(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 August 2011 (25.08.2011)

PCT

(10) International Publication Number
WO 2011/101267 A1

(51) International Patent Classification:
C07K 14/755 (2006.01) *A61K 38/37* (2006.01)

(21) International Application Number:
PCT/EP2011/051723

(22) International Filing Date:
7 February 2011 (07.02.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10153718.1 16 February 2010 (16.02.2010) EP
61/305,608 18 February 2010 (18.02.2010) US

(71) Applicant *(for all designated States except US)*: **NOVO NORDISK A/S** [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BEHRENS, Carsten** [DK/DK]; Lundtoftegade 107, 1 th, DK-2200 København N (DK). **BUCHARDT, Jens** [DK/DK]; Vangedevej 115, DK-2820 Gentofte (DK). **ZUNDEL, Magali** [FR/DK]; Plantevej 27, 3 t.h., DK-2870 Dyssegaard (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2011/101267 A1

(54) Title: CONJUGATED FVIII VARIANTS

(57) Abstract: The present invention relates to conjugated Factor VIII variants. The present invention in particular relates to conjugated FVIII variants comprising different polymeric groups as well as use thereof.

CONJUGATED FVIII VARIANTS

BACKGROUND

Haemophilia A is an inherited bleeding disorder caused by deficiency or dysfunction
5 of coagulation factor VIII (FVIII) activity. The clinical manifestation is not on primary haemostasis – formation of the blood clot occurs normally – but the clot is unstable due to a lack of secondary thrombin formation.

Haemophilia A is currently treated by intravenously injection of coagulation factor FVIII which is either isolated from blood or produced recombinantly. Treatment can be either
10 on-demand or prophylactic. Recent published data support that prophylaxis has significant advantages over on-demand treatment. These include reduction in bleeding frequency and lower risk of developing haemophilic arthropathy, both resulting in a better quality of life for the patients. However, while prophylaxis enables a virtually symptom-free life for the patients, it requires frequent injections in a peripheral vein, typically three times a week,
15 which is known to be painful, difficult, and time consuming. In addition, repeated venipuncture is not always possible in young children. Consequently, a product supporting less frequent administration and/or administration would to a greater extent enable regular prophylactic treatment.

It has long been known that coupling of polymers like for example
20 polyethyleneglycol (PEGs) or polysialic acids (PSAs) to a protein leads to increased circulation time, increased resistance towards proteases and reduced immunogenicity. There is, however, still a need in the art for FVIII variants having a prolonged circulatory half life.

SUMMARY

The present invention relates to FVIII variant conjugated with at least one PEG
25 polymer and at least one polysaccharide as well as use thereof. It is shown herein that such heteroconjugated FVIII variants have an improved increase in circulatory half life over FVIII variants conjugated with e.g. two PEG molecules or two polysaccharide molecules.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows an example of synthesis of a FVIII variant according to the present
30 invention.

DESCRIPTION

Definitions:

Factor VIII molecules: FVIII/Factor VIII is a large, complex glycoprotein that primarily is produced by hepatocytes. Human FVIII consists of 2351 amino acids, including signal peptide, and contains several distinct domains, as defined by homology. There are three A-domains, a unique B-domain, and two C-domains. The domain order can be listed as NH2-A1-A2-B-A3-C1-C2-COOH. FVIII circulates in plasma as two chains, separated at the B-A3 border. The chains are connected by bivalent metal ion-bindings. The A1-A2-B chain is termed the heavy chain (HC) while the A3-C1-C2 is termed the light chain (LC).

FVIII circulates associated with von Willebrand Factor (VWF). VWF is a large multimeric glycoprotein that serves as a carrier for FVIII and is required for normal platelet adhesion to components of the vessel wall. The plasma-half life of FVIII in complex with VWF is approximately 12 hours.

"Native FVIII" is the full length human FVIII molecule as shown in SEQ ID NO. 1 (amino acid 1-2332). The B-domain is spanning amino acids 741-1648 in SEQ ID NO 1.

SEQ ID NO 1:

ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTLFVEFT
DHLFNIAKPRPPWMGLLGPTIQAEVYDTVVITLKNMASHPVSLHAVGVSYWKASEGAEYDD
QTSQREKEDDKVFPGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALL
VCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGY
VNRSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLL
MDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRF
DDDNSPSFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGR
KYKKVRFMAYTDETFKTREAIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRP
LYSRRLPKGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLI
GPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLEDPEFQA
SNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMVYEDTLTLFPF
SGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKN
NAIEPRSFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTPMPKIQNVSSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESGLQL
RLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP

LIHDRMLMDKNATALRLNHMSNKTTSSKNMEMVQQKKEGPIPPDAQNPDMSFFKMLFLPES
ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
MVFPSSRNLFLTNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF
LLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVE
5 KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTL
TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
PAASYRKKDSGVQESSHFLQGAKKNNLSLAILTLEMTGDQREVGSLGTSATNSVTYKKVEN
TVLPKPDLPKTSGKVELLPKVHIYQKDLFPTETSNGSPGHLDLVEGSLLQGTEGAIKWNEAN
RPGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEKSPEKTAFKKKDTILSL
10 NACESNHAIAAINEGQNKPEIEVTWAKQGRTERLCSQNPPVLKRHQREITRTTLQSDQEEID
YDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQS
GSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFY
SSLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQHHMAPTKDEFDCKAWAYFSDVDLEKD
VHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQME
15 DPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEE
YKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHI
RDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFS
SLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIR
STLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPSKARLHLQGRSNAW
20 RPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGK
VKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY

"FVIII variants" according to the present invention may be FVIII derived from blood plasma and/or recombinant FVIII. FVIII variants according to the invention may be e.g. B domain truncated FVIII molecules wherein e.g. the remaining domains correspond closely to
25 the sequence as set forth in amino acid no 1-740 and 1649-2332 in SEQ ID NO. 1. B domain truncated FVIII variants according to the invention may differ slightly from the sequence set forth in SEQ ID NO 1, meaning that the remaining domains (i.e. the three A-domains and the two C-domains) may differ slightly e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9 , or 10 amino acids, alternatively may differ about 1%, 2%, 3%, 4% or 5% from the amino acid sequence as set
30 forth in SEQ ID NO 1 (amino acids 1-740 and 1649-2332) due to the fact that mutations can be introduced in order to e.g.reduce vWF binding capacity. Furthermore, it is plausible that amino acid modifications (substitutions, deletions, etc.) are introduced other places in the molecule in order to modify the binding capacity of Factor VIII with various other components such as e.g. LRP, various receptors, other coagulation factors, cell surfaces, introduction
35 and/or abolishment of glycosylation sites, etc. FVIII variants according to the present

invention have FVIII activity, meaning the ability to function in the coagulation cascade in a manner functionally similar or equivalent to FVIII, induce the formation of FXa via interaction with FIXa on an activated platelet, and support the formation of a blood clot. The activity can be assessed *in vitro* by techniques well known in the art such as e.g. chromogenic assay,
5 clot analysis, endogenous thrombin potential analysis, etc. FVIII variants according to the invention have FVIII activity being at least about 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, and 100% or even more than 100% of that of native human FVIII.

10 B domain: The B-domain in Factor VIII spans amino acids 741-1648 in SEQ ID NO 1. The B-domain is cleaved at several different sites, generating large heterogeneity in circulating plasma FVIII molecules. The exact function of the heavily glycosylated B-domain is unknown. What is known is that the domain is dispensable for FVIII activity in the coagulation cascade. This apparent lack of function is supported by the fact that B domain
15 deleted/truncated FVIII appears to have *in vivo* properties identical to those seen for full length native FVIII. That being said there are indications that the B-domain may reduce the association with the cell membrane, at least under serum free conditions.

B domain truncated/deleted Factor VIII molecule: Endogenous full length FVIII is
20 synthesized as a single-chain precursor molecule. Prior to secretion, the precursor is cleaved into the heavy chain and the light chain. Recombinant B domain-deleted FVIII can be produced from two different strategies. Either the heavy chain without the B-domain and the light chain are synthesized individually as two different polypeptide chains (two-chain strategy) or the B-domain deleted FVIII is synthesized as a single precursor polypeptide
25 chain (single-chain strategy) that is cleaved into the heavy and light chains in the same way as the full-length FVIII precursor.

In a B domain-deleted FVIII precursor polypeptide, the heavy and light chain moieties are normally separated by a linker. To minimize the risk of introducing immunogenic epitopes in the B domain-deleted FVIII, the sequence of the linker is preferably derived from
30 the FVIII B-domain. As a minimum, the linker must comprise a recognition site for the protease that separates the B domain-deleted FVIII precursor polypeptide into the heavy and light chain. In the B domain of full length FVIII, amino acid 1644-1648 constitutes this recognition site. The thrombin site leading to removal of the linker on activation of B domain-deleted FVIII is located in the heavy chain. Thus, the size and amino acid sequence of the
35 linker is unlikely to influence its removal from the remaining FVIII molecule by thrombin

activation. Deletion of the B domain is an advantage for production of FVIII. Nevertheless, parts of the B domain can be included in the linker without reducing the productivity. The negative effect of the B domain on productivity has not been attributed to any specific size or sequence of the B domain.

5 The truncated B-domain may contain several O-glycosylation sites. However, according to a preferred embodiment, the molecule comprises only one, alternatively two, three or four O-linked oligosaccharides in the truncated B-domain.

According to a preferred embodiment, the truncated B domain comprises only one potential O-glycosylation sites and a hydrophilic polymer is covalently conjugated to this O-

10 glycosylation site. The O-linked oligosaccharides in the B-domain truncated molecules according to the invention may be attached to O-glycosylation sites that were either artificially created by recombinant means and/or by exposure of “hidden” O-glycosylation sites by truncation of the B-domain. In both cases, such molecules may be made by designing a B-domain truncated Factor VIII amino acid sequence and subsequently subjecting the amino

15 acid sequence to an *in silico* analysis predicting the probability of O-glycosylation sites in the truncated B-domain. Molecules with a relatively high probability of having such glycosylation sites can be synthesized in a suitable host cell followed by analysis of the glycosylation pattern and subsequent selection of molecules having O-linked glycosylation in the truncated B-domain.

20 Suitable host cells for producing recombinant factor VIII protein are preferably of mammalian origin in order to ensure that the molecule is glycosylated. In practicing the present invention, the cells are mammalian cells, more preferably an established mammalian cell line, including, without limitation, CHO (e.g., ATCC CCL 61), COS-1 (e.g., ATCC CRL 1650), baby hamster kidney (BHK), and HEK293 (e.g., ATCC CRL 1573; Graham et al., J.

25 Gen. Virol. 36:59-72, 1977) cell lines. A preferred BHK cell line is the tk- ts13 BHK cell line (Waechter and Baserga, Proc.Natl.Acad.Sci.USA 79:1106-1110, 1982), hereinafter referred to as BHK 570 cells. The BHK 570 cell line is available from the American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852, under ATCC accession number CRL 10314. A tk- ts13 BHK cell line is also available from the ATCC under accession number

30 CRL 1632. A preferred CHO cell line is the CHO K1 cell line available from ATCC under accession number CCl61 as well as cell lines CHO-DXB11 and CHO-DG44.

Other suitable cell lines include, without limitation, Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1); DUKX cells (CHO cell line) (Urlaub and

35 Chasin, Proc. Natl. Acad. Sci. USA 77:4216-4220, 1980) (DUKX cells also being referred to

as DXB11 cells), and DG44 (CHO cell line) (Cell, 33: 405, 1983, and Somatic Cell and Molecular Genetics 12: 555, 1986). Also useful are 3T3 cells, Namalwa cells, myelomas and fusions of myelomas with other cells. In some embodiments, the cells may be mutant or recombinant cells, such as, e.g., cells that express a qualitatively or quantitatively different spectrum of enzymes that catalyze post-translational modification of proteins (e.g., glycosylation enzymes such as glycosyl transferases and/or glycosidases, or processing enzymes such as propeptides) than the cell type from which they were derived. DUKX cells (CHO cell line) are especially preferred.

Currently preferred cells are HEK293, COS, Chinese Hamster Ovary (CHO) cells, Baby Hamster Kidney (BHK) and myeloma cells, in particular Chinese Hamster Ovary (CHO) cells.

N-linked and O-linked oligosaccharides: Both N-glycans and O-glycans are attached to proteins by the cells producing the protein. The cellular N-glycosylation machinery recognizes and glycosylates N-glycosylation signals (N-X-S/T motifs) in the amino acid chain, as the nascent protein is translocated from the ribosome to the endoplasmic reticulum and continues until after transportation to the Golgi apparatus (Kiely et al. JBC (1976) 251(18), 5490; Glabe et al. JBC(1980)255(19), 9236 , Lenting et al. Haemophilia (2010) 16(suppl.5), 194)). N-linked FVIII oligosaccharide may be naturally occurring, which have been described in the art (Lenting et al. Haemophilia (2010) 16(Suppl 5), 194 and references cited herein), or it may be introduced by genetic engineering.

Likewise, O-glycans are attached to specific O-glycosylation sites. The commonly found mucin-type O-linked glycosylation involves the attachment of N-acetyl galactosamine moieties to Ser and Thr residues, a process that occurs when the protein has reached the Golgi apparatus. (Lenting *et al*. 2010).

O-glycans are attached to specific O-glycosylation sites in the amino acid chain, but the motifs triggering O-glycosylation are much more heterogenous than the N-glycosylation signals, and our ability to predict O-glycosylation sites in amino acid sequences is still inadequate (Julenius *et al*. Glycobiology (2005), 15(2), 153 and Julenius *et al* Bioinformatics for Glycobiology and Glycomics (2009) 163).

An O-linked oligosaccharide in a truncated Factor VIII B domain may thus be covalently linked to a naturally occurring O-linked glycosylation sequence or an O-linked glycosylation sequence which has been artificially constructed by recombinant techniques.

An example thereof is a B-domain truncated Factor VIII variant wherein the B-domain corresponds to amino acids 742-763 in SEQ ID NO1. This variant comprises an O-glycosylation site in the B domain linker.

Another example is “N8”, a B-domain deleted Factor VIII, the Factor VIII heavy chain comprising amino acid 1-740 of full length human Factor VIII, and Factor VIII light chain comprising amino acid 1649-2332 of full length human Factor VIII. The heavy and light chain sequences are connected by a 21 amino acid linker (SFSQNSRHPSQNPPVLKRHQR – SEQ ID NO 2) comprising the sequence of amino acid 741-750 and 1638-1648 of full length human Factor VIII (Thim *et al.* Haemophilia (2010) 16, 349)

Sialyltransferase: Sialyltransferases are enzymes that transfer sialic acid to nascent oligosaccharide. Each sialyltransferase is specific for a particular sugar substrate. Sialyltransferases add sialic acid to the terminal portions of the sialylated glycolipids (gangliosides) or to the N- or O-linked sugar chains of glycoproteins. There are about twenty different sialyltransferases which can be distinguished on the basis of the acceptor structure on which they act and on the type of sugar linkage they form. Preferred sialyltransferases according to the present invention are ST3Gal-I (specific for O-glycans) and ST3Gal-III (specific for N-glycans). It is thus possible to engineer the structure of the conjugated Factor VIII molecules according to the present invention by e.g. selection of a specific sialyltransferase and/or engineering of a Factor VIII molecule with a particular glycosylation pattern.

Glyco-conjugation of polymers to O-linked or (N)-linked oligosaccharides: The biosynthesis of O-glycans can be modified and terminated with the addition of sialic acid residues relatively early in biosynthesis. Certain sialyltransferase enzymes are capable of acting on GalNAcα-Ser/Thr, or early O-glycan core subtypes after Core 1 GalT action. The term T antigene is associated with the presence of the Galβ1–3GalNAcα-Ser/Thr disaccharide. Production of these structures involves a competition among glycosyltransferases for the same substrate and thus the expression levels and subcellular distributions of glycosyltransferases within the Golgi apparatus determine the structural outcome in O-glycan biosynthesis and diversification. Only the Galβ1–3GalNAcα-Ser/Thr disaccharide is amenable for glyco-derivatization

However, the available amount of this structure may be greatly enhanced through treatment of the protein with a sialidase or Core1 GalT or a combination thereof. As a result of the process ofglyco-conjugation of polymer the sialic acid polymer is added to the terminal

Gal moiety through an α2,3 bond to the Galβ1–3GalNAcα-Ser/Thr disaccharide of the target protein (WO03031464 and WO09108806).

Many hydrophilic polymers can be attached to O-linked oligosaccharides. The basic requirement for enzymatically conjugating hydrophilic polymers to FVIII via the O-glycan is the ability to couple them to the cytidine monophosphate -5'-Glycyl-neuraminic acid (GSC) derivative via the free amino group as disclosed in WO03031464. This may be achieved through a large variety of coupling chemistries known to those skilled in the art. Examples of activated biocompatible polymer includes polyalkylene oxides such as without limitation polyethylene glycol (PEG), 2-(methacryloyloxy)ethyl phosphorylcholine (mPC) polymers (as described in WO03062290), dextrans, colominic acids or other carbohydrate based polymers, polymers of amino acids or of specific peptides sequences, biotin derivatives, polyvinyl alcohol (PVA), polycarboxylates, polyvinylpyrrolidone, polyethylene-co-maleic acid anhydride, polystyrene-co-malic acid anhydride, polyoxazoline, poly-acryloylmorpholine, heparin, albumin, celluloses, hydrolysates of chitosan, starches such as hydroxyethyl-starches and hydroxy propyl-starches, glycogen, agaroses and derivatives thereof, guar gum, pullulan, inulin, xanthan gum, carrageenan, pectin, alginic acid hydrolysates, other bio-polymers and any equivalents thereof.

Side groups can be attached to N-linked oligosaccharides by sialyltransferase mediated methods as disclosed in e.g. WO0331464. Such methods frequently result in attachment of several side groups to the Factor VIII molecule.

Side groups attached to N-linked oligosaccharides of FVIII will be described as (N)-side group FVIII. Side groups attached to O-linked oligosaccharides will be described as (O)-side group FVIII. For example, (O)-PEG(40kD) (N)-PSA(20kD) FVIII means that PEG(40KD) is attached to O-linked oligosaccharides, and PSA(20kD) is attached to N-linked oligosaccharides.

Chemical conjugation: The FVIII variants according to the present invention may be conjugated with PEG and polysaccharide polymers using various chemo-enzymatic methods.

Chemical conjugation of relevant moieties to drugs has usually employed techniques like random derivatization of lysine residues by acylation or reductive alkylation, but the utility of these methods is generally limited, due to heterogeneicity of the product and the most often decreased bioactivity of the products obtained.

Site-selective conjugation methods are essential to be able to exploit the protein structural and biological knowledge available to choose sites which will not affect the protein biological activity, and at the same time obtain the desired effect on stability, pharmacokinetic parameters, immunogenicity, binding to biological partners etc..

5 N-terminal specific, or at least N-terminal preferential conjugation, can be achieved using the fact that the N-terminal primary amino has a pKa of 7.8, whereas that of the ε-amino groups of lysine side chain is much higher.

A more narrow application method uses the introduction of a glyoxyl group at the amino-terminus of a protein. It is however restricted to proteins which can tolerate a harsh 10 periodate oxidation reaction and which contain N-terminal serine or threonine residues.

Thiol selective conjugation to an unpaired cysteine residue is potentially also a useful procedure to achieve site-selective conjugation using a maleimide or haloacetate derivative of the relevant moiety to conjugate. The conjugation can be done on:

- either a naturally free cysteine – free cysteine are rare residues in proteins - but 15 since cysteine is a quite hydrophobic amino acid, it is often buried inside the protein structure, and thus poorly accessible to reagents

- or, more likely, a cysteine residue introduced into the protein by site-directed mutagenesis, but with all the potential problems of possible protein structure change and immunogenicity.

20

Enzymatic conjugation methods are also used and can be a valuable tool for accessing a restricted number of amino acid residues in a protein. For example, out of the thirteen glutamine residues of the human growth hormone, only two are substrates for the microbial transglutaminase enzyme (WO06/134148). (Fontana et al, Adv. Drug Delivery Rev. 25 (2008) 60, 13-28 and references cited therein, Bonora et al. (2009), Post –translational Modification of Protein Biopharmaceuticals, Wiley, 341 and references cited therein)).

PEG: The term “PEG” in connection with the present invention includes poly(ethylene glycol) in any of its forms, including alkoxy PEG, difunctional PEG, multiarmed 30 PEG, forked PEG, branched PEG, pendent PEG (i.e. PEG or related polymers having one or more functional groups pendent to the polymer backbone), or PEG with degradable linkages therein.

The polymer backbone can be linear or branched. Branched polymer backbones are generally known in the art. Typically, a branched polymer has a central branch core moiety 35 and a plurality of linear polymer chains linked to the central branch core. PEG is commonly

used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, pentaerythritol and sorbitol. The central branch moiety can also be derived from several amino acids, such as lysine or cysteine. In one example, the branched poly(ethylene glycol) can be represented in general form as R(-PEG-OH)m in which R represents the core moiety, such as glycerol or pentaerythritol, and m represents the number of arms. Multi-armed PEG molecules, such as those described in U.S. Patent No. 5,932,462, which is incorporated by reference herein in its entirety, can also be used as the polymer backbone.

Although the molecular weight of each chain of the polymer backbone can vary, it is typically in the range of from about 100 Da to about 160,000 Da, such as e.g. from about 5,000 Da to about 100,000 Da. More specifically, the size of each conjugated hydrophilic polymer according to the present invention may vary from about 500 Da to about 80,000 Da, such as e.g. about 1000 Da to about 80,000 Da; about 2000 Da to about 70,000 Da; about 5000 to about 70,000 Da; about 5000 to about 60,000 Da; about 10,000 to about 70,000 Da; about 20,000 to about 60,000 Da; about 30,000 to about 60,000 Da; about 30,000 to about 50,000 Da; or about 30,000 to about 40,000 Da. It should be understood that these sizes represent estimates rather than exact measures. According to a preferred embodiment, the molecules according to the invention are conjugated with a heterogenous population of hydrophilic polymers, such as e.g. PEG of a size of e.g. 10,000, 40,000, or 80,000 Da +/- about 5000, about 4000, about 3000, about 2000, or about 1000 Da.

Polysaccharide

A polysaccharide in connection with the present invention is a polymer based on polysaccharides, including homo- or hetero-polysaccharides, consisting of monomers units like glucose, galactose, sulfo-galactose, N-acetyl-galactose, fucose, fructose, xylose, arabinose, glucuronic acid, sulfo-glucuronic acid, iduronic acid, sulfo –iduronic acid, galacturonic acid, mannuronic acid, glucosamine, N-acetyl-glucosamine, sulfo-glucosamine, galactosamine, N-acetyl-galactosamine, N-acetyl-galactosamine-sulfate, N-acetyl-galactosamine-di sulfate N-acetyl-galactosamine-sulfate, N-acetyl-neuraminic acid (Neu5Ac), Sulfo-N-acetyl-neuraminic acid, N-glycolyl-neuraminic acid (Neu5Gc), 2-keto-3-deoxy-nonulosonic acid (KDN).

Examples of polysaccharides in connection with the present invention include: lactose, starch, hydroxyethyl starch (HES), amylase, dextran sulfate, dextran, dextrins, glycogen, hyaluronic acid, polysorbitol, polymannitol, heparin, heparan sulfate, chondroitin sulfate,

dermatan sulfate, keratin sulphate, heparin or chondroitin sulphate, sulfated polysialic acid and polysialic acid (PSA).

A preferred polysaccharide according to the invention is PSA. PSA is a polymer which is present in mammals, i.e. it is not (or very weakly) immunogenic. There are no known PSA receptor in mammals. PSA has been shown to provide therapeutic proteins with increased resistance to protease degradation. Preferably, most or all of the saccharide residues are N-acetyl-neuraminic acid (Neu5Ac) residues, preferably only Neu5Ac residues. Polysialic acids produced by bacteria are preferred sources of polysialic acids. They include the serogroup C capsular polysaccharide C from N. meningitidis C and the polysaccharide K92 from E.coli K92, and the serogroup B capsular polysaccharide from *Neisseria meningitidis* B and *Escherichia coli* K1, *Moraxella nonliquifaciens*, *Mannheimia haemolytica* A2 (formerly known as *Pasteurella haemolytica* A2). The polysaccharide from *E. coli* K92 comprises alternating alpha2,8 and alpha2,9 linked Neu5Ac units. Polysaccharide C from *N. meningitidis* group C has alpha2,9 linked Neu5Ac units. The prefered polysialic acids are from group B; they comprise 2,8-alpha linked Neu5Ac. The molecular weight of the PSA is preferably higher than or equal to 20kDa, 25 kDa, 30 kDa, 35 kDa, 40 kDa 45 kDa, 50 kDa, 55 kDa, 60 kDa, 65 kDa, 70 kDa, 75 kDa, 80 kDa, 85 kDa, 90 kDa, 95 kDa, or 100 kDa. PSA polymers in connection with the present invention are preferably of a narrow molecular weight distribution.

In the method of the present invention, the reactive aldehyde of the PSA is preferably at the non-reducing end of the polysaccharide. However, the reactive aldehyde may also be provided at the reducing end, as described in US4356170 for example.

In another aspect of the invention, the polysialylated moiety may be generated enzymatically, using a combination of a sialyltransferase and a polysialyltransferase. The sialyltransferase is preferably the *Campylobacter jejuni* sialyltransferase CstII (Gilbert *et al*. JBC (2002) 277, 327) using either the (O)-asialo glycan of N8 as the substrate, or the complex type (N)-glycans of N8 as the substrate.

The resulting glycans carrying an alpha2,3-alpha2,8 linked disialyl end motif can then be used as the substrate for a bacterial polysialyltransferase like the alpha2,8-polysialyltransferase of *N. meningitidis* or *E. coli* K1 (Willis et al., Glycobiology (2008) 18(2) 177, WO 2008/151448 A1, Cho and Troy, PNAS (1994), 91, 11427).

Alternatively, the polysialylated moiety may be generated enzymatically, using a fusion protein comprising a bifunctional sialyltransferase and a polysialyltransferase, as

described in WO 2007/087711 A1. Alternatively, the polysialylated moiety may be generated enzymatically using mammalian alpha2,8-polysialyltransferases like STX (ST8Sia II) and /or PST (ST8Sia IV) using (N)-glycans of N8 as the substrate (Angata *et al.* JBC 277(39)36808 and references cited therein)

5

Pharmaceutical composition: A pharmaceutical composition is herein preferably meant to encompass compositions comprising Factor VIIII antibodies according to the present invention optionally in combination with Factor VIII molecules suitable for parenteral administration, such as e.g. ready-to-use sterile aqueous compositions or dry sterile
10 compositions that can be reconstituted in e.g. water or an aqueous buffer. The compositions according to the invention may comprise various pharmaceutically acceptable excipients, stabilizers, etc.

Additional ingredients in such compositions may include wetting agents, emulsifiers, antioxidants, bulking agents, tonicity modifiers, chelating agents, metal ions, oleaginous
15 vehicles, proteins (e.g., human serum albumin, gelatine or proteins) and a zwitterion (e.g., an amino acid such as betaine, taurine, arginine, glycine, lysine and histidine). Such additional ingredients, of course, should not adversely affect the overall stability of the pharmaceutical formulation of the present invention. Parenteral administration may be performed by subcutaneous, intramuscular, intraperitoneal or intravenous injection by means of a syringe,
20 optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a solution or suspension for the administration of the FVIII antibody compound in the form of a nasal or pulmonal spray. As a still further option, the pharmaceutical compositions containing the FVIII compound of the invention may also be adapted to transdermal administration, e.g. by
25 needle-free injection or from a patch, optionally an iontophoretic patch, or transmucosal, e.g. buccal, administration.

The term “treatment”, as used herein, refers to the medical therapy of any human or other animal subject in need thereof. Said subject is expected to have undergone physical
30 examination by a medical practitioner, who has given a tentative or definitive diagnosis which would indicate that the use of said specific treatment is beneficial to the health of said human or other animal subject. The timing and purpose of said treatment may vary from one individual to another, according to the status quo of the subject’s health. Thus, said treatment may be prophylactic, palliative, and/or symptomatic..

35

While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skilled in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

In a first aspect, the present invention relates to a FVIII variant conjugated with at least one PEG polymer and at least one polysaccharide. Such “heteroconjugated” variants surprisingly have an in vivo circulatory half life that is improved in comparison with “homo-conjugated” FVIII variants (e.g. FVIII-PEG-PEG or FVIII-PSA-PSA variants).

In one embodiment of the present invention, the polysaccharide is PSA.

In another embodiment, said FVIII variant according to the invention is a B domain truncated molecule covalently conjugated with a PEG polymer or a PSA polymer via an O-linked oligosaccharide in the truncated B domain, wherein FVIII activation results in removal of said O-linked polymer.

In another embodiment, said variant is covalently conjugated with at least one PEG polymer or a PSA polymer via an N-linked oligosaccharide. This N-linked oligosaccharide may be naturally occurring or it may be introduced by genetic engineering..

In another embodiment, said FVIII variant is covalently conjugated with a PEG polymer via an O-linked oligosaccharide in the truncated B domain and wherein said variant is covalently conjugated with at least one PSA polymer via an N-linked oligosaccharide. In its activated stage, this FVIII variant may be similar to endogenous activated FVIII if the polymeric groups are conjugated to glycans in the B domain.

In another embodiment, said FVIII variant comprises two to four PSA polymers linked to one double-branched N-linked oligosaccharide in the A1 domain and one double-branched N-linked oligosaccharide in the A3 domain.

In another embodiment, said FVIII variant is covalently conjugated with at least one PEG polymer or a PSA polymer via an N-linked oligosaccharide.

In another embodiment, said FVIII variant is covalently conjugated with a PEG polymer via the O-linked oligosaccharide in the truncated B domain and wherein said variant is covalently conjugated with at least one PSA polymer via an N-linked oligosaccharide.

5 In another embodiment, said FVIII variant comprises two to four PSA polymers linked to one double-branched N-linked oligosaccharide in the A1 domain and one double-branched N-linked oligosaccharide in the A3 domain.

In one embodiment, said FVIII variant comprises one or two PSA polymers linked to one 10 double-branched N-linked oligosaccharide in the A1 domain.

In one embodiment, said FVIII variant comprises one or two PSA polymers linked to one double-branched N-linked oligosaccharide in the A3 domain.

15 In one embodiment, said FVIII variant is covalently conjugated with a PSA polymer via the O-linked oligosaccharide in the truncated B domain and wherein said variant is covalently conjugated with at least one PEG polymer via an N- linked oligosaccharide.

In one embodiment, said FVIII variant is covalently conjugated with a PEG polymer via 20 the O-linked oligosaccharide in the truncated B domain and wherein said variant is covalently conjugated with at least one PSA polymer via an N- linked oligosaccharide.

In one embodiment, said FVIII variant comprises two to four PEG polymers linked to one double-branched N-linked oligosaccharide in the A1 domain and one double-branched N-25 linked oligosaccharide in the A3 domain.

In one embodiment, said FVIII variant comprises one to two PEG polymers linked to one double-branched N-linked oligosaccharide in the A1 domain.

30 In one embodiment, said FVIII variant comprises one to two PEG polymers linked to one double-branched N-linked oligosaccharide in the A3 domain.

In another embodiment, said FVIII variant comprises a PEG polymer having a size of 30-50 kDa.

35

In another embodiment, said FVIII variant comprises a PSA polymer having a size of 15-50 kDa.

In another embodiment, said FVIII variant comprises a PSA polymer having a size of 40-50 kDa.

In another embodiment, said FVIII variant is a B domain truncated FVIII variant, wherein the B-domain comprises the amino acid sequence as set forth in SEQ ID NO 2.

In another embodiment , the polysaccharide is hydroxyethyl starch (HES).

A second aspect relates to a method of making a FVIII variant according to the invention, wherein said method comprises conjugating a FVIII molecule with at least one PEG polymer and at least one polysaccharide.

In one embodiment, at least one of the conjugation steps in said method is an enzymatic process.

A third aspect relates to FVIII variants obtained by or obtainable by a method according to the invention.

A fourth aspect relates to a pharmaceutical composition comprising a FVIII variant according to the invention and optionally one or more pharmaceutically acceptable excipients. Such composition is preferably intended for IV or subcutaneous administration.

A fifth aspect relates to use of a FVIII variant or a pharmaceutical composition according to the invention as a medicament.

A sixth aspect relates to use of a FVIII variant or a pharmaceutical composition according to the invention as a medicament for treating haemophilia A.

A seventh aspect relates to a method of treating haemophilia A comprising administering a therapeutically effective amount of a FVIII variant or pharmaceutical composition according to the invention to a patient.

EXAMPLES

Abbreviations:

DIC: Diisopropyl carbodiimide

HOBt: 1-Hydroxy-benzotriazole

5 THF: Tetrahydrofuran

DCM: Dichloromethane

DMF: Dimethyl formamide

TFA: Trifluoro acetic acid

HC, LC: Heavy and Light Chains of N8

10 CMP: Cytidine monophosphate

GSC: Cytidine monophosphate -5'-Glycyl-neuraminic acid

GSC-ONH_2: 5'-(2-(12-((aminoxymethylcarbonyl)amino)-4,7,10-trioxadodecanoyl)-aminoethanoyl)neuraminic acid cytidine monophosphate

HOAt: 1-Hydroxy-7-aza-benzotriazole

15 PSA: Polysialic acid. Examplified here with α2,8-polysialic acid (colominic acid)

NAN-CMP: N-acetyl neuraminic acid cytidine monophosphate

SEC-MALS: Size-exclusion chromatography with Multi-Angle-Light Scattering detection.

IEX: Ion exchange

20 CV: Column volume

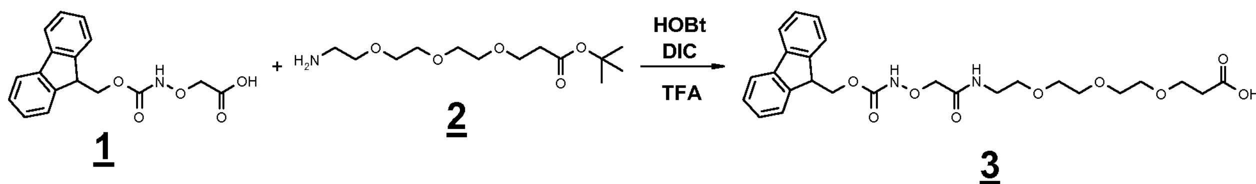
Synthesis of N8 conjugates of the type (O)-PEG40 (N)-PSA-N8

General description: a commercial colominic acid was fractionated on anion exchange column, and the fractions having a molecular weight of either about 20kD or about 25 45kD were pooled. The obtained material was oxidized with sodium periodate. The oxidized PSA was coupled to the GSC-hydroxylamine derivative 5'-(2-(12-((aminoxymethylcarbonyl)amino)-4-7-10-trioxadodecanoyl)aminoethanoyl)-neuraminic acid cytidine monophosphate to give the GSC-ON=PSA reagent which was used as the donor in the ST3Gal-III catalyzed polysialylation of N-asialo (O)-PEG40 N8 (PSA was thus coupled on 30 N-glycans).

A detailed description of the synthesis of the conjugates of this type is given below:

Example 1: Synthesis of 12-((Fmoc-aminoxymethylcarbonyl)amino)-4-7-10-trioxadodecanoic acid **3**:

35



Fmoc-aminoxyacetic acid **1** (1000 mg, 3.2 mmol), 12-amino-4-7-10-trioxadodecanoate t-butyl ester **2** (885 mg; 3,2 mmol), and HOBt (431.5mg; 3.2 mmol) were solubilized in THF (5ml). DIC (402mg; 3.2 mmol) was then added. The mixture was stirred overnight at ambient temperature.

LC-MS analysis showed that the desired product had been formed (m/z =574).

The reaction mixture was partitioned between DCM and sodium hydrogenocarbonate. The organic phase was washed twice with sodium hydrogenocarbonate, dried on sodium sulfate and evaporated.

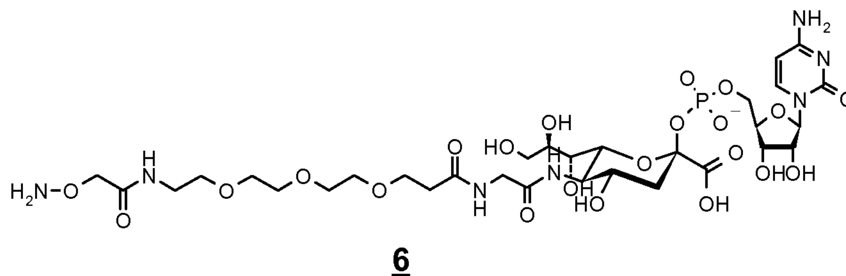
The residue was dissolved in 20% TFA-DCM (10ml), stirred at ambient temperature for 30min, and evaporated. LC-MS analysis showed the presence of the desired product 12-((Fmoc-aminoxymethylcarbonyl)amino)-4-7-10-trioxaundecanoic acid **3** (m/z = 517).

The oily residue was purified by flash chromatography on silica, using solvents A: DCM and solvent B: 5%CH_3OH in DCM, at a flow rate of 40ml/min. The gradient was: 0%B over 0.5CV, o to 100%B over 11.5CV, 100%B over 2.5CV. The product eluted between 90 and 100%B. The relevant fractions were checked on TLC, and the pure fractions pooled and evaporated, giving a colorless oil with a yield of 75%.

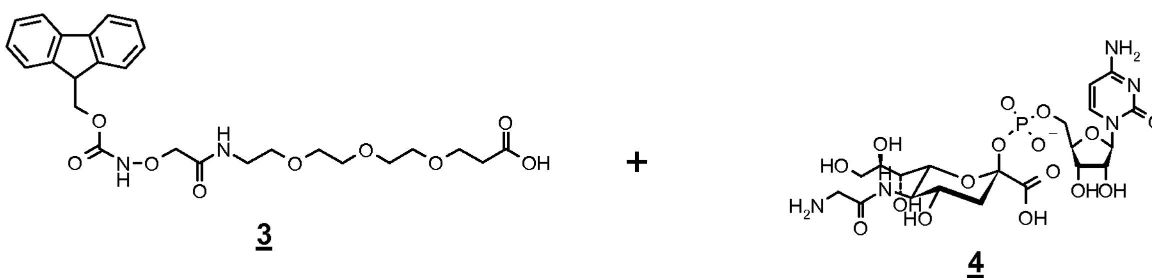
LC-MS: m/z =517

^{1}H-NMR ($CDCl_3$; 400 MHz): δ 2.55 ppm (t, 2H); 3.45-3.75 (m, 10H); 4.22 (t, 1H); 4.42 (s, 2H); 4.52 (d, 2H); 7.32 (t, 2H); 7.41 (t, 2H); 7.57 (d, 2H); 7.75 (d, 2H); 8.07 (bs, 1H); 8.79 (bs, 1H).

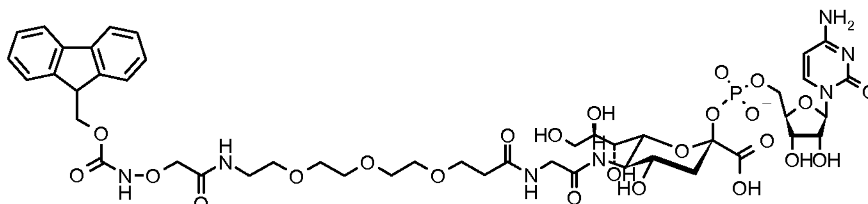
Example 2: Synthesis of the GSC derivative: (5'-(2-(12-((aminoxymethylcarbonyl)amino)-4-7-10-trioxadodecanoyl)-aminoethanoyl)-neuraminic acid cytidine monophosphate) **6 ("GSC-ONH$_2$")**



1st step:



1. HOAt/DIC
2. GSC



5

To a solution of the carboxylic acid **3** (0.52g, 1mmol) in dry THF (5ml) are added HOAt (2.2ml, 1.1 equiv of a 0.5M solution in NMP) and DIC (0.205ml, 1.3mmol, 1.3 equiv). The reaction mixture was stirred for 0.5h at ambient temperature.

The same amount of DIC was then added, followed by a freshly prepared solution of GSC **4** (0.69g, 1.1mmol) in aqueous 100mM HEPES buffer (10ml). The reaction mixture turned yellow. A further addition of DIC (1.1 equiv) was done after 5.5h reaction time.

The reaction mixture was then incubated overnight at ambient temperature.

LC-MS analysis showed that the expected product **5** had been formed (m/z = 1128.7).

The reaction mixture was filtered through a PTFE filter, and purified by HPLC on a reverse phase C18 column using acetonitrile and 250mM ammonium hydrogen carbonate as solvents. The relevant fractions were pooled and lyophilized. The purity was checked before

and after lyophilization by analytical HPLC, on a reverse phase C18 column (Waters Symmetry C18, 5µ, 3.9x150mm), using the solvents A: acetonitrile, B: H_2O, C: 0.5M NH4HCO3 pH7.9. The linear gradient started with a mixture of B:C (90:10) and ended with a mixture A:B:C: (60:30:10) over 15min, at a flow rate of 1ml/min. The column oven was set at
5 a temperature of 42°C. A minor decomposition occurred under lyophilisation (less than 4%).

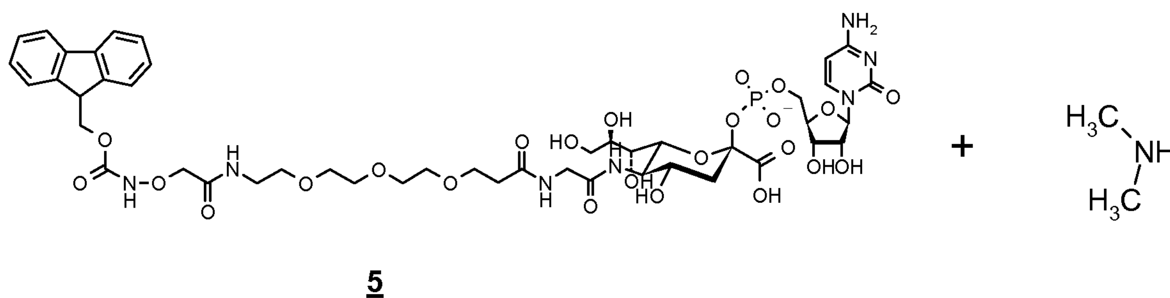
Ammonium cations were then exchanged to sodium using a Dowex 50W resin as follows: Dowex 50WX2, 100-200 mesh (H+ form) (12g) was placed in a 20 ml filter syringe. The resin was washed with 1N NaOH until the eluate was basic (25 ml). The resin was then washed with water until the eluate was pH-neutral. The product was dissolved in THF:H_2O
10 (1:10) (11ml), applied on the resin, and eluted dropwise (7x5ml H_2O). The fractions were spottet on TLC (Mercks Silica gel 60 F_{254} nm); relevant fractions were pooled and lyophilized.

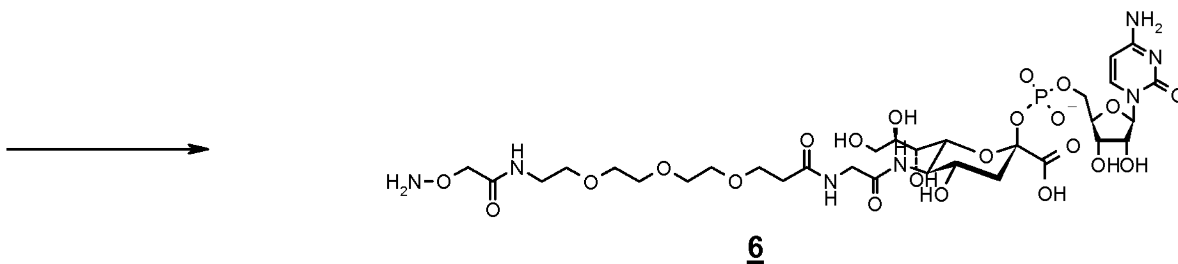
The product was quantified on an HPLC equiped with a nitrogen detector, running the product on a reverse phase Phenomenex Jupiter C18 100x4.6mm, 5µ, 300Å column.

The solvents were A: H2O, B: 2-propanol, C: 1% TFA. The gradient started with a
15 mixture of A:C (90:10), and ended with a mixture (A:B:C) (10:80:10), The flow was 1ml/min. The yield was 46%.

2nd step:

The product was then deprotected with dimethylamine:





20

5 (200 mg) was dissolved in 10% aqueous methanol (3.3 ml). Dimethylamine (3 ml of a 40% solution in H2O), and the reaction mixture stirred for 1.5h at ambient temperature. The mixture became cloudy after 10-15min. The reaction was monitored by LC-MS. The reaction was completed after 1h at 20°C.

The reaction mixture was diluted with water (5ml) and washed with dichloromethane (4x5ml). Both phases were checked on LC-MS. The aqueous phase contained the product, and the fluorene moiety could not be detected. In the organic phase, no product could be detected. The aqueous phase was lyophilized, giving the GSC derivative 5'-(2-(12-((aminoxymethylcarbonyl)amino)-4-7-10-trioxadodecanoyl)aminoethanoyl)-neuraminic acid cytidine monophosphate ("GSC-ONH_2") **6** as a colorless solid.

Example 3: Colominic acid fractionation to get a material of about 20kDa in molecular weight:

The colominic acid used was the commercial compound from Sigma-Aldrich (α2,8 polysialic acid sodium salt, (PSA) from *Escherichia coli*). In order to get a more homogenous material (regarding its molecular weight), it was fractionated on an ion exchange column according to WO 2008/074032. The fraction corresponding to a molecular weight of about 20kD was used in the subsequent experiments.

Example 4: Sodium periodate oxidation of the 20kD PSA material isolated in example 3

:

The sodium periodate oxidation of the polyol at the non reducing end of the PSA polymer was performed essentially as described in the literature (for example: Jain *and al.*, BBA (2003) 1622, 42-49), with some modifications To a solution of 20kD PSA (40mg in 2.24ml H_2O) was added a sodium periodate solution (0.96mg in 2.244ml H2O). The reaction was incubated for 15min at 23°C in the dark.

The excess of periodate was quenched by 3-methylthio-1-propanol (4.7µl). The reaction was further incubated for 2h at 23°C.

The reaction mixture was buffer shifted to water by ultra filtration on Millipore Ultra, 5kD cut-off and lyophilized. The lyophilized material was used as such in the next step, where it was reacted with GSC-ONH2..

Example 5: Coupling of sodium periodate oxidized PSA(20kD) to GSC-ONH2 to yield the sialyltransferase ST3GalIII substrate GSC-ON=PSA(20kD):Solutions:

- Reaction buffer: 100mM imidazole pH6.8
- GSC-ONH2 (from example X2):8.2mg/ml in reaction buffer
- Periodate oxidized PSA(20kD): 175mg/ml in reaction buffer
- aniline (MW= 93.13, d=1.0217)

- Methylhydroxylamine hydrochloride: 58.5mg/ml in reaction buffer

Procedure:

To the periodate oxidized PSA(20kD) solution in reaction buffer (200µl, 35mg, 1.75µmole)
5 was added the GSC-ONH2 solution in reaction buffer (400µl, 3.26mg, 3.6µmoles, about 2 equiv.). The pH was adjusted to 6.9 by addition of 1M HCl (5.5µl) under vigorous magnetic stirring. Aniline (0.56µl, 6nmoles) was then added. The yellowish and slightly cloudy mixture was incubated at 25°C. Some precipitation was observed after 10-15min.

The reaction progress was followed by analysis on a size exclusion column Waters Biosuite
10 125, HR ESC 300x7.8mm (+guard column), with 100mM phosphate buffer pH6.8 buffer as eluent, a flow of 0.6ml/min, at ambient temperature, with a DAD detector at 212 and 272nm. An analysis was run after 30min, 2h and 18h reaction time.

GSC-ONH2 elutes at 18.5min in this system. The product elutes as a broad “peak” at a retention time of about 13.8min.

15 Since both PSA(20kD) and the product GSC-ON=PSA(20kD) elute at the same retention time, the progress of the reaction was monitored by looking at the ratio: (area of product peak at 272nm) over (area of product peak at 212nm) (PSA absorbs only at 212nm, GSC absorbs at 272nm). The ratio increased from 30min to 2h, and remained constant until 18h reaction time.

20 After 19h reaction time, any unreacted aldehyde was quenched by addition of the methylhydroxylamine solution, (25µl, 10 equiv), and the mixture incubated for 1h at ambient temperature.

The reaction mixture was then filtered on 0.45µ filter (Millipore Millex-HV (PVDF)), and further purified on ProSpin CS-800 (Princeton Separations) conditioned in 1.5 g/l L-Histidine,
25 3 g/l Sucrose, 18 g/l NaCl, 0.1 g/l Tween 80; 0.25 g/l $CaCl_2$,$2H_2O$, pH7.3 buffer, to get rid of low molecular weight reagents.

The quantification of the final product was done relative to CMP (Sigma C1006): a standard curve was done by measuring the absorption of CMP solutions of known concentrations at 272nm.

30 GSC-ON=PSA(20kD) was obtained with a yield of 45% relative to periodate oxidized PSA.

Example 6: Preparation of (N)-PSA (20kD)-(O)-PEG (40kD)-N8 by sialyltransferase ST3Gal-III catalyzed reaction of (N)-asialo (O)-PEG(40kD) N8 with GSC-ON=PSA(20kD):

35 1st step:

(O)-PEG(40kD) (N)-asialo-N8 N8

The compound was synthesized according to the procedure disclosed in Patent WO2009/108806 A1.

5 2nd step:

ST3Gal-III catalyzed PSAylation of (O)-PEG(40kD) (N)-asialo N8 with GSC-ON=PSA(20kD):

Solutions:

- Reaction buffer: 1.5 g/l L-Histidine, 3 g/l Sucrose, 18 g/l NaCl, 0.1 g/l Tween 80; 0.25 g/l $CaCl_2,2H_2O$, pH7.3
10 - GSC-ON=PSA(20kD): 0.78mM in reaction buffer
- ST3Gal-III: (rat enzyme): 1.42mg/ml (1.34U/mg)
- (O)-PEG(40kD)-(N)-asialo N8: 1.76mg/ml in reaction buffer

Procedure:

To the (O)-PEG(40kD) (N)-asialo N8 solution (272µl, 0.48mg protein, 2.71nmoles) was
15 added the GSC-ON=PSA solution (36.5µl, 28.5nmoles, 10.5 equiv). Reaction buffer (104µl) was added. The reaction was started by addition of the enzyme (63.2µl, 89.6µg, about 120mU). The reaction mixture was incubated at 32°C for 22h.

The product was capped by addition of a solution of NAN-CMP (1mg) in 10µl reaction buffer. The reaction mixture was incubated for 1h at 32°C

20 Work-up and purification:

The buffers used were:

- Buffer A: 20mM imidazole buffer pH 7.4 containing 10mM CaCl2, 1M glycerol, 0.02% Tween 80, without NaCl
- Buffer B. buffer A + 1M NaCl
25 - Reaction buffer: 1.5 g/l L-Histidine, 3 g/l Sucrose, 18 g/l NaCl, 0.1 g/l Tween 80, 0.25 g/l $CaCl_2,2H_2O$, pH7.3

Work-up and purification:

After dilution in buffer A (8ml), the reaction mixture was purified by ion exchange on a Vivapure Q Mini M device according to the manufacturer instructions. The product was
30 recovered in buffer B.

The product was further run on the size exclusion column Superdex 200 10x300 GL (GE Healthcare), using the reaction buffer as eluent.

The protein recovery was 32%.

Product characterization:

35 - SDS PAGE analysis:

The recovered product was run on a 7% Tris acetate SDS gel (150V, 1h10) (Invitrogen) under reducing conditions, using Coomassie blue staining. The protein standard was the HiMark unstained HMW Protein Standard from Invitrogen.

The pegylated heavy chain band of (O)-PEG (40kD) (N)-asialo N8, appeared at about 240kD
5 and the light chain at about 83kD.After PSAylation, a band assumed to correspond to the PSAylated heavy chain appeared at a higher MW, (between 260 and 280kD, as expected. In addition, a wide and diffuse band, assumed to correspond to the PSAylated light chain, appeared at between 97 and 116kD.

No remaining band corresponding to the heavy chain of N8 could be detected, and only
10 traces of the light chain could be seen, showing that PSA was indeed transferred on both heavy and light chain of (O)-PEG40kD-N8. - Analysis on reverse phase HPLC:

The analysis was run on a reverse phase Daiso 300Å, 250x2.1, 5μ column. The eluents were: A: H2O/TFA 0.1%, and B: H2O/ACN/TFA (80:20:0.09%), the flow 0.25ml/min, and the temperature of the column oven 40C. The gradient was from 35% to 84% over 30min. The
15 HPLC was equipped with two detectors: a DAD detector (280nm) and a fluorescence detector with the excitation wawelength at 280nm, and the emission wawelength at 348nm. The retention times of the heavy chain and light chain of the product were as indicated in the table below. The retention times of the heavy chain and light chain of FVIII and of the intermediate (O)-PEG (40kD)-N8 are indicated for comparison:

20

Sample / Rt	N8	(O)-PEG(40kD) N8-	(O)-PEG(40kD) (N)-PSA(20kD)-N8-
Rt LC	19.98min	19.95min	19.85min
Rt HC	24.35min	23.84min	23.64min

Thus, as expected for the more polar final product [(N)-PSA (20kD)-(O)-PEG (40kD) N8], the retention times of the heavy and light chains are shorter than the retention times for the HC and LC of the starting and intermediate compounds. It is somewhat surprising that one does
25 not obtain a larger effect on the retention time after coupling of the polysialic acid. But the system does not reflect physiological conditions, as the acid present in the eluent is neutralizing the negative charge from the carboxylic acids moieties of PSA.

- Activity:

The activity of the final product was measured in the chromogenic assay CoA test SP FVIII from Chromogenix: compared to the starting FVIII, more than 80% of the activity was recovered.

5

Example 7: Colominic acid fragmentation to get a material of about 45kD in molecular weight:

The colominic acid used was the commercial compound from Sigma-Aldrich (α2,8 polysialic acid sodium salt, (PSA)). In order to get a more homogenous material (regarding its

10 molecular weight), it was fractionated on a HiPrep 16/10 Q FF anion exchange column (GE Healthcare) using buffers A and B:

A: 10mM Triethanol amine pH7.4, 25mM NaCl

B: 10mM Triethanol amine pH7.4, 1M NaCl

After equilibration of the column with 8CV of buffer A, the colominic acid was fractionated

15 (5ml fractions) using a gradient from 17.5% to 100%B over 24CV with a flow of 2ml/min. The UV detection was at 210nm. The fractions were buffer shifted to water by ultrafiltration on Millipore Amicon Ultra 3kD cut-off, lyophilized, and analysed by SEC-MALS and UV. The fractions corresponding to a molecular weight of about 45kD (molecular weight at maximum UV absorption), with a molecular weight range of 38-77kD ("45kD PSA") were pooled and

20 used in the subsequent experiments.

Example 8: Sodium periodate oxidation of 45kD PSA:

To an aqueous solution of the material obtained in example 7("45kD PSA") (13.5mg in 0.5ml water) was added a 4mM aqueous sodium periodate solution (167µl, 2.24 molar quivalents).

25 The reaction was incubated for 15min at ambient temperature in the dark.

The excess of sodium periodate was quenched by 3-methylthio-1-propanol (0.7µl, 12 molar equivalents). The reaction was further incubated for 2h at ambient temperature.

The reaction mixture was buffer shifted to water by ultra filtration on Millipore Amicon Ultra-4, 5kD cut-off and lyophilized. The lyophilized material was used as such in the next step.

30

Example 9: Coupling of sodium periodate oxidized PSA(45kD) to GSC-ONH2 to yield the sialyltransferase ST3GalIII substrate GSC-ON=PSA(45kD):

Solutions:

- Reaction buffer: 100mM imidazole pH6.8

35 - GSC-ONH2 (from example 2): 8.3mg/ml in reaction buffer (pH adjusted to 6.8).

- Periodate oxidized 45kDa PSA (from example Y2): 355mg/ml in reaction buffer
- saturated aqueous aniline solution (about 0.38M)
- Methylhydroxylamine hydrochloride: 58.5mg/ml in reaction buffer

Procedure:

5 The reaction is run essentially as described in example 5. The detailed description is included below: To the periodate oxidized 45kD PSA (from example 8) solution in reaction buffer (92µl, 32.7mg, 0.73µmole) was added the GSC-ONH2 (from example 2) solution in reaction buffer (168µl, 1.4mg, 1.5µmoles, about 2 equiv.). Saturated aqueous solution of aniline (6.8µl, 2.58µmoles) was then added. The reaction mixture was incubated at ambient 10 temperature.

The reaction progress was followed by HPLC analysis using a size exclusion column Waters Biosuite 125, HR ESC 300x7.8mm (+guard column). The eluent was 100mM phosphate buffer pH6.8 buffer, the flow was 0.6ml/min. The analysis was run at ambient temperature, with a DAD detector at 214 (PSA and GSC moiety detection) and 272nm (GSC 15 moiety detection). GSC-ONH2 eluted at 18.5min, the oxidized 45kD PSA and the reaction product eluted at the same retention time of 10.3min.

Since both oxidized 45kD PSA and the product GSC-ON=PSA elute at the same retention time, the progress of the reaction was monitored by looking at the ratio: (area of oxidized 45kD PSA/GSC-ON=PSA peak at 272nm) over (area of oxidized 45kD PSA/GSC-20 ON=PSA at 212nm).

An analysis was run after 1h, 1h45, 3h, 4h30, 5h30, 10h and 23h30 reaction time.

The ratio increased from 1h to 1h45, but did not change between 1h45 and 3h. More GSC-ONH2 reagent was thus added (32µl, 267µg) at 4h reaction time. Likewise, more reagent was added after 10h reaction time (0.9mg) and the mixture was left at ambient 25 temperature for a total of 23h30.

The reaction mixture was purified on a Superdex 200 10/300 GL (GE Healthcare) column using a Micro Äkta system (GE Health care). The eluent was 20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, 0.5M NaCl, with a flow of 0.4ml/min, with fraction volume of 0.5ml. Detection was at 210 and 272nm. The relevant fractions were 30 pooled, upconcentrated by ultra filtration on Millipore Amicon Ultra cut off 5kD and used as such in next step. The concentration of the final product was estimated to be 0.27mM (by comparison of a CMP (Sigma C1006) standard curve at 272nm).

Example 10: Preparation of N-PSA(45kD) (O)-PEG(40kD) N8 glycan by sialyltransferase ST3Gal-III catalyzed reaction of (N-asialo) (O)-PEG(40kD) N8 with GSC-ON=PSA(45kD):

1st step:

5 (O)-PEG40kD (N)-asialo N8:

The compound was synthesized according to the procedure described in Patent WO2009/108806 A1.

2nd step:

ST3Gal-III catalyzed PSAylation of (N)-asialo (O)-PEG(40kD) (N)-asialo N8 with GSC-

10 ON=PSA(45kD):Solutions:

- Reaction buffer: 20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, 0.5M NaCl.
- asialo-[O]-PEG40kD-N8: 2.78mg/ml
- GSC-ON=PSA(45kD 0.27mM in reaction buffer

15 - ST3Gal-III: (rat enzyme): MBP-SBP-ST3Gal III: 1mg/ml

Procedure:

To the (N) asialo (O)-PEG40kD-N8 solution (325µl, 0.9mg protein, 5.13nmoles) was added the GSC-ON=PSA(45kD) solution (190µl, 51.3nmoles, 2.3mg, 10 equiv). The reaction was

20 started by addition of the enzyme (116.3µl, 116.3µg). The reaction mixture was incubated at 32°C for 17h.

The product was capped by addition of a solution of NAN-CMP (1.2mg) in 15µl reaction buffer. The reaction mixture was incubated for 1h at 32°C.

Work-up and purification:

25 The reaction mixture was diluted to 16ml with 20mM imidazol buffer pH7.3, 10mM CaCl2, 1M glycerol, 0.02% Tween 80, 25mM NaCl before purification by ion exchange on MonoQ 5/50 GL (GE Healthcare). The buffers used were: buffer A: 20mM imidazole buffer pH 7.4 containing 10mM CaCl2, 1M glycerol, 0.02% Tween 80 (no NaCl), and buffer B: 20mM imidazole buffer pH 7.4 containing 10mM CaCl2, 1M glycerol, 0.02% Tween 80, 1M NaCl.

30 The flow was 0.7ml/min. The column was equilibrated for 20CV. The elution profile was as follows: 0%B over 3CV, 0-20%B over 5CV, 20%B over 15CV, 20 to 100%B over 15CV, 100%B over 10CV. The UV detection was at 280nm. The fractionation was run at ambient temperature. The enzyme is eluted first, the product elutes later as a peak with a small shoulder. The fractions corresponding to the major peak were pooledand further purified and

35 buffer exchanged on on the size exclusion column Superdex 200 10x300 GL (GE

Healthcare), using a buffer containing 1.5 g/l L-Histidine, 3 g/l Sucrose, 18 g/l NaCl, 0.1 g/l Tween 80; 0.25 g/l $CaCl_2$,$2H_2O$, pH7.3 as the eluent. The flow was 0.5ml/min. The UV detection was at 280nm. The product eluted as a major peak, followed by a minor peak, with base line separation between the peaks. The fraction corresponding to the major peak were
5 pooled and upconcentrated by ultrafiltration on Millipore Amicon Ultra, 50kD cut off. The protein recovery was 52%.

Product characterization:

- SDS PAGE analysis:

The recovered product was run on a 7% Tris acetate SDS gel (150V, 1h10) (Invitrogen)
10 under reducing conditions, using Coomassie blue staining. The protein standard was the HiMark unstained HMW Protein Standard from Invitrogen.

With (O)-PEG (40kD)-N8, the pegylated heavy chain band appeared at about 240kD. After PSAylation, a band at higher MW appeared at about 290kD. In addition, a wide and diffuse band (assumed to correspond to the PSAylated light chain) appeared at between120 and
15 160kD.

A very faint band corresponding to the molecular weight of the heavy chain of FVIII could be detected, and traces of a band corresponding to the the light chain of FVIII could be seen.

- Analysis on HPLC:

The analysis was run on a reverse phase Daiso 300Å, 250x2.1, 5µ column. The eluents
20 were: A: H2O/TFA 0.1%, and B: H2O/ACN/TFA (80:20:0.09%), the flow 0.25ml/min, and the temperature of the column oven 40C. The gradient was from 35% to 84% over 30min. The HPLC was equiped with two detectors: a DAD detector (214nm). The retention times of the heavy chain and light chain of the product were as indicated in the table below. The retention times of the heavy chain and light chain of N8 and of the intermediate (O)-
25 PEG(40kD)-N8 are indicated for comparison:

Sample / Rt	N8	(O)-PEG(40kD)-(N)-asialo N8	(N)-PSA (45kD)-(O)-PEG (40kD)-N8
Rt LC	25.50min	25.46min	25.49min

Rt HC	29.95min	29.47min	29.38min

The same general profile is obtained for (N)-PSA (45kD)- (O)-PEG (40kD)-N8 as for [(N)-PSA (20kD)-(O)-PEG (40kD) N8 (cf example 6), i.e., the more polar final product (N)-PSA (45kD)-(O)-PEG (40kD) N8], shows retention times of the heavy and light chains that are shorter than the retention times for the HC and LC of the starting and intermediate compounds as expected. Activity:

The activity of the final product was measured in the chromogenic assay CoA test SP FVIII from Chromogenix according to the manufacterer instructions: compared to the starting N8 , about 55% activity was recovered.

Example 11: Synthesis of N8 conjugates of the type (O)-PSA (N)-PSA-N8

General description: N8 was desialylated (reaction catalyzed by the sialidase from *Arthrobacter ureafaciens*) to give the (O)-asialo (N)-asialo N8. PSA was transferred onto the (O)-asialo glycan by the ST3Gal-I catalyzed reaction of GSC-ON=PSA (examples 5 or 9) with (O)-asialo (N)-asialo N8. After purification by ion exchange, the GSC-ON=PSA reagent was used as the donor in the ST3Gal-III catalyzed polysialylation of N-asialo (O)-PEG40 N8. Finally, any remaining unreacted galactose moiety was capped by adding NAN-CMP to the reaction mixture.

A detailed description of the synthesis of the conjugate of this type is given below:,

1st step: Preparation of (O)-PSA820kD) (N)-asialo N8 by desialylation of N8 and ST3Gal-I catalyzed transfer of PSA onto (O)-asialo glycans of N8 (one pot reactions):

Solutions:

- Reaction buffer: 20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, 0.5M NaCl.
- N8: 5.7mg/ml in reaction buffer (8650U/mg)
- Sialidase: from *Arthrobacter ureafaciens* . 0.4mg/ml, 242U/mg
- GSC-ON=PSA(20kD) : 25mg/ml in (3 g/l Sucrose, 1.5 g/l L-Histidine, 18 g/l NaCl, 0.1 g/l Tween 80; 0.25 g/l $CaCl_2$; pH7.3).
- His-ST3Gal-I; AA46-343; 2.5mg/ml in (50mM Tris pH8, 100mM NaCl)

Procedure:

To a solution of N8 in reaction buffer (1.5mg, 8.5nmoles, 263µl) was added a solution of the *A. ureafaciens* sialidase (7µl, 678mU, 1.5U/ml final) and a solution of the ST3Gal-I enzyme (108µl, 0.27mg). A solution of GSC-ON=PSA(20kD) was added (68µl, 1.7mg, about 85nmoles, about 10 equiv). The reaction mixture was incubated at 23°C for 24h.

5

Work-up and purification:

The reaction mixture was diluted twenty times with a buffer containing (20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol), and purified on an ion exchange column (MonoQ 5/50GL, GE Healthcare). The elution buffers were: buffer A: 20mM
10 imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, and buffer B: buffer A added 1.5M NaCl. The flow was 0.35ml/min. The purification was run at 15°C. The detection was done by UV, 280nm. The elution was as follows: from 0 to 20% B over 5CV, from 20 to 100%B over 25CV, 100%B for 5CV. 1ml fractions were collected in a 96 deep well plate. Relevant fractions were analyzed by SDS PAGE (7% Tris acetate SDS gel (150V, 1h10)
15 (Invitrogen) under reducing conditions, using silver staining. The protein standard was the HiMark unstained HMW Protein Standard from Invitrogen). Fractions corresponding to the main peak contain a mixture of N8 (about 35% of N8 is not O-glycosylated (Thim *et al.* Haemophilia (2010), 16(Suppl 5), 194)) and (O)-PSAylated N8. Traces of the sialidase or the ST3Gal-I enzyme could not be detected.
20 Fractions corresponding to the main peak were pooled and upconcentrated by ultrafiltration (Millipore Amicon Ultra, cut off 50kD), giving a solution with a protein concentration of 5.5mg/ml according to reverse phase HPLC analysis (for HPLC method details: see example 10). The protein recovery was about 79%.

25 **2nd step**: : Synthesis of (O)-PSA(20kD) (N)-PSA(20kD) N8 by ST3Gal-III catalyzed transfer of PSA onto (N)-asialo glycans of (O)-PSA(20kD) (N)-asialo N8:

Solutions:

- Mixture of (O)-PSA(20kD)-(N)-asialo N8 and N8 (from step 1): 5.5mg protein/ml
- GSC-ON=PSA(20kD) : 25mg/ml in (3 g/l Sucrose, 1.5 g/l L-Histidine, 18 g/l NaCl, 0.1 g/l
30 Tween 80; 0.25 g/l $CaCl_2$; pH7.3).
- ST3Gal-III: (MBP-SBD-ST3Gal-III) 0.33mg/ml in (14mM Hepes pH7, 140mM NaCl, 50% glycerol). 0.54U/ml. Upconcentrated (about 15times) by ultrafiltration on Millipore Biomax cut off 5kD.
- NAN-CMP: 50mg/ml in 20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M
35 glycerol

Procedure:

To the mixture of (O)-PSA(20kD)-(N)-asialo N8 and N8 obtained in the first step (210µl, 6.4nmoles) is added a solution of GSC-ON=PSA(20kD) (26µl, 32nmoles). The reaction was started by the addition of the ST3Gal-III enzyme solution (40µl, 324mU, 198µg). The reaction mixture was incubated at 32°C. After 3h reaction time, a new portion of the GSC-ON=PSA(20kD) solution was added (20µl, 25nmoles) The reaction mixture was incubated for 21h.

Capping:

To the reaction mixture above was added a solution of NAN-CMP (10µl, 0.5mg). The mixture was incubated at 32°C for 2h.

Work-up and purification:

The reaction mixture was diluted twenty times in 20mM imidazole buffer pH7.4, 10mM CaCl2, 0.02% Tween 80, 1M glycerol.

It was then purified on an IEX membrane (Sartorius Vivapure Q Mini M) according to the manufacterer instructions, using buffer A (20mM imidazole buffer pH7.4, 10mM CaCl2, 0.02% Tween 80, 1M glycerol) as the washing buffer and buffer B (buffer A added NaCl to1M concentration) as the elution buffer.

The eluted product was upconcentrated by ultra filtration (Millipore Amicon Ultra device, cut-off 50kD) before purification and buffer shift on a size exclusion column (Superdex 200 10/30GL, GE Healthcare). The buffer contained sucrose (3 g/l), L-Histidine (1.5 g/l), NaCl (18 g/l), Tween 80 (0.1g/l), and $CaCl_2$ (0.25 g/l) pH7.3. The flow was 0.4ml/min, the detection was by UV at 280nm. 0.5ml fractions were collected.

Remaining ST3Gal-III (probably aggregates) appeared as a shoulder eluting before the main peak. Fractions corresponding to the main peak (and not containing St3Gal-III) were pooled. and quantified by HPLC (see example 10 for HPLC method details). The overall protein recovery (starting from N8) was 28%.

Product characterization:

- SDS PAGE analysis:

The recovered product was run on a 7% Tris acetate SDS gel (150V, 1h10) (Invitrogen) under reducing conditions, using Coomassie blue staining. The protein standard was the HiMark unstained HMW Protein Standard from Invitrogen.

A very wide and diffuse band appeared between 97kD and 160kD: this is assumed to correspond to the PSAylated heavy and light chains. Traces of underivatized heavy chain and the light chain are detectable. The band appearing between 240 and 280kD was assumed to correpond to the PSAylated single chain N8..

5 - Analysis on HPLC:

The analysis was run as indicated in example 10.

The retention times of the heavy chain and light chain of the product were as indicated in the table below. The retention times of the heavy chain and light chain of N8 are indicated for comparison:

Sample / Rt	N8	(O)-PSA(20kD) N-PSA(20kD) N8
Rt LC	25.44min	25.39min
Rt HC	29.89min	29.61min

10

Thus, the PSAylated HC retention time decreases (and the peak appears wider) as expected for a more polar protein. The effect is less obvious for the PSAylated light chain, also reflecting the fact that only two potential derivatization sites are available, while three are available for the heavy chain ((O)- and (N)-glycans of the HC).

15 - Activity:

The activity of the final product was measured in the chromogenic assay CoA test SP FVIII from Chromogenix: compared to the starting FVIII: about 55% activity was recovered.

Examples 12 and 13: Synthesis of N8 conjugates of the type (N)-PSA-N8

20 General description: N8 was desialylated using the sialidase from *Clostridium perfringens* to give the (N)-asialo N8. The GSC-ON=PSA reagent was used as the donor in

the ST3Gal-III catalyzed polysialylation of N-asialo (O)-PEG40 N8. Finally, any remaining unreacted galactose moiety was capped by adding NAN-CMP to the reaction mixture.
A detailed description of the synthesis of the conjugates of this type is given below:,

5 **Example 12:** Synthesis of (N)-PSA(45kD) N8:

1st step: Desialylation of N8 by *C. perfringens* sialidase:

Solutions:

- Reaction buffer: 20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, 0.5M NaCl.
10 - sialidase: 0.3mg/ml 200U/ml
- N8: 5.7mg/ml in reaction buffer

Procedure:

To the N8 solution (350µl, 2mg) was added the reaction buffer (350µl) and the enzyme
15 solution (20µl, 4U). The mixture was incubated for 45min at 23°C.

Work-up and purification:

The reaction mixture was diluted ten times with (20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, 0.15M NaCl). The solution obtained was purified on an anion
20 exchange column (MonoQ 5/50 GL, GE Healthcare) on an Äkta Purifier (GE Healthcare). The buffers used were: buffer A: 20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, 25mM NaCl, and buffer B: buffer A with 1M NaCl. The flow was: 0.5ml/min, the detection was by UV, 280nm. The elution was done as follows: from 0 to 20%B over 5CV, 20%B over 10CV, 100%B over 10CV. The eluate was collected in 0.5ml fractions in the
25 last gradient step. The protein recovery was 45%.

2nd step: Synthesis of (N)-PSA(20kD) N8 by ST3Gal-III catalyzed transfer of PSA onto (N)-asialo N8:

30 Reagents:

- Reaction buffer: 20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, 0.5M NaCl.
- [N]-asialo-N8: 2.39mg/ml in 20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, 0.25M NaCl
35 - GSC-ON=PSA(45kD): about 0.27mM in reaction buffer (12.1mg/ml)

- ST3Gal III: (rat enzyme): MBP-SBP-ST3Gal III, 1mg/ml, 1.2U/mg

Procedure:

To the solution of [N]-asialo-N8 (364µl, 0.87mg protein) was added the solution of GSC-ON=PSA(45kD) (183µl, 2.22mg), The reaction was started by addition of the enzyme (122µl, 122µg, 146mU). The mixture was incubated overnight at 32°C.

Capping:

A solution of NAN-CMP (1.3mg in 15µl reaction buffer) was added and the resulting mixture incubated for 1h at 32°C.

Work-up and purification:

The reaction mixture was diluted ten times with 20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, 25mM NaCl, and purified by anion exchange (MonoQ 5/50 GL, GE Healthcare) on an Äkta Purifier system (GE Healthcare). The buffers used were: buffer A: 20mM imidazole buffer pH7.3, 10mM CaCl2, 0.02% Tween 80, 1M glycerol, 25mM NaCl, and buffer B: buffer A with 1M NaCl. The flow was: 0.7ml/min, the detection was by UV, 280nm. The purification was run at 15°C. The elution was done as follows: from 0 to 20%B over 5CV, 20%B over 15CV, 20 to 100%B over 15CV, 100%B over 10CV. The eluate was collected in 0.5ml fractions. The protein recovery was 32%.

Characterization of the product:

- SDS PAGE analysis:

The recovered product was run on a 7% Tris acetate SDS gel (150V, 1h10) (Invitrogen) under reducing conditions, using Coomassie blue staining. The protein standard was the HiMark unstained HMW Protein Standard from Invitrogen.

A rather wide and diffuse band appeared between about 125 and 165kD: this is assumed to correspond to the PSAylated heavy and light chains. Traces of underivatized heavy chain and light chain are detectable.

- Reverse phase HPLC analysis:

The analysis was run as indicated in example 10.

The retention times of the heavy chain and light chain of the product were as indicated in the table below. The retention times of the heavy chain and light chain of N8 are indicated for comparison:

Sample / Rt	N8	(N)PSA(45kD) N8
Rt LC	25.46min	25.43min
Rt HC	29.92min	29.72min

Thus, the PSAylated HC retention time decreases (and the peak appears wider) as expected for a more polar protein. The effect is almost negligeable on the retention time of the PSAylated light chain.

5 - Activity:

The activity of the final product was measured in the chromogenic assay CoA test SP FVIII from Chromogenix: compared to the starting FVIII: about 60% activity was recovered.

Example 13: Synthesis of (N)-PSA(20kD) N8

10 The synthesis was performed similarly to the synthesis of (N)-PSA(45kD) N8. The protein recovery was 39%.

Characterization:

-SDS-PAGE analysis: performed as in example 12.

15 A very wide and diffuse band appeared between about 97 and 160kD: this is assumed to correspond to the PSAylated heavy and light chains. Bands corresponding to traces of underivatized heavy chain (traces) and light chain (sizable amounts) are detectable.

- Reverse phase HPLC analysis:

The analysis was run on a reverse phase Daiso 300Å, 250x24, 5µ column. The eluents were:

20 A: H2O/TFA 0.1%, and B: H2O/ACN/TFA (80:20:0.09%), the flow was 1ml/min, and the temperature of the column oven 40C. The gradient was from 35% to 84% over 30min. The HPLC was equiped with two detectors: a DAD detector (214nm). The retention times of the heavy chain and light chain of the product were as indicated in the table below. The retention times of the heavy chain and light chain of N8 are indicated for comparison:

Sample / Rt	N8	(N)-PSA(20kD) N8
Rt LC	17.48min	17.49min
Rt HC	21.92min	21.77min

Thus, as for the (N)-PSA(45kD) N8 compound, the PSAylated HC retention time decreases (and the peak appears wider) as expected for a more polar protein. The effect is negligeable on the retention time of the PSAylated light chain.

- Activity:

The activity of the final product was measured in the chromogenic assay CoA test SP FVIII from Chromogenix: compared to the starting FVIII: about 88% activity was recovered.

3. PK studies in FVIII KO mice: Comparison of half-lives of various N8 glyco-PEG/PSA derivatives.

Example 14: Pharmacokinetic characterisation of N8 glyco-conjugates:

The pharmacokinetics of rFVIII variants were evaluated in FVIII-deficient mice (FVIII exon 16 knock out (KO) mice with C57Bl/6 background. The FVIII-KO mice had no detectable FVIII:C. A mixture of male and female (approximately 1:1) with an approximate weight of 25 grams and age range of 16-28 weeks were used. The mice received a single i.v. injection of rFVIII (280 IU/kg) in the tail vein. Blood was taken from the orbital plexus at time points up to 64 hours after dosing using non-coated capillary glass tubes. Three samples were taken from each mouse, and 2 to 4 samples were collected at each time point. Blood was immediately stabilized with sodium citrate and diluted in four volumes FVIII Coatest SP buffer (50mM Tris, 150mM NaCl, 1% BSA, pH7.3, with preservative) before 5 min centrifugation at 4000 × g. Plasma obtained from diluted blood was frozen on dry ice and kept at -80°C. The FVIII:C was

determined in a chromogenic assay using Coatest SP reagents (Chromogenix) according to the manufacturer instructions. Pharmacokinetic analysis was carried out by non-compartmental methods (NCA) using WinNonlin Pro software. The table below shows estimates for half-lifves (T½).

5

Compound #	Compound	Chromogenic activity (% N8)	T1/2 (h)	T1/2 prolongation
A	(N)-PSA(20kD) N8	88	11.0	x1.6
B	(N)-PSA(45kD) N8	60	15.0	x2.2
C	(O)-PEG(40kD) N8	>90	14.0*	x2.0
D	(O)-PSA(20kD) (N)-PSA(20kD) N8	92%	12.6	x1.9
E	(O)-PEG40kD) (N)-PEG(40kD) N8	n.d.	13.0	x1.9
F	(O)-PEG40kD) (N)-PSA(20kD) N8	93	17.7	x2.6
G	(O)-PEG40kD) (N)-PSA(45kD) N8	55	19.5*	X2.9
H	N8	100	6. 8*	x1.0

* when the same compound was tested several times, the value of the half-life indicated in the table is the average of the half-lives obtained for each experiment.

The compound C, where the (branched) PEG40kD moiety is linked to the (O)-glycan, has a
10 half-life of 14h, i.e. the half-life of N8 is prolonged by a factor 2. Further conjugation of polymers on the N-glycans have markedly different effects on the half-life of the resulting compounds:

conjugation of another PEG40kD moiety does not have any effect on the resulting compound E ($T_{1/2}$ = 13h)

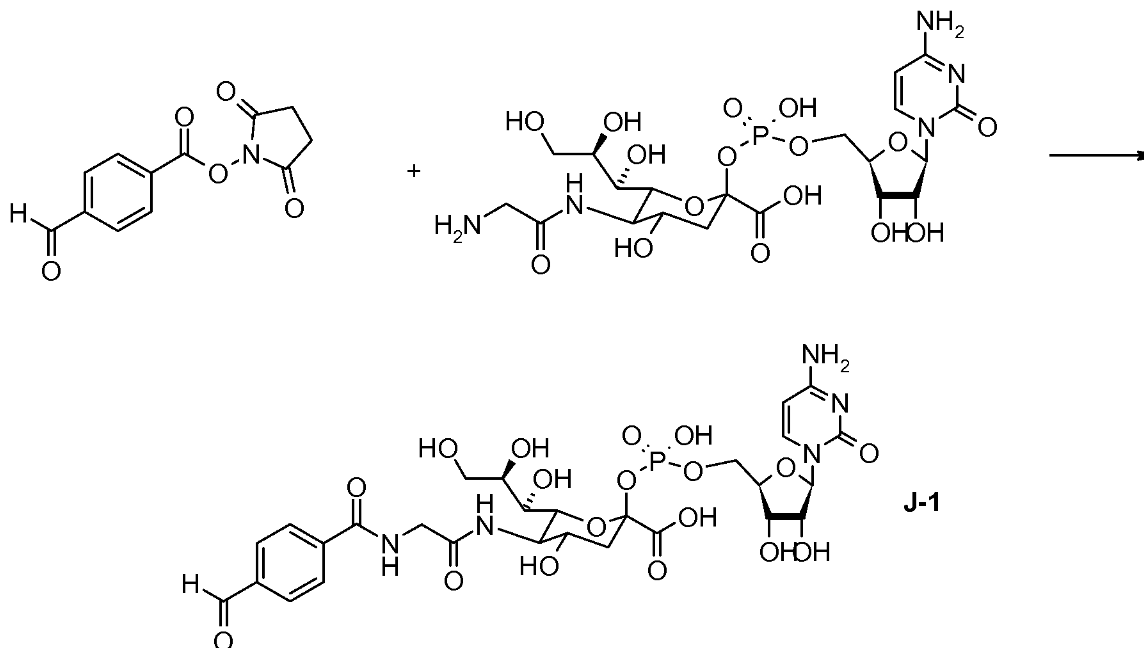
15 - while the conjugation of (linear) PSA (of either 20 or 45kD molecular weight) does have a marked effect on the resulting compounds: compounds F and G have half lives of respectively 17.7h and 19.5h, prolonging the haf-life of of the original N8 molecule by a factor 2.6, respectively 2.9. (The last one at the expense of half of the activity, though)

Likewise, N8 derivatized with PSA(20kD) on both (O)- and (N)-glycans (compound D) shows a half-life which is identical to the half-life of the N8 derivatized with PEG(40kD) on both (O)- and (N)-glycans (compound E): T1/2 = 12.6h vs 13h; this is only a modest improvement compared to the half-life of the N8 derivatized solely at the (N)-glycans
5 (compound A) (T1/2 = 11h). However, when PEG(40kD) is present on O)-glycan instead of PSA(20kD), the half-life of the resulting compound F ((O)-PEG40kD) (N)-PSA(20kD) N8) is markedly increased: 17.7h vs 12.6h for compound D.

These results strongly suggest that the combination of PEG and PSA for glyco derivatization is superior to the use of only one polymer type.

10 These results are surprising: the branched PEG 40kD was expected to have a prolonging effect due to its ability to cover the surface of the protein, thereby preventing access or make access more difficult for proteases to N8 surface or prevent/decrease binding of N8 to clearance receptors. As a branched polymer, it is expected to do so more effectively than a linear polymer (Veronese et al. J. Bioactive and Compatible Polymers (1997)12, 196). If only
15 the steric parameters are at play, one would have expected an even better protection of the N8 surface by a branched polymer than by a linear polymer, and a fortiori a linear polymer of half the molecular way of the branched poymer. Both polymers are highly hydrated, their structures are similar (mostly random coil).

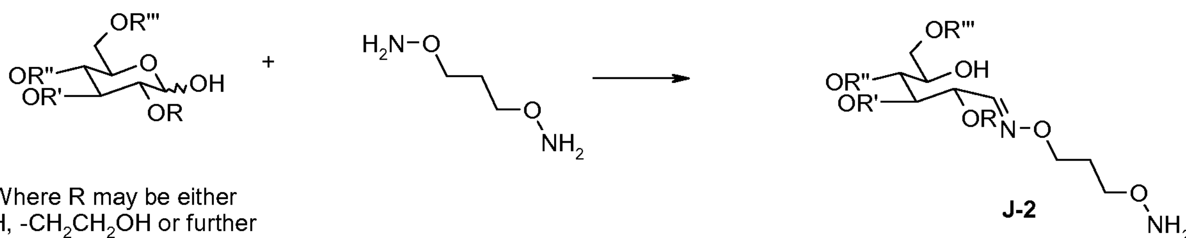
Example 15: Preparation of sialyltransferase substrate (4-formylbenzoyl)glycyl sialic
20 acid cytosine 5'-monophosphate ester (aldehyde-GSC, **J-1**)



Succinimidyl 4-formylbenzoate (100 mg, 0.41 mmol) was dissolved in THF (3 ml) and TRIS buffer (100 mM, pH 8.5, 4 ml) was added. Glycyl sialic acid cytidine 5'-monophosphate ester (GSC, 250 mg, 0.34 mmol) was weighed out and added to the solution of NHS-ester and allowed to react at rt. for a period of 2.5 h. The reaction mixture was diluted to 4 ml with 15 ml 10 mM ammonium bicarbonate buffer and purified by RP HPLC. System: Waters 2545 gradient controller, 2489 UV detector. Column: C18, Ø 2 cm. Gradient 0->30% CH_3CN with 10 mM ammonium bicarbonate. Relevant fractions were identified by LCMS and freezedried. The product was then re-purified by RP-HPLC. Yield: 62 mg. The product was identified by LCMS.

Using the above protocol, a sialyl transferase substrate carrying a chemoselective aldehyde functional group was prepared.

Example 16: Preparation of alkoxylamine functionalised hydroxyethyl starch (HES-ONH_2)

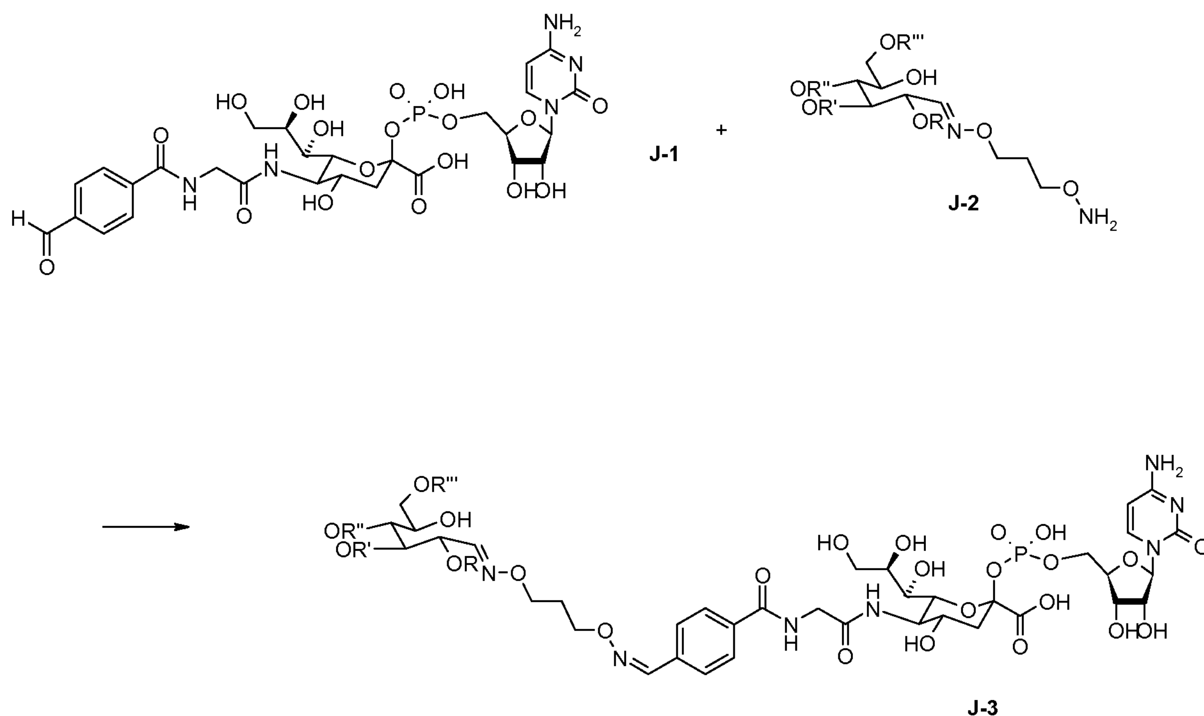


HES 200/0.5 infusion liqiud ("HyperHAES", Fresenius Kabi, 80 ml, 60 g/l, 4.8 g, 24 μmol) was mixed with a solution of 1,3-bisaminoxypropane.2HCl (1.8 g, 10.2 mmol, 425 eq.), bringing pH to 1.66. The mixture was stirred at ambient temperature overnight. An amount of 20 ml of the reaction mixture was diluted with 250 ml of water. The diluted sample was purified by tangential filtration against 5 l of water using a Vivaflow 50 system (Sartorius, 10 kDa MWCO PES membrane, pressure after pump approx. 2.5, waste: 7 ml/min). Finally, it was concentrated to 50 ml and the system was flushed with 50 ml water. After freeze drying, 690 mg of product was obtained.

In a similar fashion, HES-ONH_2 was prepared from HES 130/0.4 starting from "Voluven" infusion liquid (Fresenius Kabi)

Using the above protocol, a hydroxyethyl starch with a chemoselective alkoxylamine functional group was prepared.

Example17: Coupling of aldehyde-GSC (**J-1**) with alkoxylamine funtionalised hydroxyethyl starch HES-ONH$_2$ (**J-2**) to obtain a HES-GSC conjugate (**J-3**)



The HES-ONH$_2$ (**J-2**) (100 mg, 0.5 μmol) was dissolved in 1000 ul of PBS-buffer pH 7.4 and the aldehyde-GSC (**J-1**) (31 mg, 41 μmol) was added. The reaction was allowed to proceed at r.t. for a period of 22 h after which the reaction mixture was diluted with 100ml with PBS-Buffer pH 7.3. The diluted sample was purified by tangential filtration against 4 l of PBS buffer using a Vivaflow 50 system (Sartorius, 10 kDa MWCO RC membrane). The product was obtained in 100 ml buffer containing 140 mg HES-GSC. The product was characterised by SEC (Column: BioSep-SEC-S3000, 5 μm, 290 Å column 300 x 7.8mm, buffer: PBS-buffer pH 7.3, flow: 1 ml/min) with detection at 276 nm for cytidine. Only high molecular weight cytidine-derivatives were detected in the product by this method, and it was concluded that the product was essentially free of the starting material aldehyde-GSC.

Using the above protocol, a sialyl transferase substrate was prepared which is useful for the attachment of hydroxyethyl starch to de-sialylated glycans of glycoproteins.

Example 18: Modification of wt B-domain deleted human FVIII (N8) with HES on the O-glycan using HES-GSC substrate **J-3** and ST3Gal-I to obtain a HES-FVIII conjugate

HES-GSC (10 eq., 45 mg, 50 ml, 1 mg/ml in PBS-buffer) was concentrated and buffer exchanged to 20 mM imidazol, 10 mM CaCl$_2$, 0.02% Tween 80, 1 M glycerol, pH 7.3, 1

M NaCl using Amicon Ultra ultrafiltration vial. Final volume 2.2 ml. The HES-GSC reagent was mixed with N8 (4 mg, 22 nmol, 5.7 mg/ml), sialidase *A. Urifaciens* (40 µl, 130 U/ml, 0.43 mg/ml, 5.2 U), and His-ST3Gal-I (400 µl, 2.5 mg/ml) and incubated at 32°C. After a period of 22 h, SDS PAGE analysis showed product formation as a smeared band migrating at higher MW than both HC and LC FVIII bands. The reaction mixture was diluted with approx. 50 ml of buffer 20 mM imidazol, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, pH 7.3, 25 mM NaCl to lower the conductivity and purified by anion exchange chromatography. Column: MonoQ 5/50 GL, start buffer: 20 mM imidazol, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, pH 7.3, 25 mM NaCl, elution buffer: 20 mM imidazol, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, pH 7.3, 1 M NaCl. Relevant fractions containing the desired product was identified from SDS PAGE analysis as having three main bands: an intact LC, an HC band with very reduced intensity, and a smeared band of high MW representing HES conjugated to HC. The isolated pooled fractions contained 1.11 mg product (based on FVIII A280, 0.275 mg/ml). The pooled fractions (4 ml) were mixed with 100 µl of CMP-NAN (25 mg/ml in buffer 20 mM imidazol, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, pH 7.3, 25 mM NaCl) and ST3Gal-III (100 µl, 1.2 U/ml) and incubated for 1 hour at 32°C. The reactionmixture was then diluted with a buffer 20 mM imidazol, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, pH 7.3, 25 mM NaCl, and loaded to a Vivapure Q, Maxi M spin filter (Sartorius). The filter was washed with 2 x 15 ml 20 mM imidazol, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, pH 7.3, 25 mM NaCl and eluted using first 2 x 15 ml 20 mM imidazol, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, pH 7.3, 200 mM NaCl (to remove ST3Gal-III) and then 3 x 0.5 ml 20 mM imidazol, 10 mM $CaCl_2$, 0.02% Tween 80, 1 M glycerol, pH 7.3, 1 M NaCl to elute the product. The two first fractions contained the desired product, with 800 µg and 110 µg, respectively (based on FVIII A280). These two fractions were purified separately by SEC (column Superdex 200 10/300 GL, buffer: histidine (1.5 mg/ml), $CaCl_2$ (0.25 mg/ml), Tween 80 (0.1 mg/ml), NaCl (18 mg/ml), sucrose (3 mg/ml)) resulting in recovery of 218 µg and 66 µg, respectively (based on FVIII A280). Protein concentration determination by HPLC gave yields of 130 µg and 40 µg, respectively (based on FVIII absoprtion at 280 nm).

Using the above protocol, a HES-FVIII conjugate was prepared in which the HES was coupled to FVIII via the O-glycan of the B-domain linker. This conjugation strategy lead to a site-selectively HESylated FVIII-molecule. Moreover, the sialyltransferase mediated conjugation is mild.

Example 19. Modification of wt B-domain deleted human FVIII (N8) with HES on the O-glycan using HES-GSC substrate **J-3** and ST3Gal-I, and PEG on the N-glycans using

PEG-GSC and ST3Gal-III to obtain a simultaneous PEGylated and HESylated FVIII conjugate

The conjugate prepared according to Example 18 is treated with an immobilised sialidase, PEG-GSC and ST3Gal-III in a one-pot reaction in an aqueous buffer. After complete reaction, the sialidase is removed by filtration and a large excess of CMP-NAN is added to the reaction mixture to block any terminal galactose. After complete reaction, the conjugate is purified by anion-exchange and SEC chromatography to separate the product from the ST3Gal-III and sialyltransferase substrates.

Using this protocol a FVIII conjugated with HES and PEG on O- and N-glycans, respectively, are produced.

Example 20. Modification of wt B-domain deleted human FVIII (N8) with HES on the N-glycans using HES-GSC substrate **J-3** and ST3Gal-III, and PEG on the O-glycans using PEG-GSC and ST3Gal-I to obtain a simultaneous PEGylated and HESylated FVIII conjugate

An O-glycan PEGylated FVIII is prepared according to WO 2009/108806 A1. This conjugate is treated with an immobilised sialidase, HES-GSC **J-3** of Example 17 and ST3Gal-III in a one-pot reaction in an aqueous buffer. After complete reaction, the sialidase is removed by filtration and a large excess of CMP-NAN is added to the reaction mixture to block any terminal galactose. After complete reaction, the conjugate is purified by anion-exchange and SEC chromatography to separate the product from the ST3Gal-III and sialyltransferase substrates.

Using this protocol a FVIII conjugated with HES and PEG on N- and O-glycans, respectively, are produced.

Example 21: Preparation of sulfated PSA

The preparation is done according to published procedures (for exampke Kunou *et al.*Biomacromolecules (2000), 1, 451 and references cited therein). The starting material is either a PSA of molecular weight about 20kD, or a PSA of molecular weight about 45kD, obtained as described in examples 3 and 7.

Briefly, the sodium salt of PSA is changed to the tri-n-butylammonium salt in order to increase its solubility in organic solvents. This is done on resin ion exchange (Amberlite IR120B, H+ type). Sulfation of the lyophilized tributyl ammonium salt is performed in anhydrous DMF under inert atmosphere at 0°C, using SO3-pyridine complex as sulfation reagent. The reaction is terminated by addition of water and adjustment of pH to 9. The product is recovered by adding the reaction mixture dropwise to a large volume of acetone. The product is recovered by centrifugation of the resulting precipitate. The product is further purified by gel filtration and the eluate is lyophilized.

Example 22: Sodium periodate oxidation of sulfated PSA
The periodate oxidation is performed in the same way as in example 8, starting with the sulphated PSA obtained in example 21.

Example 23: Coupling of sodium periodate oxidized sulfated PSA to GSC-ONH2 to yield the sialyltransferase ST3Gal-III substrate GSC-ON=sulfated PSA
The coupling is done according to example 9, using GSC-ONH2 from example 2 and the oxidized sulfated PSA from example 22 as starting compounds.

Example 24: Preparation of (N)-sulfated PSA-(O)-PEG (40kD) N8 by sialyltransferase ST3Gal-III catalyzed reaction of (N)-asialo (O)-PEG(40kD) N8 with GSC-ON=sulfated PSA:
The compound is prepared according to example 10, using (N)-asialo (O)-PEG(40kD) N8 as acceptor and GSC-ON=sulfated PSA as donor in presence of ST3Gal-III.

CLAIMS

1. A FVIII variant conjugated with at least one PEG polymer and at least one polysaccharide.

5

2. A FVIII variant according to claim 1, wherein the polysaccharide is PSA.

3. A FVIII variant according to any one of claims 1-2, wherein said variant is a B domain truncated FVIII molecule covalently conjugated with a PEG polymer or a PSA polymer
10 via an O-linked oligosaccharide in the truncated B domain, wherein FVIII activation results in removal of said O-linked polymer.

4. A FVIII variant according to any one of claims 1-3, wherein said variant is covalently conjugated with a PEG polymer via the O-linked oligosaccharide in the truncated B
15 domain and wherein said variant is covalently conjugated with at least one PSA polymer via an N-linked oligosaccharide.

5. A FVIII variant according to claim 4, wherein said variant comprises two to four PSA polymers linked to one double-branched N-linked oligosaccharide in the A1 domain
20 and one double-branched N-linked oligosaccharide in the A3 domain.

6. A FVIII variant according to claim 4, wherein said variant comprises one or two PSA polymers linked to one double-branched N-linked oligosaccharide in the A1 domain.

25 7. A FVIII variant according to claim 4, wherein said variant comprises one or two PSA polymers linked to one double-branched N-linked oligosaccharide in the A3 domain.

8. A FVIII variant according to any one of the preceding claims, wherein the size of the PEG polymer is 30-50 kDa.

30

9. A FVIII variant according to any one of the preceding claims, wherein the size of the PSA polymer is 40-50 kDa.

10. A FVIII variant according to any one of the preceding claims, wherein the FVIII variant is a B domain truncated FVIII variant, wherein the B-domain comprises the amino acid sequence as set forth in SEQ ID NO 2.

11. A method of making a FVIII variant according to any one of the preceding claims, wherein said method comprises conjugating a FVIII molecule with at least one PEG polymer and at least one polysaccharide.

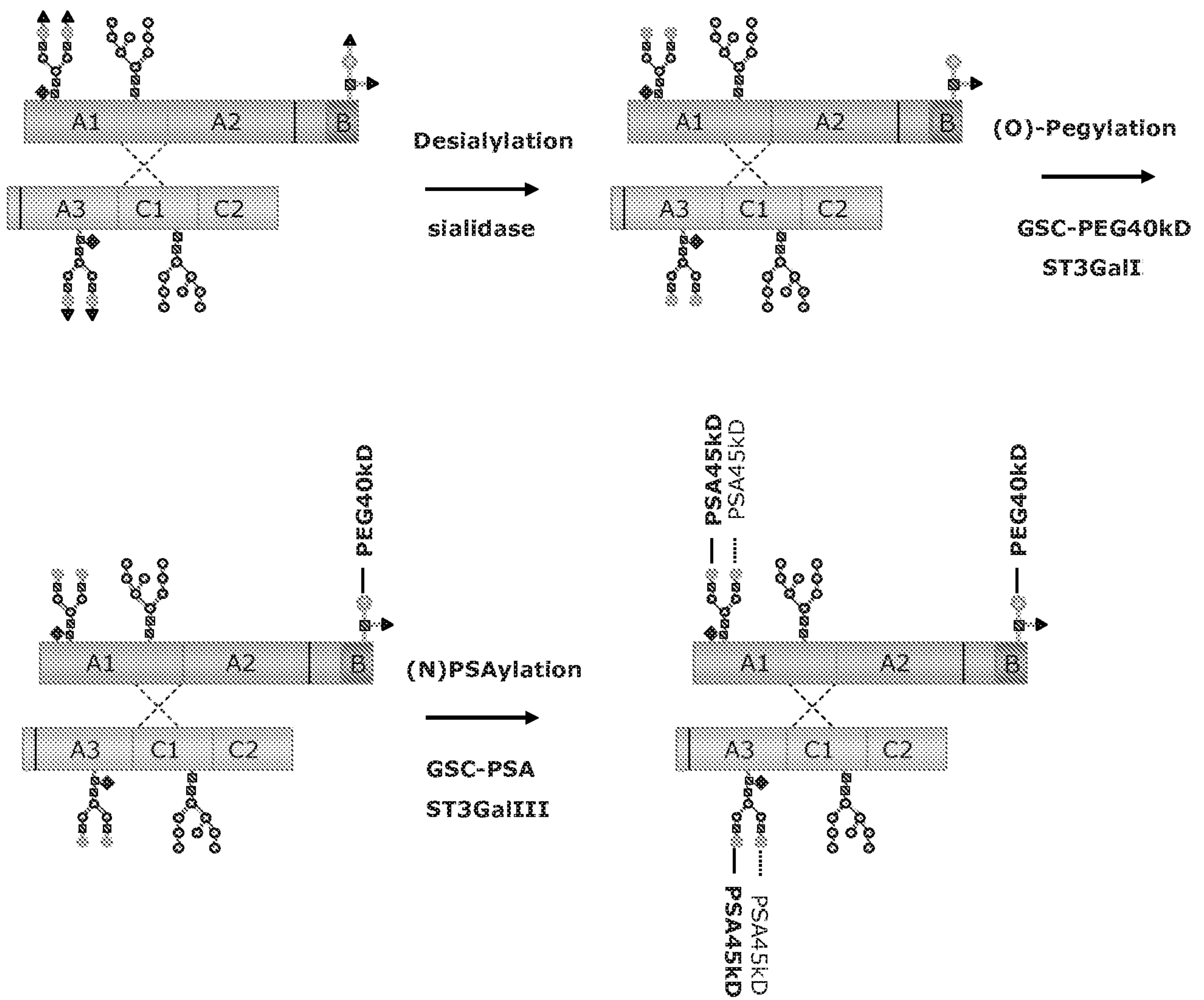
12. A FVIII variant obtainable by a method according to claim 11.

13. A pharmaceutical composition comprising a FVIII variant according to any one of claims 1-10 or 12 and optionally one or more pharmaceutically acceptable excipients.

14. Use of a FVIII variant according to any one of claims 1-10, or 12, or a pharmaceutical composition according to claim 13 as a medicament.

15. Use of a FVIII variant according to any one of claims 1-10, or 12, or a pharmaceutical composition according to claim 13 as a medicament for treating haemophilia A.

Fig. 1



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/051723

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/755 A61K38/37
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/108806 A1 (NOVO NORDISK AS [DK]; DEFREES SHAWN [US]) 3 September 2009 (2009-09-03) cited in the application sequence 2 paragraphs [0037], [0 38] example 2 -----	1-15
X,P	WO 2010/102886 A1 (NOVO NORDISK AS [DK]; ZUNDEL MAGALI [DK]; PESCHKE BERND [DK]; KARPF DI) 16 September 2010 (2010-09-16) page 12; sequence 1 -----	1-15

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 2011	21/07/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schmitz, Till

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THIM L ET AL: "Purification and characterization of a new recombinant factor VIII (N8)", HAEMOPHILIA 2010 BLACKWELL PUBLISHING LTD GBR LNKD- DOI:10.1111/J.1365-2516.2009.02135.X, vol. 16, no. 2, 11 November 2009 (2009-11-11), pages 349-359, XP002583862, figures 1, 5 page 358, left-hand column, line 2 - line 12 table 1 page 356	1-15
X	WO 2008/011633 A2 (NEOSE TECHNOLOGIES INC [US]; DEFREES SHAWN [US]) 24 January 2008 (2008-01-24) figure 5 paragraph [0034] paragraph [0518]	1-15
X	SAENKO E L ET AL: "Strategies towards a longer acting factor VIII", HAEMOPHILIA, BLACKWELL SCIENCE, OXFORD, GB, vol. 12, no. SUPPL. 3, 1 January 2006 (2006-01-01), pages 42-51, XP002434557, ISSN: 1351-8216, DOI: DOI:10.1111/J.1365-2516.2006.01260.X the whole document	1-15
X	WO 2008/151258 A2 (NEOSE TECHNOLOGIES INC [US]; DEFREES SHAWN [US]) 11 December 2008 (2008-12-11) sequence 15	1-15
X	"Abstracts from XXII ISTH congress", JOURNAL OF THROMBOSIS AND HAEMOSTASIS, vol. 7, no. supplement 2, July 2009 (2009-07), pages 508-517, XP002583863, Abstracts PP-MO-558, 565, 569, 570, 580,	1-15
X	WO 2009/089396 A2 (NEOSE TECHNOLOGIES INC [US]; DEFREES SHAWN [US]) 16 July 2009 (2009-07-16) sequence 6 figure 5	1-15

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HIGUCHI M ET AL: "Characterization of mutations in the factor VIII gene by direct sequencing of amplified genomic DNA", GENOMICS, ACADEMIC PRESS, SAN DIEGO, US LNKD- DOI:10.1016/0888-7543(90)90448-4, vol. 6, no. 1, 1 January 1990 (1990-01-01), pages 65-71, XP024797421, ISSN: 0888-7543 [retrieved on 1990-01-01] the whole document -----	1-15

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/051723

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009108806 A1	03-09-2009	EP 2257311 A1	08-12-2010
WO 2010102886 A1	16-09-2010	NONE	
WO 2008011633 A2	24-01-2008	EP 2049144 A2	22-04-2009
		JP 2009544327 A	17-12-2009
		US 2008280818 A1	13-11-2008
		US 2008255040 A1	16-10-2008
		US 2008248959 A1	09-10-2008
		US 2008274958 A1	06-11-2008
		US 2008242607 A1	02-10-2008
WO 2008151258 A2	11-12-2008	CN 101778937 A	14-07-2010
		EP 2162535 A2	17-03-2010
		JP 2010531135 A	24-09-2010
WO 2009089396 A2	16-07-2009	CA 2711503 A1	16-07-2009
		CN 102037004 A	27-04-2011
		EP 2242505 A2	27-10-2010
		JP 2011512121 A	21-04-2011
		US 2010286067 A1	11-11-2010