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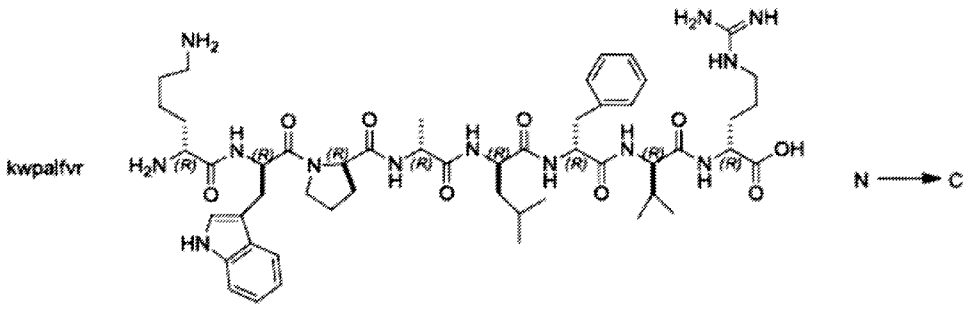
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Fig. 1A

(D-Lys)-(D-Trp)-(D-Pro)-(D-Ala)-(D-Leu)-(D-Phe)-(D-Val)-(D-Arg).

Fig. 1B



(57) Abstract: Inventors have synthesized peptides allowing an engagement of intact CD31 molecules on all the healthy endothelial cells and resting blood platelets and leukocytes that can enter in contact with an implanted device. Those cells can therefore receive the "leave-me-alone" signal delivered by the trans-homophilic engagement of CD31, which is essential to maintain the homeostasis in the circulation and vascularized tissues. Thrombotic or life-threatening occurrence of hemorrhagic or thromboembolic complications have impaired the use of endovascular devices. The devices bearing the mimicking peptides of the present invention are rapidly integrated, because they are perceived by blood platelets and leukocytes as a healthy endothelium, a "self' component. Furthermore, their ability to be rapidly endothelialized with a physiologic endothelial cell phenotype also limits platelet and leukocyte activation at the site of device implantation in the long-term. Accordingly, the present invention relates to peptides mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction.

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CD31 MIMETIC COATING FOR ENDOVASCULAR STENT

FIELD OF THE INVENTION

The present invention finds application in the medical field and in particular to improve the integration and performance of implantable medical devices.

BACKGROUND OF THE INVENTION

Strategies aimed at improving the biocompatibility of medical devices are needed to prevent the reaction of living tissues to the implantation of medical devices, potentially driving unexpected risk for the patients.

A "foreign body response" is caused by the injury of the tissue at the site of implantation and initiated by the contact of the non-biocompatible material with the blood.

The adherence and activation of platelets followed by the recruitment of inflammatory cells is followed by an aberrant wound healing sequence characterized by a chronic inflammation and the formation of granulomatous tissue.

Upon the occurrence of an injury, the interruption of the endothelial barrier allows the activation of platelets, to seal off the damage, and infiltration of leukocytes to clear the debris and prepare the site for tissue regeneration.

Medical devices however cannot be cleared.

As a consequence, in the absence of a prompt endothelialization, there is a strong activation of leucocytes and platelets at the site of implantation.

The excessive platelet adhesion and aggregation can cause the obstruction of the blood flow (ischemia) whereas the proteases released by activated platelets and leukocytes can lead to the rupture of the vascular wall (hemorrhage).

One of such condition is triggered by the presence of heart valves and vascular devices, which are seen as foreign elements to the body.

That has and is hampering the use of valvular and vascular devices for the prevention and treatment of severe cardiovascular conditions.

CD31 is a transmembrane glycoprotein expressed constitutively and exclusively on platelets, leukocytes, and endothelial cells (EC). Under healthy conditions, the trans-homophilic CD31-CD31 interaction allows the recognition of the "self" by endothelial cells, platelets, and leukocytes preventing their inappropriate activation.

The favorable issue of blood-contacting implantable devices can be achieved by treatments rendering their surface "biocompatible" to allow the rapid formation of a functional endothelial layer on them and ensuring a proper integration of the biomaterial.

The abundant expression of CD31 by the healthy endothelium exerts an essential role for the maintenance of the homeostasis in the circulation and vascularized tissues.

Cortese et al (Stroke, February 2021) discloses that the immobilization of a CD31-mimetic peptide P8RI (kwpalfvr) reduces the blood element reaction, increases the adhesion of endothelial cells *in vitro* and enhances the integration of endovascular devices *in vivo*.

Diaz-Rodriguez et al (EHJ, 2021) discloses that the soluble peptide referred to as P8RI acts like a CD31 agonist; therefore, it has been studied the effect of CD31-mimetic metal stent coating on the *in vitro* adherence of endothelial cells and blood elements and the *in vivo* endothelial strut coverage and foreign body response-driven neointimal growth.

CD31 is a type I transmembrane glycoprotein composed of 6 extracellular Ig-like domains, numbered starting from the membrane distal N-terminus, a short transmembrane fragment, and a cytoplasmic tail.

The engagement of CD31 depends upon a trans-homophilic interaction between domains 1 and 2 of the molecules expressed by a cell and the same domains expressed by the interacting cell.

The trans-homophilic interaction of CD31 domains 1 and 2 drives the clusterisation of the molecules by lateral displacement and strong cis-homophilic interaction of the transmembrane and juxtamembrane extracellular sequence.

This cis-homophilic interaction occurs at sites of cell activation and is essential for allowing the regulatory function of CD31 because this protein is not able to autophosphorylate.

CD31 phosphorylation depends on the ability of the molecules to remain clustered close to activated tyrosine kinase receptors.

Upon cell activation, the activation of cell membrane proteases drives the cleavage and shedding of most of the extracellular portion of CD31 proteins.

CD31 shedding invalidates the trans-homophilic engagement of the molecule because its trans-homophilic portion (comprised between domain 1 and 2) is lost, resulting in the dissolution of the clusters of CD31.

The amino acids included in the P8RI sequence are issued from the cis-homophilic juxta-membrane portion of CD31. P8RI co-clusters with this sequence and up-holds the regulatory signaling properties of truncated CD31 molecules in activated endothelial cells, platelets and leukocytes at site of inflammation or thrombosis.

SUMMARY OF THE INVENTION

The inventors of the present patent application have surprisingly found some peptides endowed with the property of mimicking the trans-homophilic (domain 1 and 2) CD31-CD31 intercellular interaction.

OBJECT OF THE INVENTION

According to a first object there are disclosed peptides having the property of mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction.

As per preferred aspects, said peptides may have three different general structures.

As per other aspects, there are disclosed derivatives of said peptides.

According to a second object, there is disclosed the medical use of the peptides or the peptide derivatives disclosed.

As per a preferred aspect, the medical use is for the prevention of complications associated with the implantation of medical devices to treat heart and vascular pathologies.

As per another preferred aspect, the medical use is for the treatment of heart vascular pathologies.

According to a third object of the invention, it is disclosed a coating comprising the disclosed biomimetic peptides.

According to a fourth object of the invention, it is disclosed a method for the preparation of the coating comprising the disclosed biomimetic peptides.

According to a fifth object, there are disclosed devices comprising a portion coated with the peptides or the coating of the invention.

According to a sixth object, there is disclosed a method for the prevention or for the treatment or for the diagnosis of vascular pathologies comprising the use of the biomimetic peptides of the invention.

According to another object, there is disclosed the use of the biomimetic peptides of the invention for the adhesion to the surface of a device.

As per an embodiment, there is disclosed the use of the biomimetic peptides of the invention for the adhesion to a vascular device.

According to a further object, there is disclosed the use of the biomimetic peptides of the invention for promoting the endothelialization of an arterial vessel, for preventing the neointimal growth and for the integration of a device in the target vessel.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

For the purposes of the present invention, the term "biomimetic" is to be intended as the mimicking of a natural effect.

Within the present invention, the biomimetic peptides are endowed with the property of mimicking the natural effect of the endothelium, i.e. the layer of endothelial cells (ECs) coating the interior wall of the vessels and particular of arteries.

In particular, there is mimicked the natural effect of the endothelium under healthy conditions provided by the trans-homophilic CD31-CD31 intercellular interaction.

Said mimicking property can also be provided to a surface, which is covered with the peptides of the invention.

For the purposes of the present patent application, the peptides of the invention mimic the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction.

According to a first aspect, the biomimicking peptides of the invention are based on the following sequence IA:

HQMLFYKDDVLFYNISS	Seq. ID. No. 4

According to a second aspect, the biomimicking peptides of the invention are based on the following sequence IB:

YKSTVIVNNKEKTTAE	Seq. ID. No. 5

According to a third aspect, the biomimicking peptides of the invention are based on the following sequence IC:

PRVTLDKKEAIQGGIVRVNSSVPEEK	Seq. ID. No. 6

For the purposes of the present invention, the biomimicking peptides also include cyclic peptides; in particular, the cysteine comprising biomimicking peptides may be in the cyclic form through disulfide bond.

For the purposes of the present invention, the biomimicking peptides may comprise linkers and/or spacer and/or a tail, optionally represented by an aminoacidic sequence; in particular, said sequence may be linked to a peptidic terminus or to one amino acid residue.

In a particularly preferred embodiment, said aminoacidic sequence may be represented by the following sequence:

GGSGGSGG	SEQ. ID No. 27

For the purposes of the present invention, the biomimicking peptides also include heteropeptides comprising two of the above described peptidic sequence.

In particular, the heteropeptides may comprise:

- a peptide having a sequence based on structure IA and a peptide having a sequence based on structure IB, or
- a peptide having a sequence based on structure IA and a peptide having a sequence based on structure IC.

The heteropeptides may comprise peptidic sequences linked through acetyl thioether linkage between amino acids of different sequences or between linkers and/or spacers.

As per preferred aspect, the peptides of the invention are based on the following sequences:

Aminoacidic sequence (IA)	**Seq. ID No.**
HQMLFYKDDVLFYNISSC	7
SHQMLFYKDDVLFYNISSS	8
QHQMLFYKDDVLFYNISSMK	9
CHQMLFYKDDVLFYNISSCK	10
YKDDVLFYNISSMKST	11
PQHQMLFYKDDVLFYNISSMK	12
FADVSTTSHVKPQHQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYK	13
PQHQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV	14
YKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV	15
HQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV	16
LFYKDDVLFYNISSMKSTESYF	17
PQHQMLFYKDDVLFYNISSMK	18
QHQMLFYhCDDVLFYNISSMK	28
hC=homocysteine	

As per preferred aspect, the peptides of the invention are based on the following sequences:

Aminoacidic sequence (IB)	**Seq. ID No.**
YKSTVIVNNKEKTTAE	19
DDVLFYNISSMK	20
QHQMLFY	21
CYKSTVIVNNKEKTTAEYC	22
hCYKSTVIVNNKEKTTAEYhC	23
GTYKSTVIVNNKEKTTAEYQ	24
hC=homocysteine	

As per preferred aspect, the peptides of the invention are based on the following structures:

Aminoacidic sequence (IC)	**Seq. ID No.**
PRCTLDKKEAIQGGIVRVNCSVPEEK	25

As per preferred aspect, the peptides of the invention are based on the following structures:

Aminoacidic sequence	**Seq. ID No.**
RDQNFVILEFP	26

As per a particularly preferred aspect, the heteropeptides of the invention are characterized by comprising the following peptides:

Aminoacidic sequences	**Seq. ID No.**
GTYKSTVIVNNKEKTTAEYQ and QHQMLFYhCDDVLFYNISSMK	24 and 28 (SEQ ID No.37)
PRVTLDKKEAIQGGIVRVNSSVPEEK and QHQMLFYhCDDVLFYNISSMK	6 and 28 (SEQ ID No.38)

According to a preferred aspect, the biomimicking peptides of the invention may comprise any of the below modifications:

- a cysteine residue may be substituted by the corresponding homocysteine residue,
- an L-amino acid may be substituted by the corresponding D-amino acid.

According to a preferred embodiment of the present invention, the biomimicking peptides of the invention may comprise a spacer and/or a linker and/or a tail, selected from:

GGSGGSGG	SEQ. ID no. 27
one or more PEG4 units	
one or more Ttds units	
KN_3	

and combinations thereof, such as for instance:

$K(N_3)(Ttds)_4$
$(Ttds)_2$-$K(N_3)$
$(Ttds)_4$-$K(N_3)$
$K(N_3)$GGSGGSGG

For the purposes of the present invention, the above disclosed structures may comprise modifications at the C-terminus and/or at the N-terminus.

In particular, said modifications may comprise:

Ac-
Br-CH_2-CO-
CH_2-CO-
CH_2-$CONH_2$-
Ac-KN_3
-NH_2
-CO-NH_2

According to a preferred embodiment of the invention, there are disclosed the following biomimicking sequences:

Aminoacidic sequence	**SEQ. ID No.**
QHQMLFYCDDVLFYNISSMK-$CONH_2$	1
($BrCH_2$CO-GTYKSTVIVNNKEKTTAEYQ-$(Ttds)_4$-$K(N_3)$-NH_2)	2
Ac-$K(N_3)$-$(Ttds)_4$-PRVTLDKKEAIQGGIVRVNSSVPEEK-Ttds-K($BrCH_2$CO)-	3

$CONH_2$	
Ac-c*HQMLFYKDDVLFYNISSC*-GGSGGSGG-K(N_3)-$CONH_2$	29
SHQMLFYKDDVLFYNISSS-GGSGGSGG-K(N_3)-$CONH_2$	30
Ac-KKKc*HQMLFYKDDVLFYNISSC*-Ttds-Ttds-K(N_3)-$CONH_2$	31
QHQMLFYKDDVLFYNISSMK-Ttds-Ttds-Ttds-Ttds-K(N_3)-$CONH_2$	32
hCHQMLFYKDDVLFYNISShCK-Ttds-Ttds-Ttds-Ttds-K(N_3)-$CONH_2$	33
YKSTVIVNNKEKTTAE-PEG4-K(N_3)-$CONH_2$	34
Ac-K(N_3)-PEG4-PRVTLDKKEAIQGGIVRVNSSVPEEK-$CONH_2$	35
Ac-K(N_3)-(Ttds)$_4$-PRhCTLDKKEAIQGGIVRVNhCSVPEEK-$CONH_2$	36
[$^{\#}CH_2CONH_2$GTYKSTVIVNNKEKTTAEYQ-(Ttds)$_4$-K(N_3)-NH_2]-[QHQMLFYhC$^{\#}$DDVLFYNISSMK-$CONH_2$]	37
[Ac-K(N_3)-(Ttds)$_4$-PRVTLDKKEAIQGGIVRVNSSVPEEK-Ttds-K$^{\#}$ (CH_2CO)-$CONH_2$]-[QHQMLFYhC$^{\#}$DDVLFYNISSMK-$CONH_2$]	38
Ac-K(N_3)-GGSGGSGG-YKDDVLFYNISSMKST-NH_2	39
Ac-YKDDVLFYNISSMKST-GGSGGSGG-K(N_3)-NH_2	40
Ac-PQHQMLFYKDDVLFYNISSMK(GGSGGSGG-K(N_3))STESYFI-$CONH_2$	41
Ac-K(N_3)-GGSGGSGG-FADVSTTSHVKPQHQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYK-	42

$CONH_2$	
Ac-K(N_3)-GGSGGSGG-PQHQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV-$CONH_2$	43
Ac-K(N_3)-GGSGGSGG-YKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV-$CONH_2$	44
Ac-K(N_3)-GGSGGSGG-HQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV-$CONH_2$	45
Ac-LFYKDDVLFYNISSMKSTESYF-GGSGGSGG-K(N_3)-$CONH_2$	46
Ac-LFYKDDVLFYNISSMKSTESYF-Ttds-Ttds-K(N_3)-$CONH_2$	47
Ac-PQHQMLFYKDDVLFYNISSMK(GGSGGSGG-K(N_3))STESYFIKK-$CONH_2$	48
RDQNFVILEFP-PEG4-K(N_3)-NH_2	49
wherein hC: homoCysteine; c: D-Cysteine; ** position for disulfide bridge formation of cyclic peptides #acetyl thioether linkage	

For the purposes of the present invention, the biomimicking peptides of the invention comprise peptides having at least 95% identity of the above disclosed structure, preferably at least 97% and even more preferably at least 99%.

According to a second object, there is disclosed the medical use of the peptides disclosed.

As per a preferred aspect, the medical use is for the prevention of vascular pathologies.

In particular, according to the present invention, said pathologies are selected from the group comprising: heart valve pathology, atherosclerosis, thrombosis, ischemia, hemorrhage, restenosis, aneurysm.

In addition to the above, there is disclosed the medical use for the prevention of a pathological condition represented by the in-stent stenosis.

As per another preferred aspect, the medical use is for the treatment of vascular pathologies.

The above medical uses are disclosed for humans as well as for animals in the veterinary field.

According to a third object of the invention, it is disclosed a coating comprising the disclosed biomimetic peptides.

As per an embodiment of the present invention, the coating is a mono-layered coating, while as per an alternative embodiment of the invention it is a multi-layer coating.

According to a fourth object of the invention, it is disclosed a method for the preparation of the coating comprising the disclosed biomimetic peptides.

For the purposes of the present invention, the coating of a device or of a portion thereof can be obtained with dip-coating, by immersion of the portion to be coated within a coating bath.

As per a preferred embodiment, the method is a three-step dip coating method comprising:

- a first step for a polydopamine coating,
- a second step for the grafting of a suitable linker, and
- a third step for the coating with the invention peptide.

According to a fifth object, there are disclosed devices comprising a portion coated with the biomimetic peptides or the coating of the invention.

Coating may comprise the whole surface of a device or of a part of a device.

For the purposes of the present invention, devices, which can be fully or partially covered with the biomimetic peptides of the invention comprise: coronary stent, flow diverting stent, aortic tubes, cardiac valves or any portions thereof.

The fully or partially coated devices may be in any suitable material, such as, for instance:

- metal or alloys: stainless steel, cobalt-chromium alloy, platinum-chromium alloy or nickel-titanium alloy;
- synthetic polymeric materials: Dacron®, Teflon®;
- clinical-grade biological tissues, such as pericardial sheets for heart valves bioprostheses.

According to a sixth object, there is disclosed a method for the prevention or for the treatment of heart and vascular pathologies comprising the use of the biomimetic peptides of the invention.

According to a particular aspect, said method for the prevention or for the treatment of heart and vascular pathologies comprises the use of a device fully or partially coated with the coating of the invention.

In particular, said pathologies are represented by: heart valve pathology, atherosclerosis, thrombosis, ischemia, hemorrhage, restenosis, aneurism.

In addition to the above, there is disclosed a method for the prevention of a condition represented by the in-stent stenosis.

The above methods for the prevention or for the treatment are disclosed for humans as well as for animals in the veterinary field.

According to another object, there is disclosed the use of the biomimetic peptides of the invention for the adhesion to the surface of a device.

As per an embodiment, there is disclosed the use of the biomimetic peptides of the invention for the adhesion to vascular device.

According to a further object, there is disclosed the use of the biomimetic peptides of the invention for promoting the endothelialization of a vessel, for preventing the neointimal growth and for the integration of a device in the target vessel.

In particular, a target vessel is represented by an arterial vessel.

According to an even further object, there is disclosed the use of the biomimetic peptides of the invention for improving the biocompatibility of a device.

According to an even further object, there is disclosed the use of the biomimetic peptides of the invention for endowing the stented segment with recovered endothelial anti-inflammatory and anti-thrombotic properties.

According to an even further object, there is disclosed the use of the biomimetic peptides of the invention for endowing the stented segment with improved adaptive remodelling allowing recovery of its functional properties.

The present invention will be further disclosed in the following non-limitative examples.

EXAMPLES:

Brief description of the figures

Figures 1A and 1B show the structure of the octa-peptide P8RI.

Figure 2 shows the strategy used to analyze the biocompatibility of nitinol disks interacting with human endothelial cells.

Figure 3 shows representative images and quantification of the F-actin staining of HAEC growing onto bare and coated disks.

Figure 4 shows representative images and quantification of the CD31 staining of HAEC growing onto bare and coated disks.

Figure 5 shows quantitative analysis of F-actin and CD31 expression on HAEC growing onto coated vs bare control nitinol disks.

Figure 6 shows the ratio between CD31 and F-actin expression.

Figures 7 to 17 show the structure of the preferred peptides of the invention.

Figure 18 shows the structure of the intermediate A.

Figure 19 shows the structure of the intermediate B.

Figure 20 shows the structure of the intermediate C.

Figure 21 shows the structures of linkers according to the present invention.

Abbreviations

Certain abbreviations are used in the examples and elsewhere herein:

“AA” refers to amino acid;

“Alloc” refers to allyloxycarbonyl

“Boc” refers to tert-butyloxycarbonyl;

“tBu” refers to tertiary butyl;

“DCM” refers to dichloromethane;

“DIC” refers to N,N'-diisopropylcarbodiimide;

“DIPEA” refers to N, N-Diisopropiletylamine

“DMF” refers to dimethylformamide;

“Fmoc” refers to fluorenylmethyloxycarbonyl;

“HOAt” refers to 1-hydroxy-7-azabenzotriazole;

“HPLC” refers to High Performance Liquid Chromatography;

"LCMS" refers to Liquid Chromatography/Mass Spectrometry;

"UPLC" refers to High Performance Chromatography;

"RP-HPLC" refers to reversed-phase high performance liquid chromatography;

"MS" refers to mass spectrometry;

"OtBu" refers to O-tert-butyl;

"Oxyma" refers to ethyl cyanohydroxyiminoacetate;

"Pbf" refers to 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl;

"TIPS" refers to triisopropylsilane;

"TFA" refers to trifluoroacetic acid;

"Trt" refers to trityl

Materials and Methods

The peptides were synthesized by standard Solid-phase Peptide Synthesis (SPPS) using Fmoc/t-Bu chemistry. DMF was used as the solvent. The following starting materials and methods were employed in the synthetic procedures described in the examples.

Fmoc-protected natural amino acids were purchased from Novabiochem, Iris Biotech, Bachem or Chem-Impex International. The following standard amino acids were used in the syntheses: Fmoc-L-Ala-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Asp(OMpe)-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-Glu(OtBu)-OH, Fmoc-L-Gly-OH, Fmoc-L-Ile-OH, Fmoc-L-Leu-OH, Fmoc-L-Lys(Boc)-OH, Fmoc-L-Pro-OH, Fmoc-L-Ser(tBu)-OH, Fmoc-L-Thr(tBu)-OH, Fmoc-L-Tyr(tBu)-OH, Fmoc-L-Val-OH.

In addition, the following amino acids were purchased from the same suppliers as above: Fmoc-K-(N_3)OH; Fmoc-K(alloc)OH; Fmoc-c-OH, Fmoc-hC-OH, Fmoc-Ttds-COOH, Fmoc-NH-PEG4-COOH.

Analytical characterization.

Crude and purified peptides were analyzed by Ultra-high performance liquid chromatography with UV and mass spectrometry detection (UPLC-UV-MS). Analytical UPLC was performed according to one of the following methods:

Method A:

Detection at 214 nm

Column: Acquity Waters BEH130 C4, 1.7 μm (2.1x100 mm) at 45°C

Solvent: H_2O+0.1%TFA: ACN+0.1%TFA (flow 0.4 ml/min)

Gradient: 85:15 (0 min) to 85:15 (1 min) to 65:35 (5 min) to 10:90 (5.2 min) to 10:90 (5.5 min) to 85:15 (5.7 min) to 85:15 (6 min)

Mass analyzer: Waters SQ Detector with electrospray ionization in positive ion detection mode

Method B:

Detection at 214 nm

Column: Acquity Waters BEH130 C4, 1.7µm (2.1x100 mm) at 45°C

Solvent: H2O+0.1%TFA: ACN+0.1%TFA (flow 0.4 ml/min)

Gradient: 80:20 (0 min) to 80:20 (1 min) to 60:40 (5 min) to 10:90 (5.2 min) to 10:90 (5.5 min) to 80:20 (5.7 min) to 80:20 (6 min)

Method C:

Detection at 214 nm

Column: Acquity Waters BEH130 C4, 1.7µm (2.1x100 mm) at 45°C

Solvent: H2O+0.1%TFA: ACN+0.1%TFA (flow 0.4 ml/min)

Gradient: 75:25 (0 min) to 75:25 (1 min) to 55:45 (4 min) to 10:90 (4.2 min) to 10:90 (4.5 min) to 80:20 (4.7 min) to 75:25 (5 min)

Mass analyzer: Waters SQ Detector with electrospray ionization in positive ion detection mode

Mass analysis was performed on a Waters SQ Detector with electrospray ionization in positive ion detection mode and the scan range of the mass-to-charge ratio was 400-1800.

Table 3: Analytical method, retention time, calculated and found masses, of the example compounds

Cpd ID (SP)	Exact Mass (monoisotopic)	Observed [M+2H]2+	Observed [M+3H]3+	Observed [M+4H]4+	Observed [M+5H]5+	Retention Time (min)	Method
1	3034.32	1519.2	1013.5	ND	ND	3.27	C
2	2962.37	1483.8	989.4	ND	ND	2.85	C
3	3506.78	1756.2	1171.0	878.6	ND	2.90	C
4	3868.04	ND	1291.8	969.2	775.5	4.20	B
5	3843.58	ND	1282.8	962.4	770.2	4.05	B
6	2225.21	1114.0	743.0	ND	ND	2.97	A

7	3580.90	ND	1195.4	897.1	ND	3.57	A
8	3249.83	ND	1085.0	814.0	651.6	3.65	A
9	4298.36	ND	1435.4	1076.8	861.8	3.23	B
10	6169.14	ND	ND	1545.5	ND	4.35	B
11	7216.85	ND	ND	ND	1445.9	4.59	B
ND: Not Detected							

General procedure for peptide synthesis on solid support

The synthesis of all the peptides was performed by standard Fmoc stepwise solid phase synthesis (SPPS) on a Liberty Blue microwave synthesizer (CEM corp.). The assembly was performed using a Protide Rink-amide (4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucylaminomethyl resin, CEM, 200 μmol, 100-200 Mesh; loading 0.2 mmol/g) on a 0.2 mmol scale, with DIC/Oxyma activation. DMF was used as the solvent.

All the amino acids were dissolved at a 0.4 M concentration in DMF. The acylation reactions were performed for 3 min at 90°C under microwave irradiation with 5 folds excess of activated amino acids over the resin free amino groups. The amino acids were activated with equimolar amounts of 0.5 M solution of DIC in DMF and Oxyma solution 1 M in DMF.

The following conditions were employed:

Standard deprotection: 20% piperidine in DMF for 2 x 120 s, 90°C

Washes: 4 x DMF.

Standard Single coupling: 5 eq. AA 0.4 M / 5 eq. DIC 1M/ 5 eq. Oxyma 1M, 120 s, 90°C

Standard Double coupling: standard single coupling repeated twice.

Washes: 4 x DMF.

At the end of the assembly the resin was washed with DMF, MeOH, DCM, Et2O.

Cleavage of the peptides from the resin was performed using the following cleavage cocktail:

Mix 1) 87.5% TFA, 5% phenol, 5% water, 2.5% TIPS for 1.5 to 2.5 hours at RT (30 ml).

Mix 2) 87.5% TFA, 5% phenol, 5% water, 2.5% Thioanisole for 1.5 to 2.5 hours at RT (20 ml).

The resin employed in the synthesis was such that the C-terminal was cleaved from the resin as a primary amide.

The cleavage mixture was collected by filtration, the crude peptides were precipitated in methyl tert-butyl ether, centrifuged, the supernatant was removed, fresh diethyl ether was added to the peptides and re-centrifuged, twice; the crude peptides were then lyophilized.

Peptides were analyzed by analytical UPLC and verified by ESI+ mass spectrometry. Crude peptides were purified by a conventional preparative RP-HPLC purification on a preparative Waters 2489 HPLC system, (UV detection at wavelength 214 nm); the following solvents as eluents: acetonitrile + 0.1% TFA (mobile phase A) and water + 0.1% TFA (mobile phase B). Product containing fractions were collected and lyophilized to obtain the purified product as a TFA salt. Unless otherwise described the compounds were tested as TFA salt.

Procedure for the synthesis of Seq ID 29:

Ac-c*HQMLFYKDDVLFYNISSC*-GGSGGSGG-K(N_3)-$CONH_2$

The structure of the peptide is shown in Figure 7.

Seq ID 1 (PepSP722) spans region His71-Ser87 of CD31 IgL1-A with an acetylated N-terminal D-Cysteine and a Cysteine at the C-terminal position of Ser87, with the two thiol groups engaged in a disulfide bond.

After the synthesis and the cleavage performed according to the general procedure (Mix 1), the mixture was analyzed by analytical UPLC and verified by ESI+ mass spectrometry and it was lyophilized to afford the crude title compound (Y= 64%). Crude material was dissolved in a mixture of 8/2 acetonitrile/water at 1 mg/mL concentration: aqueous NH_3 added to reach pH 9 and the mixture was stirred for 72 hours at room temperature. TFA added to the mixture and freeze dried. The crude peptide was purified by reverse-phase HPLC using preparative Reprosil Gold C4 (250x40mm, 120Å, 5µm) column. The following gradient of eluent B was used: 20%B to 20%B over 5min, to 40%B over 25 min, flow rate 60 mL/min, wavelength 214 nm. Seq ID 1 was isolated as an amorph white freeze-dried solid (Y =5%), TFA salt. The purified peptide was solubilized at 1 mg/mL concentration in a mixture of acetonitrile/water 8/2: HCl 50 mM (10 eq) was added, and mixture stirred for 1 hour at room temperature. After lyophilization, the peptide was dissolved in acetonitrile/water 8/2 and freeze dried again. The peptide was analyzed by LC/MS (Method C). $[M+3H]^{3+}$ mass signal found under the

peak with retention time 3.27 min revealed the peptide mass 1013.5 which is in line with the expected value of molecular weight 3036.37.

Procedure for the synthesis of Seq ID 30:

SHQMLFYKDDVLFYNISSS-GGSGGSGG-K(N_3)- $CONH_2$

The structure of the peptide is shown in Figure 8.

Seq ID 2 (PepSP745) spans the same region His71-Ser87 of CD31 IgL1-A, common with Seq ID 1 but it is a linear sequence with replacement of the two cysteine residues in seq ID1 in Serines.

After the synthesis and the cleavage performed according to the general procedure (Mix 1), the mixture was analyzed by analytical UPLC and verified by ESI+ mass spectrometry and it was lyophilized to afford the crude title compound (Y= 66%). The crude peptide was purified by reverse-phase HPLC using preparative Reprosil Gold C4 (250x40mm, 120Å, 5μm) column. The following gradient of eluent B was used: 20%B to 20%B over 5min, to 40%B over 20 min, flow rate 60 mL/min, wavelength 214 nm. Seq ID 2 was isolated as an amorph freeze-dried solid (Y =4%), TFA salt. The peptide was solubilized at 1 mg/mL concentration in a mixture of acetonitrile/water 8/2: HCl 50 mM (10 eq) was added, and mixture stirred for 1 hour at room temperature. After lyophilization, the peptide was dissolved in acetonitrile/water 8/2 and freeze dried again. The peptide was analyzed by LC/MS (Method C). $[M+3H]^{3+}$ mass signal found under the peak with retention time 2.85 min revealed the peptide mass 989.4 which is in line with the expected value of molecular weight 2964.22.

Procedure for the synthesis of Seq ID 31:

Ac-KKKc*HQMLFYKDDVLFYNISSC*-Ttds-Ttds-K(N_3)- $CONH_2$

The structure of the peptide is shown in Figure 9.

Seq ID 3 (PepSP765) spans the same region His71-Ser87 of CD31 IgL1-A and similar disulfide connectivity of seq ID 1 but with a solubilizing tail at the N-terminus of 3 lysine residues.

The synthesis was performed according to the general procedure. At the end of the assembly the resin was treated with Ac_2O (10 eq) in DMF for 30 mins. The cleavage was performed according to the general procedure using Mix 1, the mixture was analyzed by analytical UPLC and verified by ESI+ mass spectrometry and freeze-dried (Y= 61%). Crude material was dissolved in a mixture of 8/2 acetonitrile/water at 1 mg/mL concentration: aqueous NH_3 added to reach pH 9 and the mixture was stirred for 72 hours

at room temperature. TFA added to the mixture and freeze dried. The crude peptide was purified by reverse-phase HPLC using preparative Reprosil Gold C4 (250x40mm, 120Å, 5µm) column. The following gradient of eluent B was used: 25%B to 25%B over 5 min, to 40%B over 25 min, flow rate 60 mL/min. Seq ID 3 was isolated as an amorph freeze-dried solid (Y =2%), TFA salt. The peptide was solubilized at 1 mg/mL concentration in a mixture of acetonitrile/water 8/2: HCl 50 mM (10 eq) was added, and mixture stirred for 1 hour at room temperature. After lyophilization, the peptide was dissolved in acetonitrile/water 8/2 and freeze dried again. The peptide was analyzed by LC/MS (Method C). $[M+3H]^{3+}$ mass signal found under the peak with retention time 2.90 min revealed the peptide mass 1171.0 which is in line with the expected value of molecular weight 3509.17.

Procedure for the synthesis of Seq ID 32:

QHQMLFYKDDVLFYNISSMK-Ttds-Ttds-Ttds-Ttds-K(N_3)- $CONH_2$

The structure of the peptide is shown in Figure 10.

Seq ID 4 (PepSP1375) spans the same region Gln70-Lys89 of CD31 IgL1-A and a long linker of Ttds units (see Table 1)

During peptide assembly on solid phase, double acylation reactions were performed for Val and Leu. Fmoc deprotections were performed with a double treatment of the resin with 20%(V/V) piperidine in DMF for 120 at 90 °C up to the Asp residue in position 9: after this residue deprotections were performed at room temperature to minimize aspartimide formation. The cleavage was performed according to the general procedure using Mix 2. The mixture was analyzed by analytical UPLC and verified by ESI+ mass spectrometry and it was lyophilized to afford the crude title compound (Y= 70%).

Purification of the crude material was performed using an XBridge C18 (250x50mm, 120A, 10um) column and a gradient of Solvent B as follows: 15% B for 5 min; 15% of B to 35% in 20 min; flow rate: 80 mL/min. Product-containing fractions were collected and lyophilized to obtain the purified product as a TFA salt (Y =10%). The purified peptide was analyzed by LC/MS (Method B). $[M+4H]^{4+}$ mass signal found under the peak with retention time 4.20 min revealed the peptide mass 969.2 which is in line with the expected value of molecular weight 3870.58.

Procedure for the synthesis of Seq ID 33:

hCHQMLFYKDDVLFYNISShCK-Ttds-Ttds-Ttds-Ttds-K(N_3)- $CONH_2$

The structure of the peptide is shown in Figure 11.

Seq ID 5 (PepSP1374) spans the same region Gln70-Lys89 of CD31 IgL1-A of Seq ID 4 with mutations to homocysteines: Gln70hCys and Lys89hCys that results in a disulfide bond with a cyclic structure.

During peptide assembly, double acylation reactions were performed for Val and Leu. Fmoc deprotections were performed with a double treatment of the resin with 20%(V/V) piperidine in DMF for 120 seconds at 90°C up to the Asp residue in position 9: after this residue deprotections were performed at room temperature to minimize aspartimide formation. The cleavage was performed according to the general procedure using Mix 2. The mixture was analyzed by analytical UPLC and verified by ESI+ mass spectrometry and it was lyophilized to afford the crude title compound (Y= 70%). Crude peptide was analyzed by analytical UPLC and verified by ESI+ mass spectrometry. Crude material was dissolved in a 9/1 mixture of DMSO/H_2O at 1 mg/mL concentration: aqueous NH_3 added to reach pH 9 and the mixture was stirred for 48 hours at room temperature. After this time the formation of the disulfide bridge was confirmed by UPLC and the mixture was freeze-dried. Purification of the crude material was performed with a Waters XBridge C18 (250x50mm, 120A, 10um) column using the following gradient: 15% B for 5 min; 15% of B to 35% in 20 min; flow rate 80 mL/min. Product-containing fractions were collected and lyophilized to obtain the purified product as a TFA salt (Y =10%). The purified peptide was analyzed by LC/MS (Method B). $[M+4H]^{4+}$ mass signal found under the peak with retention time 4.05 min revealed the peptide mass 962.4 which is in line with the expected value of molecular weight 3843.58.

Procedure for the synthesis of Seq ID 34:

YKSTVIVNNKEKTTAE-PEG4-K(N_3)- $CONH_2$

The structure of the peptide is shown in Figure 12.

Seq ID 6 (PepSP1072) spans the region Tyr107-Glu122 of CD31 IgL1-A.

During the assembly, double acylation reactions were performed for all natural amino acids and Fmoc-NH-PEG4-COOH. After the synthesis and the cleavage performed according to the general procedure (Mix 1), the mixture was analyzed by analytical UPLC and verified by ESI+ mass spectrometry; lyophilized to afford the crude title compound (Y= 70%). LCMS anal. calc. for C96H166N27O33: 2225.21 Da; found; 1114.0 $(M+2)^{2+}$. The crude peptide was purified by reverse-phase HPLC using preparative Waters XBridge C18 (250x50mm, 120A, 10um) column and the following gradient of eluent B: 5%B to 5%B over 5min, 5%B to 25%B over 20min, flow rate 80

mL/min. Seq ID 6 was isolated as an amorph freeze-dried solid (Y =25%) as TFA salt. The purified peptide was analyzed by LC/MS (Method A). $[M+3H]^{3+}$ mass signal found under the peak with retention time 2.97 min revealed the peptide mass 743.0 which is in line with the expected value of molecular weight 2226.54.

Procedure for the synthesis of Seq ID 23:

hCYKSTVIVNNKEKTTAEYhC-(Ttds)$_4$-K(N$_3$)-NH$_2$

The structure of the peptide is shown in Figure 13.

Seq ID 7 (PepSP1376) spans the region 106-124 where position 106 and 124 are mutated to Homocysteines that results in a disulfide bond with a cyclic structure.

Synthesis and cleavage were performed according to the general procedure (Mix 1); cleaved the mixture was analyzed by analytical UPLC and verified by ESI+ mass spectrometry and it was lyophilized to afford the crude title compound (Y= 65%). Crude material was dissolved in a 9/1 mixture of DMSO/H_2O at 1 mg/mL concentration: aqueous NH_3 added to reach pH 9 and the mixture was stirred for 48 hours at room temperature. The formation of the disulfide bridge was confirmed by UPLC and the mixture was freeze-dried. The crude peptide was purified by reverse-phase HPLC using XBridge C18 (250x50mm, 120A, 10um) column and the following gradient of eluent B: 5%B to 5%B over 5min, to 25%B over 20min, flow rate 80 mL/min. Product-containing fractions were collected and lyophilized to obtain the purified product as a TFA salt (Y =28%). The purified peptide was analyzed by LC/MS (Method A). $[M+3H]^{3+}$ mass signal found under the peak with retention time 3.57 min revealed the peptide mass 1195.4 which is in line with the expected value of molecular weight 3583.22.

Procedure for the synthesis of Seq ID 35:

K(N$_3$)-PEG4-PRVTLDKKEAIQGGIVRVNSSVPEEK-CONH$_2$

The structure of the peptide is shown in Figure 14.

Seq ID 8 (PepSP1071) spans the region Pro133-Lys158 of CD31 IgL2-A.

Synthesis and cleavage performed according to the general procedure (Mix 1); the cleaved mixture was analyzed by analytical UPLC and verified by ESI+ mass spectrometry and it was lyophilized to afford the crude title compound (Y= 54%). The crude peptide was purified by reverse-phase HPLC using preparative double Waters DeltaPack C18 cartridges (100x40mm, 300Å, 15µm). The following gradient of eluent B was used: 5%B to 5%B over 5min, to 25%B over 20 min, flow rate 80 mL/min. Product-containing fractions were collected and lyophilized to obtain the purified product as a

TFA salt (Y =35%). The purified peptide was analyzed by LC/MS (Method A). $[M+3H]^{3+}$ mass signal found under the peak with retention time 3.57 min revealed the peptide mass 1085.0 which is in line with the expected value of molecular weight 3251.76.

Procedure for the synthesis of Seq ID 36:

Ac-K(N_3)-(Ttds)$_4$-PRhCTLDKKEAIQGGIVRVNhCSVPEEK-CONH$_2$

The structure of the peptide is shown in Figure 15.

Seq ID 9 (PepSP1380) spans the region Pro133-Lys158 of CD31 IgL2-A as seq ID 8 but with mutations homocysteines at position 35 and 52 that results in a disulfide bond with a cyclic structure.

Synthesis performed according to the general procedure. During peptide assembly, double acylation reactions were performed for the residues in the fragments Ser-Val-Pro, Ile-Val-Arg-Val and Pro-Arg-homoCys-Thr-Leu. Fmoc deprotections were performed with a double treatment of the resin with 20%(V/V) piperidine in DMF for 120 seconds at 90°C up to Asp in position 6: after this residue deprotections were performed at room temperature to minimize aspartimide formation. The four Ttds and the terminal Lys-azide residues were introduced manually using HOAT (5 eq) and DIPC (5q) as coupling reagents in DMF: triple coupling of the last Ttds residue was performed to afford a full conversion. At the end of the assembly the resin was treated with Ac2O (10 eq) in DMF for 30 mins. The cleavage was performed according to the general procedure using Mix 1, the mixture was analyzed by analytical UPLC and verified by ESI+ mass spectrometry and it was lyophilized to afford the crude title compound (Y =73%). Crude material was dissolved in a 9/1 mixture of DMSO/H_2O at 1 mg/mL concentration: aqueous NH3 added to reach pH 9 and the mixture was stirred for 72 hours at room temperature. After this time the formation of the disulfide bridge was confirmed by UPLC, TFA added to quench the reaction mixture and then it was freeze dried. The crude peptide was purified by RP-HPLC using Reprosil C8 (250x40mm, 200Å, 5μm) column and the following gradient of eluent B was used: 20% B for 5 min; 20% of B to 40% in 25 min; flow rate 60 mL/min. Product-containing fractions were collected and lyophilized to obtain the purified product as a TFA salt (Y =5%). The purified peptide was analyzed by LC/MS (Method B). $[M+4H]^{4+}$ mass signal found under the peak with retention time 3.23 min revealed the peptide mass 1076.8 which is in line with the expected value of 4301.09 molecular weight.

Synthesis of Seq ID 10:

[$^{\#}$CH$_2$CONH$_2$GTYKSTVIVNNKEKTTAEYQ-(Ttds)$_4$-K(N$_3$)-NH$_2$]-[QHQMLFYhC$^{\#}$DDVLFYNISSMK-CONH$_2$]

The structure of the peptide is shown in Figure 16.

Seq ID 10 is the heterodimer obtained through a thioether linkage between hCys8 of intermediate A (INT A) (QHQMLFYhC#DDVLFYNISSMK-CONH2) and the bromoacetyl group of intermediate B (INT B) [BrCH$_2$CO-G#TYKSTVIVNNKEKTTAEYQ-(Ttds)4-K(N$_3$)-NH$_2$].

A 5 mM solution of INT B (1 eq) in DMSO was added dropwise to a 2 mM solution of INT A (1 eq) in DMSO in the presence of DIPEA (5 eq). The reaction was stirred at room temperature 1 hour during which time reaction progression was monitored by LCMS. TFA was added to reaction mixture to reach pH 4 and crude purified by RP-HPLC using Reprosil C8 (250x40mm, 200Å, 5µm) column. The following gradient was employed: 25% B for 5 min; 25% of B to 45% in 25 min; flow rate: flow rate 40 mL/min. Product-containing fractions were collected and lyophilized to obtain the purified product as a TFA salt (Y = 21%). The purified peptide was analyzed by LC/MS (Method B). $[M+4H]^{4+}$ mass signal found under the peak with retention time 4.35 min revealed the peptide mass 1545.5 which is in line with the expected value of molecular weight 6173.13.

Synthesis of Seq ID 38:

[Ac-K(N$_3$)-(Ttds)$_4$-PRVTLDKKEAIQGGIVRVNSSVPEEK-Ttds-K$^{\#}$ (CH$_2$CO)-CONH$_2$]-[QHQMLFYhC$^{\#}$DDVLFYNISSMK-CONH$_2$]

The structure of the peptide is shown in Figure 17.

Seq ID 11 is the heterodimer obtained through a thioether linkage between hCys8 of intermediate A (INTA (QHQMLFYhCDDVLFYNISSMK-CONH$_2$) and the bromoacetyl group of intermediate C (INT C) [Ac-K(N$_3$)-(Ttds)$_4$-PRVTLDKKEAIQGGIVRVNSSVPEEK-Ttds-K(BrCH$_2$CO)-CONH$_2$].

A 5 mM solution of INT C (1 eq) in DMSO was added dropwise to a 2 mM solution of INT A (1 eq) in DMSO in the presence of DIPEA (5 eq). The reaction was stirred at room temperature 1 hour during which time reaction progression was monitored by LCMS. TFA was added to reaction mixture to reach pH 4 and crude purified by RP-HPLC using Reprosil C8 (250x40mm, 200Å, 5µm) as column. The following gradient of eluent B was employed: 25% B for 5 min; 25% of B to 45% in 25 min; flow rate: flow rate 40 mL/min.

Product-containing fractions were collected and lyophilized to obtain the purified product as a TFA salt (Y = 5%). The purified peptide was analyzed by LC/MS (Method B). $[M+5H]^{5+}$ mass signal found under the peak with retention time 4.59 min revealed the peptide mass 1445.9 which is in line with the expected value of 7221.47 molecular weight.

INT A: QHQMLFYhCDDVLFYNISSMK-$CONH_2$

The structure of the intermediate A is shown in Figure 18.

The linear precursor common intermediate for heterodimers Seq ID 10 and 11 was prepared following the general procedure for the synthesis and cleavage: all the Fmoc deprotections were performed at room temperature. The crude peptide was purified using Reprosil C4 (250x40mm, 120Å, 5µm) as column and the following gradient of eluent B: 20% B for 5 min; 20% of B to 40% in 25 min; flow rate: flow rate 60 mL/min, wavelength 214 nm. Product-containing fractions were collected and lyophilized to obtain the purified product as a TFA salt (Y = 2%).

INT B: ($BrCH_2CO$-GTYKSTVIVNNKEKTTAEYQ-$(Ttds)_4$-$K(N_3)$-NH_2)

The structure of the intermediate B is shown in Figure 19.

Linear precursor for heterodimer Seq ID 10, was prepared following the general procedure for the synthesis. At the end of the assembly, the free amino group on the last residue on the resin (gly) was treated with bromoacetic acid (5 eq) according to the DIC/HOAt method (4 equivalent excess with respect to resin loading) in DMF. The mixture was shaken at room temperature for 1 h and the reaction was monitored by Kaiser Test. The resin was filtered and washed with DMF/DCM/DMF (6/6/6 time each) and treated with the cleavage mixture (Mix 1) as reported in the general synthetic procedure. The crude peptide was purified by RP-HPLC using Reprosil C4 (250x40mm, 120Å, 5µm) column and the following gradient: 20% B for 5 min; 20% of B to 40% in 25 min; flow rate: flow rate 60 mL/min, wavelength 214 nm. Product-containing fractions were collected and lyophilized to obtain the purified product as a TFA salt (Y = 30%).

INT C: Ac-$K(N_3)$-$(Ttds)_4$-PRVTLDKKEAIQGGIVRVNSSVPEEK-Ttds-$K(BrCH_2CO)$-$CONH_2$

The structure of the intermediate C is shown in Figure 20.

Linear precursor C for heterodimer Seq ID 11 was prepared following the general synthetic procedure. The first amino acid loaded onto the resin was Lys protected with

alloc on the side chain amino group. Double acylations were performed on the following residues: SVP, IVRV and PRVTL: after the PRVTL residues all Fmoc deprotections were performed at room temperature.

At the end of the assembly the resin was suspended in DCM: phenylsilane (24 eq) and $Pd(PPh_3)_4$ (0.25 eq) were added and the resin was shaken for 30 minutes. The cycle was repeated twice. At the end of the second cycle a solution of 0.5% DIPEA and 0.5% Sodium dithiocarbamate in DMF is percolated to the resin to remove Palladium residues. Coupling of the Lys deprotected amino group with bromoacetic acid (5 eq) was performed with DIC/HOAt method (4 equivalent excess with respect to resin loading) in DMF. The mixture was shaken at room temperature for 1 h and the reaction was monitored by Kaiser Test. The resin was filtered and washed with DMF/DCM/DMF (6/6/6 time each) and treated with the cleavage mixture (Mix 1) as reported in the general synthetic procedure. The crude peptide was purified: Reprosil C4 (250x40mm, 120Å, 5µm) column and the following gradient: 20% B for 5 min; 20% of B to 40% in 25 min; flow rate: flow rate 60 mL/min, wavelength 214 nm. Product-containing fractions were collected and lyophilized to obtain the purified product as a TFA salt (Y = 30 %).

Protocol

Nitinol disks are left untouched (bare metal) or coated with a domain 1, domain 2 or domain 1 and 2 CD31 analog peptide. The tested peptide is grafted to the nitinol disk following a polydopamine, DBCO-PEG4-amine and azide CD31 derivative peptide using a sequential dip-coating layering procedure. After this step, all the disks are incubated with Human aortic endothelial cells for 48h. Disks are then rinsed and the cells are fixed in a formalin solution before staining. The staining is fulfilled using phalloidin coupled to AlexaFluor®488 dye (green fluorescence for Actin), DAPI is used for DNA staining and a domain 1 CD31 antibody coupled to APC is used for external Domain 1 staining. The cell adhesion and Actin/CD31 expression was evaluated by image analysis of the surface after staining.

The results are shown in Figures 2 through to 6.

In particular, as per Figure 2 the bare or coated nitinol disks were placed at the bottom of culture wells. Primary Human Aortic Endothelial Cells (HAEC) were allowed to grow on the disks for a period of 48h. The disks were thereafter rinsed with phosphate buffered saline, fixed in formalin, and processed for fluorescence microscopy. Disk-

adherent cells were immersed in a solution comprising Hoechst 33342 (1 µg/ml, to stain the nuclei), phalloidin coupled to AlexaFluor®488 dye (0,5µM, to stain F-actin fibers) and a monoclonal antibody directed against CD31 domain 1, coupled to allophycocyanin (to detect the presence and localization of intact CD31 at the cell surface) for 1 hour at 37°C, washed and mounted face down on glass bottom imaging chambers for imaging with an inverted fluorescence microscope. Representative images show that HAEC growing on bare material displayed a strong F-actin staining, reflecting enhanced formation of stress fibers whereas the expression of intact CD31 was rather faint. At the opposite, disks coated with CD31-mimetic peptides displayed little stress fibers signal and an intense and uniform CD31 expression at the cell borders, reflecting a more physiologic endothelial phenotype.

Figure 3 shows quantitative analysis of the signal on individual disk images is expressed as "Integrated Density" (signal Density multiplied by the area of positive staining, as reported by ImageJ analysis open software). Surface type is indicated on the Y axis, data are expressed as average±SD (N=6/surface type). Each dot corresponds to a specific surface modification. The F-actin expression relates the cellular stress of the cell in presence of the exogenous material. All surface modification lead to a decrease in cellular stress compared to the bare metal, except for the 1070 peptide, which does not show an influence on the cellular stress compared to the bare metal.

In Figure 4 the surface type is indicated on the Y axis. Each dot corresponds to a specific surface modification. Data are expressed as "Integrated Density" average±SD (N=6/surface type). CD31 expression relates the capacity of endothelial cells to adopt a physiologic phenotype while growing on a given substrate. An important variability is observed in terms of CD31 expression by HAEC according to the surface modification of the nitinol disks. Four of the displayed CD31 analog peptide (peptide 1072-1374-1375 and 1380) exert an evident positive effect in terms of CD31 expression as compared to the other modified disks and bare controls.

The "mirror" bars of Figure 5 report the average Integrated Density of F-actin (left) and of CD31 (right) staining for each of the indicated (Y axis) type of peptide coated onto the disk surface and bare disks. Data are sorted (from the top to the bottom) according to the increase in the cellular stress (actin expression) of each coating. As shown on the figure, the degree of cellular stress (as detected by a high actin expression) and impaired endothelial phenotype (documented by a low CD31 expression) is evident

on HAEC grown onto bare metal disks. On the contrary, the CD31-mimetic surfaces, such the disks coated with the 1374 peptide, lead to a low cellular stress (low actin expression) and consistent functional endothelial phenotype (high CD31 expression).

The data shown in Figure 6 are expressed as the ratio between CD31 and F-actin expression. As compared to bare metal and disks coated with PDA only, cells growing onto surfaces coated with peptides 1072, 1074, 1075 and 1080 clearly show a higher CD31/actin ratio, reflecting a more physiological endothelial cell phenotype.

Coating

The coating of the device or of device-suitable components is achieved through a three-step dip-coating during which the sample is allowed to be completely coated by dynamic dipping.

Gentle vertical movements with static immersions periods of 2 minutes in upper and 2 minutes in lower position into a long tube filled with the solution is performed. The first bath is composed of Polydopamine (2 mg/ml in tris buffer, pH 8.5) over a period of 22±2 hours. The second bath allows the grafting of the click-chemistry-suitable linker (for instance DBCO-PEG 4 –Amine, 300 μM). The third bath consists in a solution of the chosen Azide-derivatized CD31 analog peptide (domain 1-2 or 1 and 2).

From the above description, the advantages of the present invention will be clear to the person skilled in the art.

Whereas the P8RI sequence can uphold the clusterisation of truncated CD31 molecules expressed by activating cells, the presence of the invention peptides allows the engagement of intact CD31 molecules on all the healthy endothelial cells and resting blood platelets and leukocytes that can enter in contact with an implanted device.

Those cells can therefore receive the “leave-me-alone” signal delivered by the trans-homophilic engagement of CD31, which is essential to maintain the homeostasis in the circulation and vascularized tissues.

Thrombotic or life-threatening occurrence of hemorrhagic or thromboembolic complications have impaired the use of endovascular devices.

The devices bearing the mimicking peptides of the present invention are rapidly integrated, because they are perceived by blood platelets and leukocytes as a healthy endothelium, a “self” component.

Furthermore, their ability to be rapidly endothelialized with a physiologic endothelial cell phenotype also limits platelet and leukocyte activation at the site of device implantation in the long-term.

CLAIMS

1. A peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction.

2. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to claim 1 comprising one of the following sequences:

Amino acidic sequence	**Seq. ID. No.**
HQMLFYKDDVLFYNISS	4
YKSTVIVNNKEKTTAE	5
PRVTLDKKEAIQGGIVRVNSSVPEEK	6
HQMLFYKDDVLFYNISSC	7
SHQMLFYKDDVLFYNISSS	8
QHQMLFYKDDVLFYNISSMK	9
CHQMLFYKDDVLFYNISSCK	10
YKDDVLFYNISSMKST	11
PQHQMLFYKDDVLFYNISSMK	12
FADVSTTSHVKPQHQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYK	13
PQHQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV	14
YKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV	15
HQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV	16
LFYKDDVLFYNISSMKSTESYF	17
PQHQMLFYKDDVLFYNISSMK	18
QHQMLFYhCDDVLFYNISSMK, being hC=homocysteine	28
YKSTVIVNNKEKTTAE	19
DDVLFYNISSMK	20
QHQMLFY	21
CYKSTVIVNNKEKTTAEYC	22
hCYKSTVIVNNKEKTTAEYhC, being hC=homocysteine	23
GTYKSTVIVNNKEKTTAEYQ	24
PRCTLDKKEAIQGGIVRVNCSVPEEK	25
RDQNFVILEFP	26

3. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to claim 1, which comprises two of said sequences.

4. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to claim 1, which comprises:

- a peptide of structure (IA) and a peptide of structure (IB), or
- a peptide of structure (IA) and a peptide of structure (IC).

5. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to the preceding claim, which is characterized by comprising the followings sequences:

Aminoacidic sequences	**Seq. ID No.**
GTYKSTVIVNNKEKTTAEYQ and QHQMLFYhCDDVLFYNISSMK	24 and 28 (SEQ ID No.37)
PRVTLDKKEAIQGGIVRVNSSVPEEK and QHQMLFYhCDDVLFYNISSMK	6 and 28 (SEQ ID No.38)

6. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to any one of the preceding claims, which is a cyclic peptide.

7. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to any one of the preceding claims, which comprises a linker and/or a spacer and/or a tail at any terminus or linked to an aminoacidic residue.

8. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to the preceding claim, wherein said linker and/or a spacer and/or a tail is represented by:

GGSGGSGG	SEQ. ID No. 27
one or more PEG4 units	
one or more Ttds units	

or combinations thereof.

9. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to any one of the preceding claims, which comprises a modification at the C-terminus and/or at the N-terminus.

10. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to the preceding claim, wherein said modification is selected from:

Ac-
Br-CH_2-CO-
CH_2-CO-
CH_2-$CONH_2$-
Ac-KN_3
-NH_2
-CO-NH_2

11. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to any one of the preceding claims, which comprises any one of the modifications:

- a cysteine residue may be substituted by the corresponding homocysteine,
- an L-amino acid residue may be substituted by the corresponding D-amino acid residue.

12. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to any one of the preceding claims, which comprises a peptide having at least 95% identity of the sequence, preferably at least 97% and even more preferably at least 99% identity of the sequence.

13. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to any one of the preceding claims, which is characterized by having one of the following structures:

Aminoacidic sequence	**SEQ. ID No.**
QHQMLFYCDDVLFYNISSMK-$CONH_2$	1
($BrCH_2CO$-GTYKSTVIVNNKEKTTAEYQ-$(Ttds)_4$-K(N_3)-NH_2)	2
Ac-K(N_3)-$(Ttds)_4$-PRVTLDKKEAIQGGIVRVNSSVPEEK-Ttds-K($BrCH_2CO$)-$CONH_2$	3
Ac-c*HQMLFYKDDVLFYNISSC*-GGSGGSGG-K(N_3)-$CONH_2$	29
SHQMLFYKDDVLFYNISSS-GGSGGSGG-K(N_3)-$CONH_2$	30

Ac-KKKc*HQMLFYKDDVLFYNISSC*-Ttds-Ttds-K(N_3)-$CONH_2$	31
QHQMLFYKDDVLFYNISSMK-Ttds-Ttds-Ttds-Ttds-K(N_3)-$CONH_2$	32
hCHQMLFYKDDVLFYNISShCK-Ttds-Ttds-Ttds-Ttds-K(N_3)-$CONH_2$	33
YKSTVIVNNKEKTTAE-PEG4-K(N_3)-$CONH_2$	34
Ac-K(N_3)-PEG4-PRVTLDKKEAIQGGIVRVNSSVPEEK-$CONH_2$	35
Ac-K(N_3)-(Ttds)$_4$-PRhCTLDKKEAIQGGIVRVNhCSVPEEK-$CONH_2$	36
[$^{\#}CH_2CONH_2$GTYKSTVIVNNKEKTTAEYQ-(Ttds)$_4$-K(N_3)-NH_2]-[QHQMLFYhC$^{\#}$DDVLFYNISSMK-$CONH_2$]	37
[Ac-K(N_3)-(Ttds)$_4$-PRVTLDKKEAIQGGIVRVNSSVPEEK-Ttds-K$^{\#}$ (CH_2CO)-$CONH_2$]-[QHQMLFYhC$^{\#}$DDVLFYNISSMK-$CONH_2$]	38
Ac-K(N_3)-GGSGGSGG-YKDDVLFYNISSMKST-NH_2	39
Ac-YKDDVLFYNISSMKST-GGSGGSGG-K(N_3)-NH_2	40
Ac-PQHQMLFYKDDVLFYNISSMK(GGSGGSGG-K(N_3))STESYFI-$CONH_2$	41
Ac-K(N_3)-GGSGGSGG-FADVSTTSHVKPQHQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYK-$CONH_2$	42
Ac-K(N_3)-GGSGGSGG-PQHQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV-$CONH_2$	43

Ac-K(N_3)-GGSGGSGG-YKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV-$CONH_2$	44
Ac-K(N_3)-GGSGGSGG-HQMLFYKDDVLFYNISSMKSTESYFIPEVRIYDSGTYKSTV-$CONH_2$	45
Ac-LFYKDDVLFYNISSMKSTESYF-GGSGGSGG-K(N_3)-$CONH_2$	46
Ac-LFYKDDVLFYNISSMKSTESYF-Ttds-Ttds-K(N_3)-$CONH_2$	47
Ac-PQHQMLFYKDDVLFYNISSMK(GGSGGSGG-K(N_3))STESYFIKK-$CONH_2$	48
RDQNFVILEFP-PEG4-K(N_3)-NH_2	49
wherein hC: homoCysteine; c: D-Cysteine; ** position for disulfide bridge formation of cyclic peptides #acetyl thioether linkage	

14. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to any one of the preceding claims for medical use.

15. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to any one of the preceding claims for the prevention or the treatment of a vascular pathology.

16. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to the preceding claim for the prevention or the treatment of a vascular pathology selected from the group comprising: heart valve pathology, atherosclerosis, thrombosis, ischemia, hemorrhage, restenosis, aneurism.

17. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to any one of the preceding claims 14 to 16 for medical use for the prevention of a pathological condition represented by the in-stent stenosis.

18. The peptide mimicking the trans-homophilic CD31-CD31 domain 1 and 2 intercellular interaction according to any one of the preceding claims 14 to 17 for medical use in humans or in animals.

19. A coating comprising one or more of the peptides according to any one of claims 1 to 13.

20. The coating according to the preceding claim, which is a mono-layered or a multi-layered coating.

21. A medical device partially or fully coated with the coating according to the preceding claim 19 or 20.

22. The medical device according to the preceding claim, which is selected in the group comprising: coronary stent, flow diverting stent, aortic tubes, cardiac valves or any portions thereof.

23. A method for the prevention or for the treatment of heart and vascular pathologies comprising the use of a peptide according to any one of claims 1 to 13.

24. A method for the prevention or for the treatment of heart and vascular pathologies comprising the use of a medical device according to claim 21 or 22.

25. A method for the prevention of a condition represented by the in-stent stenosis comprising the use of a peptide according to any one of claims 1 to 13.

26. A method for the prevention of a condition represented by the in-stent stenosis comprising the use of a medical device according to claim 21 or 22.

27. The method according to any one of claims 23 to 26 which is performed on a human or on an animal.

28. Use of a biomimetic peptide according to any one of claims 1 to 13 for the adhesion to vascular device.

29. Use of a biomimetic peptide according to any one of claims 1 to 13 for promoting the endothelialization of a vessel, for preventing the neointimal growth or for the integration of a vascular device in the target vessel.

30. Use of a biomimetic peptide according to any one of claims 1 to 13 for improving the biocompatibility of a vascular device.

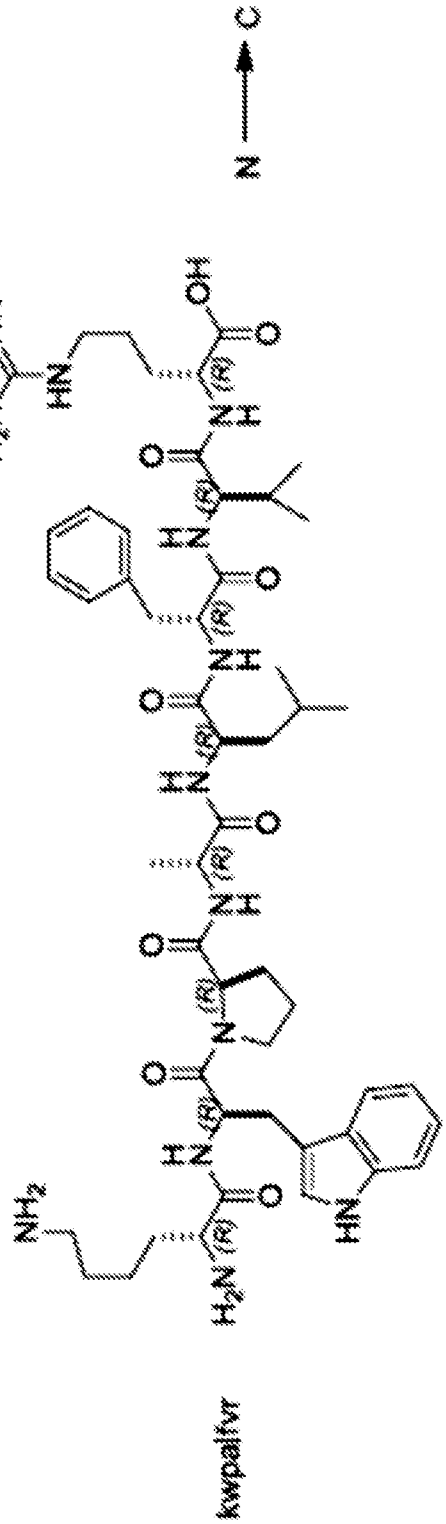
31. Use of a biomimetic peptide according to any one of claims 1 to 13 for endowing the stented segment with recovered endothelial anti-inflammatory and anti-thrombotic properties.

32. Use of a biomimetic peptide according to any one of claims 1 to 13 for endowing the stented segment with improved adaptive remodelling allowing recovery of its functional properties.

Fig. 1A

(D-Lys)-(D-Trp)-(D-Pro)-(D-Ala)-(D-Leu)-(D-Phe)-(D-Val)-(D-Arg).

Fig. 1B



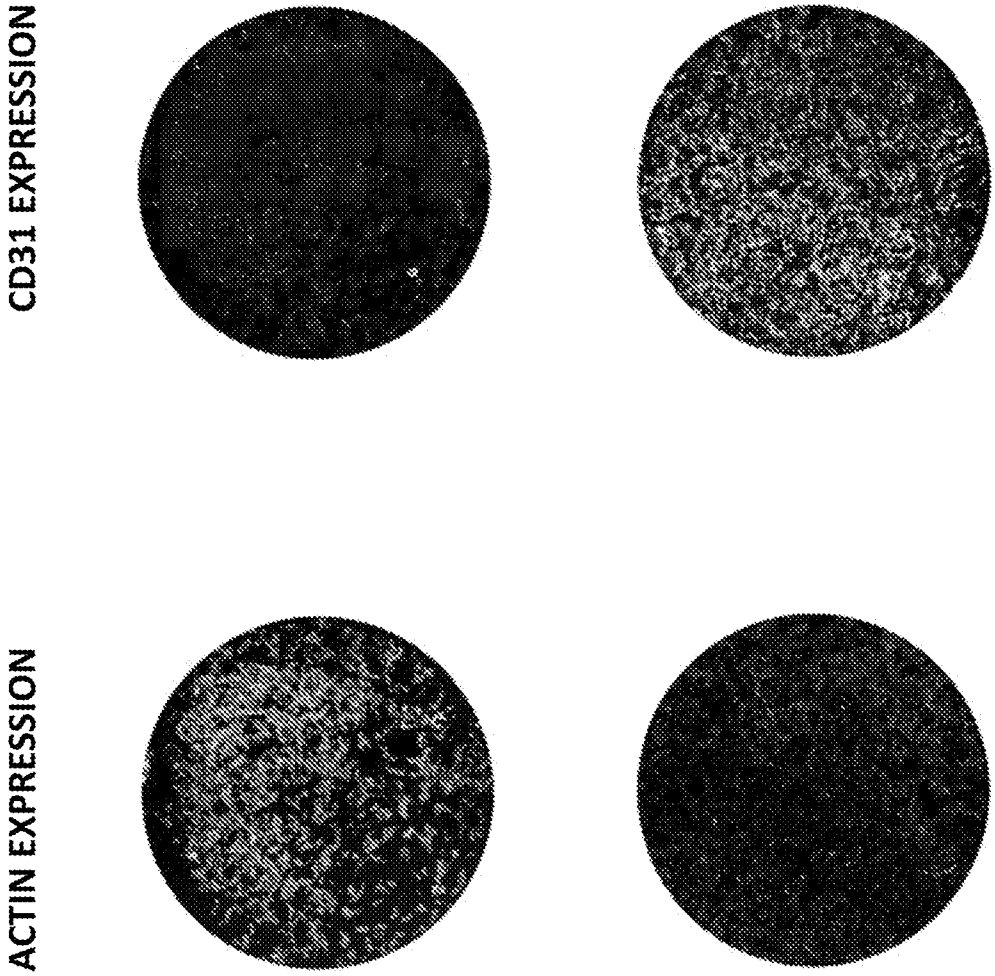

CD31 EXPRESSION
ACTIN EXPRESSION
BARE MATERIAL
COATED DIKS (P-1375)

Fig. 2

Fig. 3

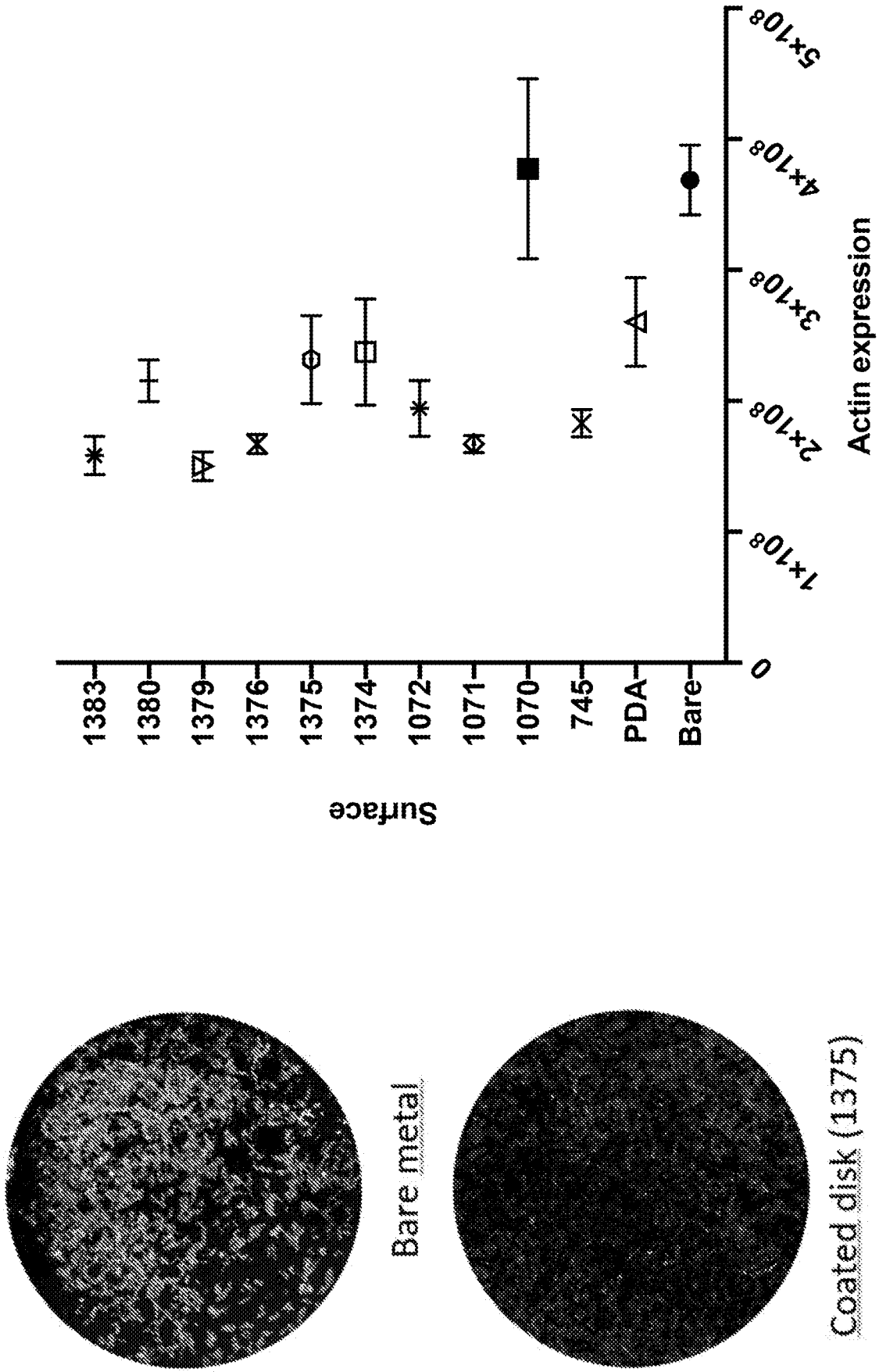

Surface
1383
1380
1379
1376
1375
1374
1072
1071
1070
745
PDA
Bare
0
1*10^8
2*10^8
3*10^8
4*10^8
5*10^8
Actin expression
Bare metal
Coated disk (1375)

Fig. 4

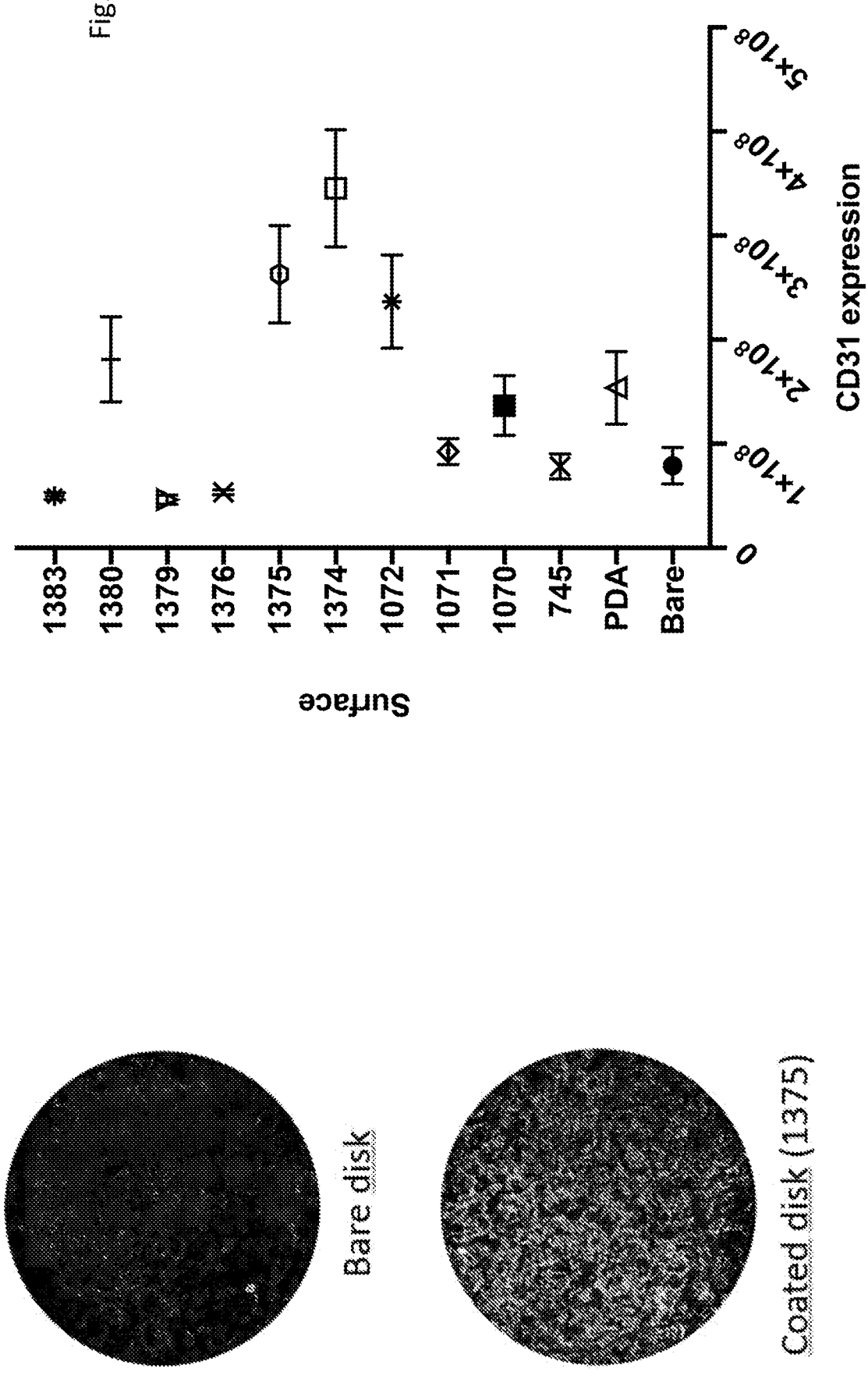
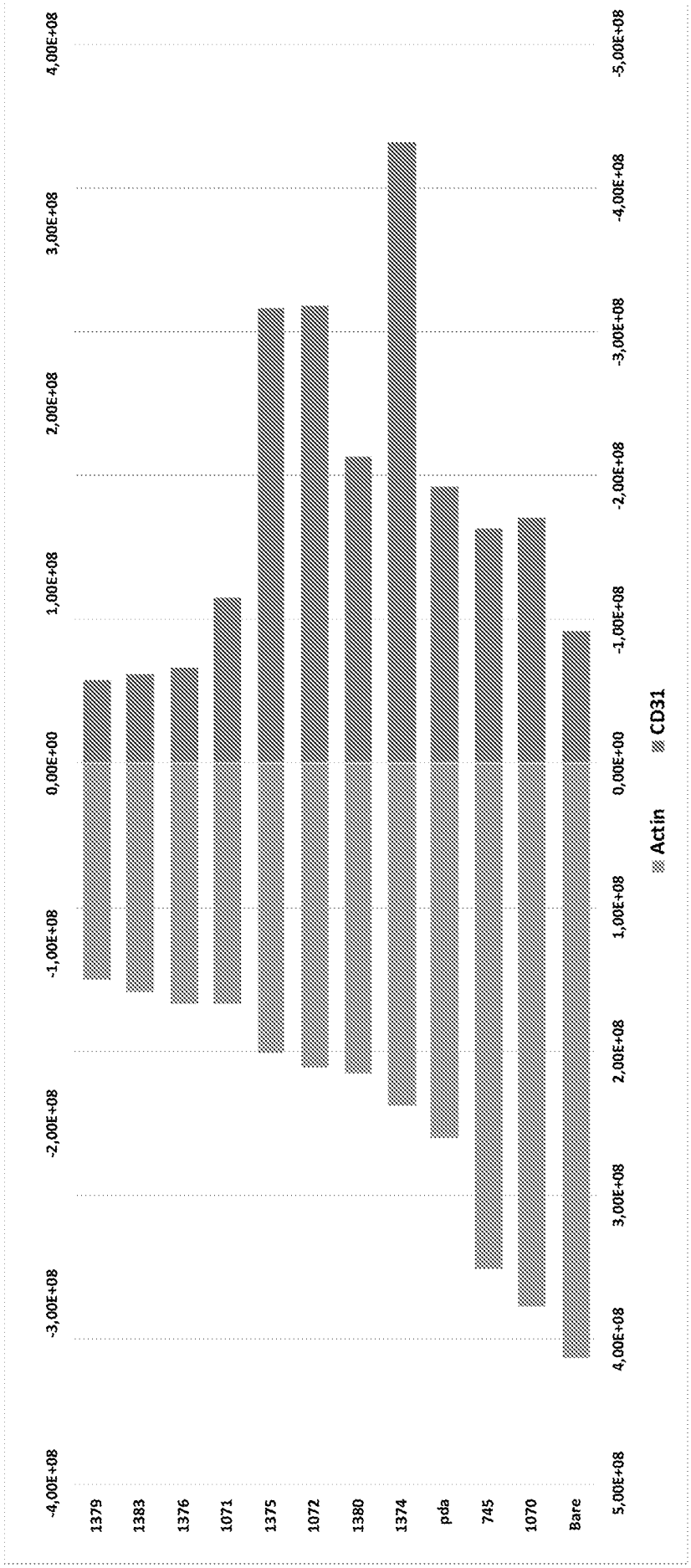

CD31 expression
0
1*10^8
2*10^8
3*10^8
4*10^8
5*10^8
Surface
Bare
PDA
745
1070
1071
1072
1374
1375
1376
1379
1380
1383
Bare disk
Coated disk (1375)

Fig. 5


-4,00E+08
-3,00E+08
-2,00E+08
-1,00E+08
0,00E+00
1,00E+08
2,00E+08
3,00E+08
4,00E+08
5,00E+08
4,00E+08
3,00E+08
2,00E+08
1,00E+08
0,00E+00
-1,00E+08
-2,00E+08
-3,00E+08
-4,00E+08
-5,00E+08
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pda
745
1070
Bare
Actin
CD31

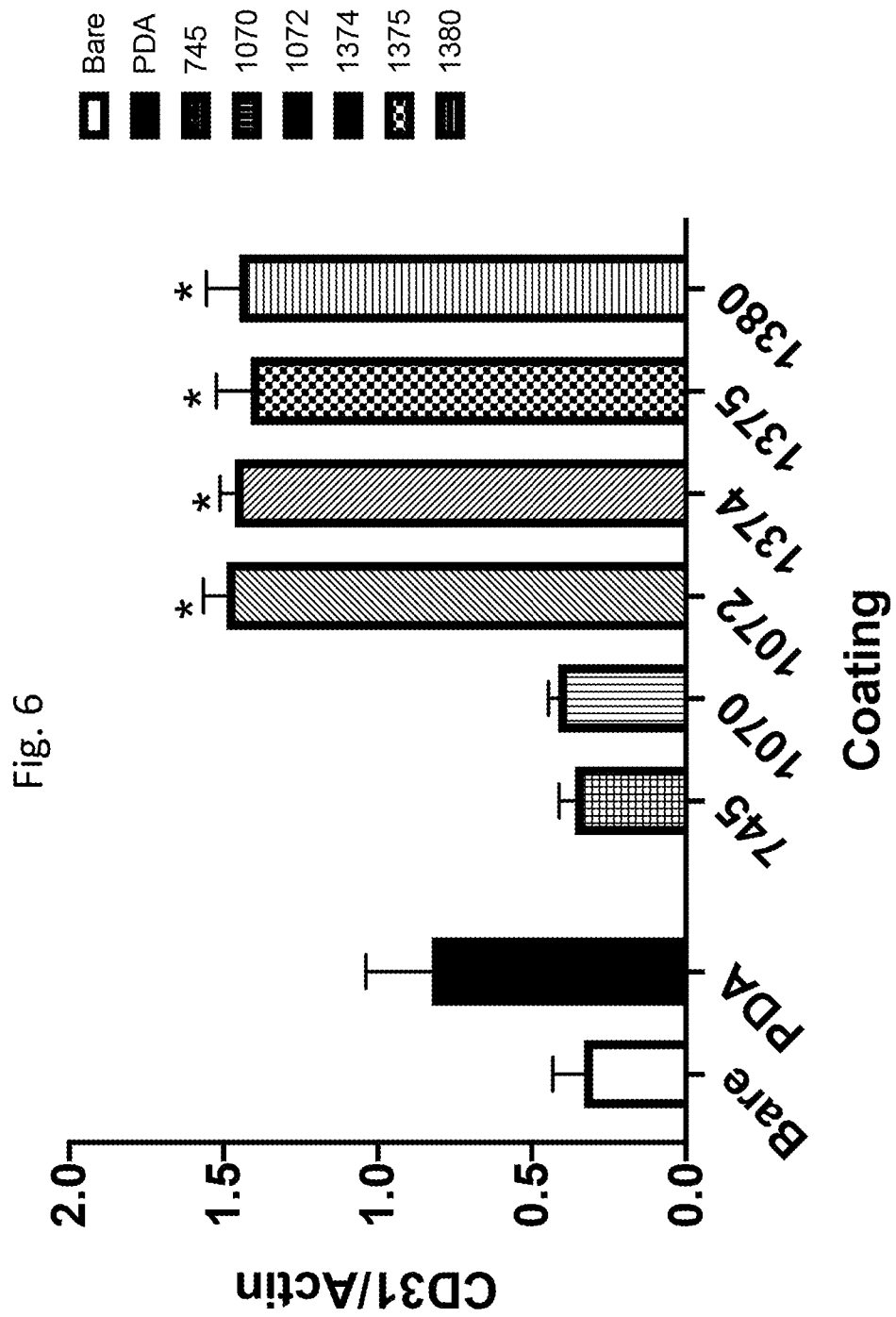
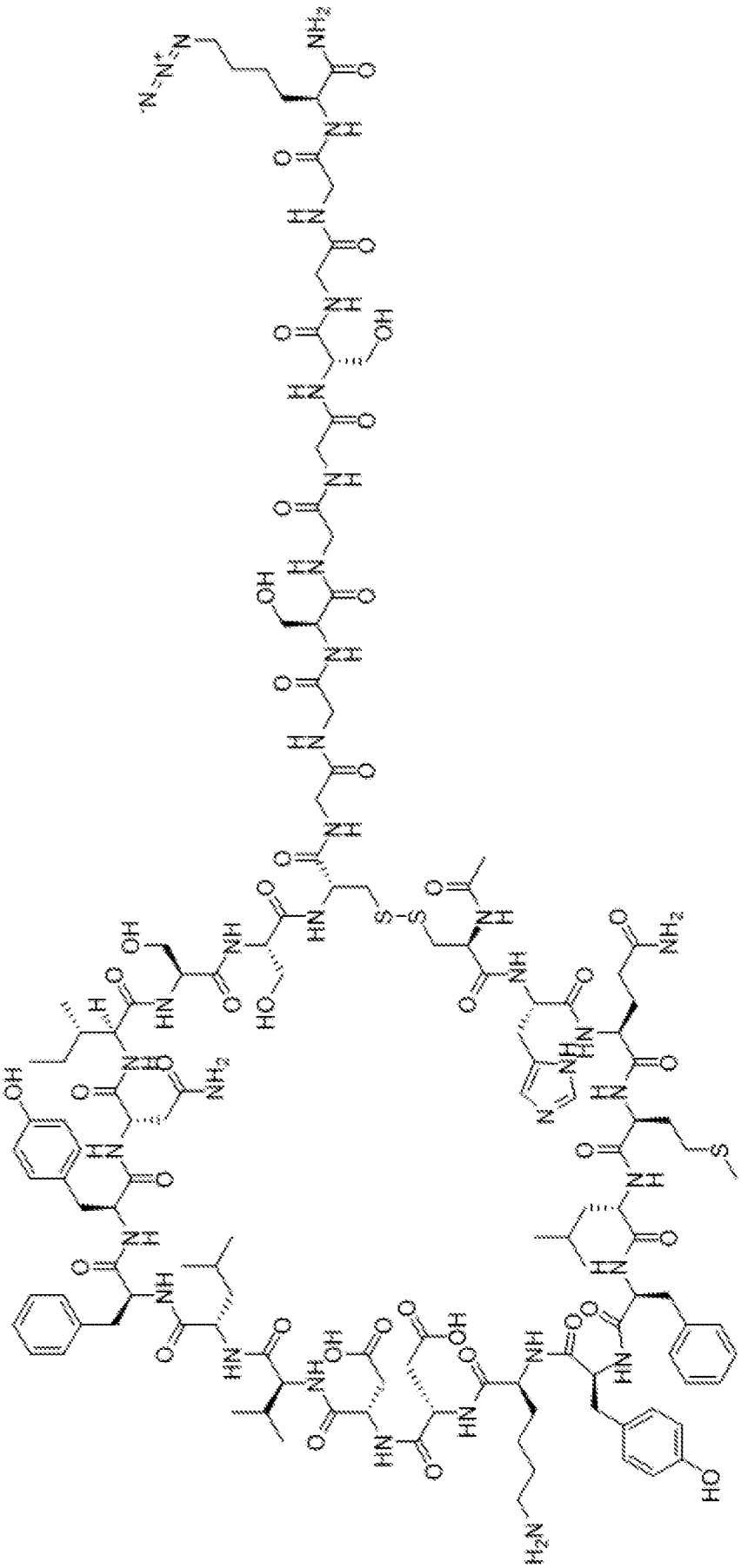

Bare
PDA
745
1070
1072
1374
1375
1380
CD31/Actin
2.0
1.5
1.0
0.5
0.0
Bare
PDA
745
1070
1072
1374
1375
1380
Coating
*
*
*
*

Fig. 6

Fig. 7



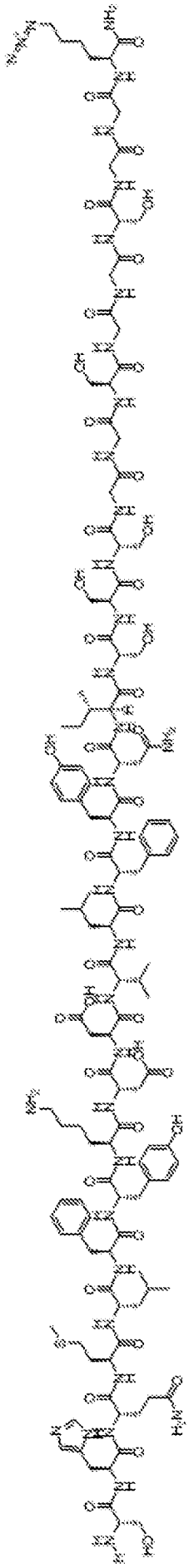


Fig. 8

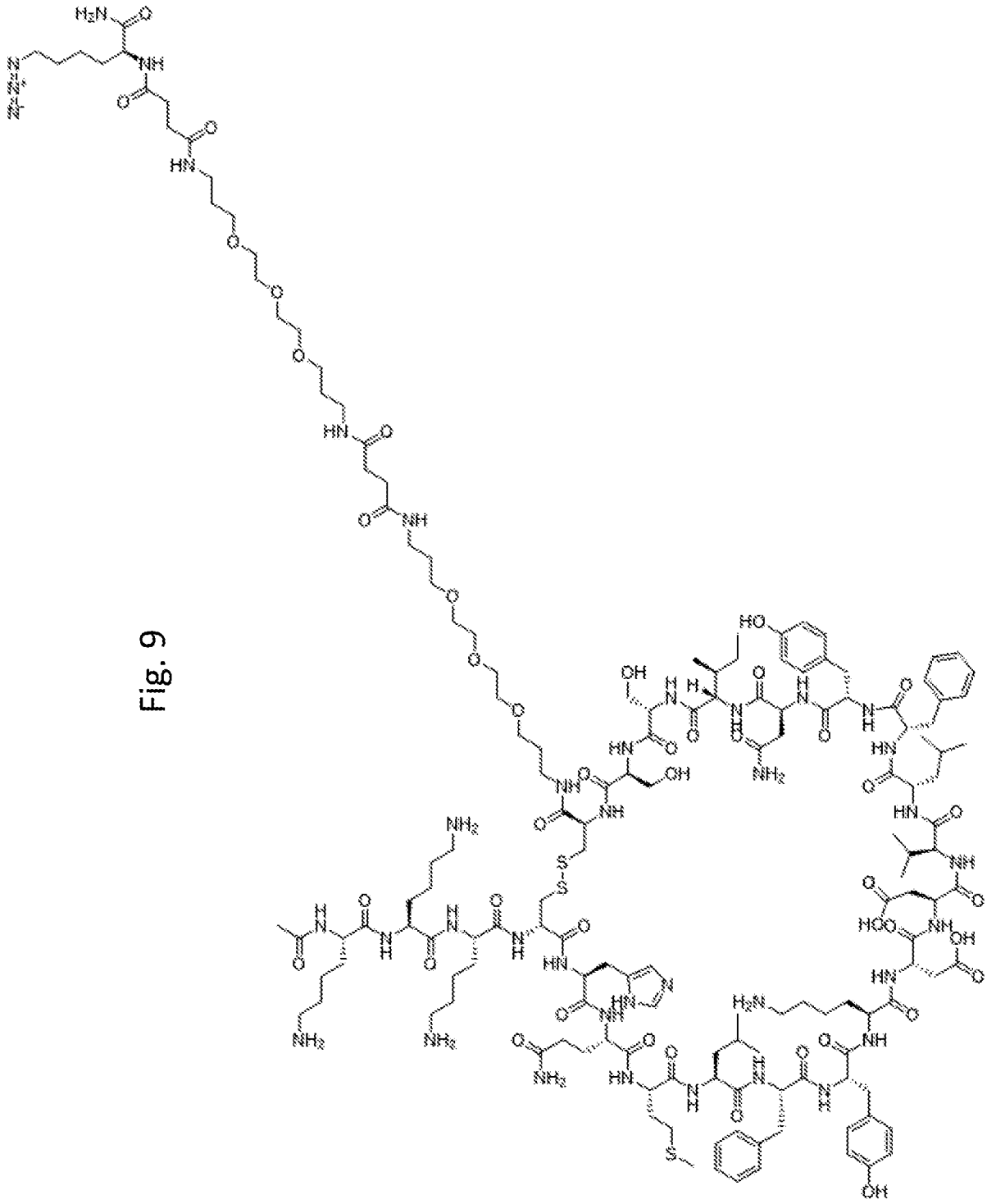


Fig. 9

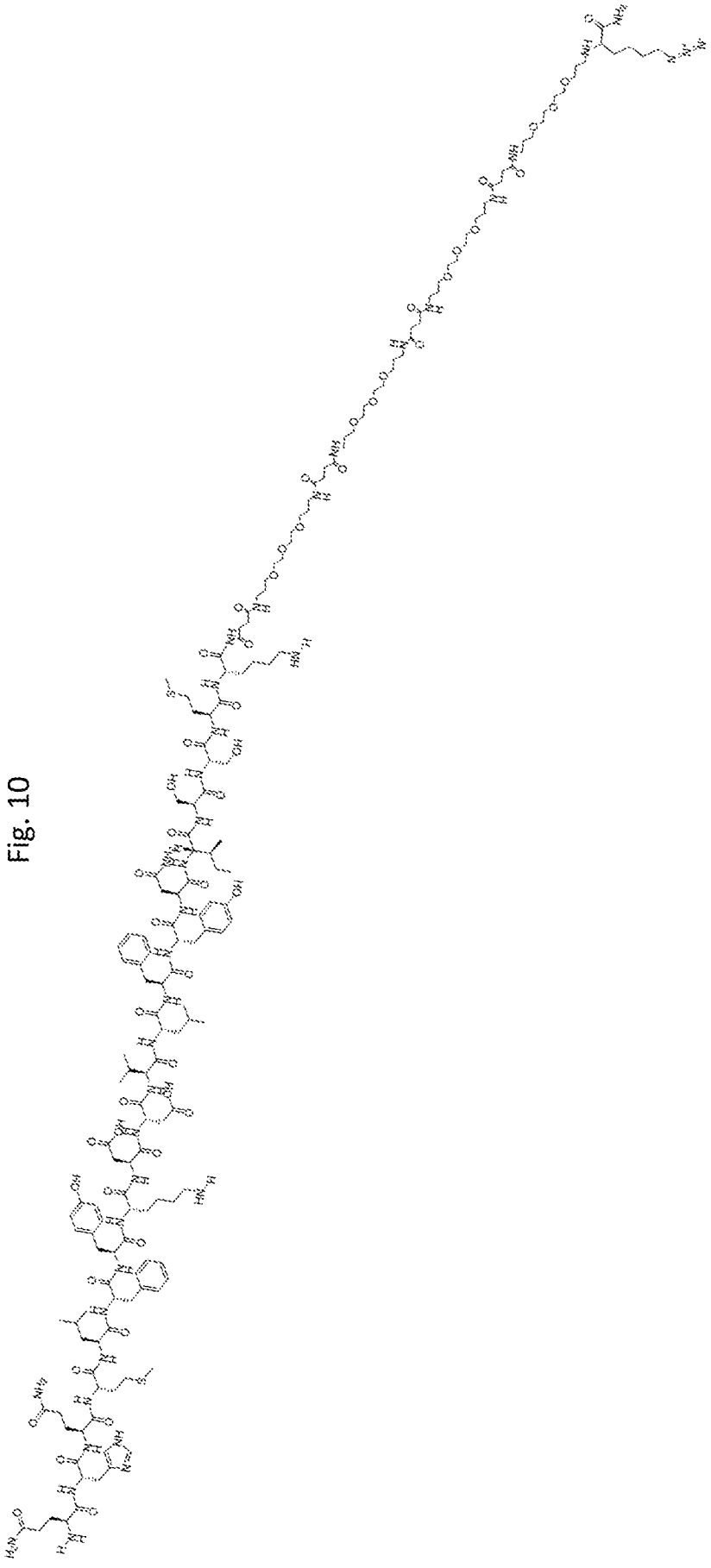


Fig. 10

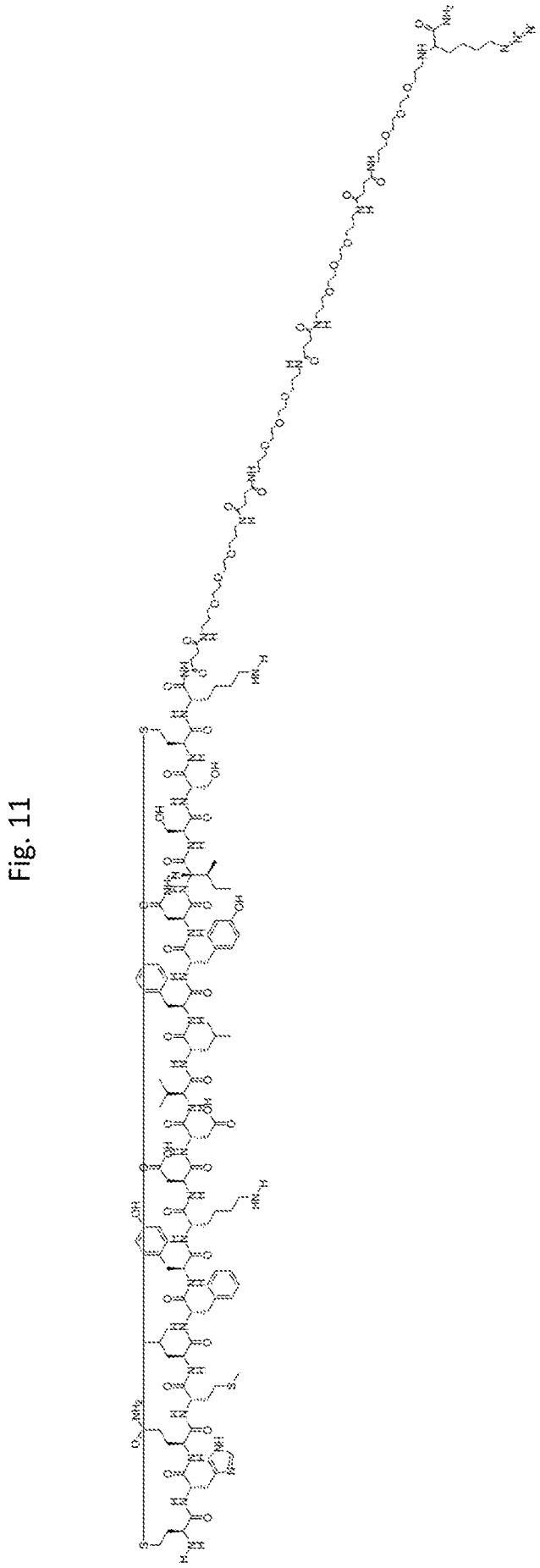


Fig. 11

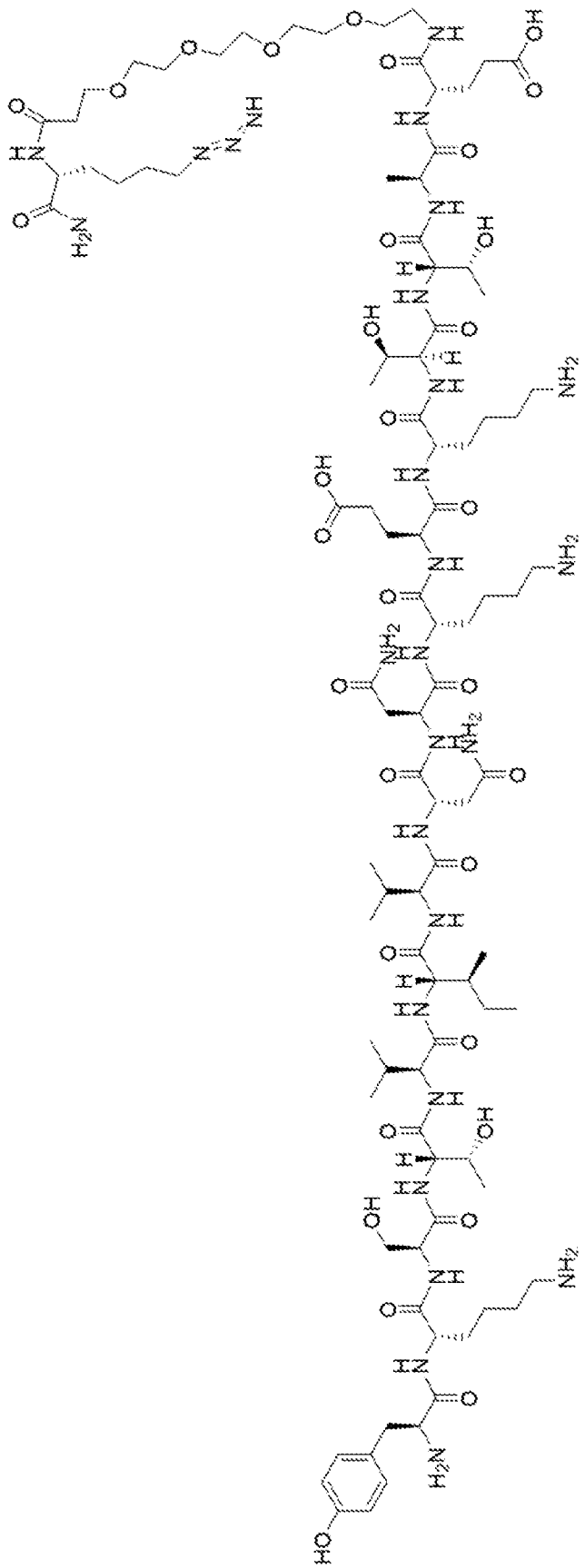


Fig. 12

Fig. 13

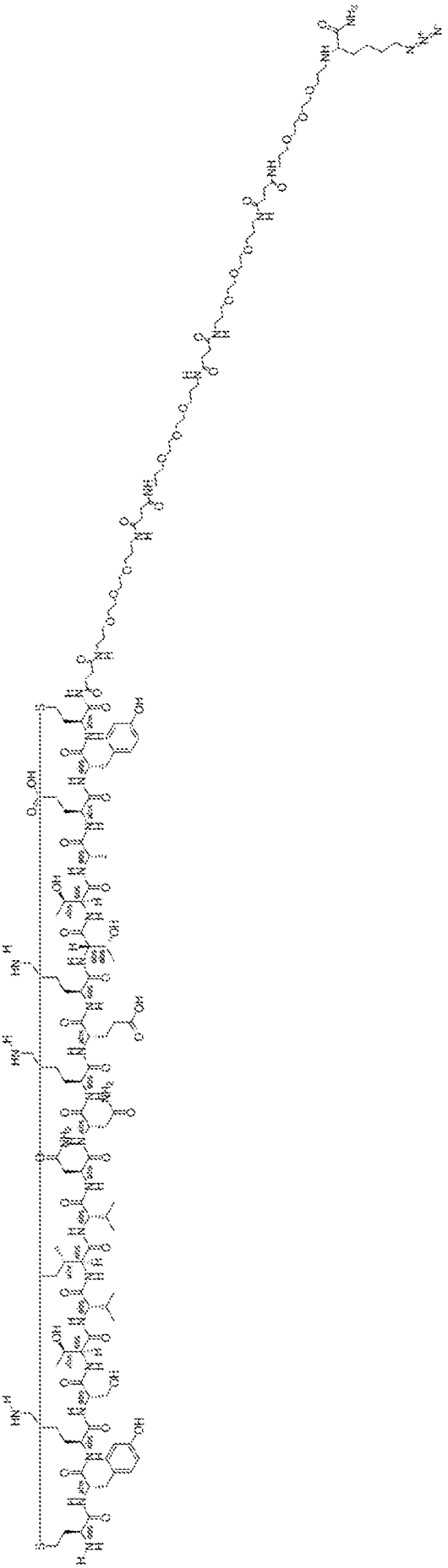
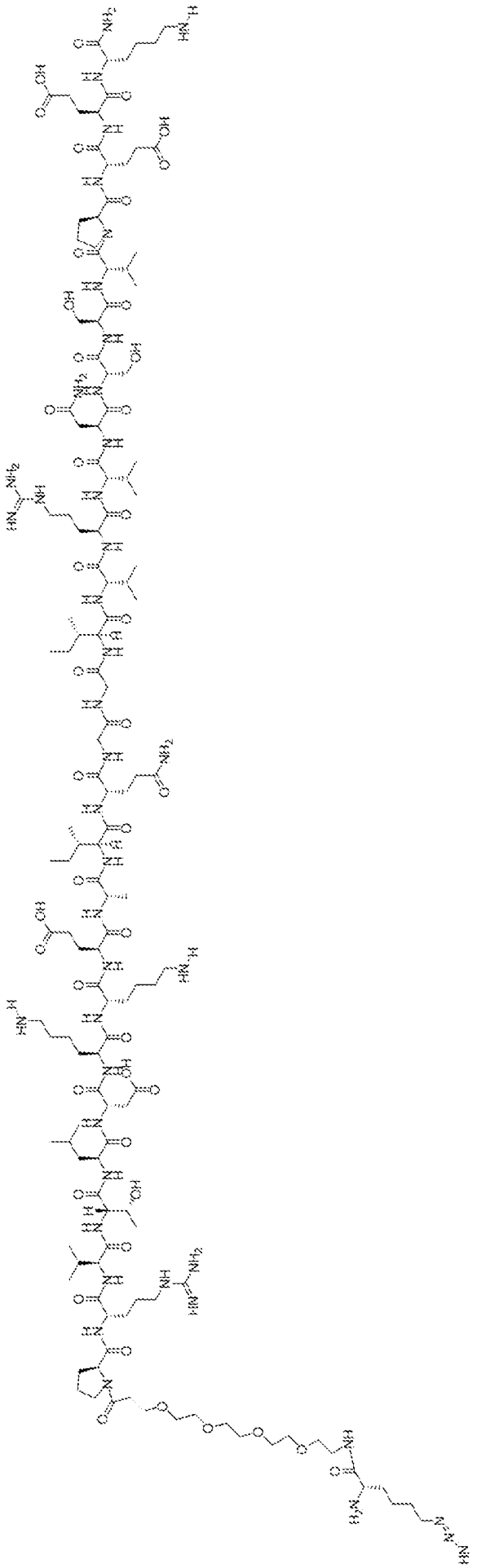


Fig. 14



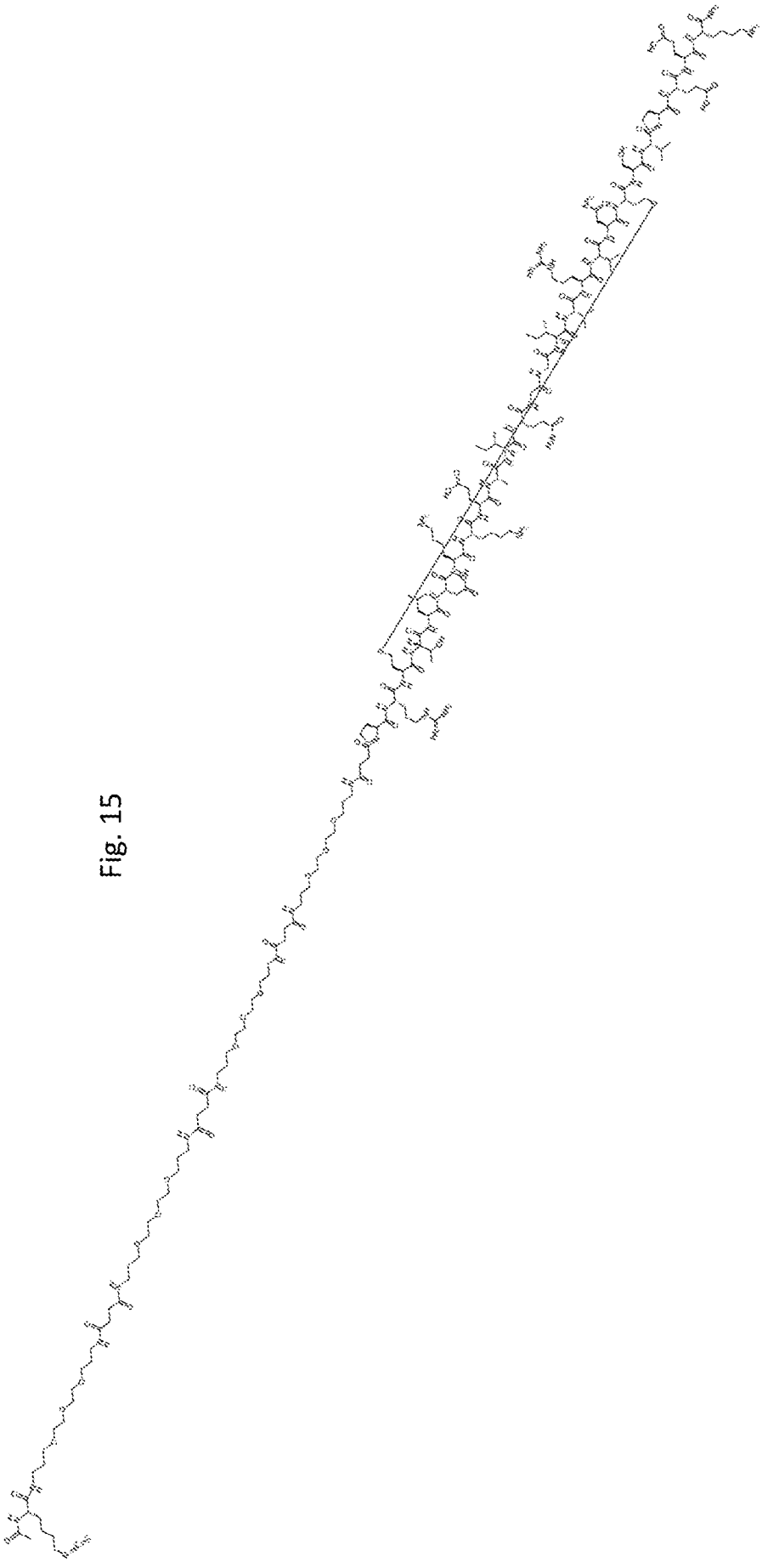


Fig. 15

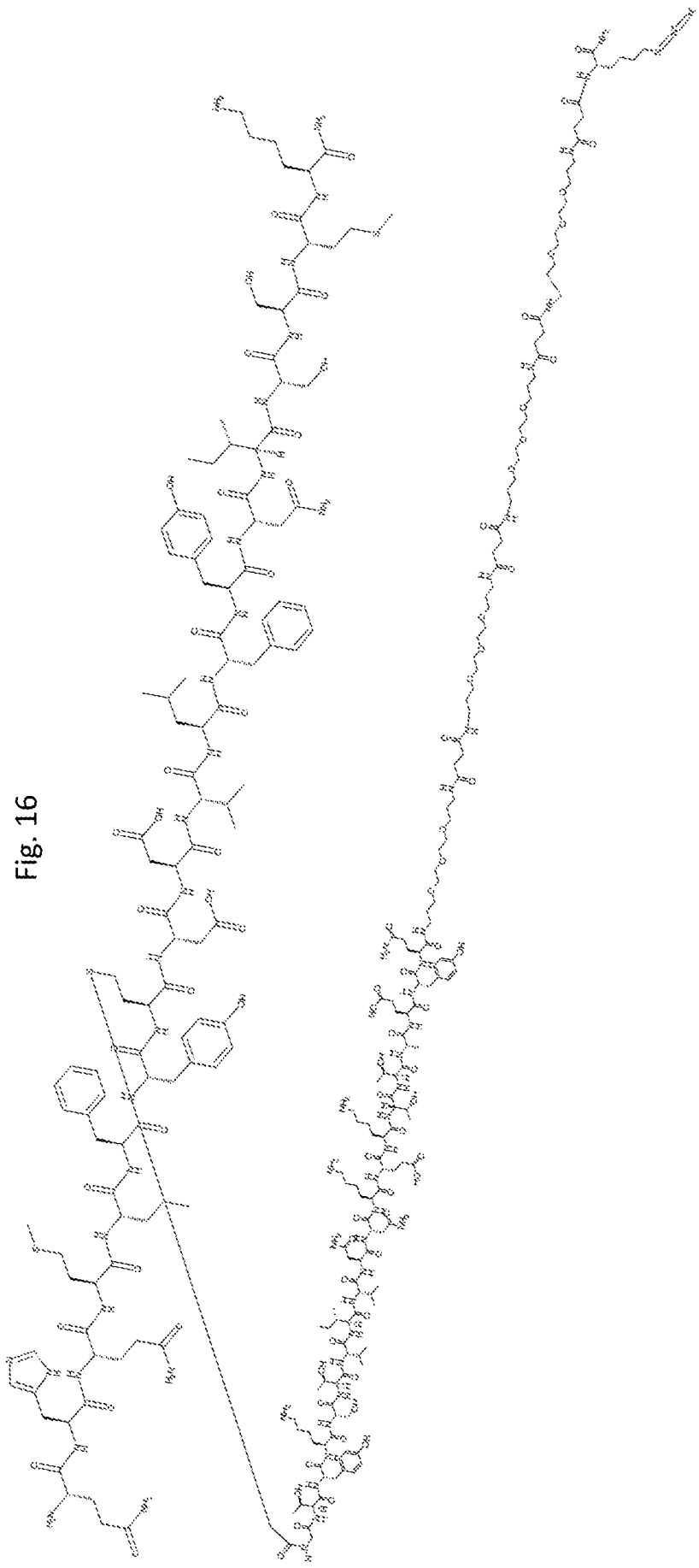


Fig. 16

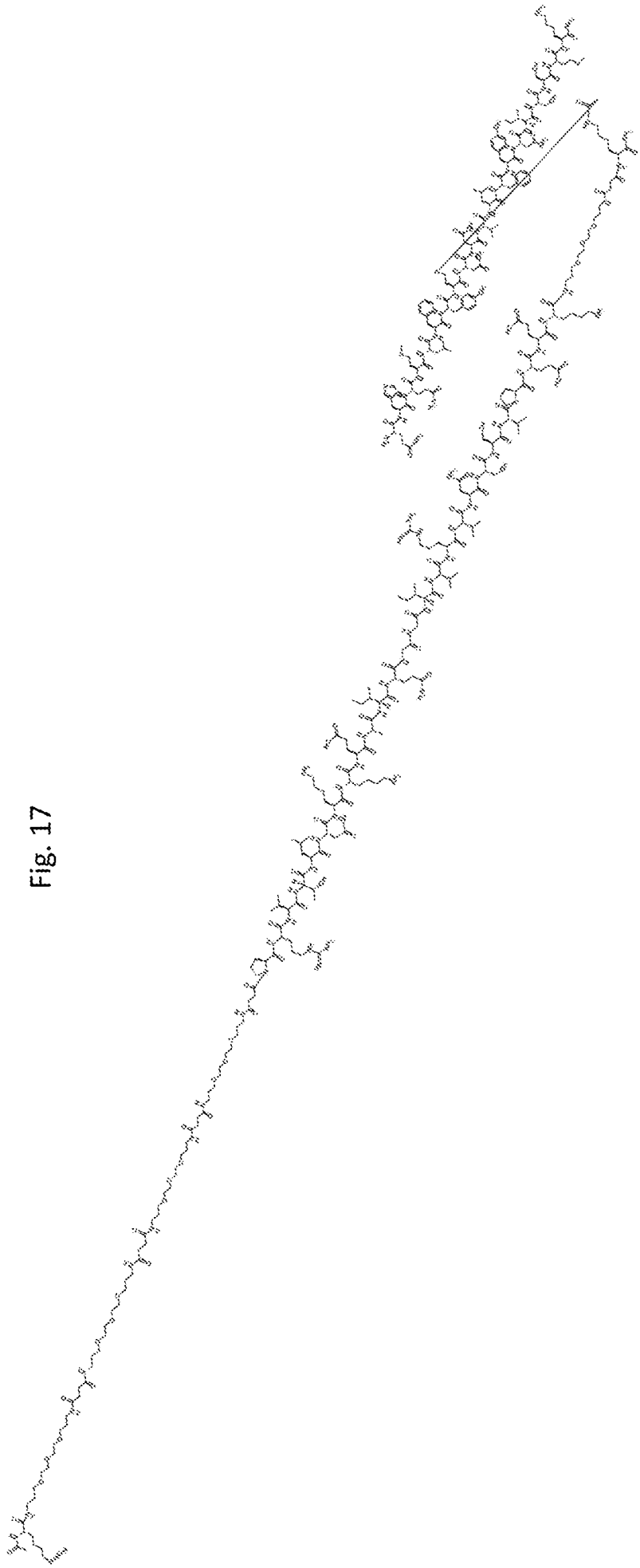


Fig. 17

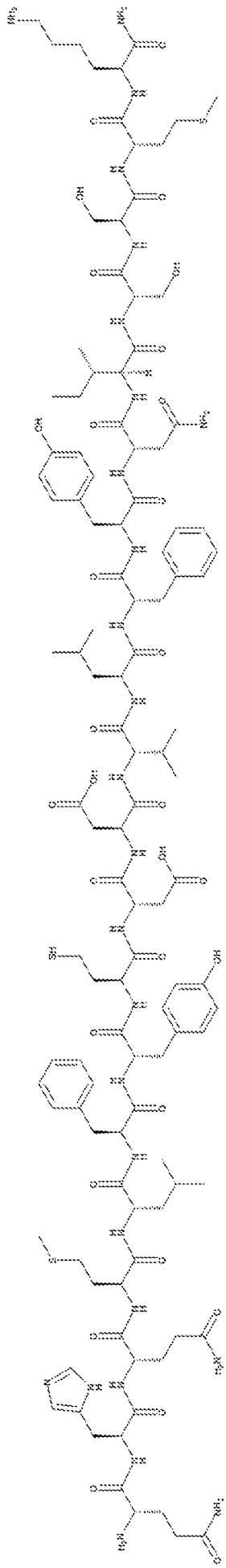


Fig. 18

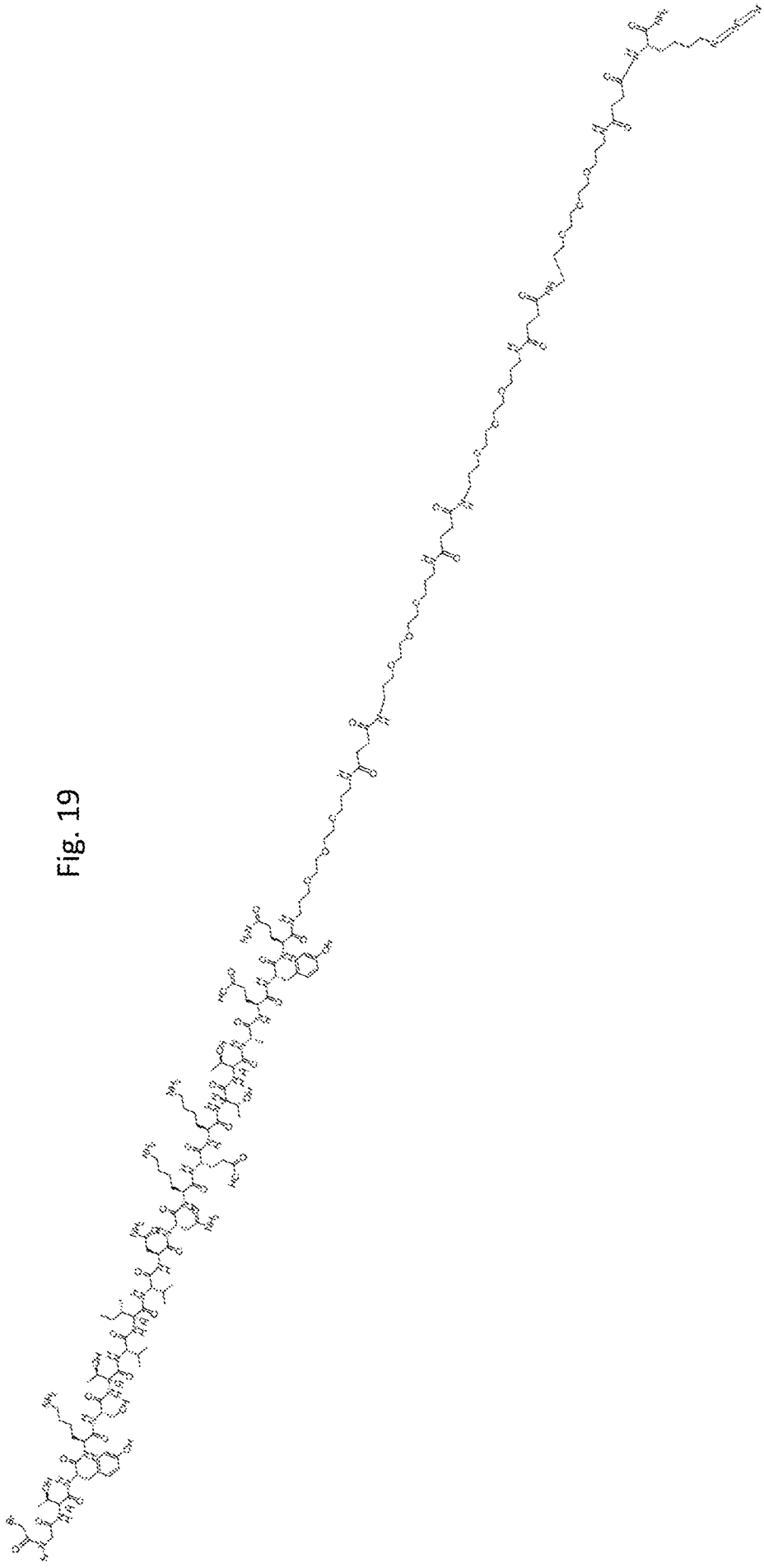


Fig. 19

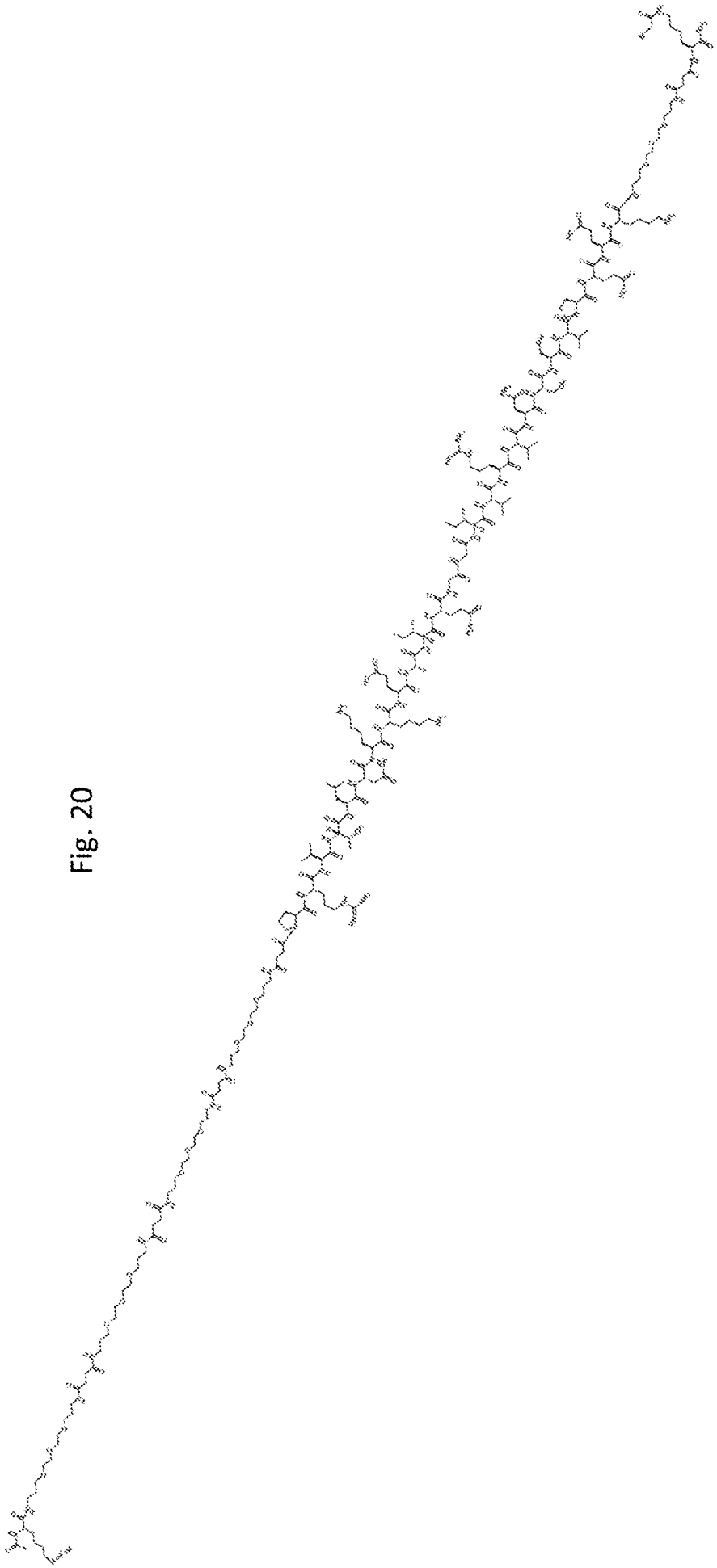
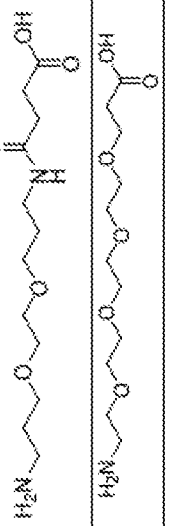
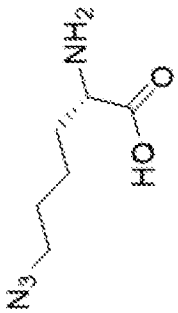
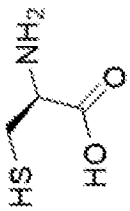
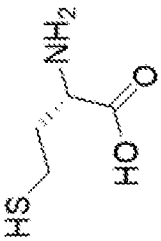


Fig. 20

Fig. 21

Abbreviation	STRUCTURE
Ttds	
PEG4	
K(N3)	
c	
hC	

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/000860

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61L31/00 A61K38/00 A61P9/10 C07K14/705
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)
A61L A61P A61K C07K

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 practicable, search terms used)
EPO-Internal, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DIAZ-RODRIGUEZ SERGIO ET AL: "Coronary stent CD31-mimetic coating favours endothelialization and reduces local inflammation and neointimal development in vivo.", EUROPEAN HEART JOURNAL 07 05 2021, vol. 42, no. 18, 7 May 2021 (2021-05-07), pages 1760-1769, XP55945712, ISSN: 1522-9645 figure 3 page 1763, paragraph bridging column 1 and 2	1,3,4, 6-12, 14-32
A	WO 2020/109833 A1 (INST NAT SANTE RECH MED [FR]; UNIV PARIS 13 PARIS NORD [FR] ET AL.) 4 June 2020 (2020-06-04) page 25, line 1 - page 40, line 20 page 3, line 9 - line 29	1-32

-/--

[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 application or patent 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
27 July 2022	09/08/2022

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	Voigt-Ritzer, Heike

2

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/000860

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	QUOCTHANG HOANG ET AL: "Peptide binding to cleaved CD31 dampens ischemia/reperfusion-induced intestinal injury", INTENSIVE CARE MEDICINE EXPERIMENTAL, BIOMED CENTRAL LTD, LONDON, UK, vol. 6, no. 1, 15 August 2018 (2018-08-15) , pages 1-12, XP021259664, DOI: 10.1186/S40635-018-0192-3 the whole document -----	1-32
A	WO 2010/000741 A1 (INST NAT SANTE RECH MED [FR]; CALIGIURI GIUSEPPINA [FR] ET AL.) 7 January 2010 (2010-01-07) example 10 page 2, line 20 - page 3, line 1 sequence 1 -----	1-32
A	WERNER HELD ET AL: "Cis-trans interactions of cell surface receptors: biological roles and structural basis", CELLULAR AND MOLECULAR LIFE SCIENCES, BIRKHÄUSER-VERLAG, BA, vol. 68, no. 21, 24 August 2011 (2011-08-24), pages 3469-3478, XP019964063, ISSN: 1420-9071, DOI: 10.1007/S00018-011-0798-Z the whole document -----	1-32

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2021/000860

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      [ ] on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2021/000860

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