabilizing agents,
15 preservatives, isotonicifiers, non-ionic detergents, antioxidants, and other miscellaneous additives. See, Remington's Pharmaceutical Sciences, 16th edition (Osol, ed. 1980). Such additives must be nontoxic to the recipients at the dosages and concentrations employed.

- Buffering agents help to maintain the pH in the range which approximates physiological
20 conditions. They can present at concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid- monosodium succinate mixture, succinic acid-sodium hydroxide mixture,
25 succinic acid- disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium
30 hydroxide mixture, gluconic acid-potassium glyconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide
35 mixture, etc.). Additionally, phosphate buffers, histidine buffers and trimethylamine salts such as Tris can be used.

Preservatives can be added to retard microbial growth, and can be added in amounts ranging from 0.2%- 1% (w/v). Suitable preservatives include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalconium halides (e.g., chloride, bromide, and iodide), hexamethonium chloride, and alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, and 3-pentanol. Isotonicifiers sometimes known as "stabilizers" can be added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinositol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur containing reducing agents, such as urea, glutathione, thiocetic acid, sodium thioglycolate, thioglycerol, α -monothioglycerol and sodium thio sulfate; low molecular weight polypeptides (e.g., peptides of 10 residues or fewer); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophylic polymers, such as polyvinylpyrrolidone monosaccharides, such as xylose, mannose, fructose, glucose; disaccharides such as lactose, maltose, sucrose and trisaccharides such as raffinose; and polysaccharides such as dextran. Stabilizers can be present in the range from 0.1 to 10,000 weights per part of weight active protein.

Non-ionic surfactants or detergents (also known as "wetting agents") can be added to help solubilize the therapeutic agent as well as to protect the therapeutic protein against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stressed without causing denaturation of the protein. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic polyols, polyoxyethylene sorbitan monoethers (TWEEN®-20, TWEEN®-80, etc.). Non-ionic surfactants can be present in a range of about 0.05 mg/ml to about 1.0 mg/ml, or in a range of about 0.07 mg/ml to about 0.2 mg/ml.

Additional miscellaneous excipients include bulking agents (e.g., starch), chelating agents (e.g., EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E), and cosolvents.

The formulation herein can also contain a second therapeutic agent in addition to a glycoprotein of the invention. Examples of suitable second therapeutic agents are provided below.

- 5 The dosing schedule can vary from once a month to daily depending on a number of clinical factors, including the type of disease, severity of disease, and the patient's sensitivity to the glycoprotein of the invention. In specific embodiments, a glycoprotein of the invention is administered, twice weekly, every 5 days, once weekly, every 10 days, every two weeks, every three weeks, every four weeks or once a month, or in any range
10 between any two of the foregoing values, for example from every four weeks to every month, from every 10 days to every two weeks, or from two to three times a week, etc.

The dosage of a glycoprotein of the invention to be administered will vary according to the particular glycoprotein, the subject, and the nature and severity of the disease, the
15 physical condition of the subject, the therapeutic regimen (e.g., whether a second therapeutic agent is used), and the selected route of administration; the appropriate dosage can be readily determined by a person skilled in the art.

It will be recognized by one of skill in the art that the optimal quantity and spacing of
20 individual dosages of a glycoprotein of the invention will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the age and condition of the particular subject being treated, and that a physician will ultimately determine appropriate dosages to be used. This dosage can be repeated as often as appropriate. If side effects develop the amount and/or frequency of the dosage
25 can be altered or reduced, in accordance with normal clinical practice.

Combination Therapy

Preferably, the patient being treated with the glycoprotein of the invention is also treated
30 with a conventional therapy of coagulation disorders. For example, a patient suffering from hemophilia is typically also being treated with Factor VIII.

In accordance with this invention, the patient being treated with the glycoprotein of the invention is also treated with Factor VIII. The glycoprotein of the invention and the Factor
35 VIII molecule may be administered simultaneously or in a sequential fashion, both modes of administration being encompassed by the term "combination therapy" and "co-

administration". The glycoprotein of the invention and the Factor VIII molecule may be administered as a mixture, i.e. within the same composition, or separately, i.e. as separate compositions.

- 5 The concentration of Factor VIII in the composition used according to the present invention is typically in the range of 10-10,000 IU/mL. In different embodiments, the concentration of FVIII in the compositions of the invention is in the range of 10-8,000 IU/mL, or 10-5,000 IU/mL, or 20-3,000 IU/mL, or 50-1,500 IU/mL, or 3,000 IU/mL, or 2,500 IU/mL, or 2,000 IU/mL, or 1,500 IU/mL, or 1,200 IU/mL, or 1,000 IU/mL, or 800 IU/mL, or
10 750 IU/mL, or 600 IU/mL, or 500 IU/mL, or 400 IU/mL, or 300 IU/mL, or 250 IU/mL, or 200 IU/mL, or 150 IU/mL, or 125 IU/mL, or 100 IU/mL, or 62.5 IU/mL, or 50 IU/mL.

"International Unit," or "IU," is a unit of measurement of the blood coagulation activity (potency) of FVIII as measured by a FVIII activity assay such as a one stage clotting
15 assay or a chromogenic substrate FVIII activity assay using a standard calibrated against an international standard preparation calibrated in "IU". One stage clotting assays are known to the art, such as that described in N Lee, Martin L, et al., An Effect of Predilution on Potency Assays of FVIII Concentrates, Thrombosis Research (Pergamon Press Ltd.) 30, 511 519 (1983). Principle of the one stage assay: The test is executed as a modified
20 version of the activated Partial Thromboplastin Time (aPTT)-assay: Incubation of plasma with phospholipids and a surface activator leads to the activation of factors of the intrinsic coagulation system. Addition of calcium ions triggers the coagulation cascade. The time to formation of a measurable fibrin clot is determined. The assay is executed in the presence of Factor VIII deficient plasma. The coagulation capability of the deficient plasma is
25 restored by Coagulation Factor VIII included in the sample to be tested. The shortening of coagulation time is proportional to the amount of Factor VIII present in the sample. The activity of Coagulation Factor VIII is quantified by direct comparison to a standard preparation with a known activity of Factor VIII in International Units.

30 Another standard assay is a chromogenic substrate assay. Chromogenic substrate assays may be purchased commercially, such as the coamatic FVIII test kit (Chromogenix-Instrumentation Laboratory SpA V. le Monza 338 - 20128 Milano, Italy). Principle of the chromogenic assay: In the presence of calcium and phospholipid, Factor X is activated by Factor IXa to Factor Xa. This reaction is stimulated by Factor VIIIa as
35 cofactor. FVIIIa is formed by low amounts of thrombin in the reaction mixture from FVIII in the sample to be measured. When using the optimum concentrations of Ca²⁺,

phospholipid and Factor IXa and an excess quantity of Factor X, activation of Factor X is proportional to the potency of Factor VIII. Activated Factor X releases the chromophore pNA from the chromogenic substrate S-2765. The release of pNA, measured at 405 nm, is therefore proportional to the amount of FXa formed, and, therefore, also to the Factor VIII activity of the sample.

5

The nucleotide and amino acid sequences shown in the sequence listing are summarized in the following table:

Table 1:

SEQ ID NO:	Remarks
1	DNA sequence encoding a polypeptide comprising amino acids 1 to 1242 of human VWF, a glycine/serine linker and human albumin; nucleotide positions (nt): nt 1-6: EcoRI restriction enzyme cleavage site nt 32 – 3757: coding sequence for VWF amino acids 1 to 1242 nt 3758 – 3850: coding sequence for glycine/serine linker nt 3851 – 5608: coding sequence for human albumin nt 5609 – 5616: NotI restriction enzyme cleavage site
2	Amino acid sequence encoded by SEQ ID NO:1 (mature form): amino acid positions (aa): aa 1 – 479: VWF D'D3 region (VWF amino acids 764 – 1242) aa 480 – 510: glycine/serine linker aa 511 – 1195: human albumin
3	PCR primer α -2,6 sialyltransferase
4	PCR primer α -2,6 sialyltransferase
5	nested PCR primer α -2,6 sialyltransferase
6	nested PCR primer α -2,6 sialyltransferase
7	cDNA sequence encoding human α -2,6 sialyltransferase
8	DNA sequence encoding the pre-pro form of human native VWF
9	Amino acid sequence encoded by SEQ ID NO:8
10	Amino acid sequence of a single chain Factor VIII molecule
11	Amino acid sequence of mature human serum albumin
12	cDNA sequence encoding human α -2,3 sialyltransferase

The following examples illustrate the invention but should not be construed as limiting the present invention to the specific embodiments described herein below.

Example 1: Generation of D'D3 albumin fusion protein (D'D3-FP)

5

The expression cassette for D'D3-FP consisting of cDNA encoding VWF amino acids 1 to 1242, a glycine/serine linker and the cDNA of human albumin was prepared by custom gene synthesis (Eurofins Genomics, Ebersberg, Germany). Through flanking restriction sites (EcoRI, NotI) the expression cassette was excised from the cloning vector supplied
10 and inserted into a pIRESneo3 vector (BD Biosciences, Franklin Lakes, NJ, USA) linearized with EcoRI and NotI. The resulting expression plasmid contained nucleotide sequences encoding the VWF propeptide, D' and D3 (VWF amino acids 1 to 1242 of SEQ ID NO:9) fused to the albumin coding sequence through a short linker coding sequence under CMV promoter control. The nucleotide sequence of the coding sequence is
15 displayed as SEQ ID NO:1, the amino acid sequence of the mature D'D3-FP is shown as SEQ ID NO:2.

Example 2: Transfection of plasmids and stable expression of D'D3-FP dimer in Chinese Hamster Ovary (CHO) cells

20

The expression plasmid as described above was grown up in XL10 Gold (Agilent Technologies) and purified using standard protocols (Qiagen, Hilden, Germany).

CHO K1 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) and
25 grown up in serum-free medium (CD-CHO, Invitrogen) in the presence of 500-1000 µg/ml Geneticin. An expression plasmid encoding PACE/furin (pFu-797) as described in WO2007/144173 was cotransfected to maximize propeptide cleavage efficacy. Single cell derived clones were grown up and selected according to their D'D3-FP expression yield as quantified by an albumin specific enzyme immunoassay (see below). The cell line
30 finally selected for D'D3-FP fermentation was called T2050-CL3.

Example 3: Coexpression of an α -2,6 sialyl transferase

During a cell line generation process as described in example 2 a plasmid carrying an expression unit encoding an α -2,6 sialyl transferase to support the attachment of non-
5 rodent sialic acids can be cotransfected.

The coding sequence for human α -2,6 sialyl transferase is amplified from a human liver cDNA library (Ambion) using primers We2556 (SEQ-ID NO. 3) and We 2558 (SEQ-ID NO. 4) for a first and primers We2553 (SEQ-ID NO. 5) and We 2559 (SEQ-ID NO. 6) for a
10 second PCR in a nested PCR setup. For the first PCR 2 μ L of the Ambion human liver cDNA library are mixed with 34.5 μ L of water, 10 μ L 5x PCR buffer Phusion GC (New England Biolabs), 1 μ L of 10mM dNTPs, 1 μ L of We2556 (10 pmol), 1 μ L of We2558 (10 pmol) and 0.5 μ L of Phusion DNA polymerase (New England Biolabs) and amplified using a touchdown protocol of initial 60 seconds at 98°C, 15 cycles of a) 15 seconds of
15 denaturation at 98°C, b) 30 seconds of annealing at 64°C and c) 2 minutes of elongation at 72°C, wherein the temperature of the annealing step is reduced by 0.3°C per cycle, followed by 25 cycles of a) 25 seconds of denaturation at 98°C, b) 30 seconds of annealing at 62°C and c) 2 minutes of elongation at 72°C, followed by a final extension step for 10 minutes at 72°C, after which the reaction is stopped by cooling down and
20 holding at 4°C. For the nested PCR 2 μ L of the first PCR reaction are mixed with 34.5 μ L of water, 10 μ L 5x PCR buffer Phusion GC, 1 μ L of 10mM dNTPs, 1 μ L of We2553 (10 pmol), 1 μ L of We2559 (10 pmol) and 0.5 μ L of Phusion DNA polymerase and amplified using the touchdown protocol as described for the first PCR. The nested PCR adds an NheI restriction enzyme cutting site to the 5'-end and a BamH1 site to the 3'-end of the
25 PCR fragment. This fragment is cut by NheI and BamH1 and ligated into expression vector pIRESneo3 which had been opened by the same enzymes. The resulting expression vector then can be used for cotransfection.

Example 4: Production of D'D3-FP in bioreactors

30 The fermentation process for the production of D'D3-FP started with the thaw of cell line T2050-CL3 followed by cell expansion in shake flasks and finally a fermentation process in perfusion mode using the Sartorius BioStat B-DCU 5 L bioreactor and the BioStat STR 50L single-use bioreactors. The BioSeps 10L or 200L (Applikon), respectively, were used
35 as cell retention devices. Cell culture media were either PowerCHO3 (Lonza BESP1204)

with 8 mM L-glutamine and 1 μM CuSO_4 or ProCHO5 (Lonza BESP1072) with 10 mM L-glutamine and 1 μM CuSO_4 .

5 The seed trains in shake flasks were performed at 37°C, 7.5% CO_2 at a shaker speed of 160 rpm.

10 The 5L bioreactor was inoculated with a target VCD of 2.5×10^5 cells/mL. The cells were cultivated in PowerCHO3 with 8 mM L-glutamine and 1 μM CuSO_4 at a temperature of +37.0°C, a pH of 7.00, and at 30 % oxygen saturation. A temperature shift to +34.0 °C (evaluated range +31°C to +35°C) was performed after initial harvests from the bioreactor run at +37°C had been taken. The pH was controlled using CO_2 sparged as acid and NaHCO_3 as base. The overlay air flow rate was set to 0.5 L/min. A ring sparger was used as a sparging unit. The agitation rate was 150 rpm with a 2fold pitch blade impeller in down pull mode.

15 The 50L bioreactor was inoculated with a target VCD of 3.0×10^5 cells/mL. The cells were cultivated in ProCHO5 medium with 10 mM L-glutamine and 1 μM CuSO_4 at a temperature of +37.0°C, a pH of 6.90, and at 30 % oxygen saturation. A temperature shift to +34.0 °C was performed after the initial one or two harvests. PH control as above, the overlay air flow rate was set to 2 L/min. A micro sparger was used as a sparging unit. The
20 agitation rate was 90 rpm with a 2fold pitch blade impeller in down pull mode.

The perfusion was initiated when the VCD in the bioreactor was $\geq 1.0 \times 10^6$ cells/mL. The perfusion rate was set to 1.0 volume/volume/day. The BioSep was operated in back flush mode with 5 (10) minutes runtime and 10 seconds back flush at a power input of 7 (30) W
25 (numbers in brackets refer to the 50L bioreactor). The perfusate and the bleed were filtered inline and collected in bags over 48 hours at +2 to +8°C. The VCD was controlled by active bleeding using a turbidity probe using glucose consumption as parameter with a target of 2 g/L glucose. Harvest and bleed were filtered inline, the harvest system consisting of a disposable filter and disposable bag was changed every second day.

30

To prepare material for the PK analyses described below harvests taken before and after the respective temperature shifts were purified by affinity and size exclusion chromatography.

Example 5: Purification of D'D3-FP dimer using affinity chromatography and size exclusion chromatography

The cell-free harvest from the bioreactor was concentrated 30-fold using a TFF system (e.g. Pall Centramate 500 S) with a 30 kD membrane (e.g. Pall Centramate OS030T12). That concentrate was spiked with NaCl and EDTA to a final concentration of 0.75 M NaCl and 5 mM EDTA and loaded overnight on a CaptureSelect Human Albumin column (Life Technologies) which was pre-equilibrated with 20 mM Tris buffer pH 7.4. After washing the column with equilibration buffer D'D3-FP was eluted with elution buffer (20 mM Tris, 2 M MgCl₂, pH 7.4). The eluate was then 10-fold concentrated and dialyzed against 50 mM Tris, 150 mM NaCl, pH 7.4 using Ultra Centrifugal Filters with a 30 kD cut-off (e.g. Amicon. UFC903024). To separate the D'D3-FP dimer from the monomer portion that material was loaded on a Superdex 200 pg column (GE Healthcare Code: 17-1069-01) pre-equilibrated with 50 mM Tris, 150 mM NaCl, pH 7.4 and the peak fractions containing the D'D3-FP dimer were pooled. The area under the curve for the dimer and monomer peak fractions were used to calculate dimer to monomer ratio.

Example 6: Total sialylation assay**Materials and Methods:**

Acetic acid was from Sigma-Aldrich (Prod. 338826). Acetonitrile was from Burdick and Jackson (Prod. LC015). 2-aminobenzamide (2-AB) was from Aldrich (Prod. A89804). Ammonium hydroxide was from Sigma-Aldrich (Prod. 338818). Ammonium bicarbonate was from Fluka (Prod. 09830). Dimethyl sulfoxide was from Sigma Prod. (D2650). Dithiothreitol (DTT) was from Sigma (Prod. 646563). Formic acid was from Thermo (Prod. 28905). N-Glycosidase F (PNGase 250U) was from Roche (Prod. 11 365 193 00). Sodium cyanoborohydride was from Aldrich (Prod. 156159). Oasis HLB 3cc 60 mg SPE cartridges were from Waters (Part No: WAT094226). 50KDa Amicon Ultra 4 centrifugal ultrafilters were from Millipore (Cat. No. UFC805008). Zeba Spin 7K MWCO columns 2mL were from Thermo (No. 89889).

PNGase F enzymatic glycan release:

About 700µg of D'D3-FP was reduced with DTT in approximately 70 mM ammonium bicarbonate, pH 8.5 at 60°C for 30 min. The reduced sample was cooled to room temperature and alkylated with iodoacetamide at RT in the dark for 30 min. The alkylated sample was buffer exchanged into 50mM ammonium bicarbonate pH 8.6 using a 2mL

Zeba Spin 7K MWCO column. To the buffer exchanged sample, 40U of PNGase was added and the sample incubated at 37°C for 14 hours. An additional 40U of PNGase was added and the sample incubated for a further 6 hours at 37°C. The PNGase digested sample was centrifuged through a 50KDa Amicon Ultra 4 ultrafilter. The filtrate was dried in a CentriVap.

2-AB labelling of released N-glycans:

To prepare the 2-AB labelling reagent, 23 mg of 2-aminobenzamide was dissolved in 350 µL of DMSO and 150 µL glacial acetic acid was added. The resulting solution was added to 32 mg of sodium cyanoborohydride and mixed thoroughly until dissolved.

50 µL of the 2-AB reagent was added to the dried sample and incubated in the dark at 65°C for 3.5 hours.

A Waters Oasis HLB 3cc 60 mg SPE cartridge was conditioned with 3 mL 95% acetonitrile the 3mL 35% acetonitrile then 3 mL 95% acetonitrile. The 2-AB labelled sample was diluted by adding 1.95 mL of 95% v/v acetonitrile and immediately loaded onto the HLB cartridge and allowed to drain under gravity. Sample was washed under gravity with 3x 3mL of 95% v/v acetonitrile and the eluted with 3mL of 35% v/v acetonitrile. The eluate was dried in a Centrivap. The dried 2-AB derivatised sample was dissolved by the addition of 35 µL of Milli Q water and vortex mixing. After dissolution, 85 µL of Acetonitrile was added and mixed briefly. Sample was transferred to a HPLC vial for analysis.

2-AB Glycan analysis:

High performance liquid chromatography was performed on a Thermo Dionex Ultimate 3000 system consisting of an RS Binary Pump, Autosampler, RS Column Compartment and RS Fluorescence detector. The separation of 2-AB glycan derivatives was achieved using a Dionex GlycanPac AXH-1, 1.9 µm, 2.1 x 150 mm column (P/N 082472). Mobile phase A consisted of 100% acetonitrile, Mobile phase B consisted of 50mM Formic acid adjusted to pH 4.0 with 5M ammonium hydroxide solution. The column was maintained at 50° C and the flow rate was 0.200 mL/min. The column was equilibrated with 15% B. After injection of 6 µL of sample, the mobile phase composition was changed linearly to 40% B over 50 minutes, then to 80% B over 10 minutes, then to 95% B over 0.1 minutes, then maintained at 95% B for 4.9 minutes, and then back to 15% B over 0.1 minutes. The column was requilibrated at 15% B for 14.9 minutes. Fluorescence detection was performed with an excitation wavelength of 320 nm and an emission wavelength of 420 nm.

Results:**Table 2:** Lots of D'D3-FP provided for PK analysis:

Lot #	Asialo [%]	Mono-sialo [%]	Di - sialo [%]	Tri - sialo [%]	Tetra-sialo [%]	Sialylation [%]
B-140526 (no temperature shift)	59.4	29.0	9.7	1.9	n.d.	40.6
B-140616KS	16.4	34.6	28.1	15.3	5.6	83.6
B-140825	12.7	42.9	32.0	9.9	2.6	87.3
B-140623KS	10.2	38.7	33.8	14.2	3.0	89.8

5

D'D3-FP protein purified from harvests taken after the temperature shift from 37°C to 33°C (e.g. Lot B-140825) or to 34°C (e.g. Lot B-140623KS) showed an improved sialylation pattern in that a reduced amount of asialo and monosialo structures was detected while in particular the Di-sialo and Tri-sialo structures increased. The reduced content of asialo structures had a positive effect on the half-life of the D'D3-FP protein itself as well as on a co-administered FVIII (see example 8).

10

A further beneficial effect was found as a result of the temperature shift in that the ratio of D'D3-FP dimers increased over the monomer at lower temperatures, wherein the dimer is the preferred structure due to its increased binding to FVIII.

15

Table 3. Effect of Temperature on Dimer Content

Bioreactor temperature before harvest	% Dimer	% Monomer	Ratio Dimer:Monomer
37	52.3	47.7	1.1
35	71.0	29.0	2.45
33	71.2	28.8	2.5
32	74.6	25.4	2.94
31	77.5	22.5	3.44

As shown in Table 4 the beneficial effect of a temperature shift on the degree of sialylation was not observed with respect to full length VWF. Specifically, the content of asialostructures could not be reduced when full length wild-type VWF albumin fusion

20

("rVWF-FP") was expressed under similar bioreactor conditions to those described in example 4 and when the temperature was reduced to 33.5°C compared to the expression at the standard temperature of 37°C. Purification had been performed as described in US 2014/0072561 A1.

5

Table 4. Sialylation of full length VWF

Lot #	Sialylation
rVWF-FP only expressed at 37°C	100
rVWF-FP first expressed at 37°C then at 33.5°C	91 % of the sialylation degree of rVWF-FP above which was only expressed at 37°C

The degree of sialylation of the Lot harvested at 37°C was normalised to a nominal value of 100. The degree of sialylation determined for the Lot harvested at 33.5°C was lower than that of the Lot harvested at 37°C.

Example 7: Determination of D'D3-FP antigen concentration

Human albumin was determined by an ELISA whose performance is known to those skilled in the art. Briefly, microplates were incubated with 100 µL per well of the capture antibody (goat anti-human-albumin-IgG, Cat. No. A80-129A, Bethyl Laboratories, Inc.), diluted to 2 µg/mL in Buffer A [Sigma C3041] for 16 hours at ambient temperature. After washing plates three times with buffer B (Sigma P3563), microplates were blocked with 200 µL per well of blocking solution (Cat.No. 110500, Candor Bioscience GmbH), for 1.5 hours at ambient temperature. After washing plates again three times with buffer B (Sigma P3563), serial dilutions of the test sample in LowCross Buffer (Cat. No. 100500, Candor Bioscience GmbH,) as well as serial dilutions of N Protein Standard SL (OQIM13, Siemens Healthcare 50-0.78 ng/mL) in LowCross Buffer (volumes per well: 100 µL) were incubated for one hour at +37°C. After four wash steps with buffer B, 100 µL of a 1:40,000 dilution in blocking solution of the detection antibody (goat-anti-Human Albumin-IgG peroxidase labelled, Cat. No. A80-129P, Bethyl Laboratories, Inc.)-D, were added to each well and incubated for 45 min. at +37°C. After three wash steps with buffer B, 100 µL of substrate solution (1:10 (v/v) TMB OUVF : TMB Buffer OUVG, Siemens Healthcare) were added per well and incubated for 20 minutes at ambient temperature in the dark. Addition of 100 µL

stop solution (OSFA, Siemens Healthcare) prepared the samples for reading in a suitable microplate reader at 450 nm wavelength. Concentrations of test samples were then calculated using the standard curve with the N Protein Standard SL as reference.

5 Example 8: PK analysis

Aim

We aimed at characterizing the impact of sialylation on pharmacokinetics (PK) of the half-life extended von Willebrand Factor (VWF) fragment D'D3-FP dimer and FVIII. One aim of these studies was to determine the influence of sialylation of the D'D3-FP dimer on its PK and additionally on the PK of co-administered FVIII in rats (example 8.1). A second example covers the effect on a full-length FVIII product Advate® in rats (example 8.2). The lot # (see Table 2 above) and the degree of D'D3-FP dimer sialylation in % are indicated for each preparation.

Example 8.1: Prolongation of pharmacokinetics of FVIII by co-administration of highly sialylated D'D3-FP dimer in rats

Material and Methods

Animals: Female Crl:CD (Sprague Dawley) rats in a weight range of 230-300 g were breed at Charles River Laboratories (Sulzfeld, Germany). In house, the animals were kept at standard housing conditions, i.e. at 21-22°C under a 12 h/12 h light-darkness cycle. Animals were fed ad libitum with standard rat diet (Ssniff-Versuchsdäten, Soest, Germany). Tap water was supplied ad libitum. Animal husbandry and study procedures complied with the German Animal Welfare law and European Union regulations.

Laboratory evaluations: The test articles were administered i.v. by a single injection into the lateral tail vein at a volume of 3 mL/kg. All D'D3-FP dimer preparations were administered at a dose level of 1000 µg/kg based on human albumin values, and co-administered with 200 IU/kg rVIII-SingleChain (rVIII-SC, chromogenic activity) after incubating for approximately 30 minutes at +37°C. Animals receiving only rVIII-SC served as control (Table 5).

Blood samples were taken retro-orbitally under short term anaesthesia at 5 min, 2, 4, 8, 24, 32, 48 and 72 h after intravenous bolus injection using an alternating sampling scheme. The PK profile was taken from two cohorts of rats per group (n=3 per time-point,

n=6 per group). Blood samples were anticoagulated using sodium citrate (2 parts sodium citrate 3.13% + 8 parts blood), processed to plasma and stored at -20°C for the determination of FVIII antigen and/or albumin.

- 5 D'D3-FP dimer exposure was determined by measurement of the albumin part of the protein using an immunoassay specific for human albumin (example 7), and FVIII:Ag plasma levels were detected with the FVIII Asserachrom ELISA test kit from Stago, S.A.S., France.

10 Table 5: Treatment groups

Treatment*	Sialylation [%] of D'D3-FP	D'D3-FP dimer dose [mg albumin/kg]	FVIII dose [IU FVIII:C/kg]
rVIII-SC		-	200
D'D3-FP dimer (B-140526) & rVIII-SC	40.6	1	200
D'D3-FP dimer (B-140616KS) & rVIII-SC	83.6	1	200
D'D3-FP dimer (B-140623KS) & rVIII-SC	89.8	1	200

FVIII:C = chromogenic FVIII activity

* Lot # given in brackets

Results

- 15 D'D3-FP dimer was quantified via its albumin component, and measurements were performed up to 72 h p.a., and all measured data were well above the detection limit of the assay. Mean residence time (MRT) and clearance (CL) were estimated by non-compartmental methods and the data are presented in Fig. 1. rVIII-SC co-administered with the D'D3-FP dimer with 40.6% sialylation (B-140526) had a shorter MRT and higher
 20 clearance as when co-administered with the D'D3-FP dimer preparations with 83.6% and 89.8% sialylation (B-140616KS and B-140623KS, respectively).

- In line with this observation, the pharmacokinetic profile of the co-administered FVIII (200 IU/kg chromogenic FVIII activity), quantified as FVIII:Ag via ELISA, was modified
 25 accordingly. It shall be mentioned that not all plasma levels at 48h and 72 h could be measured, some values were below the detection limit of 57 mIU/mL. Clearly, rVIII-SC alone had the shortest MRT and highest clearance, which was generally prolonged when D'D3-FP dimer was co-administered (Fig. 2). Those D'D3-FP dimers, which had a longer exposure by themselves, also prolonged the FVIII PK profile. Thus, MRT of the D'D3-FP

dimer with 40.6% sialylation (B-140526) was shorter and clearance was higher compared to D'D3-FP dimer with sialylation >80%.

Thus, the pharmacokinetic profile of FVIII:Ag was dependent on the sialylation of D'D3-FP dimer, i.e., shortest PK was observed with 40.6% sialylation and longest PK with those of >80% sialylation.

Evaluation of PK characteristics of D'D3-FP dimer was done in more detail, i.e. additionally calculating maximal concentrations (C_{max}) and terminal half-life ($t_{1/2}$) in a non-compartmental model, as well as calculating the x-fold increases (Table 6).

Sialylation between 89.8% and 40.6% influenced clearance of D'D3-FP dimer by more than 2-fold (0.91 mL/kg/h for the 89.8% D'D3-FP dimer and 2.06 mL/kg/h for the 40.6% D'D3-FP dimer as determined by measuring the albumin concentration over time). This relates to more than 40% increase in mean residence time (MRT, i.e. 56.9 h to 81.5 h) and more than 30% increase in terminal half-life (i.e. 44.0 h to 58.6 h).

As depicted in the graphs for MRT and clearance, this translates to the PK characteristics of the co-administered FVIII, even though not as obvious as for D'D3-FP dimer (Table 6, FVIII:Ag): clearance is decreased by more than 30% (3.93 mL/kg/h to 2.95 mL/kg/h), MRT is increased by 19% (16.5 h to 19.6 h) and terminal half-life by 15% (11.4 h to 13.1 h).

Therewith, the increase in exposure over time is given by D'D3-FP dimer depending on the percentage of sialylation, as may also be seen by the fold increase of PK characteristics of rVIII-SC given alone. While 40.6% sialylation prolong FVIII PK 1.5-1.9fold, an optimized D'D3-FP dimer with 89.8% sialylation prolongs FVIII PK 2.0-2.2fold, and 83.6% sialylation leads to intermediate values. Thus, this effect correlates with the degree of sialylation within the investigated range from 40.6% to 89.8%.

Table 6: Pharmacokinetic parameters of D'D3-FP dimer and FVIII:Ag after co-administration of rVIII-SC and D'D3-FP dimer in rats (non-compartmental analysis)

Dose D'D3-FP dimer 1 mg/kg, dose rVIII-SC 200 IU/kg

Treatment*	C _{max} , extrap. IU/mL	Clearance mL/kg/h	MRT h	Half-life, terminal h
Albumin				
D'D3-FP dimer (40.6%) & rVIII-SC	18.1	2.06	56.9	44.0
D'D3-FP dimer (83.6%) & rVIII-SC	18.8	1.07	82.4	61.4
D'D3-FP dimer (89.8%) & rVIII-SC	21.3	0.91	81.5	58.6
FVIII:Ag				
rVIII-SC	4.26	6.04	8.9	6.4
D'D3-FP dimer (40.6%) & rVIII-SC	3.05	3.93	16.5	11.4
		1.5fold	1.9fold	1.8fold
D'D3-FP dimer (83.6%) & rVIII-SC	3.41	3.41	18.1	12.8
		1.8fold	2.0fold	2.0fold
D'D3-FP dimer (89.8%) & rVIII-SC	3.97	2.95	19.6	13.1
		2.0fold	2.2fold	2.0fold

* degree of D'D3-FP dimer sialylation given in brackets

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Example 8.2: Prolongation of pharmacokinetics of full-length FVIII by co-administration of highly sialylated D'D3-FP dimer in rats

10 Material and Methods

Animals: Female Crl:CD (Sprague Dawley) rats in a weight range of 220-300 g were breed at Charles River Laboratories (Sulzfeld, Germany). In house, the animals were kept at standard housing conditions, i.e. at 21-22°C under a 12 h/12 h light-darkness cycle. Animals were fed ad libitum with standard rat diet (Ssniff-Versuchsdäten, Soest, Germany). Tap water was supplied ad libitum. Animal husbandry and study procedures complied with the German Animal Welfare law and European Union regulations.

Laboratory evaluations: The test articles were administered i.v. by a single injection into the lateral tail vein at a volume of 3 mL/kg. All D'D3-FP dimer preparations were administered at a dose level of 1000 µg/kg based on human albumin values, and co-

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administered with 200 IU/kg Advate® (nominal chromogenic activity) after incubating for approximately 30 minutes at +37°C. Animals receiving only Advate® served as control (Table 7).

- 5 Blood samples were taken retro-orbitally under short term anaesthesia at 5 min, 2, 4, 8, 24, 32, 48 and 72 h after intravenous bolus injection using an alternating sampling scheme. The PK profile was taken from two cohorts of rats per group (n=3 per time-point, n=6 per group). Blood samples were anticoagulated using sodium citrate (2 parts sodium citrate 3.13% + 8 parts blood), processed to plasma and stored at -20°C for the
10 determination of FVIII antigen and/or albumin.

D'D3-FP dimer exposure was determined by measurement of the albumin part of the protein using an immunoassay specific for human albumin (example 7), and FVIII:Ag plasma levels were detected with the FVIII Asserachrom ELISA testkit from Stago, S.A.S.,
15 France.

Table 7: Treatment groups (experiment-wise)

Treatment*	Sialylation [%]	D'D3-FP dimer dose [mg albumin/kg]	FVIII dose [IU FVIII:C/kg]
Advate®		-	200
D'D3-FP dimer (B-140526) & Advate®	40.6 %	1	200
D'D3-FP dimer (B-140825) & Advate®	87.3 %	1	200

FVIII:C = chromogenic FVIII activity

* Lot # given in brackets

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Results

D'D3-FP dimer was quantified via its albumin component, and measurements were performed up to 72 h p.a., and measured data were well above the detection limit over the whole observation period. Mean residence time (MRT) and clearance (CL) were estimated
25 by non-compartmental methods and the data are presented in Fig. 3. PK characteristics of D'D3-FP dimer in the group of Advate® co-administered with the D'D3-FP dimer with 40.6 % sialylation had a shorter MRT and higher clearance as when co-administered with the D'D3-FP dimer preparation with 87.3 % sialylation.

In line with this observation, the pharmacokinetic profile of the co-administered FVIII (200 IU/kg nominal chromogenic FVIII activity), quantified as FVIII:Ag via ELISA, was modified accordingly. It shall be mentioned that samples could be measured until 4-8 h p.a. with the Advate[®]-treated group and until 24-32 h p.a. with the D'D3-FP dimer co-treated groups, thereafter the values were below the limit of detection of the assay of 117 mIU/mL. Clearly, Advate[®] alone had the shortest MRT and highest clearance, which was generally prolonged when D'D3-FP dimer was co-administered (Fig. 4). Those D'D3-FP dimers, which had a longer exposure by themselves, also prolonged the FVIII PK profile. Thus, MRT of the D'D3-FP dimer with 40.6% sialylation was shorter and clearance was higher compared to D'D3-FP dimer with sialylation >85%. Thus, the pharmacokinetic profile of FVIII:Ag was dependent on the sialylation of D'D3-FP dimer, i.e., shortest PK was observed with 40.6 % sialylation and longest PK with those of >85 % sialylation.

Evaluation of PK characteristics of D'D3-FP dimer was done in more detail, i.e. additionally calculating maximal concentrations (C_{max}) and terminal half-life ($t_{1/2}$) in a non-compartmental model, as well as calculating the x-fold increases over Advate[®] given alone (Table 8).

Sialylation between 87.3% and 40.6% influenced clearance of D'D3-FP dimer by more than 1.5-fold (1.32 mL/kg/h for the 87.3% D'D3-FP dimer and 2.17 mL/kg/h for the 40.6% D'D3-FP dimer as determined by measuring the albumin concentration over time). This relates to slight effects on mean residence time (MRT, +14%, i.e. 54.4 h to 62.0 h) and terminal half-life ($t_{1/2}$, +4%, i.e. 42.2 h to 44.0 h).

As depicted in the graphs for MRT and clearance, this translates to the PK characteristics of the co-administered FVIII, even though mostly not as obvious as for D'D3-FP dimer (Table 8, FVIII:Ag): clearance is decreased by more than 20% (12.99 mL/kg/h to 10.66 mL/kg/h), MRT is increased by 12% (10.2 h to 11.4 h) and terminal half-life by 11% (8.9 h to 9.9 h).

Therewith, also for the full-length FVIII product Advate[®], the increase in exposure over time is given by D'D3-FP dimer depending on the percentage of sialylation, as may also be seen by the fold increase of PK characteristics of Advate[®] given alone. While 40.6% sialylation prolong FVIII PK 2.3-2.9fold, an optimized D'D3-FP dimer with 87.3% sialylation prolongs FVIII PK 2.8-3.2fold.

Table 8: Pharmacokinetic parameters of D'D3-FP dimer and FVIII:Ag after coadministration of Advate® and D'D3-FP dimer in rats (non-compartmental analysis)

Dose D'D3-FP dimer 1 mg/kg, dose Advate® 200 IU/kg

Treatment*	C _{max} , extrap. IU/mL	Clearance mL/kg/h	MRT h	Half-life, terminal h
Albumin				
D'D3-FP dimer (40.6%) & Advate®	18.8	2.17	54.4	42.2
D'D3-FP dimer (87.3%) & Advate®	17.2	1.32	62.0	44.0
FVIII:Ag				
Advate®	3.29	29.55	3.5	3.1
D'D3-FP dimer (40.6%) & Advate®	3.19	12.99	10.2	8.9
		2.3fold	2.9fold	2.9fold
D'D3-FP dimer (87.3%) & Advate®	3.38	10.66	11.4	9.9
		2.8fold	3.3fold	3.2fold

* degree of D'D3-FP dimer sialylation given in brackets

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Conclusion from PK study results

These studies demonstrate that co-administration of D'D3-FP dimer and FVIII prolongs FVIII:Ag plasma exposure using different FVIII products. This prolongation is dependent on the status of sialylation of D'D3-FP dimer: generally, a better sialylation further optimizes FVIII plasma exposure. In detail, D'D3-FP dimer with a sialylation of 40.9% was inferior with regard to FVIII:Ag plasma exposure to D'D3-FP with sialylation in the range of 83.6-89.8%.

Since in the rat (in contrast to human haemophilia A patients), human and endogenous FVIII compete with D'D3-FP dimer binding sites, it may be expected that the effect on FVIII in the human haemophilia patient is even stronger.

20 Example 9: In vitro sialylation of D'D3-FP

D'D3-FP dimer was dialyzed against 35 mM sodium acetate / 35 mM Tris buffer at pH 7.0. To about 600 µg of the protein in 110 µl, 0.75 mg CMP-NANA (Roche Cat.No

05974003103) dissolved in 100 µl water as donor substrate and 10.5 µl ST6GAL-1 (60 µg, Roche Cat.No 07012250103, in water) were added. The mixture was incubated at 37°C for 6 hours and the reaction was stopped by freezing at -15°C to -25°C. This procedure was according to the manufacturer's recommendation. D'D3-FP dimer was then purified from the reagents by chromatography using SEC Superdex 200pg (GE Healthcare, Code 90-1002-10). Sialylation was determined as described above and the results are given in Table 9.

Table 9: results of an in vitro sialylation study

Lot #	Sialylation
starting material	100
after in vitro sialylation	137 % of the sialylation degree as compared to the starting material

The degree of sialylation of the starting material was normalised to a nominal value of 100. The degree of sialylation after in vitro sialylation was substantially higher than that of the starting material.

Example 10: Anion-exchange Chromatography to enrich for highly sialylated VWF fragments

D'D3-FP prepared according to example 5 is further purified using anion exchange chromatography to reduce the content of asialo N-glycan structures. Therefore, the D'D3-FP solution is diluted using 20 mM Tris x HCl pH 7.4 buffer to a conductivity low enough to allow complete binding of D'D3-FP to the column (in general below 5 mS/cm) and loaded on a chromatography column (fill height approximately 20 cm) filled with Poros XQ resin that was equilibrated using equilibration buffer containing 20 mM Tris x HCl, 20 mM NaCl pH 7.4. After washing the column with equilibration buffer, D'D3-FP is eluted using a flat linear gradient from equilibration buffer to elution buffer (20 mM Tris x HCl, 500 mM NaCl pH 7.4). The elution peak containing D'D3-FP is fractionated into approximately 10 fractions of similar volumes and the early peak fractions that contain D'D3-FP with increased amounts of asialo N-glycan structures are discarded and the later peak fractions containing asialo N-glycan structures below the desired level (e.g. 20 % or lower) are pooled.

Alternatively the purification run of D'D3-FP is performed with the difference that pooling of D'D3-FP eluate peak fractions is only done for those fractions containing D'D3-FP with an asialo N-glycan structure content of below 15% (or below 10%).

- 5 As described, by pooling of corresponding fractions suitable D'D3-FP preparations can be manufactured with a desired maximum content of asialo N-glycan structures.

Based on the results obtained with a linear gradient used for elution, step gradients with buffers containing different concentrations of NaCl can be derived that also allow removal
10 of first fractions with higher amounts of asialo N-glycan structures thus resulting in D'D3-FP eluates with content of below 15% of asialo N-glycan structures.

Example 11: Determination of FVIII affinity to VWF fragment dimer and monomer

- 15 D'D3-FP monomer and dimer were isolated as described above, and the affinity of FVIII to these preparations was assessed through surface plasmon resonance via a Biacore instrument (T200, GE Healthcare).

An anti-albumin antibody (MA1-20124, Thermo Scientific) was covalently coupled via its N-terminus to an activated CM 3 chip by NHS (N-Hydroxysuccinimide) and EDC
20 (Ethanolamine hydrochloride), both contained in the amine coupling kit (BR1000-50) from GE Healthcare. For immobilization 3 µg/mL of the antibody were diluted in sodium acetate buffer (10 mM, pH 5.0) and the antibody solution was flown over the chip for 7 min. at a flow rate of 10 µL/min. After the immobilization procedure non-coupled dextran filaments were saturated by flowing ethanolamine solution (1 M, pH 8.3) over the chip for 5 min (at a
25 flow rate of 10 µL/min). The aim of saturating the flow cell was to minimize unspecific binding of the analytes to the chip. A reference flow cell was set up by saturating an empty flow cell with ethanolamine by using the same procedure as above.

Dimeric and monomeric D'D3-FP proteins, respectively, were immobilized to the covalently coupled anti-albumin antibody by a flow of the D'D3-FP proteins (5 µg/mL) over
30 the chip for 3 min (flow rate of 10 µL/min). The captured mass of dimeric D'D3-FP was 335 RU and for monomeric D'D3-FP 147 RU, assuming one binding site both on the monomer and on the dimer D'D3-FP for FVIII.

To create binding curves for FVIII, each D'D3-FP protein preparation was diluted in running buffer (HBS-P+: 0.1 M HEPES, 1.5 M NaCl and 0.5% v/v Surfactant P20, pH 7.4;

product code BR100671, GE Healthcare) to concentrations of 0.25 nM, 0.5 nM, 1 nM, 3nM and 4 nM. By performing a single cycle kinetic, samples with ascending concentrations of each dilution were flown over the chip for 2 min (flow rate 30 μ L/min.), followed by a dissociation time of 10 min. with running buffer HBS-P+. All measurements were performed twice. The temperature for the measuring procedure was adjusted to +25°C.

Binding parameters were calculated using BiaEvaluation Software. The curve fitting methods were based on Langmuir equations. The input data for calculations were the molar mass of the analyte FVIII (rVIII-SingleChain) of 170kDa, other parameters like max. RU and slopes were automatically extracted out of the fitted association and dissociation curves. The outputs of BiaEvaluation Software are the association rate constants and the dissociation rate constants, from which the affinity constants were calculated. The results are shown in Table 10.

Table 10: FVIII affinity data for D'D3-FP dimer and monomer

D'D3-FP preparation	ka [1/Ms]	kd [1/s]	KD [M]
D'D3-FP Dimer	2,33E+07	1,37E-03	5,90E-11
D'D3-FP Monomer	4,41E+07	3,96E-03	8,99E-11

The association rate constant was slightly increased for rVIII-SingleChain to the monomeric D'D3-FP, while the dissociation rate constant of rVIII-SingleChain to D'D3-FP dimer was three times slower than to the monomer. The quotient of the dissociation rate constant and the association rate constant indicates the affinity of rVIII-SingleChain to D'D3-FP. The dimeric D'D3-FP hence shows an increased affinity to FVIII compared to the D'D3-FP monomer.

Example 12: Quantitative determination of individual N-glycan species

Lot #	Percentage of all N-glycans with two or more terminal and non-sialylated galactose residues [% of total N-glycans]	Percentage of all N-glycans with three or more terminal and non-sialylated galactose residues [% of total N-glycans]
B-140526 (no temperature shift)	38.7	6.9
B-140616KS	21.2	2.2
B-140825	17.8	1.9
B-140623KS	9.5	1.1

The N-Glycans released by PNGase F were labelled with a fluorophore 2-aminobenzamide (AB) and purified prior analysis using in-line LC-fluorescence – high resolution MS detection allowing simultaneous quantitative determination and identification of the labelled N-Glycans using accurate mass and retention time information. Using a mixed mode HILIC/RP LC-column allowed the separation of the released and AB labelled N-Glycans based on charge and structure which enabled a quantitative determination of different structures according to the number of terminal galactose and non-sialylated residues. The standard deviation of the fluorescence quantitation using the area under curve was found to be on average less than 0.5% using a reference sample (n=5). The presence of terminal and non-sialylated galactose residues in the separated AB labelled N-glycans was confirmed by treating the released AB-labelled N-glycans with β 1-4-Galactosidase and re-injecting them using the same LC-FLD-MS methods and analysing the shifted peaks.

The following methods were applied:

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PNGase F enzymatic glycan release:

About 700 μ g of the purified protein was reduced with DTT in ammonium bicarbonate, pH 8.5 at 60°C for 30 min. The reduced sample was cooled to room temperature and alkylated with iodoacetamide at RT in the dark for 30 min. The alkylated sample was buffer exchanged into 50mM ammonium bicarbonate pH 8.6 using a 2mL Zeba Spin 7K MWCO column. To the buffer exchanged sample, 40U of PNGase was added and the sample incubated at 37°C for 14 hours. An additional 40U of PNGase was added and the

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sample incubated for a further 6 hours at 37°C . The PNGase digested sample was centrifuged through a 50KDa Amicon Ultra 4 ultrafilter. The filtrate was dried in a CentriVap.

5 2-AB labelling of released N-glycans:

The 2-AB labelling reagent was prepared following the manufacture instructions. 50 µL of the 2-AB reagent was added to the dried sample and incubated in the dark at 65°C for 3.5 hours.

10 A Waters Oasis HLB 3cc 60 mg SPE cartridge was conditioned with 3 mL 95% acetonitrile the 3mL 35% acetonitrile then 3 mL 95% acetonitrile. The 2-AB labelled sample was diluted by adding 1.95 mL of 95% v/v acetonitrile and immediately loaded onto the HLB cartridge and allowed to drain under gravity. Sample was washed under gravity with 3x 3mL of 95% v/v acetonitrile and the eluted with 3mL of 35% v/v acetonitrile. The dried 2-AB derivatised sample was dissolved by the addition of 35 µL of Milli Q water and vortex
15 mixing. After dissolution, 85 µL of acetonitrile was added and mixed briefly. The sample was transferred to a HPLC vial for analysis.

2-AB Glycan analysis:

High performance liquid chromatography was performed on a Thermo Dionex Ultimate
20 3000 system consisting of an RS Binary Pump, Autosampler , RS Column Compartment and RS Fluorescence detector. The separation of 2-AB glycan derivatives was achieved using a Dionex GlycanPac AXH-1, 1.9 µm, 2.1 x 150 mm column (P/N 082472). Mobile phase A consisted of 100% acetonitrile, Mobile phase B consisted of 50mM formic acid adjusted to pH 4.0 with 5M ammonium hydroxide solution. The column was maintained at
25 50° C and the flow rate was 0.200 mL/min. Fluorescence detection was performed with an excitation wavelength of 320 nm and an emission wavelength of 420 nm.

The LC-FLD system was coupled to a high resolution orthogonal TOF-MS (MaXis, Bruker-Daltonik, Bremen, Germany). The transfer capillary was kept at a voltage of -4500 V
30 (positive ion polarity mode). The nebulizer was set to 0.8 bar using the standard ESI sprayer (Bruker, Bremen, Germany), the dry gas temperature to 180 °C and the dry gas flow-rate to 7 L/min. The ion transfer was optimized in the range m/z 200–3000 for highest sensitivity while keeping the resolution $R > 50,000$ across the whole mass range. The TOF-MS mass calibration was carried out prior the LC–MS experiment by direct infusion
35 of a 100 fold dilution of ES Tuning Mix (Agilent Technologies, Waldbronn, Germany) at 4 µL/min.

Claims

1. A method of producing a glycoprotein comprising a truncated von Willebrand Factor (VWF) comprising N-glycans with increased sialylation, which method
5 comprises (i) providing cells comprising a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF), and (ii) culturing said cells at a temperature of less than 36.0°C.
2. A method of producing a dimer of a glycoprotein comprising a truncated von
10 Willebrand Factor (VWF), or for increasing the dimerization of said glycoprotein, which method comprises (i) providing cells comprising a nucleic acid encoding the amino acid sequence of the glycoprotein, and (ii) culturing said cells at a temperature of less than 36.0°C.
- 15 3. The method of any one of the preceding claims, wherein the cells further comprise a recombinant nucleic acid encoding a sialyltransferase, preferably an α -2,6-sialyltransferase or an α -2,3-sialyltransferase.
4. The method of any one of the preceding claims, wherein prior to step (ii) the
20 cells are cultured at a temperature of 37.0°C $\pm$ 1.0°C, and step (ii) comprises culturing the cells at a temperature of 34.0°C $\pm$ 2.0°C.
5. A method of producing a glycoprotein comprising N-glycans with increased sialylation, which comprises (i) providing cells comprising a nucleic acid encoding a
25 polypeptide comprising a truncated von Willebrand Factor (VWF) and a recombinant nucleic acid encoding an α -2,6-sialyltransferase, and (ii) culturing the cells under conditions that allow expression of the glycoprotein and of the α -2,6-sialyltransferase.
6. The method of any one of the preceding claims, further comprising (i)
30 subjecting the glycoprotein obtained in any one of the preceding claims to ion exchange chromatography, whereby glycoprotein with high sialylation is separated from glycoprotein with low sialylation; and collecting the fractions eluted from the ion exchange column having high sialylation; or (ii) contacting the glycoprotein obtained in any one of the preceding claims with a sialyltransferase and a sialic acid donor *in vitro*.

7. The method of any one of the preceding claims, wherein, on average, at least 75% of the N-glycans of the obtained glycoprotein comprise at least one sialic acid moiety.

8. The method of any one of the preceding claims, wherein, on average, at least 50% of the obtained glycoprotein is present as dimer.

5 9. A glycoprotein obtainable by a method of any one of the preceding claims.

10. A glycoprotein comprising a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said glycoprotein comprises N-glycans

- 10 i) wherein at least 75%, preferably at least 80%, more preferably at least 85% of said N-glycans comprise, on average, at least one sialic acid moiety and/or
- ii) wherein less than 35% of said N-glycans comprise, on average, two or more terminal and non-sialylated galactose residues and/or
- 15 iii) wherein less than 6% of said N-glycans comprise, on average, three or more terminal and non-sialylated galactose residues.

11. The glycoprotein of claim 10, wherein at least 70% of said N-glycans comprise, on average, at least one α -2,6-sialic acid moiety or at least one α -2,3-sialic acid moiety.

12. The glycoprotein of any one of claims 9 to 11, wherein the truncated VWF comprises (a) amino acids 776 to 805 of SEQ ID NO:9 or (b) an amino acid sequence having a sequence identity of at least 90% to amino acids 776 to 805 of SEQ ID NO:9.

13. The glycoprotein of any one of claims 9 to 12, wherein the truncated VWF consists of (a) amino acids 764 to 1242 of SEQ ID NO:9, (b) an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:9, or (c) a
25 fragment of (a) or (b).

14. The glycoprotein of any one of claims 9 to 13, further comprising a half-life extending heterologous polypeptide fused to the truncated VWF, and/or a half-life-extending moiety conjugated to the glycoprotein.

15. The glycoprotein of any one of claims 9 to 14, wherein the glycoprotein is a dimer.

16. The dimeric glycoprotein of claim 15, wherein the affinity of said dimeric glycoprotein to the FVIII is greater than the affinity of a monomeric glycoprotein to said FVIII, wherein said monomeric glycoprotein has the same amino acid sequence as the dimeric glycoprotein.

5 17. A pharmaceutical composition comprising a glycoprotein of any one of claims 9 to 16 and a pharmaceutically acceptable excipient.

18. The pharmaceutical composition of claim 17, wherein at least 50% of the glycoproteins in the composition are present as dimers.

10 19. A glycoprotein as defined in any one of claims 9 to 16 for use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject an effective amount of said glycoprotein and an effective amount of a FVIII, wherein the glycoprotein is administered intravenously or subcutaneously, and the FVIII is administered intravenously or subcutaneously.

15 20. The glycoprotein for use according to claim 19, wherein the mean residence time (MRT) of the FVIII is increased and/or the clearance of the FVIII is decreased by the co-administration of the glycoprotein, as compared to a treatment with the FVIII alone; and/or wherein the frequency of administration of the FVIII is reduced as compared to a treatment with the FVIII alone.

20 21. A pharmaceutical kit comprising (i) a FVIII and (ii) a glycoprotein as defined in any one of claims 9 to 16 for simultaneous, separate or sequential use in the treatment of a blood coagulation disorder.

Figure 1

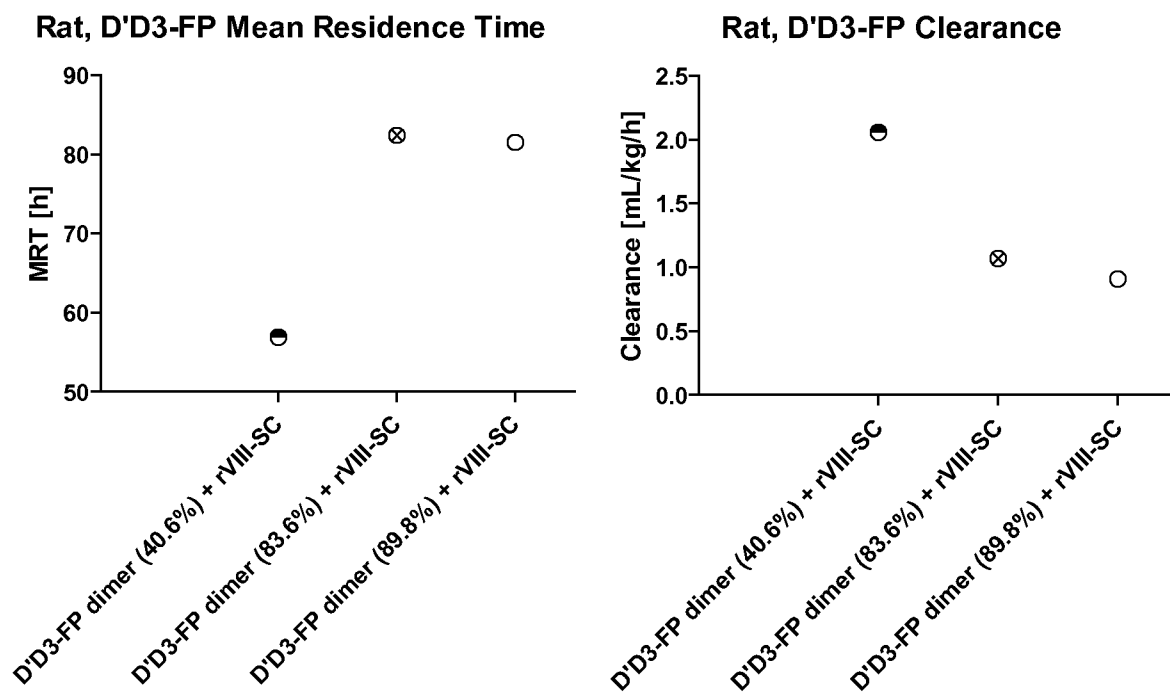


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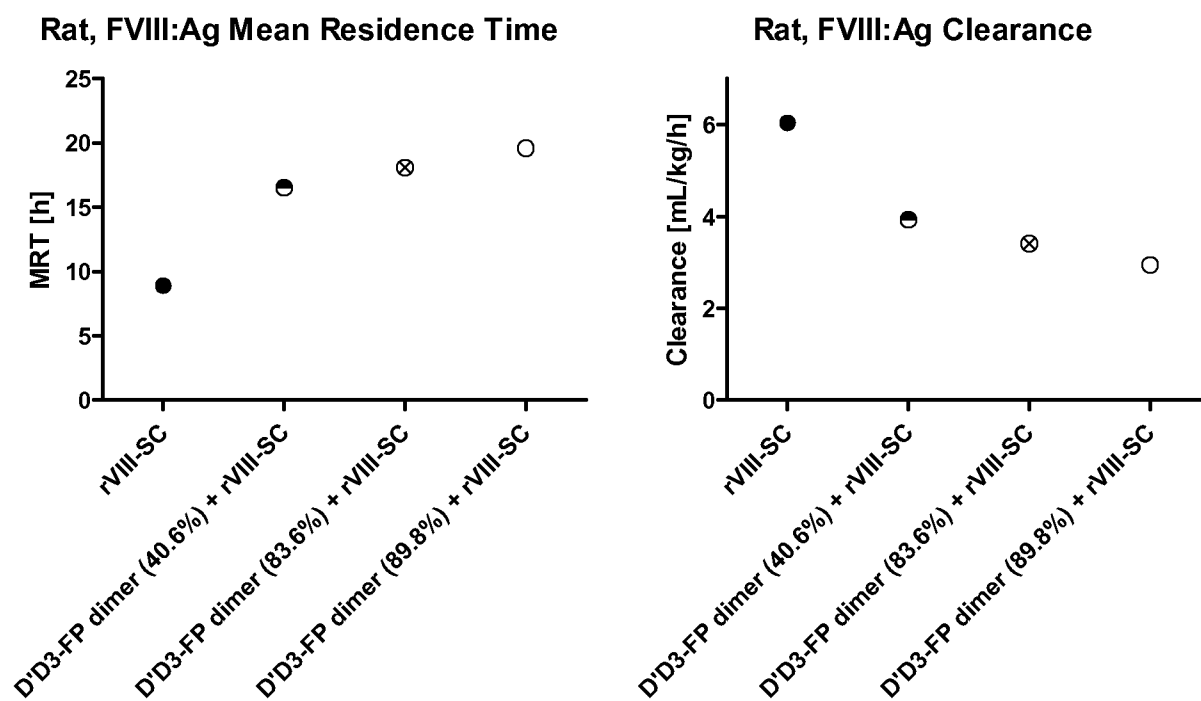
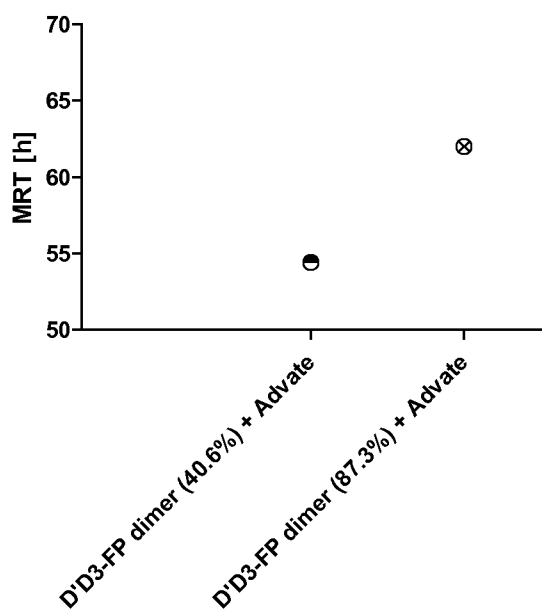


Figure 3

Rat, D'D3-FP Dimer Mean Residence Time



Rat, D'D3-FP Dimer Clearance

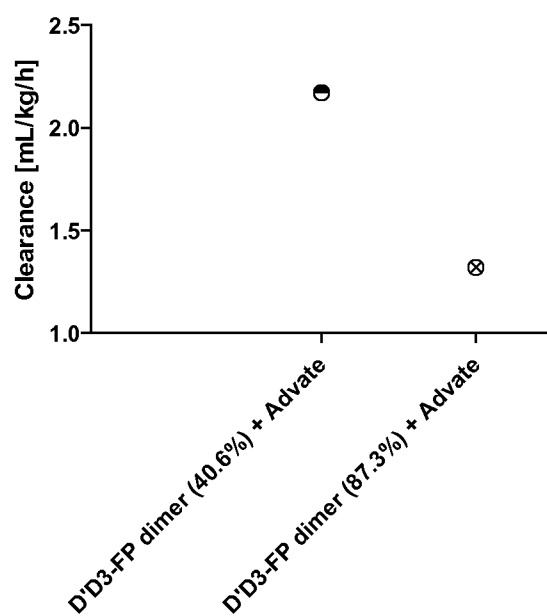
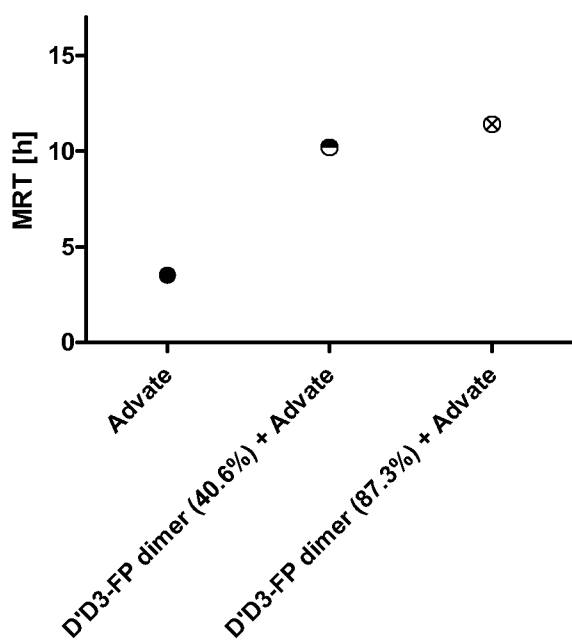


Figure 4

Rat, FVIII:Ag Mean Residence Time



Rat, FVIII:Ag Clearance

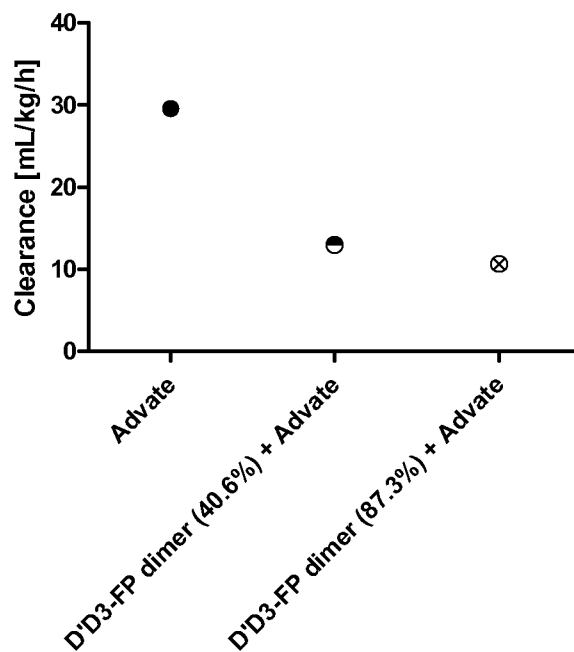


Figure 5

Legend to the glycostructures shown in figures 6 to 25

	Gal	galactose
	Man	mannose
	GlcNAc	N-acetyl glucoseamine
	Neu5Ac	N-acetyl neuraminic acid
	Neu5Gc	N-glycolyl neuraminic acid
	Fuc	fucose
	SO₄	sulfated
	Glycostructures with two or three non-sialylated terminal galactose residues	

Figure 6

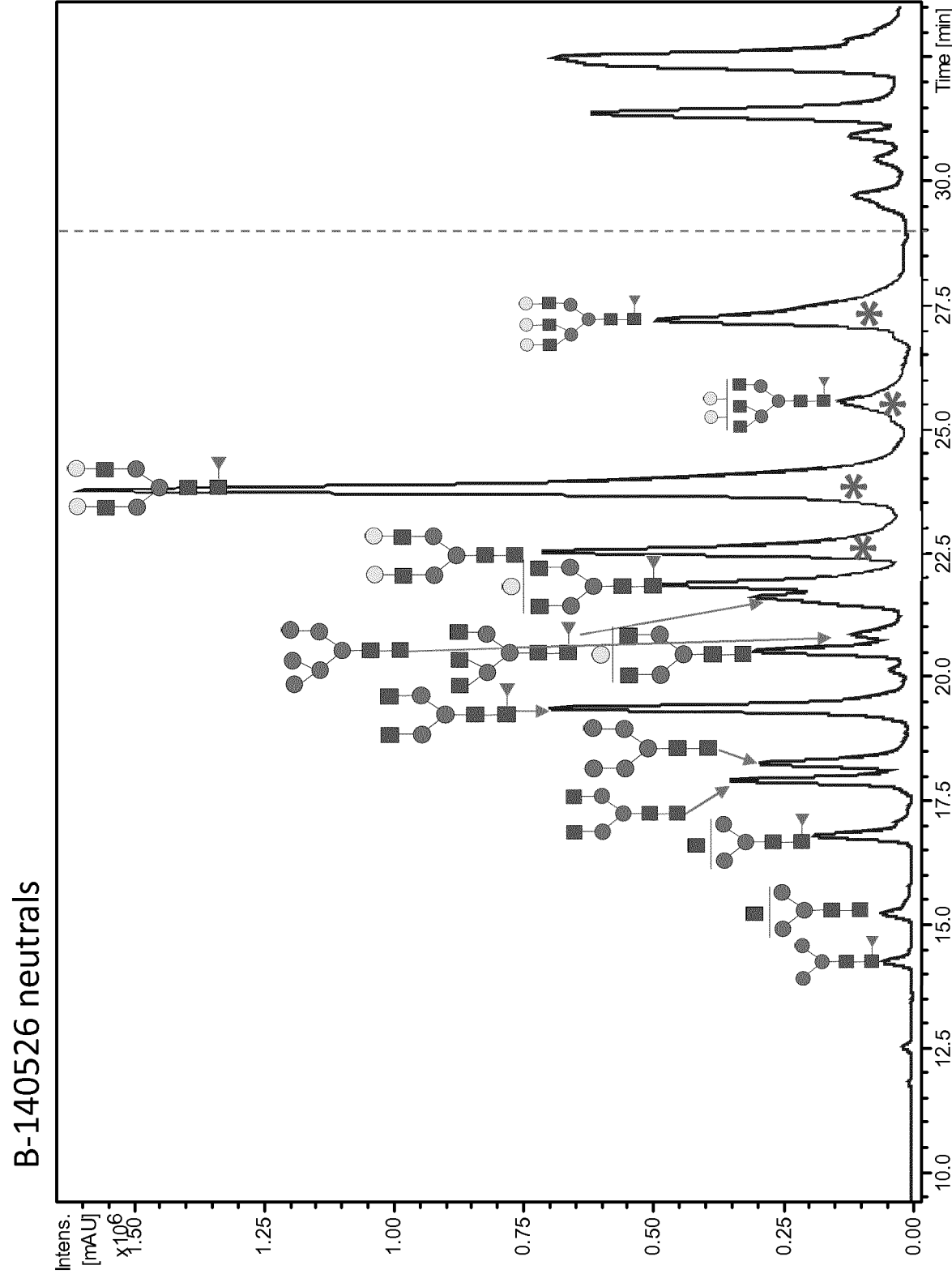


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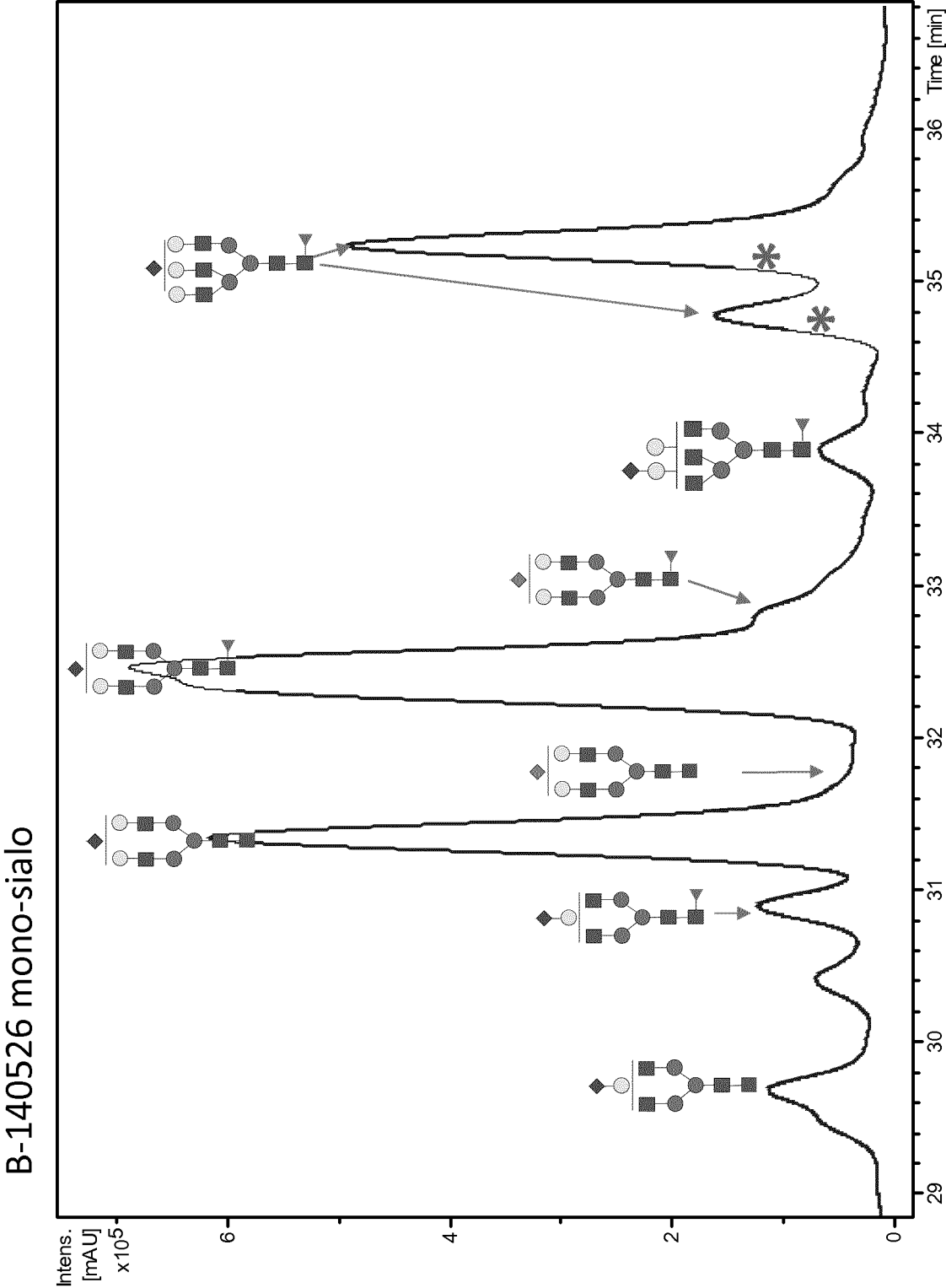


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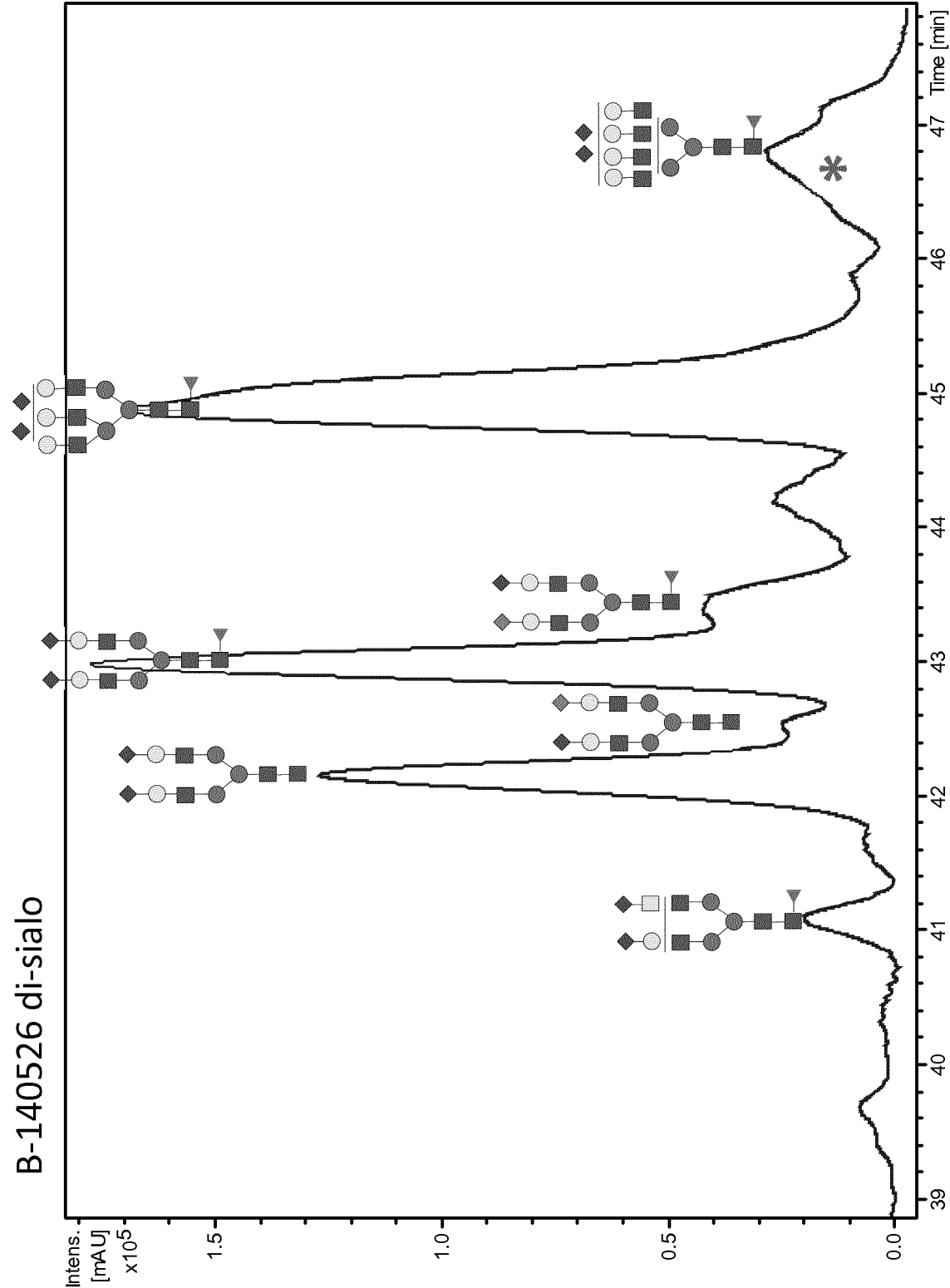


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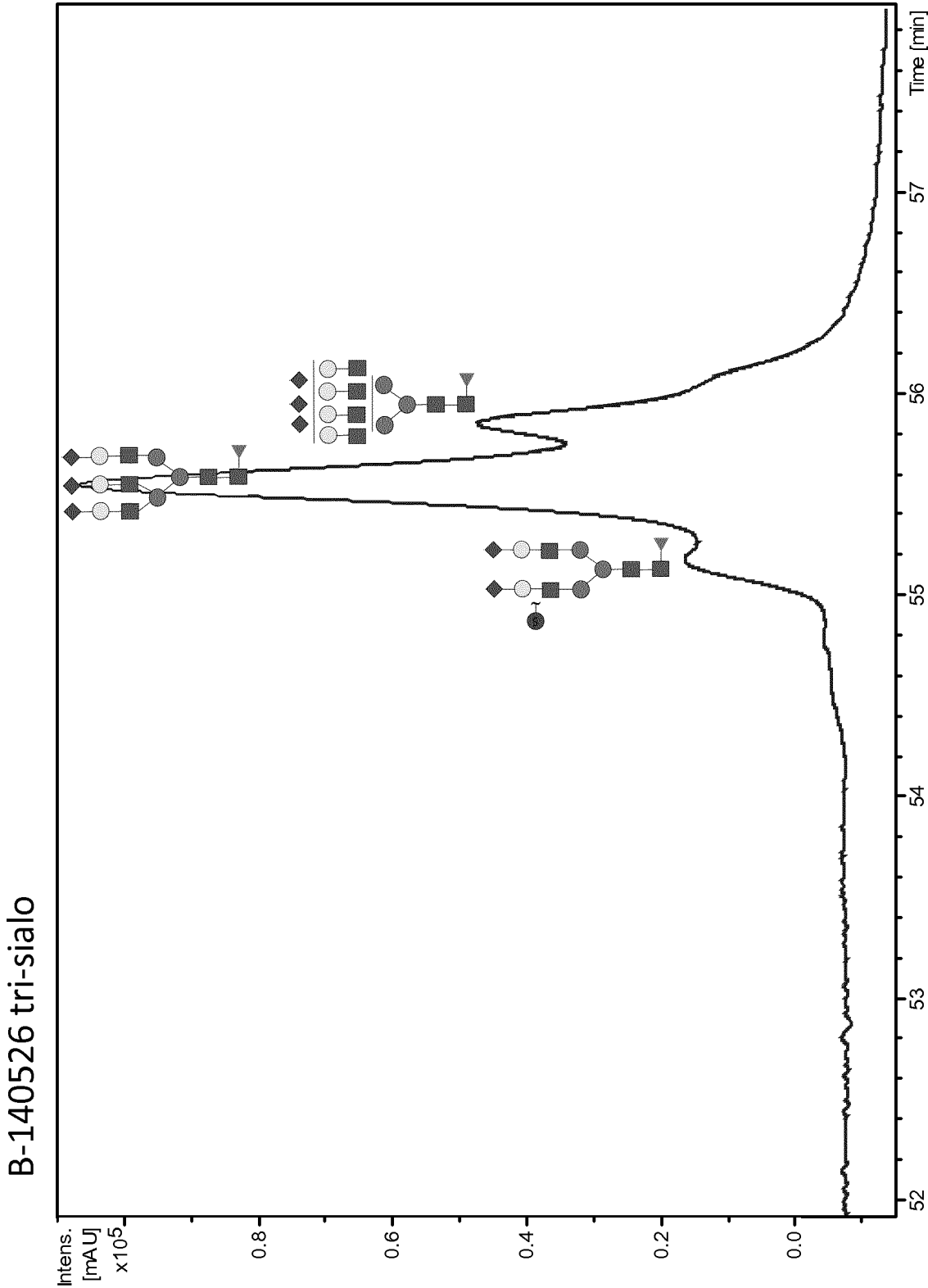


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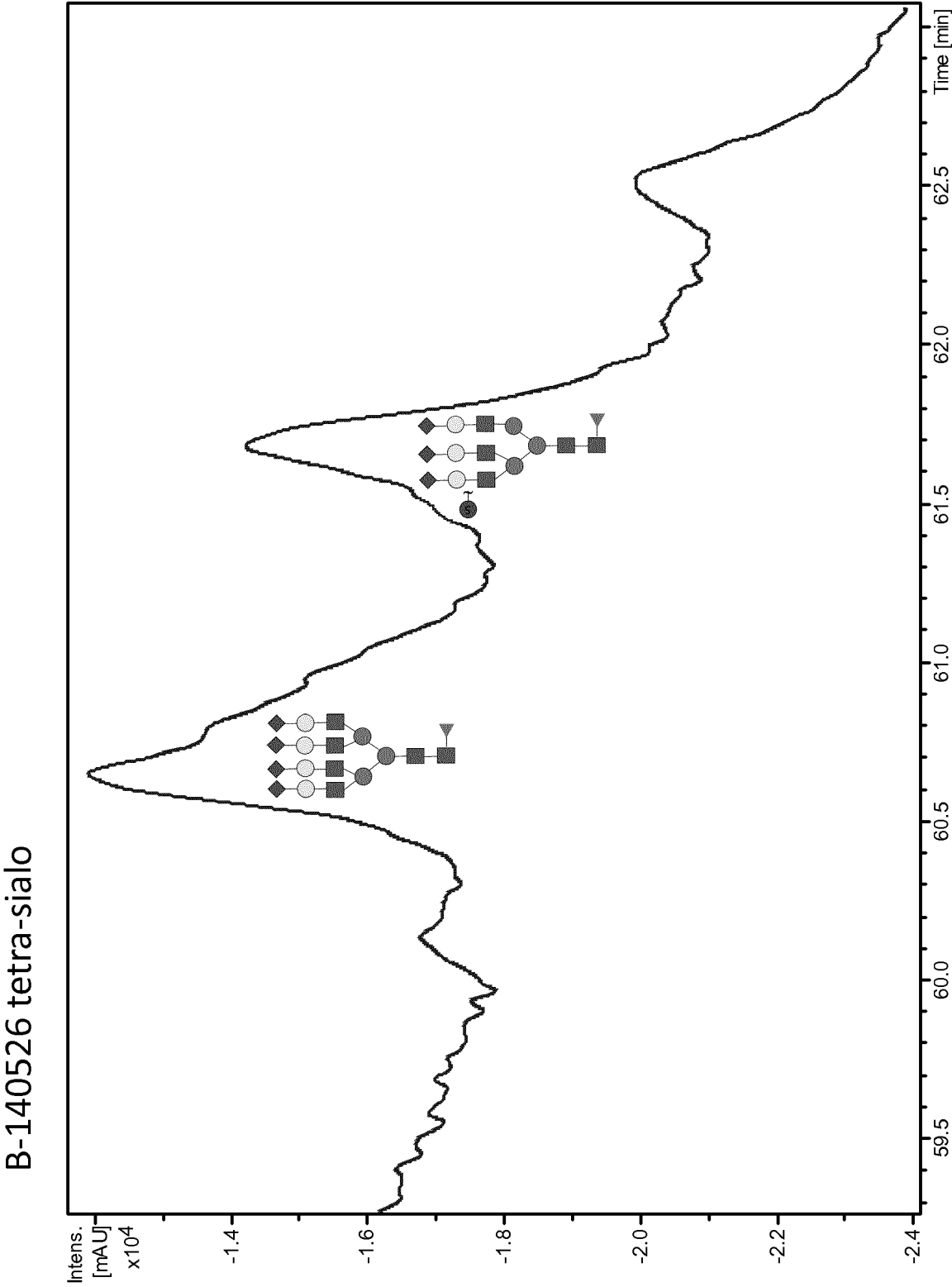


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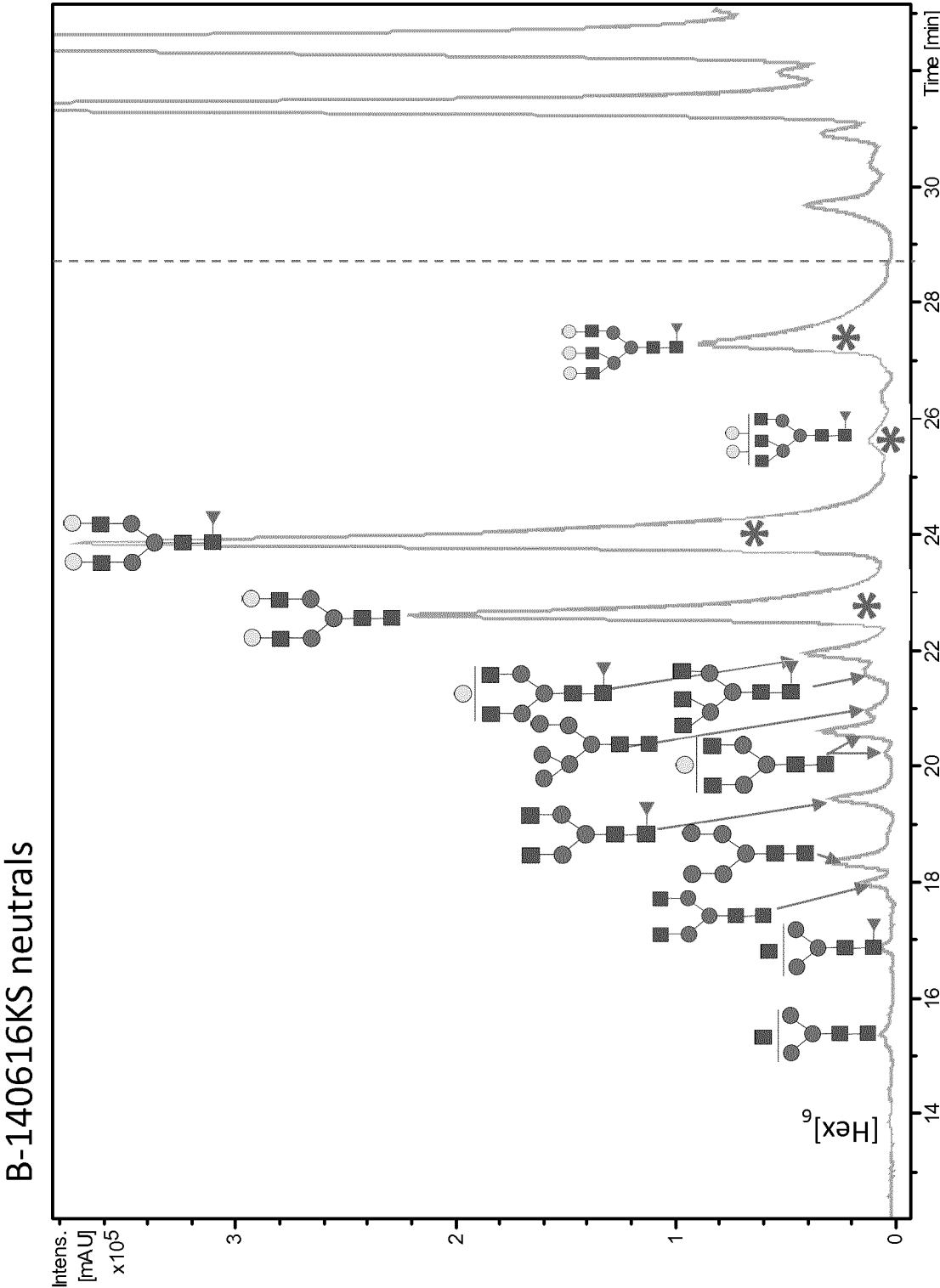


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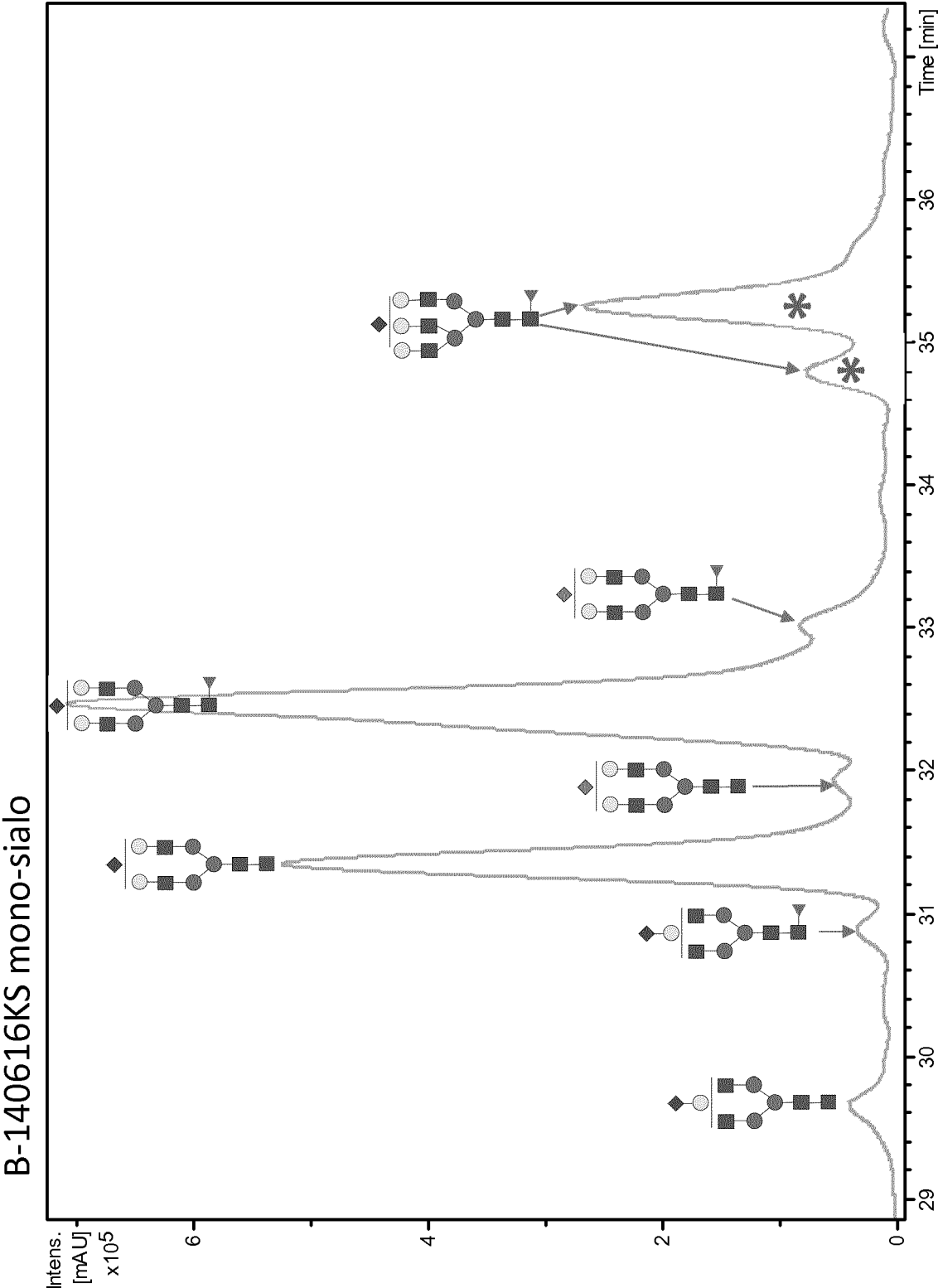


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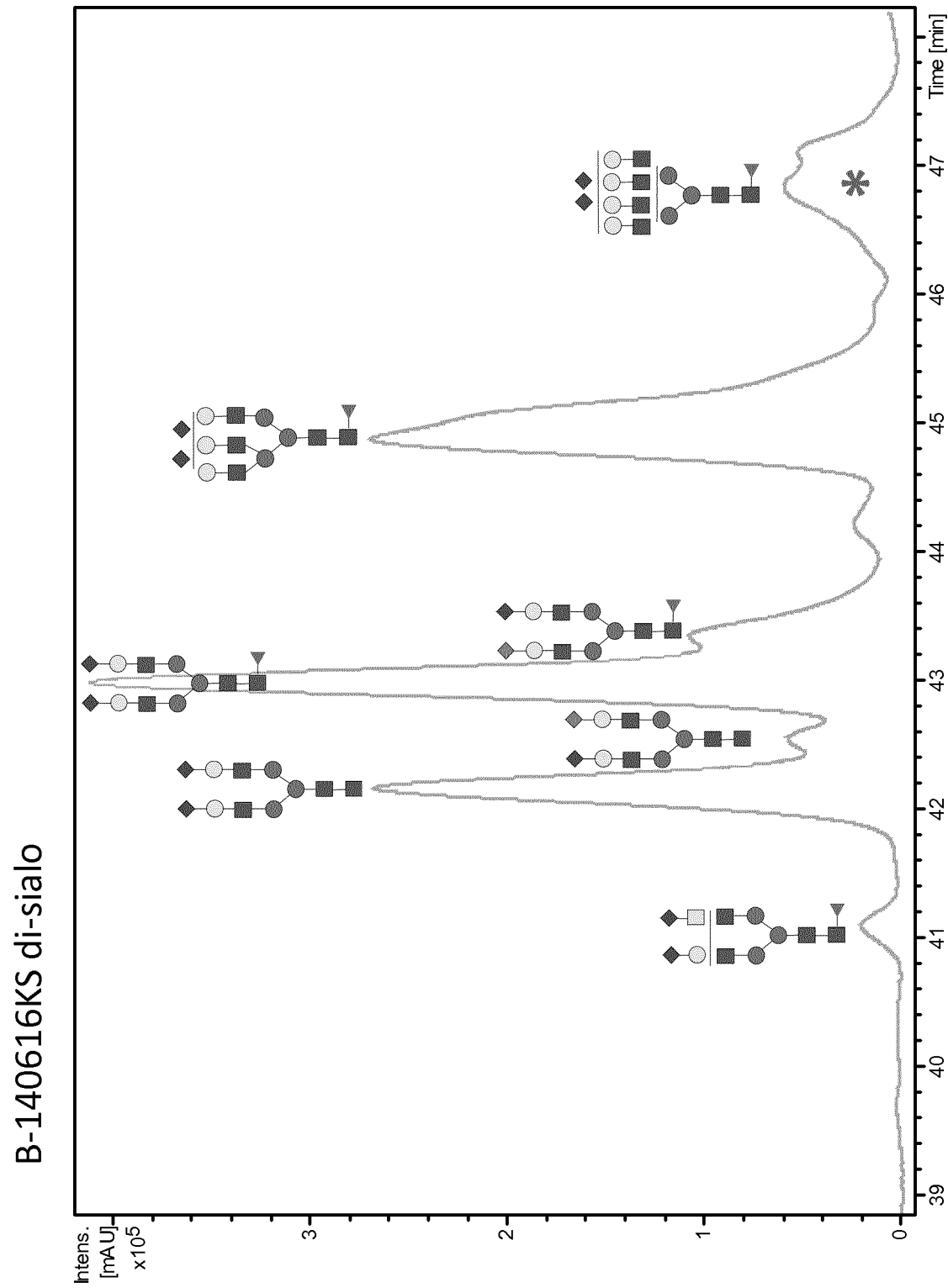


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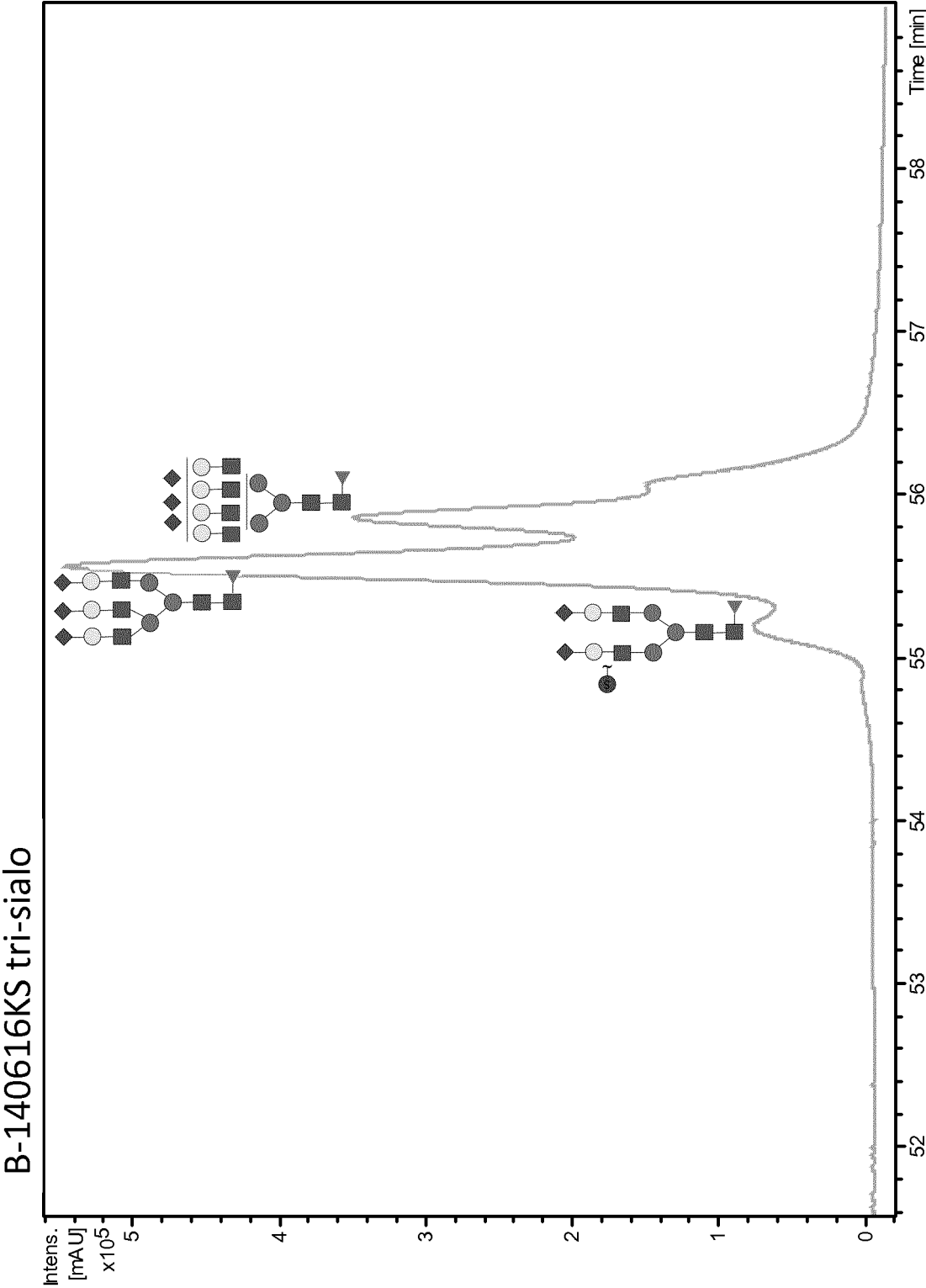


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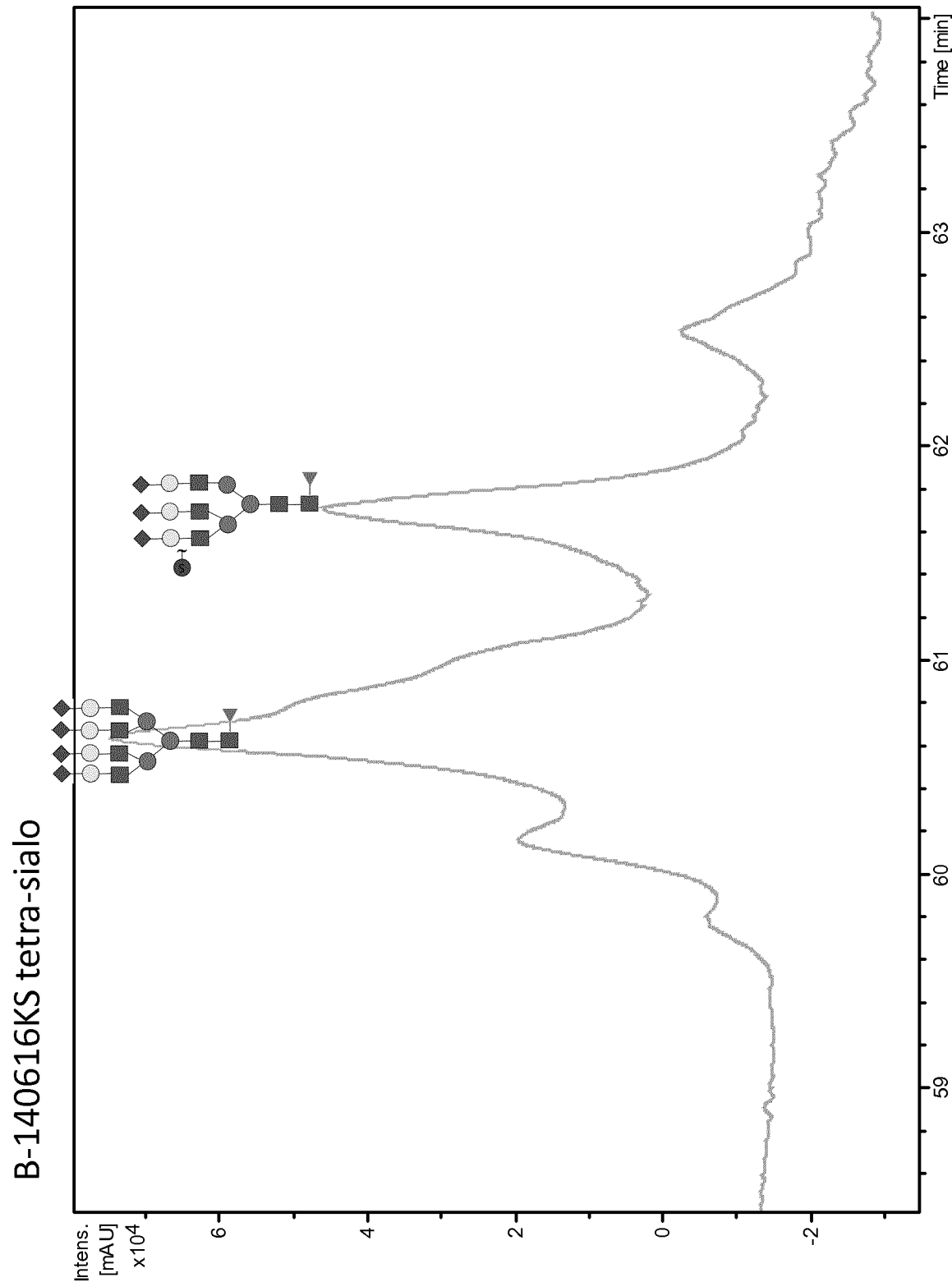


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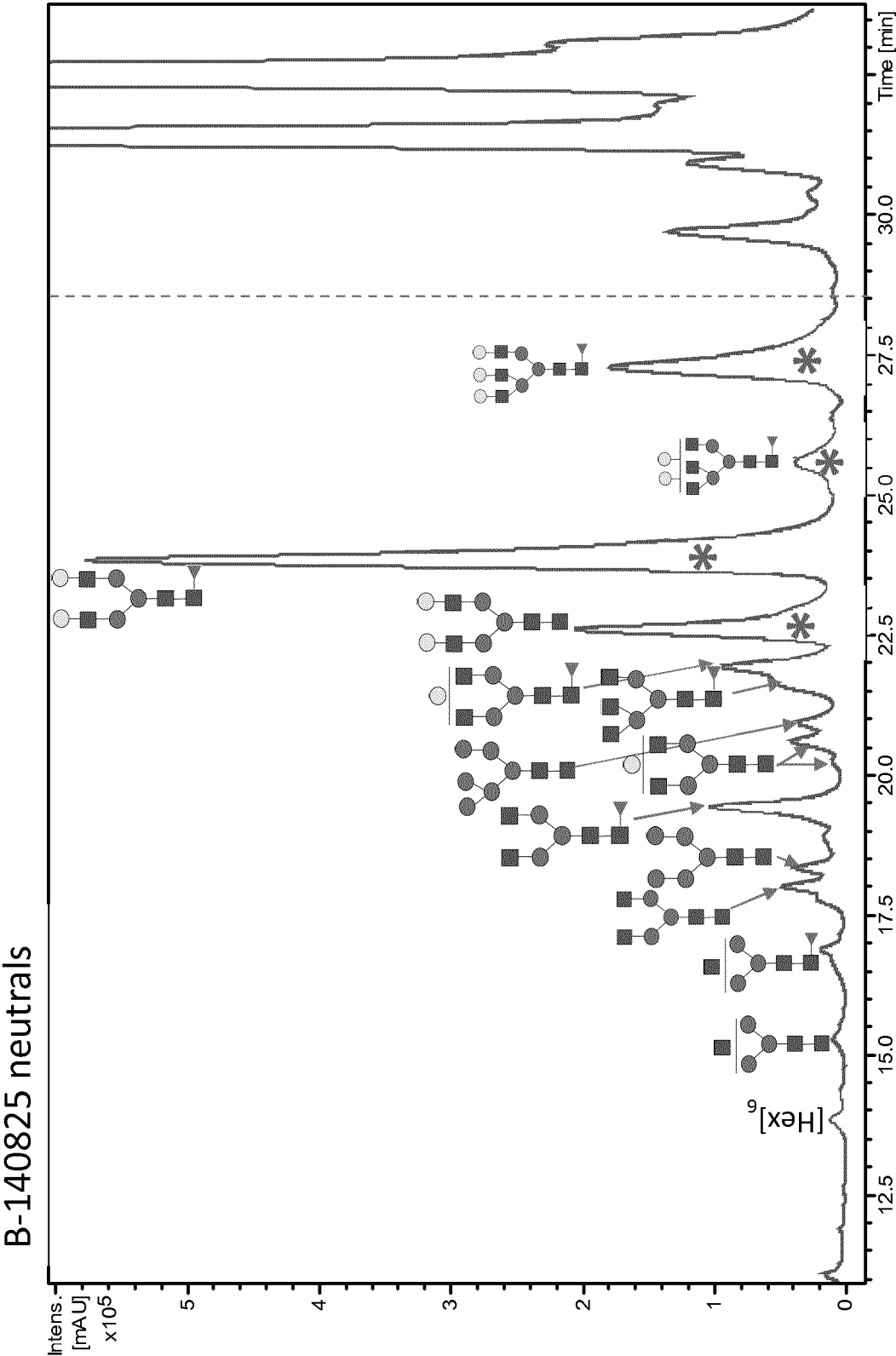


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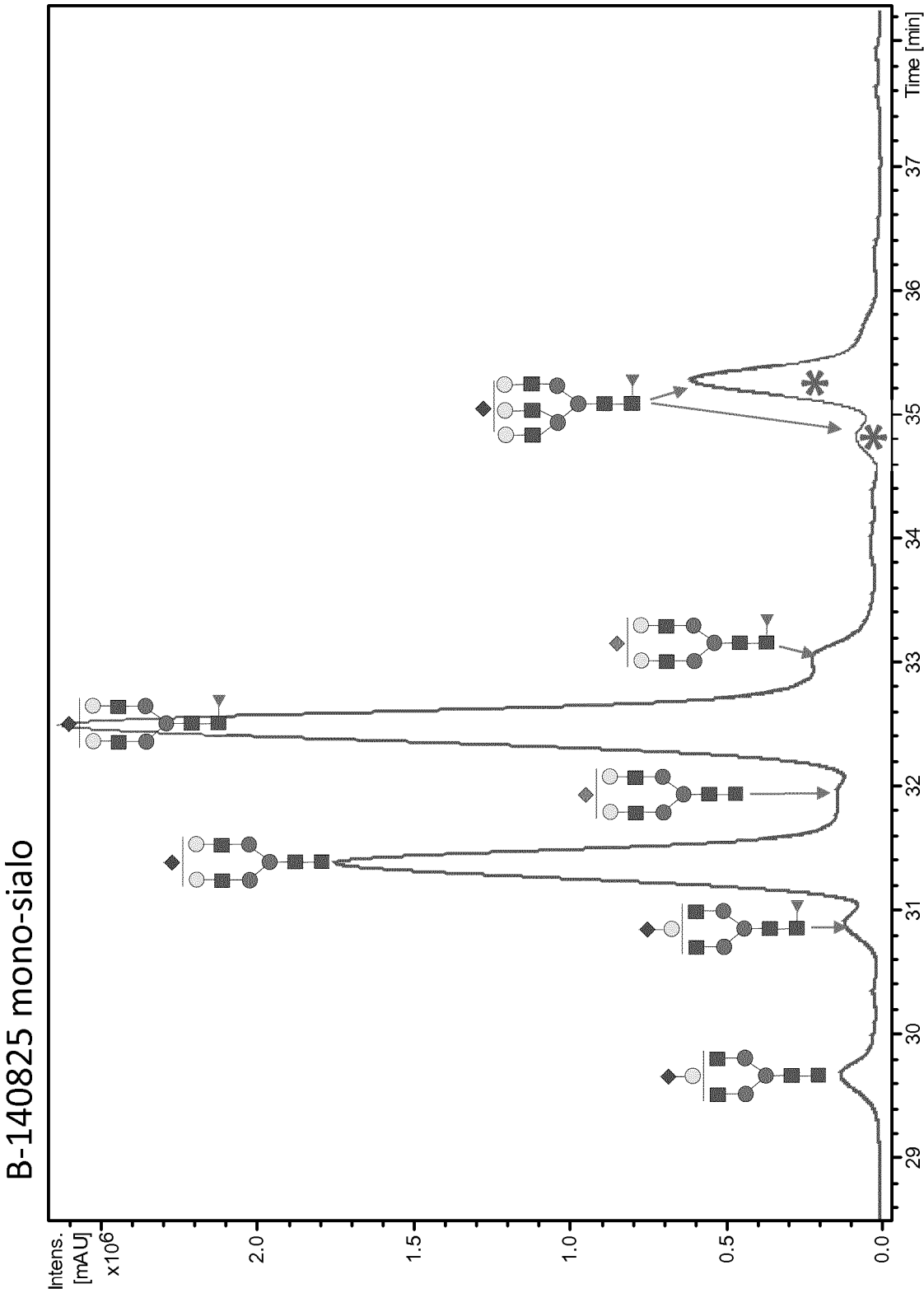


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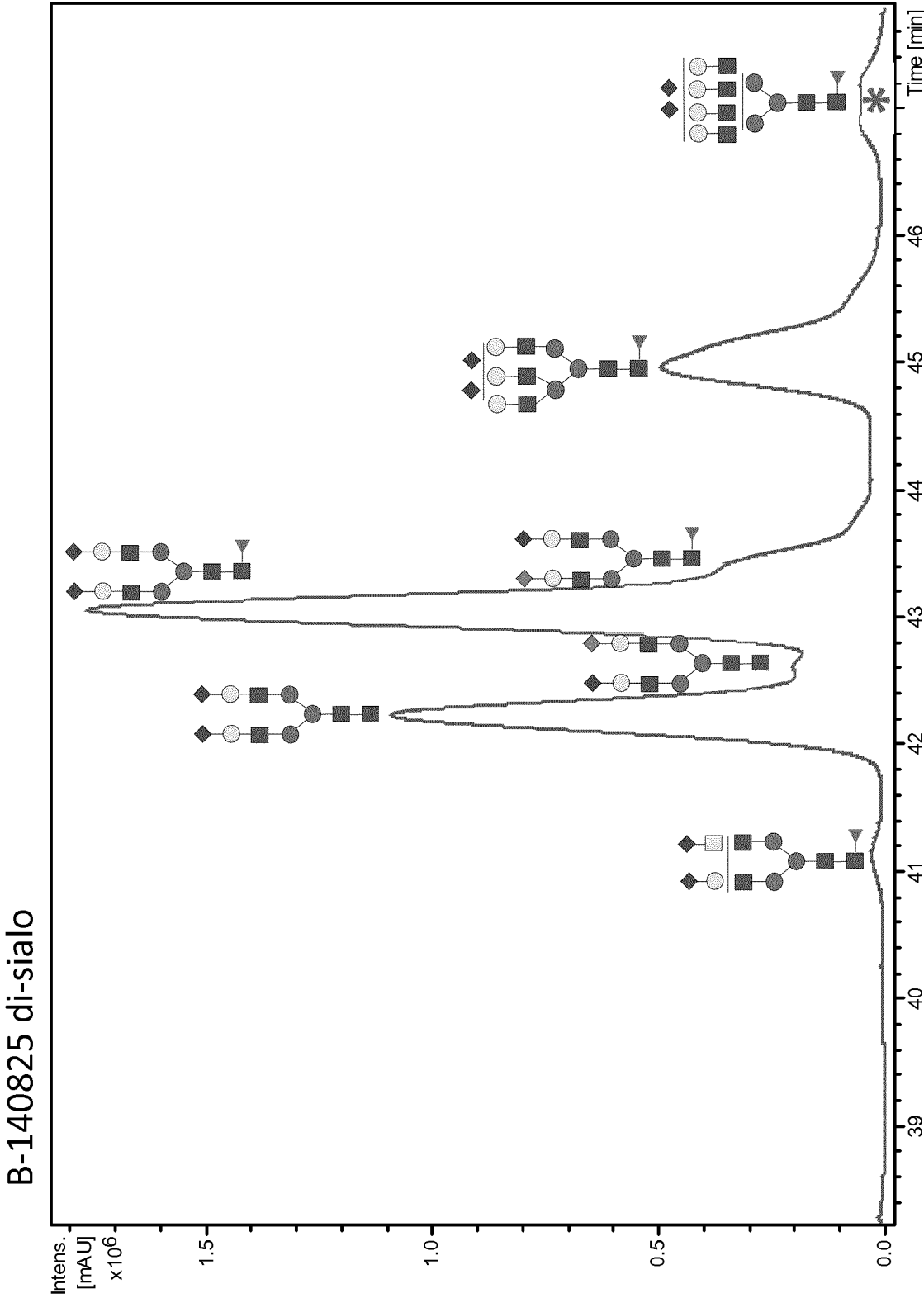


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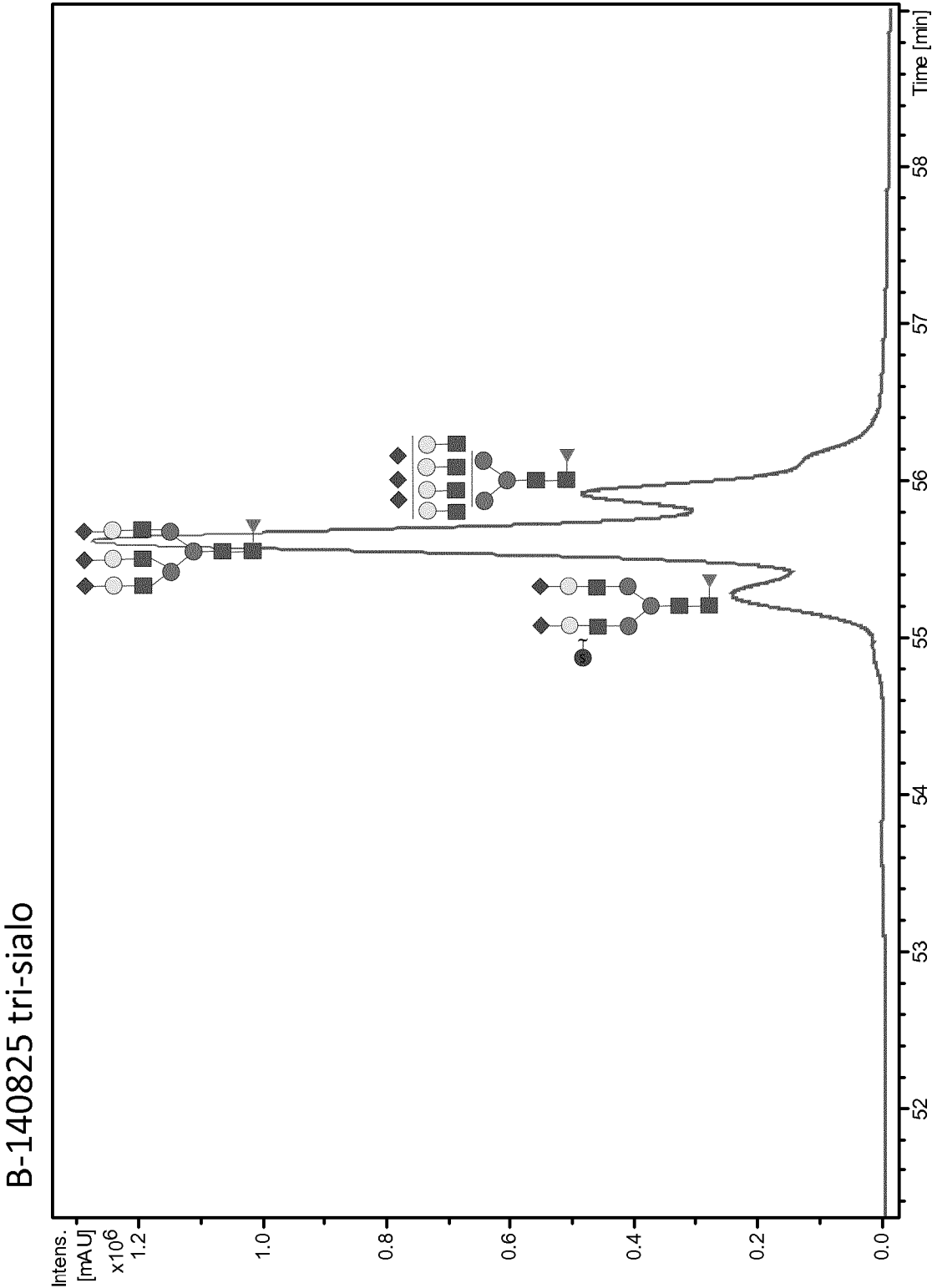


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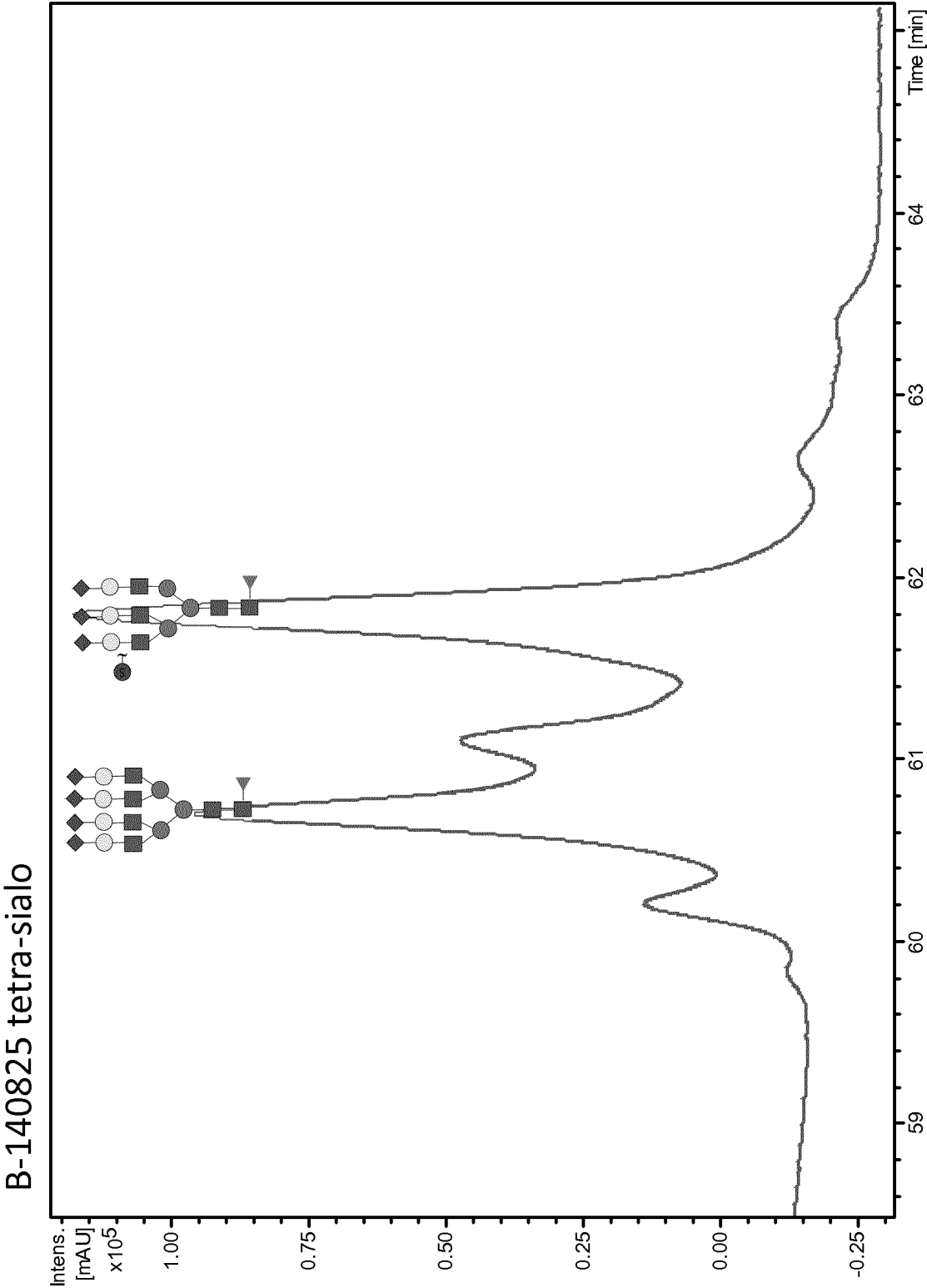


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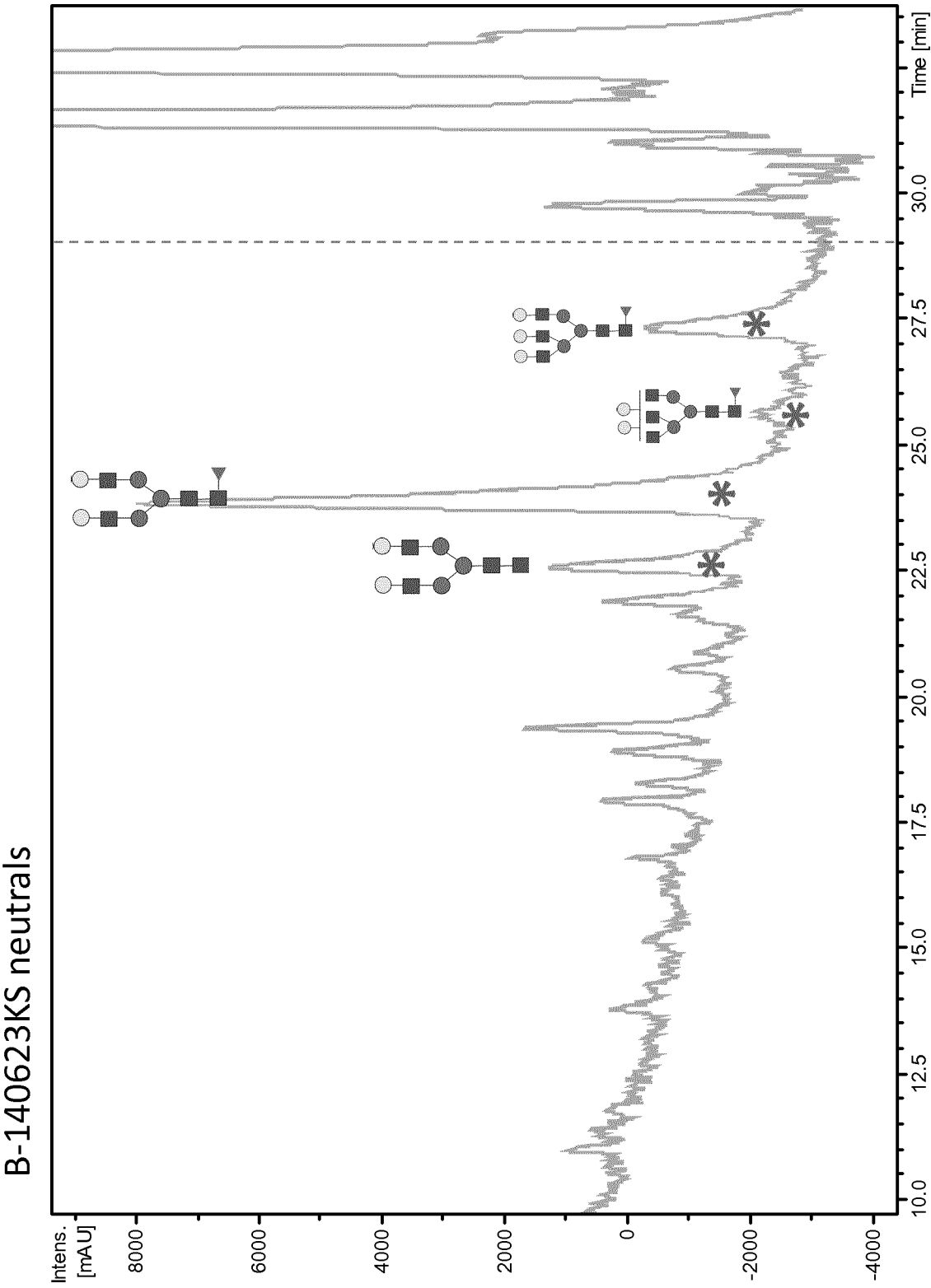


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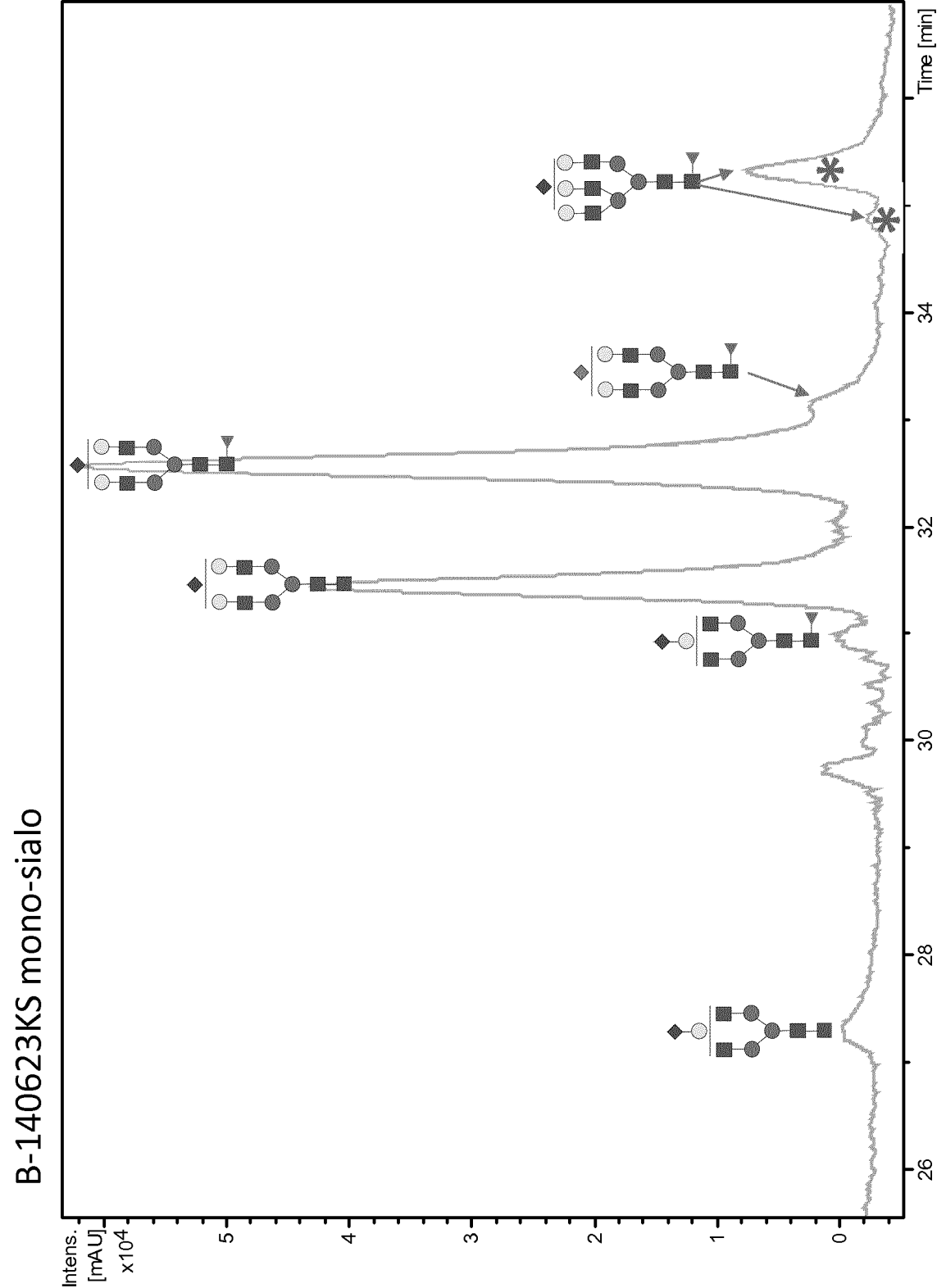


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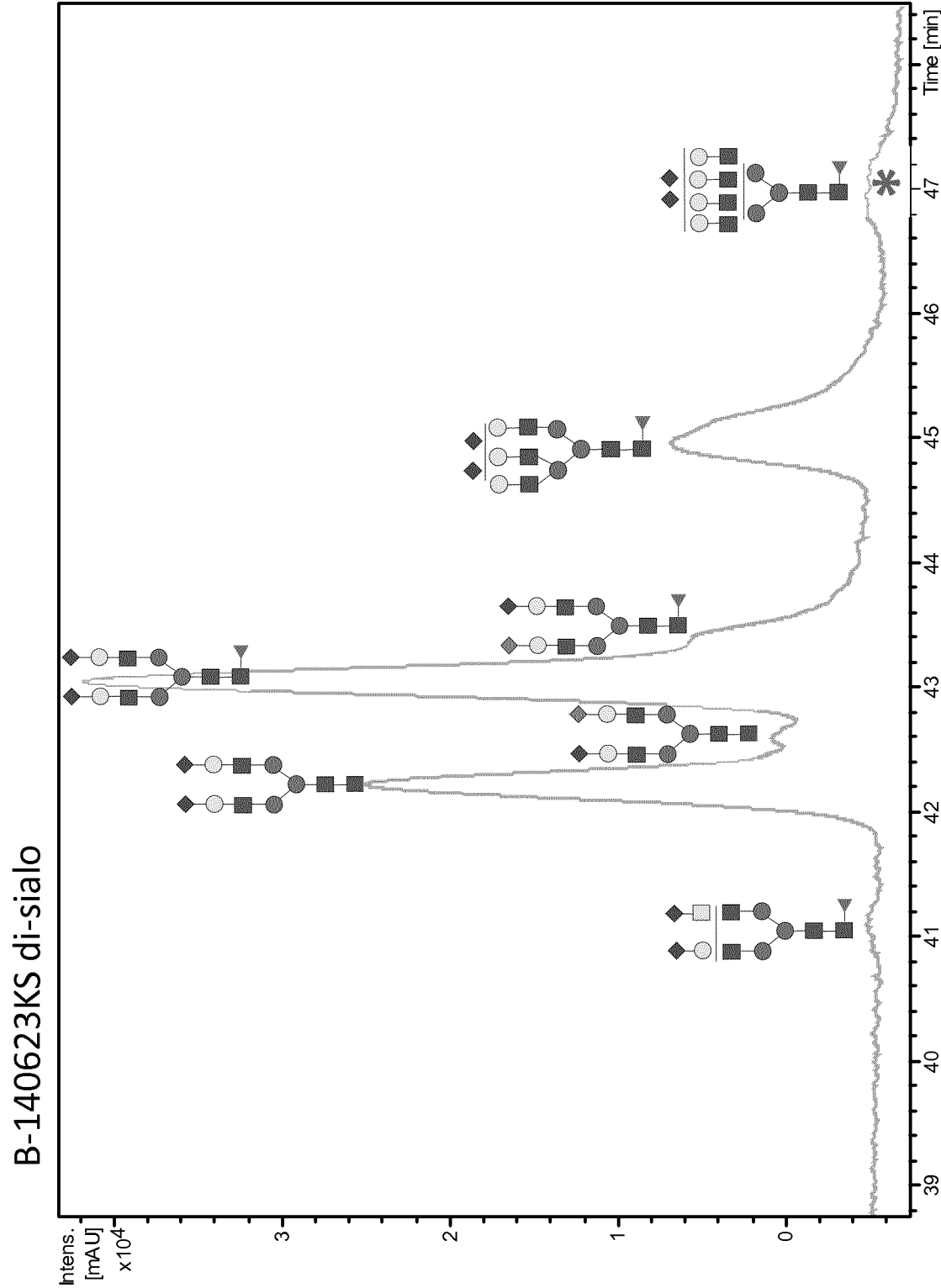


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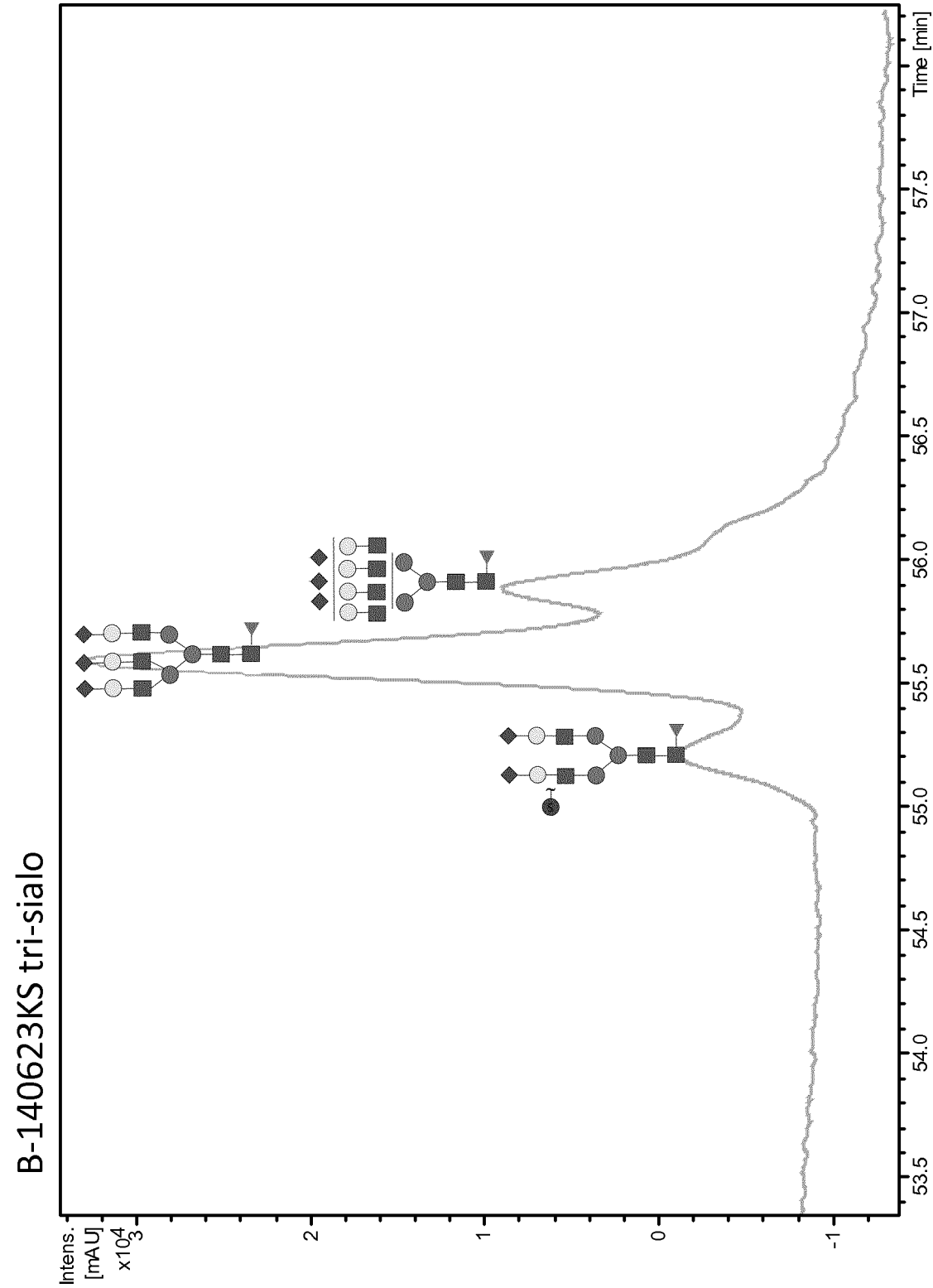


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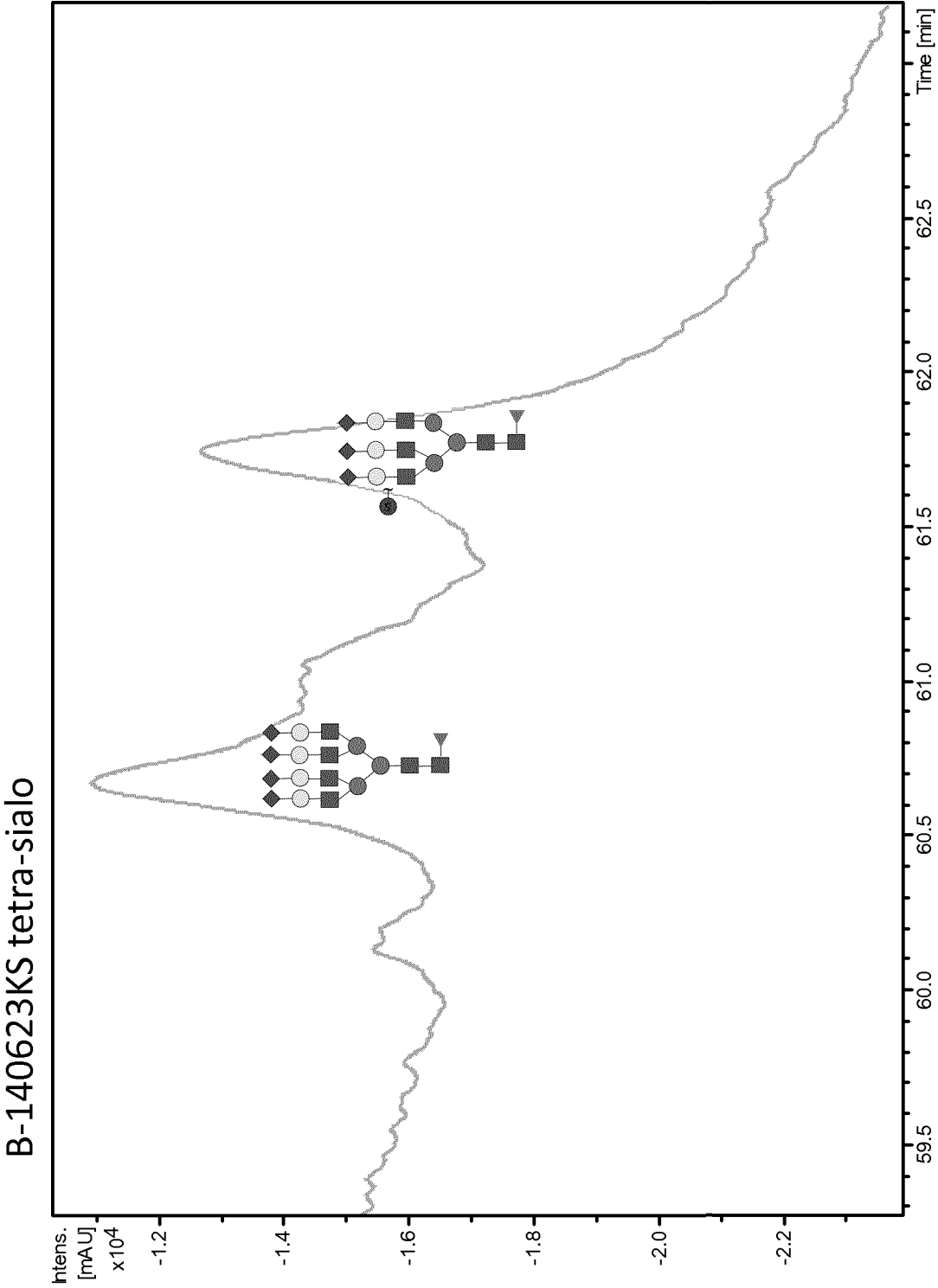


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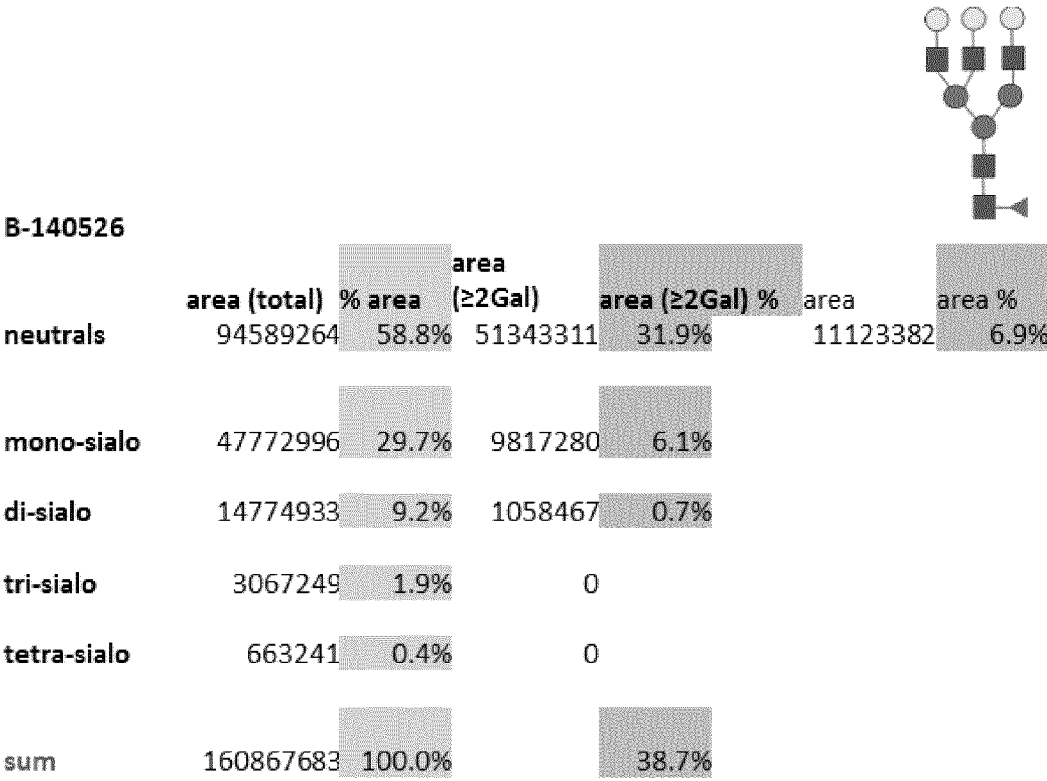


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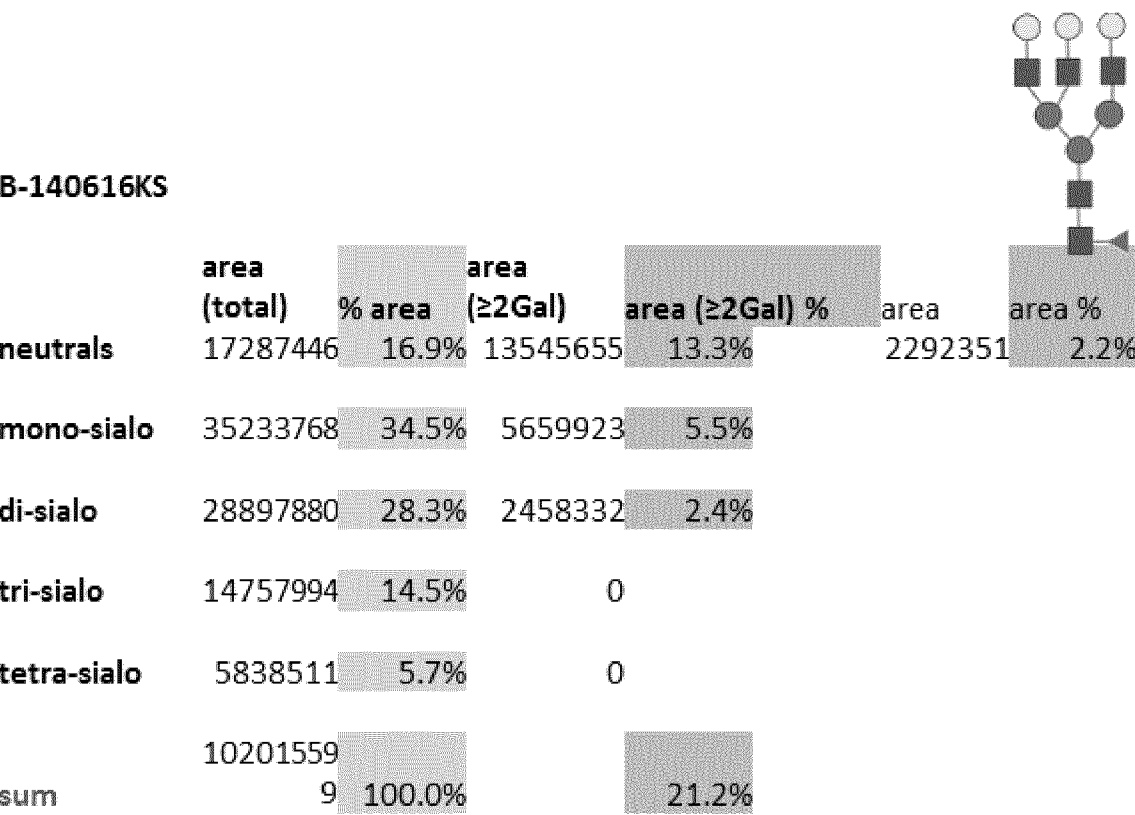


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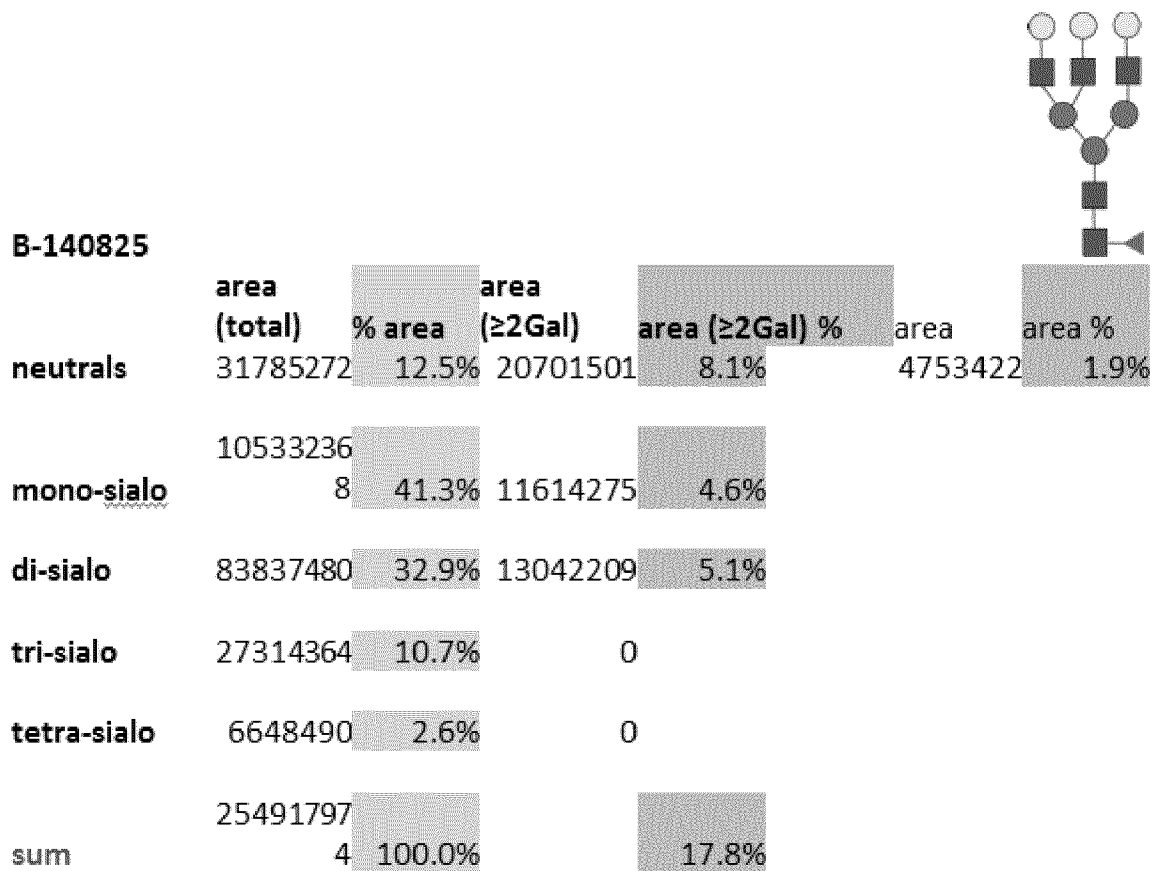
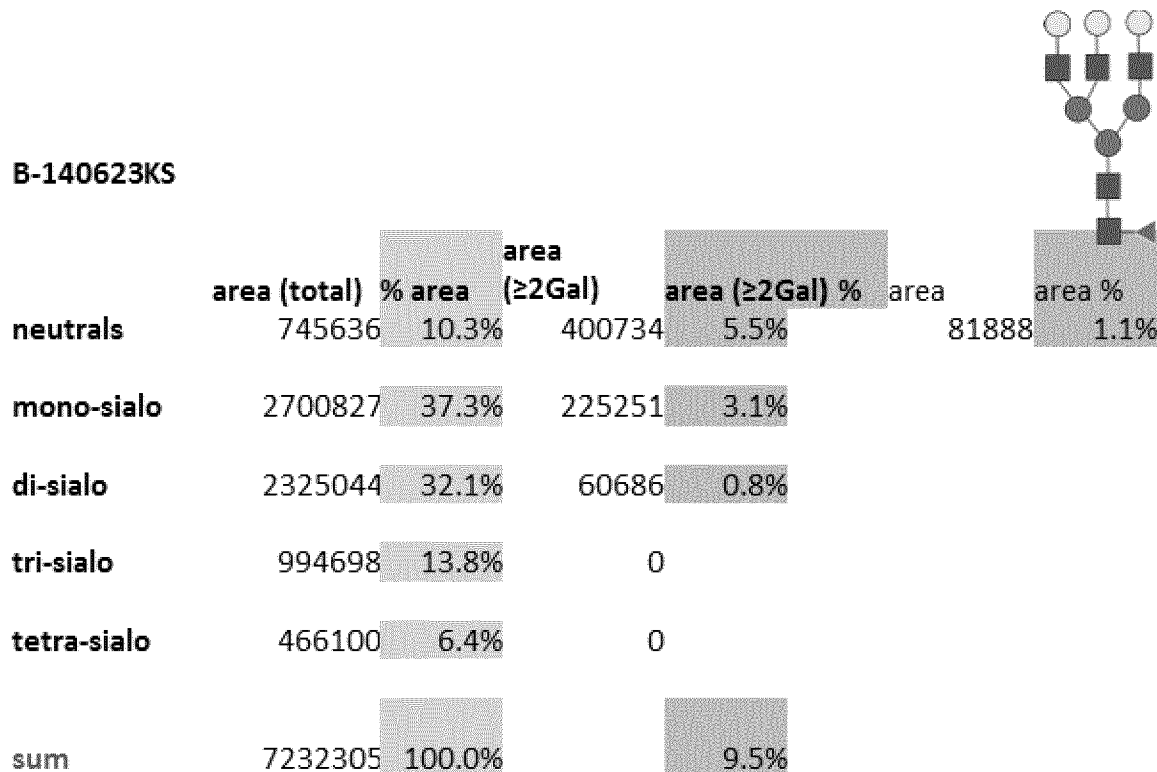


Figure 29



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 Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
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Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro Glu Arg
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Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu Pro Arg
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Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro Lys Ala
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Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys Val His
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Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp Arg Ala
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Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser Ser Lys
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Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His Cys Ile
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Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala Glu Ala
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Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg Arg His
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Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu Cys Tyr
865 870 875 880

Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro Gln Asn
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Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu Tyr Lys
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Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro Gln Val
915 920 925

Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys Val Gly
930 935 940

Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys Ala Glu
945 950 955 960

Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His Glu Lys
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Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser Leu Val
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Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp Ile
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 <212> PRT
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Tyr	Ala	Ser	Lys	Gly	Leu	Tyr	Leu	Glu	Thr	Glu	Ala	Gly	Tyr	Tyr	Lys
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Leu	Ser	Gly	Glu	Ala	Tyr	Gly	Phe	Val	Ala	Arg	Ile	Asp	Gly	Ser	Gly
	130					135					140				

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Asn Phe Gln Val Leu Leu Ser Asp Arg Tyr Phe Asn Lys Thr Cys Gly
 145 150 155 160
 Leu Cys Gly Asn Phe Asn Ile Phe Ala Glu Asp Asp Phe Met Thr Gln
 165 170 175
 Glu Gly Thr Leu Thr Ser Asp Pro Tyr Asp Phe Ala Asn Ser Trp Ala
 180 185 190
 Leu Ser Ser Gly Glu Gln Trp Cys Glu Arg Ala Ser Pro Pro Ser Ser
 195 200 205
 Ser Cys Asn Ile Ser Ser Gly Glu Met Gln Lys Gly Leu Trp Glu Gln
 210 215 220
 Cys Gln Leu Leu Lys Ser Thr Ser Val Phe Ala Arg Cys His Pro Leu
 225 230 235 240
 Val Asp Pro Glu Pro Phe Val Ala Leu Cys Glu Lys Thr Leu Cys Glu
 245 250 255
 Cys Ala Gly Gly Leu Glu Cys Ala Cys Pro Ala Leu Leu Glu Tyr Ala
 260 265 270
 Arg Thr Cys Ala Gln Glu Gly Met Val Leu Tyr Gly Trp Thr Asp His
 275 280 285
 Ser Ala Cys Ser Pro Val Cys Pro Ala Gly Met Glu Tyr Arg Gln Cys
 290 295 300
 Val Ser Pro Cys Ala Arg Thr Cys Gln Ser Leu His Ile Asn Glu Met
 305 310 315 320
 Cys Gln Glu Arg Cys Val Asp Gly Cys Ser Cys Pro Glu Gly Gln Leu
 325 330 335
 Leu Asp Glu Gly Leu Cys Val Glu Ser Thr Glu Cys Pro Cys Val His
 340 345 350
 Ser Gly Lys Arg Tyr Pro Pro Gly Thr Ser Leu Ser Arg Asp Cys Asn
 355 360 365
 Thr Cys Ile Cys Arg Asn Ser Gln Trp Ile Cys Ser Asn Glu Glu Cys
 370 375 380
 Pro Gly Glu Cys Leu Val Thr Gly Gln Ser His Phe Lys Ser Phe Asp
 385 390 395 400
 Asn Arg Tyr Phe Thr Phe Ser Gly Ile Cys Gln Tyr Leu Leu Ala Arg
 405 410 415

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Asp Cys Gln Asp His Ser Phe Ser Ile Val Ile Glu Thr Val Gln Cys
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 Ala Asp Asp Arg Asp Ala Val Cys Thr Arg Ser Val Thr Val Arg Leu
 435 440 445
 Pro Gly Leu His Asn Ser Leu Val Lys Leu Lys His Gly Ala Gly Val
 450 455 460
 Ala Met Asp Gly Gln Asp Val Gln Leu Pro Leu Leu Lys Gly Asp Leu
 465 470 475 480
 Arg Ile Gln His Thr Val Thr Ala Ser Val Arg Leu Ser Tyr Gly Glu
 485 490 495
 Asp Leu Gln Met Asp Trp Asp Gly Arg Gly Arg Leu Leu Val Lys Leu
 500 505 510
 Ser Pro Val Tyr Ala Gly Lys Thr Cys Gly Leu Cys Gly Asn Tyr Asn
 515 520 525
 Gly Asn Gln Gly Asp Asp Phe Leu Thr Pro Ser Gly Leu Ala Glu Pro
 530 535 540
 Arg Val Glu Asp Phe Gly Asn Ala Trp Lys Leu His Gly Asp Cys Gln
 545 550 555 560
 Asp Leu Gln Lys Gln His Ser Asp Pro Cys Ala Leu Asn Pro Arg Met
 565 570 575
 Thr Arg Phe Ser Glu Glu Ala Cys Ala Val Leu Thr Ser Pro Thr Phe
 580 585 590
 Glu Ala Cys His Arg Ala Val Ser Pro Leu Pro Tyr Leu Arg Asn Cys
 595 600 605
 Arg Tyr Asp Val Cys Ser Cys Ser Asp Gly Arg Glu Cys Leu Cys Gly
 610 615 620
 Ala Leu Ala Ser Tyr Ala Ala Ala Cys Ala Gly Arg Gly Val Arg Val
 625 630 635 640
 Ala Trp Arg Glu Pro Gly Arg Cys Glu Leu Asn Cys Pro Lys Gly Gln
 645 650 655
 Val Tyr Leu Gln Cys Gly Thr Pro Cys Asn Leu Thr Cys Arg Ser Leu
 660 665 670
 Ser Tyr Pro Asp Glu Glu Cys Asn Glu Ala Cys Leu Glu Gly Cys Phe
 675 680 685

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Cys Pro Pro Gly Leu Tyr Met Asp Glu Arg Gly Asp Cys Val Pro Lys
 690 695 700
 Ala Gln Cys Pro Cys Tyr Tyr Asp Gly Glu Ile Phe Gln Pro Glu Asp
 705 710 715 720
 Ile Phe Ser Asp His His Thr Met Cys Tyr Cys Glu Asp Gly Phe Met
 725 730 735
 His Cys Thr Met Ser Gly Val Pro Gly Ser Leu Leu Pro Asp Ala Val
 740 745 750
 Leu Ser Ser Pro Leu Ser His Arg Ser Lys Arg Ser Leu Ser Cys Arg
 755 760 765
 Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp Asn Leu Arg Ala Glu
 770 775 780
 Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr Asp Leu Glu Cys Met
 785 790 795 800
 Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro Pro Gly Met Val Arg
 805 810 815
 His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys Pro Cys Phe His Gln
 820 825 830
 Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys Ile Gly Cys Asn Thr
 835 840 845
 Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr Asp His Val Cys Asp
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 Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr Leu Thr Phe Asp Gly
 865 870 875 880
 Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr Val Leu Val Gln Asp
 885 890 895
 Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile Leu Val Gly Asn Lys
 900 905 910
 Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys Arg Val Thr Ile Leu
 915 920 925
 Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly Glu Val Asn Val Lys
 930 935 940
 Arg Pro Met Lys Asp Glu Thr His Phe Glu Val Val Glu Ser Gly Arg
 945 950 955 960

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Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser Val Val Trp Asp Arg
 965 970 975
 His Leu Ser Ile Ser Val Val Leu Lys Gln Thr Tyr Gln Glu Lys Val
 980 985 990
 Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln Asn Asn Asp Leu Thr
 995 1000 1005
 Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val Asp Phe Gly Asn
 1010 1015 1020
 Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg Lys Val Pro
 1025 1030 1035
 Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met Lys Gln
 1040 1045 1050
 Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val Phe
 1055 1060 1065
 Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
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 Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala
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 Cys Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln
 1100 1105 1110
 His Gly Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln
 1115 1120 1125
 Ser Cys Glu Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu
 1130 1135 1140
 Trp Arg Tyr Asn Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln
 1145 1150 1155
 His Pro Glu Pro Leu Ala Cys Pro Val Gln Cys Val Glu Gly Cys
 1160 1165 1170
 His Ala His Cys Pro Pro Gly Lys Ile Leu Asp Glu Leu Leu Gln
 1175 1180 1185
 Thr Cys Val Asp Pro Glu Asp Cys Pro Val Cys Glu Val Ala Gly
 1190 1195 1200
 Arg Arg Phe Ala Ser Gly Lys Lys Val Thr Leu Asn Pro Ser Asp
 1205 1210 1215

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Pro	Glu 1220	His	Cys	Gln	Ile	Cys 1225	His	Cys	Asp	Val	Val 1230	Asn	Leu	Thr
Cys	Glu 1235	Ala	Cys	Gln	Glu	Pro 1240	Gly	Gly	Leu	Val	Val 1245	Pro	Pro	Thr
Asp	Ala 1250	Pro	Val	Ser	Pro	Thr 1255	Thr	Leu	Tyr	Val	Glu 1260	Asp	Ile	Ser
Glu	Pro 1265	Pro	Leu	His	Asp	Phe 1270	Tyr	Cys	Ser	Arg	Leu 1275	Leu	Asp	Leu
Val	Phe 1280	Leu	Leu	Asp	Gly	Ser 1285	Ser	Arg	Leu	Ser	Glu 1290	Ala	Glu	Phe
Glu	Val 1295	Leu	Lys	Ala	Phe	Val 1300	Val	Asp	Met	Met	Glu 1305	Arg	Leu	Arg
Ile	Ser 1310	Gln	Lys	Trp	Val	Arg 1315	Val	Ala	Val	Val	Glu 1320	Tyr	His	Asp
Gly	Ser 1325	His	Ala	Tyr	Ile	Gly 1330	Leu	Lys	Asp	Arg	Lys 1335	Arg	Pro	Ser
Glu	Leu 1340	Arg	Arg	Ile	Ala	Ser 1345	Gln	Val	Lys	Tyr	Ala 1350	Gly	Ser	Gln
Val	Ala 1355	Ser	Thr	Ser	Glu	Val 1360	Leu	Lys	Tyr	Thr	Leu 1365	Phe	Gln	Ile
Phe	Ser 1370	Lys	Ile	Asp	Arg	Pro 1375	Glu	Ala	Ser	Arg	Ile 1380	Ala	Leu	Leu
Leu	Met 1385	Ala	Ser	Gln	Glu	Pro 1390	Gln	Arg	Met	Ser	Arg 1395	Asn	Phe	Val
Arg	Tyr 1400	Val	Gln	Gly	Leu	Lys 1405	Lys	Lys	Lys	Val	Ile 1410	Val	Ile	Pro
Val	Gly 1415	Ile	Gly	Pro	His	Ala 1420	Asn	Leu	Lys	Gln	Ile 1425	Arg	Leu	Ile
Glu	Lys 1430	Gln	Ala	Pro	Glu	Asn 1435	Lys	Ala	Phe	Val	Leu 1440	Ser	Ser	Val
Asp	Glu 1445	Leu	Glu	Gln	Gln	Arg 1450	Asp	Glu	Ile	Val	Ser 1455	Tyr	Leu	Cys
Asp	Leu 1460	Ala	Pro	Glu	Ala	Pro 1465	Pro	Pro	Thr	Leu	Pro 1470	Pro	His	Met

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Ala	Gln	Val	Thr	Val	Gly	Pro	Gly	Leu	Leu	Gly	Val	Ser	Thr	Leu
	1475					1480					1485			
Gly	Pro	Lys	Arg	Asn	Ser	Met	Val	Leu	Asp	Val	Ala	Phe	Val	Leu
	1490					1495					1500			
Glu	Gly	Ser	Asp	Lys	Ile	Gly	Glu	Ala	Asp	Phe	Asn	Arg	Ser	Lys
	1505					1510					1515			
Glu	Phe	Met	Glu	Glu	Val	Ile	Gln	Arg	Met	Asp	Val	Gly	Gln	Asp
	1520					1525					1530			
Ser	Ile	His	Val	Thr	Val	Leu	Gln	Tyr	Ser	Tyr	Met	Val	Thr	Val
	1535					1540					1545			
Glu	Tyr	Pro	Phe	Ser	Glu	Ala	Gln	Ser	Lys	Gly	Asp	Ile	Leu	Gln
	1550					1555					1560			
Arg	Val	Arg	Glu	Ile	Arg	Tyr	Gln	Gly	Gly	Asn	Arg	Thr	Asn	Thr
	1565					1570					1575			
Gly	Leu	Ala	Leu	Arg	Tyr	Leu	Ser	Asp	His	Ser	Phe	Leu	Val	Ser
	1580					1585					1590			
Gln	Gly	Asp	Arg	Glu	Gln	Ala	Pro	Asn	Leu	Val	Tyr	Met	Val	Thr
	1595					1600					1605			
Gly	Asn	Pro	Ala	Ser	Asp	Glu	Ile	Lys	Arg	Leu	Pro	Gly	Asp	Ile
	1610					1615					1620			
Gln	Val	Val	Pro	Ile	Gly	Val	Gly	Pro	Asn	Ala	Asn	Val	Gln	Glu
	1625					1630					1635			
Leu	Glu	Arg	Ile	Gly	Trp	Pro	Asn	Ala	Pro	Ile	Leu	Ile	Gln	Asp
	1640					1645					1650			
Phe	Glu	Thr	Leu	Pro	Arg	Glu	Ala	Pro	Asp	Leu	Val	Leu	Gln	Arg
	1655					1660					1665			
Cys	Cys	Ser	Gly	Glu	Gly	Leu	Gln	Ile	Pro	Thr	Leu	Ser	Pro	Ala
	1670					1675					1680			
Pro	Asp	Cys	Ser	Gln	Pro	Leu	Asp	Val	Ile	Leu	Leu	Leu	Asp	Gly
	1685					1690					1695			
Ser	Ser	Ser	Phe	Pro	Ala	Ser	Tyr	Phe	Asp	Glu	Met	Lys	Ser	Phe
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Ala	Lys	Ala	Phe	Ile	Ser	Lys	Ala	Asn	Ile	Gly	Pro	Arg	Leu	Thr
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Gln	Val	Ser	Val	Leu	Gln	Tyr	Gly	Ser	Ile	Thr	Thr	Ile	Asp	Val
	1730					1735					1740			
Pro	Trp	Asn	Val	Val	Pro	Glu	Lys	Ala	His	Leu	Leu	Ser	Leu	Val
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Asp	Val	Met	Gln	Arg	Glu	Gly	Gly	Pro	Ser	Gln	Ile	Gly	Asp	Ala
	1760					1765					1770			
Leu	Gly	Phe	Ala	Val	Arg	Tyr	Leu	Thr	Ser	Glu	Met	His	Gly	Ala
	1775					1780					1785			
Arg	Pro	Gly	Ala	Ser	Lys	Ala	Val	Val	Ile	Leu	Val	Thr	Asp	Val
	1790					1795					1800			
Ser	Val	Asp	Ser	Val	Asp	Ala	Ala	Ala	Asp	Ala	Ala	Arg	Ser	Asn
	1805					1810					1815			
Arg	Val	Thr	Val	Phe	Pro	Ile	Gly	Ile	Gly	Asp	Arg	Tyr	Asp	Ala
	1820					1825					1830			
Ala	Gln	Leu	Arg	Ile	Leu	Ala	Gly	Pro	Ala	Gly	Asp	Ser	Asn	Val
	1835					1840					1845			
Val	Lys	Leu	Gln	Arg	Ile	Glu	Asp	Leu	Pro	Thr	Met	Val	Thr	Leu
	1850					1855					1860			
Gly	Asn	Ser	Phe	Leu	His	Lys	Leu	Cys	Ser	Gly	Phe	Val	Arg	Ile
	1865					1870					1875			
Cys	Met	Asp	Glu	Asp	Gly	Asn	Glu	Lys	Arg	Pro	Gly	Asp	Val	Trp
	1880					1885					1890			
Thr	Leu	Pro	Asp	Gln	Cys	His	Thr	Val	Thr	Cys	Gln	Pro	Asp	Gly
	1895					1900					1905			
Gln	Thr	Leu	Leu	Lys	Ser	His	Arg	Val	Asn	Cys	Asp	Arg	Gly	Leu
	1910					1915					1920			
Arg	Pro	Ser	Cys	Pro	Asn	Ser	Gln	Ser	Pro	Val	Lys	Val	Glu	Glu
	1925					1930					1935			
Thr	Cys	Gly	Cys	Arg	Trp	Thr	Cys	Pro	Cys	Val	Cys	Thr	Gly	Ser
	1940					1945					1950			
Ser	Thr	Arg	His	Ile	Val	Thr	Phe	Asp	Gly	Gln	Asn	Phe	Lys	Leu
	1955					1960					1965			
Thr	Gly	Ser	Cys	Ser	Tyr	Val	Leu	Phe	Gln	Asn	Lys	Glu	Gln	Asp
	1970					1975					1980			

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Leu	Glu	Val	Ile	Leu	His	Asn	Gly	Ala	Cys	Ser	Pro	Gly	Ala	Arg
	1985					1990					1995			
Gln	Gly	Cys	Met	Lys	Ser	Ile	Glu	Val	Lys	His	Ser	Ala	Leu	Ser
	2000					2005					2010			
Val	Glu	Leu	His	Ser	Asp	Met	Glu	Val	Thr	Val	Asn	Gly	Arg	Leu
	2015					2020					2025			
Val	Ser	Val	Pro	Tyr	Val	Gly	Gly	Asn	Met	Glu	Val	Asn	Val	Tyr
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Gly	Ala	Ile	Met	His	Glu	Val	Arg	Phe	Asn	His	Leu	Gly	His	Ile
	2045					2050					2055			
Phe	Thr	Phe	Thr	Pro	Gln	Asn	Asn	Glu	Phe	Gln	Leu	Gln	Leu	Ser
	2060					2065					2070			
Pro	Lys	Thr	Phe	Ala	Ser	Lys	Thr	Tyr	Gly	Leu	Cys	Gly	Ile	Cys
	2075					2080					2085			
Asp	Glu	Asn	Gly	Ala	Asn	Asp	Phe	Met	Leu	Arg	Asp	Gly	Thr	Val
	2090					2095					2100			
Thr	Thr	Asp	Trp	Lys	Thr	Leu	Val	Gln	Glu	Trp	Thr	Val	Gln	Arg
	2105					2110					2115			
Pro	Gly	Gln	Thr	Cys	Gln	Pro	Ile	Leu	Glu	Glu	Gln	Cys	Leu	Val
	2120					2125					2130			
Pro	Asp	Ser	Ser	His	Cys	Gln	Val	Leu	Leu	Leu	Pro	Leu	Phe	Ala
	2135					2140					2145			
Glu	Cys	His	Lys	Val	Leu	Ala	Pro	Ala	Thr	Phe	Tyr	Ala	Ile	Cys
	2150					2155					2160			
Gln	Gln	Asp	Ser	Cys	His	Gln	Glu	Gln	Val	Cys	Glu	Val	Ile	Ala
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Ser	Tyr	Ala	His	Leu	Cys	Arg	Thr	Asn	Gly	Val	Cys	Val	Asp	Trp
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Arg	Thr	Pro	Asp	Phe	Cys	Ala	Met	Ser	Cys	Pro	Pro	Ser	Leu	Val
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Tyr	Asn	His	Cys	Glu	His	Gly	Cys	Pro	Arg	His	Cys	Asp	Gly	Asn
	2210					2215					2220			
Val	Ser	Ser	Cys	Gly	Asp	His	Pro	Ser	Glu	Gly	Cys	Phe	Cys	Pro
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Pro	Asp 2240	Lys	Val	Met	Leu	Glu 2245	Gly	Ser	Cys	Val	Pro 2250	Glu	Glu	Ala
Cys	Thr 2255	Gln	Cys	Ile	Gly	Glu 2260	Asp	Gly	Val	Gln	His 2265	Gln	Phe	Leu
Glu	Ala 2270	Trp	Val	Pro	Asp	His 2275	Gln	Pro	Cys	Gln	Ile 2280	Cys	Thr	Cys
Leu	Ser 2285	Gly	Arg	Lys	Val	Asn 2290	Cys	Thr	Thr	Gln	Pro 2295	Cys	Pro	Thr
Ala	Lys 2300	Ala	Pro	Thr	Cys	Gly 2305	Leu	Cys	Glu	Val	Ala 2310	Arg	Leu	Arg
Gln	Asn 2315	Ala	Asp	Gln	Cys	Cys 2320	Pro	Glu	Tyr	Glu	Cys 2325	Val	Cys	Asp
Pro	Val 2330	Ser	Cys	Asp	Leu	Pro 2335	Pro	Val	Pro	His	Cys 2340	Glu	Arg	Gly
Leu	Gln 2345	Pro	Thr	Leu	Thr	Asn 2350	Pro	Gly	Glu	Cys	Arg 2355	Pro	Asn	Phe
Thr	Cys 2360	Ala	Cys	Arg	Lys	Glu 2365	Glu	Cys	Lys	Arg	Val 2370	Ser	Pro	Pro
Ser	Cys 2375	Pro	Pro	His	Arg	Leu 2380	Pro	Thr	Leu	Arg	Lys 2385	Thr	Gln	Cys
Cys	Asp 2390	Glu	Tyr	Glu	Cys	Ala 2395	Cys	Asn	Cys	Val	Asn 2400	Ser	Thr	Val
Ser	Cys 2405	Pro	Leu	Gly	Tyr	Leu 2410	Ala	Ser	Thr	Ala	Thr 2415	Asn	Asp	Cys
Gly	Cys 2420	Thr	Thr	Thr	Thr	Cys 2425	Leu	Pro	Asp	Lys	Val 2430	Cys	Val	His
Arg	Ser 2435	Thr	Ile	Tyr	Pro	Val 2440	Gly	Gln	Phe	Trp	Glu 2445	Glu	Gly	Cys
Asp	Val 2450	Cys	Thr	Cys	Thr	Asp 2455	Met	Glu	Asp	Ala	Val 2460	Met	Gly	Leu
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Ser	Gly 2480	Phe	Thr	Tyr	Val	Leu 2485	His	Glu	Gly	Glu	Cys 2490	Cys	Gly	Arg

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Asp	Ser	Gln	Ser	Ser	Trp	Lys	Ser	Val	Gly	Ser	Gln	Trp	Ala	Ser
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Pro	Glu	Asn	Pro	Cys	Leu	Ile	Asn	Glu	Cys	Val	Arg	Val	Lys	Glu
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Val	Pro	Val	Cys	Pro	Ser	Gly	Phe	Gln	Leu	Ser	Cys	Lys	Thr	Ser
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Ala	Cys	Cys	Pro	Ser	Cys	Arg	Cys	Glu	Arg	Met	Glu	Ala	Cys	Met
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Leu	Asn	Gly	Thr	Val	Ile	Gly	Pro	Gly	Lys	Thr	Val	Met	Ile	Asp
	2585					2590					2595			
Val	Cys	Thr	Thr	Cys	Arg	Cys	Met	Val	Gln	Val	Gly	Val	Ile	Ser
	2600					2605					2610			
Gly	Phe	Lys	Leu	Glu	Cys	Arg	Lys	Thr	Thr	Cys	Asn	Pro	Cys	Pro
	2615					2620					2625			
Leu	Gly	Tyr	Lys	Glu	Glu	Asn	Asn	Thr	Gly	Glu	Cys	Cys	Gly	Arg
	2630					2635					2640			
Cys	Leu	Pro	Thr	Ala	Cys	Thr	Ile	Gln	Leu	Arg	Gly	Gly	Gln	Ile
	2645					2650					2655			
Met	Thr	Leu	Lys	Arg	Asp	Glu	Thr	Leu	Gln	Asp	Gly	Cys	Asp	Thr
	2660					2665					2670			
His	Phe	Cys	Lys	Val	Asn	Glu	Arg	Gly	Glu	Tyr	Phe	Trp	Glu	Lys
	2675					2680					2685			
Arg	Val	Thr	Gly	Cys	Pro	Pro	Phe	Asp	Glu	His	Lys	Cys	Leu	Ala
	2690					2695					2700			
Glu	Gly	Gly	Lys	Ile	Met	Lys	Ile	Pro	Gly	Thr	Cys	Cys	Asp	Thr
	2705					2710					2715			
Cys	Glu	Glu	Pro	Glu	Cys	Asn	Asp	Ile	Thr	Ala	Arg	Leu	Gln	Tyr
	2720					2725					2730			
Val	Lys	Val	Gly	Ser	Cys	Lys	Ser	Glu	Val	Glu	Val	Asp	Ile	His
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Tyr Cys Gln Gly Lys Cys Ala Ser Lys Ala Met Tyr Ser Ile Asp
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Ile Asn Asp Val Gln Asp Gln Cys Ser Cys Cys Ser Pro Thr Arg
2765 2770 2775

Thr Glu Pro Met Gln Val Ala Leu His Cys Thr Asn Gly Ser Val
2780 2785 2790

Val Tyr His Glu Val Leu Asn Ala Met Glu Cys Lys Cys Ser Pro
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Arg Lys Cys Ser Lys
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Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
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Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65 70 75 80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
85 90 95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100 105 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115 120 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
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145 150 155 160
 His Val Asp Leu Val₁₆₅ Lys Asp Leu Asn Ser₁₇₀ Gly Leu Ile Gly Ala₁₇₅ Leu
 Leu Val Cys Arg₁₈₀ Glu Gly Ser Leu Ala₁₈₅ Lys Glu Lys Thr Gln₁₉₀ Thr Leu
 His Lys Phe₁₉₅ Ile Leu Leu Phe Ala₂₀₀ Val Phe Asp Glu Gly₂₀₅ Lys Ser Trp
 His Ser₂₁₀ Glu Thr Lys Asn Ser₂₁₅ Leu Met Gln Asp Arg₂₂₀ Asp Ala Ala Ser
 Ala Arg Ala Trp Pro Lys₂₃₀ Met His Thr Val Asn₂₃₅ Gly Tyr Val Asn Arg₂₄₀
 Ser Leu Pro Gly Leu₂₄₅ Ile Gly Cys His Arg₂₅₀ Lys Ser Val Tyr Trp₂₅₅ His
 Val Ile Gly Met₂₆₀ Gly Thr Thr Pro Glu Val His Ser Ile Phe₂₇₀ Leu Glu
 Gly His Thr₂₇₅ Phe Leu Val Arg Asn₂₈₀ His Arg Gln Ala Ser₂₈₅ Leu Glu Ile
 Ser Pro₂₉₀ Ile Thr Phe Leu Thr₂₉₅ Ala Gln Thr Leu Leu₃₀₀ Met Asp Leu Gly
 Gln Phe Leu Leu Phe Cys₃₁₀ His Ile Ser Ser His₃₁₅ Gln His Asp Gly Met₃₂₀
 Glu Ala Tyr Val Lys₃₂₅ Val Asp Ser Cys Pro₃₃₀ Glu Glu Pro Gln Leu Arg₃₃₅
 Met Lys Asn Asn₃₄₀ Glu Glu Ala Glu Asp₃₄₅ Tyr Asp Asp Asp Leu₃₅₀ Thr Asp
 Ser Glu Met₃₅₅ Asp Val Val Arg Phe₃₆₀ Asp Asp Asp Asn Ser₃₆₅ Pro Ser Phe
 Ile Gln Ile Arg Ser Val Ala₃₇₅ Lys Lys His Pro Lys₃₈₀ Thr Trp Val His
 Tyr Ile Ala Ala Glu Glu₃₉₀ Glu Asp Trp Asp Tyr₃₉₅ Ala Pro Leu Val Leu₄₀₀
 Ala Pro Asp Asp Arg₄₀₅ Ser Tyr Lys Ser Gln₄₁₀ Tyr Leu Asn Asn Gly₄₁₅ Pro
 Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr

420

425

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Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
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Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
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Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
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Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
 485 490 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
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Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
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Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
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Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
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Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
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Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
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Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
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Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
 610 615 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
 625 630 635 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
 645 650 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
 660 665 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
 675 680 685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala

690

695

700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
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Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
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Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser Thr Arg
740 745 750

Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Thr Thr Leu Gln
755 760 765

Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met
770 775 780

Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro
785 790 795 800

Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu
805 810 815

Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn
820 825 830

Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln
835 840 845

Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu
850 855 860

Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu
865 870 875 880

Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser
885 890 895

Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala
900 905 910

Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe
915 920 925

Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys
930 935 940

Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His
945 950 955 960

Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn
Page 35

Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe
 980 985 990

Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu
 995 1000 1005

Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr
 1010 1015 1020

Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met
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Asp Thr Leu Pro Gly Leu Val Met Ala Gln Asp Gln Arg Ile Arg
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Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn Ile His Ser Ile
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His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys Glu Glu Tyr
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Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu Thr Val
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Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys Leu
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Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His
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Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp
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Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala
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Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu
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Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln
 1190 1195 1200

Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser
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Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly

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1220

1225

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His	Pro	Thr	His	Tyr	Ser	Ile	Arg	Ser	Thr	Leu	Arg	Met	Glu	Leu
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Met	Gly	Cys	Asp	Leu	Asn	Ser	Cys	Ser	Met	Pro	Leu	Gly	Met	Glu
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Ser	Lys	Ala	Ile	Ser	Asp	Ala	Gln	Ile	Thr	Ala	Ser	Ser	Tyr	Phe
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Leu	Gln	Gly	Arg	Ser	Asn	Ala	Trp	Arg	Pro	Gln	Val	Asn	Asn	Pro
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Gly	Val	Thr	Thr	Gln	Gly	Val	Lys	Ser	Leu	Leu	Thr	Ser	Met	Tyr
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Val	Lys	Glu	Phe	Leu	Ile	Ser	Ser	Ser	Gln	Asp	Gly	His	Gln	Trp
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Thr	Leu	Phe	Phe	Gln	Asn	Gly	Lys	Val	Lys	Val	Phe	Gln	Gly	Asn
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Tyr

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 Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
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 Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu
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 Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
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 Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala
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 Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
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 Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu
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 Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
 225 230 235 240
 Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
 245 250 255
 Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
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Ser Lys Leu₂₇₅ Lys Glu Cys Cys Glu₂₈₀ Lys Pro Leu Leu Glu₂₈₅ Lys Ser His
 Cys Ile₂₉₀ Ala Glu Val Glu Asn₂₉₅ Asp Glu Met Pro Ala₃₀₀ Asp Leu Pro Ser
 Leu Ala Ala Asp Phe Val₃₁₀ Glu Ser Lys Asp Val₃₁₅ Cys Lys Asn Tyr Ala₃₂₀
 Glu Ala Lys Asp Val₃₂₅ Phe Leu Gly Met Phe₃₃₀ Leu Tyr Glu Tyr Ala₃₃₅ Arg
 Arg His Pro Asp₃₄₀ Tyr Ser Val Val₃₄₅ Leu Leu Arg Leu Ala₃₅₀ Lys Thr
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 Gln Asn Leu Ile Lys Gln₃₉₀ Asn Cys Glu Leu Phe₃₉₅ Glu Gln Leu Gly Glu₄₀₀
 Tyr Lys Phe Gln Asn₄₀₅ Ala Leu Leu Val Arg₄₁₀ Tyr Thr Lys Lys Val₄₁₅ Pro
 Gln Val Ser Thr₄₂₀ Pro Thr Leu Val Glu₄₂₅ Val Ser Arg Asn Leu Gly Lys
 Val Gly Ser₄₃₅ Lys Cys Cys Lys His₄₄₀ Pro Glu Ala Lys Arg₄₄₅ Met Pro Cys
 Ala Glu Asp Tyr Leu Ser Val₄₅₅ Val Leu Asn Gln Leu₄₆₀ Cys Val Leu His
 Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys₄₇₅ Cys Cys Thr Glu Ser₄₈₀
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 Tyr Val Pro Lys₅₀₀ Glu Phe Asn Ala Glu₅₀₅ Thr Phe Thr Phe His₅₁₀ Ala Asp
 Ile Cys Thr₅₁₅ Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys₅₂₅ Gln Thr Ala
 Leu Val Glu Leu Val Lys His₅₃₅ Lys Pro Lys Ala Thr₅₄₀ Lys Glu Gln Leu

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Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys
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tga 1023