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(54) Title: MODIFIED CHEMODENERVATING AGENTS

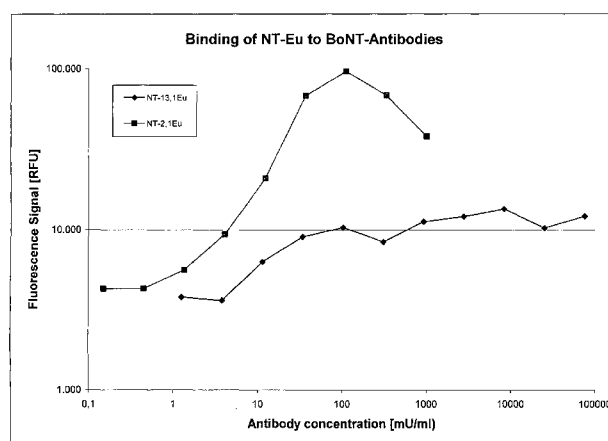


Figure 5

(57) Abstract: The present invention relates to chemodenerivating agents that are modified to carry different functional modulators via specific linker groups. In one embodiment said chemodenerivating agent is botulinum toxin. Claimed by this invention is a modified polypeptide wherein the polypeptide is represented by formula I: $\text{NT}[-\text{NH}_m(\text{W})_n]_k$ wherein NT represents a biologically active neurotoxic component or fragment thereof, which is still capable of cleaving proteins of the SNARE complex and of binding to the native HC domain receptor as well as to translocate the light chain; NH_m represents an amino group of NT; W represents a residue comprising at least one moiety that is negative charged at physiological pH; m represents an integer of 0, 1 or 2; n represents an integer of 1, 2 or 3 and $(m+n) = 2$ or 3; k represents an integer of 1 to 1000 wherein for $k > 1$, each set of m, W and n can be equal or different from each other; i represents a positive charge if $(m+n)$ is 3, wherein if $(m+n)$ is 2 then i represents no charge.

5

MODIFIED CHEMODENERVATING AGENTS**FIELD OF THE INVENTION**

[0001] The present invention relates to chemodenerivating agents that are
10 modified to carry a „residue W“ comprising at least one moiety that is
negative charged at physiological pH. In one embodiment said
chemodenerivating agent is a modified botulinum toxin.

BACKGROUND OF THE INVENTION

15

[0002] Chemodenervation refers to use of a chemical to prevent a nerve
from stimulating its target tissue, e.g. a muscle, a gland or another nerve.
Chemodenervation is for example performed with phenol, ethyl alcohol, or
botulinum toxin. Chemodenervation is for example appropriate in patients
20 with localized spasticity in one or two large muscles or several small
muscles. It may be used to alleviate symptoms such as muscle spasm and
pain, and hyperreflexia.

[0003] Chemodenerivating agents block neuromuscular transmission at the
25 neuromuscular junction, causing paralysis of the affected skeletal muscles.
This is accomplished either by acting presynaptically via the inhibition of
acetylcholine (ACh) synthesis or release, or by acting postsynaptically at the
acetylcholine receptor. There are drugs that act presynaptically (such as
botulinum toxin, tetrodotoxin and tetanus toxin (i.e. tetanospasmin)) and
30 other clinically-relevant drugs which work postsynaptically.

[0004] One chemodenerivating agent, botulinum toxin, although being one
of the most toxic compounds known to date, has in the past been used for a
large number of conditions and disorders, some of which are described in
35 e.g. PCT/EP 2007/005754. Furthermore, commercial forms of botulinum
toxin type A based on the botulinum toxin A protein complex are available
under the tradename Botox® (Allergan Inc.) and under the tradename
Dysport (Ipsen® Ltd.), respectively. A pharmaceutical composition comprising

- 5 the neurotoxic component of botulinum toxin type A in isolated form is commercially available in Germany from Merz Pharmaceuticals GmbH under the tradename Xeomin®.

[0005] The wild-type neurotoxic components display many interesting
10 characteristics, which have been used manifold in the state of the art. However, for certain medical indications a tailoring or modulation of these characteristics is desired to allow for specialized treatments. Examples for such a "modulation" might be a changed activity, half-life, stability, solubility, antigenicity, immunogenicity and many more. For these reasons some
15 approaches have been followed to modify the wild type characteristics of the neurotoxic component.

[0006] In the prior art few approaches of modifying neurotoxins are known.

- 20 [0007] In WO97/32599 a modified *Clostridium* neurotoxin is disclosed, which possesses a charged-modified tyrosine residue.

[0008] WO 99/17806; Chaddock et al., (2000), *Infect. Immun.* 68, 2587-2593; Duggan et al., (2002), *J. Biol. Chem.* 277, 34846-34852; WO 96/33273
25 and WO 2003/101484 disclose modified *Clostridium* neurotoxins, where the native binding site was changed with an non-native one to enable the binding to a different i.e. non-native target cell.

- [0009] WO2003/000193 for example discloses the covalently coupling of
30 polyethylene glycol to botulinum toxin via the amino terminal.

[00010] WO 2008/000490 discloses the high frequency application of unmodified botulinum toxins and fragments thereof.

- [00011] WO 2007/104567 discloses the modification of botulinum toxin with
35 PEG (polyethylene-glycol).

[00012] WO2001/95942 discloses a composition comprising a targeting molecule linked to an effector molecule through a connector molecule, said

- 5 connector molecule being a peptide and said targeting molecule is selected from a ligand, an antibody and a fragment of an antibody.

[00013] Mukkala et al., (1989), Anal. Biochem., 176, 319-325, discloses a method for labeling antibodies with metal ions (inter alia the lanthanide Eu^{3+}).
10 It does not disclose any use thereof for modifying a botulinum toxin, nor does it disclose any change in immunogenicity of the modified protein.

[00014] In view of the above, it was an object of the invention to provide novel polypeptides comprising a neurotoxic component or fragments thereof,
15 which exhibit one or more properties that are improved over the respective wild-type neurotoxin. One improvement of particular interest is the reduction of antigenicity, again, compared to the respective wild-type neurotoxin.

SUMMARY OF THE INVENTION

20

[00015] The present invention relates to modified polypeptides, comprising a neurotoxic component or fragments thereof, which carry residues that are negative charged at physiological pH ("negative charged residues") that are attached to one or more amino groups of the polypeptide. In one embodiment
25 said polypeptide is the neurotoxic component of botulinum toxin devoid of any complexing proteins.

[00016] The invention is based on the finding that the attachment of negative charged residues to amino groups of polypeptides in general, and in
30 particular botulinum neurotoxins, such as the neurotoxic component of botulinum toxin devoid of any complexing proteins, e.g. amino groups found in lysine-residues, may change the antigenic characteristics compared to the wild-type neurotoxin. Despite this modification the overall characteristics of the polypeptide, such as the chemodenervating activity of the botulinum
35 toxins are maintained, when being compared to the corresponding wild-type botulinum toxin. This attachment may be realized by classical chemical routes known to the person skilled in the art from, e.g. the peptide synthesis. Like building blocks in a "construction kit" the skilled person is now able to

- 5 combine the chemodenervating agent with a wide range of negative charged residues.

[00017] The term "patient" as used herein refers to mammals, human or animal, who suffered from one or more diseases or conditions specified
 10 hereinunder. There exist patients who have never been exposed to botulinum toxin but also to patients who have been exposed to botulinum toxin. The latter patient may have developed antibodies directed against the botulinum toxin complex or its components. Such antibodies may be neutralizing antibodies. Preferably, the patients do not have an antibody titer
 15 above 7mU, in particular not a titer of neutralizing antibodies above 7mU. The term "antibody titer not above ..." means less than 7mU, e.g. 1mU to 6mU or 0.01mU to 1mU.

[00018] Using the method of the invention allows the treatment of patients
 20 without inducing neutralizing antibodies directed against the neurotoxic component. Therefore, in one embodiment, the treatment of "secondary non-responders" is envisaged, i.e. patients which have developed antibodies against one or more botulinum toxin strains resulting in an active immunity against conventional neurotoxins. In this regard, it should be noted that the
 25 neurotoxins of the present invention allow also the "emergency" treatment of patients, which do not respond to other botulinumtoxin therapies.

[00019] In one embodiment the invention is a modified polypeptide wherein the polypeptide is represented by formula I:



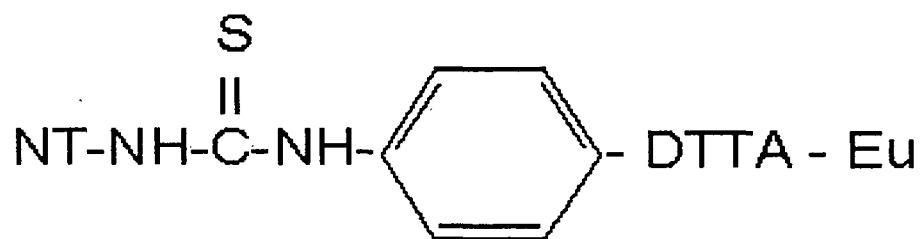
wherein

- NT represents a biologically active neurotoxic component or fragment thereof, which is still capable of cleaving proteins of the SNARE complex and of binding to the native HC domain receptor as well as to translocate the light
 35 chain;
- NH_m represents an amino group of NT;

- 5 -

- 5 - W represents a residue comprising at least one moiety that is negative charged at physiological pH;
- m represents an integer of 0, 1 or 2;
- n represents an integer of 1, 2 or 3 and
- $(m + n) = 2$ or 3;
- 10 - k represents an integer of 1 to 1000 wherein for $k > 1$, each set of m, W and n can be equal or different from each other;
- i represents a positive charge if $(m + n)$ is 3, wherein if $(m+n)$ is 2 then i represents no charge.
- 15 [00020] In another embodiment the negative charged moiety of the above mentioned polypeptide of formula I is selected from the group consisting of a carboxylate, a sulfonate, a phosphate and a combination of two or more thereof.
- 20 [00021] In yet another embodiment said negative charged moiety is derived from a multivalent carboxylic acid selected from the group consisting of citric acid, NTA, EDTA, DTTA, DTPA, TETA and derivatives thereof.

[00022] In yet another embodiment the polypeptide of the invention is
 25 represented by formula III:



Formula III

5 [00023] In yet another embodiment a counterion is bound to the above mentioned negative charged moiety.

[00024] In yet another embodiment the above mentioned counterion is selected from the group comprising any ion of the metals Zinc (Zn),
10 Manganese (Mn), Magnesium (Mg), Calcium (Ca), Copper (Cu), Nickel (Ni), Iron (Fe), Cobalt (Co), Europium (Eu), Samarium (Sm), Dysprosium (Dy) and Terbium (Tb).

[00025] In yet another embodiment the above mentioned NHm-moiety is
15 part of a surface or solvent exposed lysine residue of above mentioned neurotoxic component.

[00026] In yet another embodiment above mentioned neurotoxic component is at least 30% identical to

- 20 (a) BoNT/A - SEQ ID No. P10845;
gi|399133|sp|P10845.4|BXA1_CLOBO[399133].
- (b) BoNT/B - SEQ ID No. P10844;
gi|399134|sp|P10844.3|BXB_CLOBO[399134]
- (c) BoNT/C1 - SEQ ID No. P18640;
25 gi|115185|sp|P18640.2|BXC1_CLOBO[115185]
- (d) BoNT/D – SEQ ID No. P19321;
gi|115188|sp|P19321.1|BXD_CLOBO[115188]
- (e) BoNT/E - SEQ ID No. Q00496;
gi|399135|sp|Q00496.2|BXE_CLOBO[399135]
- 30 (f) BoNT/F - SEQ ID No. P30996;
gi|399137|sp|P30996.1|BXF_CLOBO[399137]
- (g) BoNT/G - SEQ ID No. Q60393;
gi|2499920|sp|Q60393.2|BXG_CLOBO[2499920]

- 5 (h) Neurotoxic component of neurotoxin type E; *Clostridium butyricum* - SEQ ID No. P30995; gi|399136|sp|P30995.2|BXE_CLOBU[399136]
- (i) TeNT; *Clostridium tetani* - SEQ ID No. P04958; gi|135624|sp|P04958.2|TETX_CLOTE[135624]
- (j) Neurotoxic component of neurotoxin type F; *Clostridium baratii* - SEQ
10 ID No. Q45851; gi|75346848|sp|Q45851|Q45851_9CLOT[75346848]

[00027] In yet another embodiment the above mentioned fragment is the light or heavy chain of a clostridial neurotoxic component.

- 15 [00028] In yet another embodiment the above mentioned clostridial neurotoxic component is obtainable from *C. botulinum*, *C. butyricum*, *C. baratii* and *C. tetani*.

- [00029] In yet another embodiment said *C. botulinum* is of serotype A, B, C,
20 D, E, F or G.

- [00030] In yet another embodiment a method for preparing the polypeptides as mentioned above is disclosed the modification resulting in the formation of the compound of formula I is the result of a chemical reaction comprising a
25 condensation reaction, a Michael-type reaction, native chemical ligation, sulfonation, glycosylation or phosphorylation.

[00031] In yet another embodiment a method is disclosed, comprising the steps of:

- 30 (a) translating or synthesizing the polypeptide or polypeptide fragment according to any one of claims 1 to 10;
- (b) exposing said polypeptide or polypeptide fragment in a buffer to a reactive compound; and

- 5 (c) purifying the modified polypeptide from the buffer, which contains at least one or more moieties as defined as W in claim 1.

[00032] In yet another embodiment also a composition, comprising polypeptides as defined above or the polypeptides obtainable by the
10 methods as defined above.

[00033] In yet another embodiment also the use of the above mentioned polypeptides according, or the polypeptide obtainable by the above mentioned methods according, or the above mentioned composition for
15 cosmetic treatment in disclosed.

[00034] In yet another embodiment the above mentioned polypeptides or the polypeptide obtainable by the above mentioned methods or the above mentioned compositions are suitable for the use in treatment of

- 20 (a) a disease or condition which can be ameliorated by temporarily reducing muscle activity;
- (b) a disease or condition which can be ameliorated by temporarily reducing glandular secretion;
- (c) a disease or condition during an operation or in support of wound
25 healing which can be ameliorated by temporarily reducing muscle activity.

[00035] In yet another embodiment the above mentioned polypeptides according, or the polypeptide obtainable by the above mentioned methods are suitable for the preparation of a pharmaceutical composition for

- 30 (a) treating a disease or condition which can be ameliorated by temporarily reducing muscle activity;
- (b) treating a disease or condition which can be ameliorated by temporarily reducing glandular secretion;

- 5 (c) temporarily reducing muscle activity during an operation or in support of wound healing.

BRIEF DESCRIPTION OF THE DRAWINGS

10 [00036] **Figure 1:** Chemical structure of the Eu-linking reagent, N1-(p-isothiocyanatobenzyl)-diethylene-triamine-N1,N2,N3,N3-tetraacetic acid chelated with Eu^{3+} .

[00037] **Figure 2:** The conjugation reaction between the aromatic
15 isothiocyanate group of the linking reagent and an amino group of a NT.

[00038] **Figure 3:** Anionization of NT.

[00039] **Figure 4a:** Reaction scheme for determination of primary amine
20 groups.

[00040] **Figure 4b:** Standard curve for determination of primary amine groups.

25 [00041] **Figure 5:** Binding of NT-Eu to BoNT-Antibodies.

5

DETAILED DESCRIPTION OF THE INVENTION

[00042] The present invention relates to a modified polypeptide, wherein the polypeptide is represented by formula I:



wherein

NT represents a biologically active neurotoxic component or a fragment thereof, which is still capable of cleaving proteins of the SNARE complex and of binding to the native HC domain receptor as well as to translocate the light chain;

NH_m represents an amino group of NT;

W represents a residue comprising at least one moiety that is negative charged at physiological pH;

m represents an integer of 0, 1 or 2;

n represents an integer of 1, 2 or 3 and

$(m + n) = 2$ or 3;

k represents an integer of 1 to 1000 wherein for $k > 1$, each set of m, W and n can be equal or different from each other; and

i represents a positive charge if $(m + n)$ is 3, wherein if $(m+n)$ is 2 then i represents no charge.

[00043] The terms "modification" or "modified" as used herein always refer to a comparison with the respective "unmodified" neurotoxic component, e.g. the modified wild-type neurotoxic component is compared to the wild-type neurotoxic component, whereas the modified recombinant neurotoxic component is compared with the unmodified recombinant neurotoxic component.

5 [00044] In this document the „residue W“ i.e. “the residue with at least one negative charged moiety at physiological pH” is capable of modifying the properties of the neurotoxic component. In one embodiment said property is a changed antigenicity.

10 [00045] Whereas “antigenicity” is defined herein as the capacity to react with an antibody. The antigenicity can be tested by in vivo, e.g. by antibody stimulation (i.e. antibody production upon injection) or in vitro, e.g. in binding assays of anti- antibodies against the polypeptide of the invention. Of course the skilled person is not bound to any testing method and can define the
15 antigenicity by any suitable method. The term “modified polypeptide” as used herein refers to any modification which is added to the native neurotoxin. In some embodiments such “modifications” are additional organic or inorganic residues, which are bond covalently to the neurotoxic component.

20 [00046] The “NT” within the above formula may be any biologically active “neurotoxic component”, or a fragment thereof, of a clostridial bacterium.

[00047] The “neurotoxic component” of the botulinum toxin complex is initially formed as a single polypeptide chain, having in the case of serotype
25 A a molecular weight of approximately 150 kDa. In other serotypes the neurotoxic component has been observed to vary between about 145 and about 170 kDa, depending on the bacterial source. In the case of serotype A, for example, proteolytic processing of the polypeptide results in an activated polypeptide in the form of a dichain polypeptide consisting of a heavy chain
30 and a light chain, which are linked by a disulfide bond.

[00048] The term “neurotoxic component” also includes functional homologs found in the other serotypes of *Clostridium botulinum*. In one embodiment of the present invention, the neurotoxic component is devoid of any other *C.*
35 *botulinum* protein, in another embodiment devoid of RNA, which might potentially be associated with the neurotoxic component. The neurotoxic component may be the single chain precursor protein of approximately 150kDa or the proteolytically processed neurotoxic component. In wild-type

5 the neurotoxic component comprises the light chain (L_c) of approximately 50kDa and the heavy chain (H_c) of approximately 100kDa, which may be linked by one or more disulfide bonds (for a review see e.g. *Simpson LL, Ann Rev Pharmacol Toxicol. 2004; 44:167-93*).

10 [00049] The term "botulinum toxin" as used throughout the present application, refers both to the neurotoxic component devoid of any other clostridial proteins, as well as to the "botulinum toxin complex", i.e. the neurotoxic component plus the complex proteins found in the wild-type. The term "botulinum toxin" is used herein in cases when no discrimination
15 between the toxin complex and the neurotoxic component is necessary or desired. "BoNT" or "BoNT/A" (i.e. to indicate the serotype) are common used abbreviations.

[00050] In one embodiment it is the neurotoxic component of botulinum
20 toxin. In another embodiment it is the neurotoxic component of tetanus toxin.

[00051] The precursor of the polypeptide of the invention, therefore, may be cleaved or uncleaved, however, in one embodiment the precursor has been cleaved into the heavy and the light chain. As pointed out elsewhere herein,
25 the polypeptides may be of wild-type sequence or may be modified at one or more residues.

[00052] In one embodiment the polypeptide of the invention has a biological activity of 10 to 500 LD_{50} units per ng polypeptide of the invention, as
30 determined in a mouse LD_{50} assay. In another embodiment the polypeptide of the invention has a biological activity of about 150 LD_{50} units per nanogram.

[00053] In one embodiment said polypeptide of the invention of botulinum
35 toxin is free of any complexing proteins. In addition thereto, modified as well as recombinant produced polypeptides derived from botulinum toxins including the respective mutations, deletions, etc. are also within the scope of the present invention. With respect to suitable mutants, reference is made to

- 5 WO 2006/027207 A1, WO 2006/114308 A1 and PCT/EP2008/006151, which are fully incorporated by reference herein.

[00054] In a further embodiment the neurotoxic component of botulinum toxin is botulinum toxin of the serotypes A, B, C, D, E, F or G. In another
10 embodiment the neurotoxic component of botulinum toxin derived from botulinum toxin serotype A.

[00055] Wherever the botulinum toxin serotype A, B, C, D, E, F or G are mentioned, also known variants of the serotypes are encompassed, like
15 serotypes A1, A2, A3, C1, C2, C3, etc.

[00056] The polypeptide chain of the neurotoxic component may, however, alternatively or additionally be modified by addition, substitution or deletion of one or more amino acid residues. Any changes in this regard will be referred
20 to as "variants" herein. Any variant, which has at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, and up to 60%, 70%, 80%, 90%, 100% sequence identity with the below neurotoxic components:

- (a) BoNT/A - SEQ ID No. P10845;
gi|399133|sp|P10845.4|BXA1_CLOBO[399133].
- 25 (b) BoNT/B - SEQ ID No. P10844;
gi|399134|sp|P10844.3|BXB_CLOBO[399134]
- (c) BoNT/C1 - SEQ ID No. P18640;
gi|115185|sp|P18640.2|BXC1_CLOBO[115185]
- (d) BoNT/D - SEQ ID No. P19321;
30 gi|115188|sp|P19321.1|BXD_CLOBO[115188]
- (e) BoNT/E - SEQ ID No. Q00496;
gi|399135|sp|Q00496.2|BXE_CLOBO[399135]
- (f) BoNT/F - SEQ ID No. P30996;
gi|399137|sp|P30996.1|BXF_CLOBO[399137]

- 5 (g) BoNT/G - SEQ ID No. Q60393;
gi|2499920|sp|Q60393.2|BXG_CLOBO[2499920]
- (h) Neurotoxic component of neurotoxin type E; *Clostridium butyricum* - SEQ ID No. P30995;
gi|399136|sp|P30995.2|BXE_CLOBU[399136]
- 10 (i) TeNT; *Clostridium tetani* - SEQ ID No. P04958;
gi|135624|sp|P04958.2|TETX_CLOTE[135624]
- (j) Neurotoxic component of neurotoxin type F; *Clostridium baratii* - SEQ ID No. Q45851;
gi|75346848|sp|Q45851|Q45851_9CLOT[75346848]

15 is therefore encompassed by this invention. In one embodiment 100% sequence identity is achieved. The SEQ ID Numbers above refer to the protein sequences as published in the NCBI-Database.

[00057] In a further embodiment said clostridial neurotoxic component is
20 obtainable from *C. botulinum*, *C. butyricum*, *C. baratii* and *C. tetani*. In one embodiment said *C. botulinum* is of serotype A, B, C, D, E, F or G.

[00058] Thus, in one embodiment, said polypeptide is derived from tetanus toxin, in a further embodiment it is derived from botulinum toxin, in another
25 embodiment it is derived from botulinum neurotoxin type A.

[00059] Furthermore, within the present invention, "chimeras" of various serotypes are encompassed. "Chimeras" may comprise combination of different domains derived from different serotypes, for example a polypeptide
30 of the invention comprising the heavy chain of one serotype and the light chain of another serotype (e.g. heavy chain of BoNT/A and light chain of oNT/E). In such cases as comparative reference for the polypeptide of the invention, the corresponding chimera without the modification is used.

35 [00060] Furthermore, also mixtures (e.g. in the form the wild-type or recombinant form of polypeptide of the invention or both forms thereof), as

- 5 well as mixtures of different serotypes (e.g. mixtures of botulinum neurotoxins of types A and B) may be used.

[00061] The terms "biologically active" or "biological activity" are defined herein as the ability of the polypeptide of the invention to denervate an
10 effector organ (e.g. gland or muscle). On the molecular level "biological activity" is the ability of the polypeptide to cleave proteins of the SNARE complex such as e.g. syntaxin, SNAP-25 or synaptobrevin, to bind to the native HC domain receptor and to translocate the light chain into the target cell. Accordingly, biological activity may be tested e.g. by a SNAP-25
15 protease assay, LD₅₀-Assay, HDA-Assay, and the like. It therefore encompasses the time period of action, the strength of a given amount of the polypeptide of the invention as well as the molecular capability of performing the intoxication process of the native toxin.

20 [00062] The term "fragment" is defined herein as a part of a protein, which still possesses biological activity, i.e. is capable of cleaving proteins of the SNARE complex and of binding to the native HC domain receptor as well as to translocate the light chain. Fragments, therefore, are e.g. polypeptides of which 1, 2, 3, 5, or up to 10, 50, or 100 amino acids have been deleted.
25 Wherein the deletion can be a truncation at the C- or N-terminus or an internal deletion.

[00063] The term "fragment of the LC domain", as used herein, is a fragment which is capable of cleaving a polypeptide of the SNARE complex. A
30 "fragment of the HC domain", as used herein, refers to a fragment which is still capable of binding to the native HC domain receptor, from which it is derived. Moreover, said fragment is also a fragment capable of translocating an LC domain attached to it.

35 [00064] The "NH_m" within the above formula is any amine group belonging to the neurotoxic component. In yet another embodiment the NH_m- moiety is part of surface or solvent exposed lysine residue of said neurotoxic component.

5

[00065] It is obvious to the skilled person, that to one NH_m – moiety the same or different „residues W“ can be bound. Furthermore, to different NH_m – moieties the same or different “residues W” can be bound. In fact, per single NH_m – moiety up to 3 “residues W” (e.g. if $m = 0$) can be bound.
10 Depending on the number of chemical modifications m therefore may be an integer of 0, 1 or 2.

[00066] Depending on the number of NH_m – moieties distributed over the whole polypeptide of the invention therefore 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20,
15 30, 40, 50, 60, 70, 80, 100, 1000, and up to 3000 “residues W” (e.g. 3 times 1000 NH_m - “residues W”), and any integer in between, can be bound. Furthermore the bonded “residues W” may be all independent from each other in composition, size and features.

20 [00067] For further guidance of protein purification methods useful for the production and purification of the polypeptide of the invention, (e.g. precipitation (with salt, organic solvent, acid etc.), chromatography (SEC, IEX, HIC, RP, NP, chromatofocusing, affinity chromatography etc.), dialysis, extraction), reference is made to the documents of Walker et al., 2002; Harris
25 et al. 1989 and Scopes et al., 1994, which are cited in the section “Literature” below.

[00068] The term “residue W”, which is represented by “W” in formula I refers to a chemical entity connected to the neurotoxic component “NT” (cf.
30 formula I).

[00069] In some embodiments “residue W” is the result of the reaction of NT with a molecule of the general structure $A - X - B$, wherein A represents at least one reactive group able to bind to the any amine group of the
35 neurotoxic component, B represents at least one negative charged moiety at physiological pH (i.e. at a pH of 7.3 to 7.4) and X represents either a chemical bond or at least one spacer-molecule. In another embodiment the reactive group A is generated as an intermediate. In yet another

- 5 embodiment, the primary amine of the NT itself is activated to enable the binding of the X-B moiety.

[00070] Examples for reactive groups which are comprised by A are e.g. isothiocyanates, isocyanates, acyl azides, *NHS* - esters, (n-hydroxy
10 succinimide esters), TFP esters (Tetrafluorophenyl esters), PFP esters (Pentafluorophenyl esters), sulfonyl chlorides, aldehydes and glyoxales, epoxides and oxiranes, carbonates, imidoesters, carbodiimides, anhydrides and alkylating and acylating reagents.

15 [00071] In another embodiment A comprises at least one member selected from an aldehyde group, isocyanate group, isothiocyanate group, anhydride, an aldehyde, a peroxide, a thiocyanate, a methyl isocyanate, isothiocyanate and allyl isothiocyanate.

20 [00072] In one embodiment the neurotoxic component is coupled to the „residue W“ via a primary amine. In another embodiment via a lysine-residue. In another embodiment via one of the aminoacid residues selected from the group comprising argining, asparagin, glutamin and glycin. In yet another embodiment, the secondary amin of histidin, prolin or tryptophan is used to
25 attach the linker and/or modulator moiety to the neurotoxic component

[00073] In one embodiment said reactive group A comprises N-bromosuccinimide or phtalimide (phtalic acid imide).

30 [00074] For the introduction of such reactive groups several publications are known, which are described for example in Practice and Theory of Enzyme Immunoassays, P. Tijssen, Editors: R.H. Burdon, P.H. von Knippenberg, Elsevier-Verlag, 1985. Such reagents are for example 2-Iminothiolan (Jue et al, Biochemistry 17, 5399-5405 (1978), N-Succinimidyl-
35 3-(2-pyridyldithio)propionat (Carlsson et al, Biochem. J. 173, 723-737 (1978)), Acetylmercaptobernsteinsäureanhydrid (Klotz und Heiney, Arch. Biochem. Biophys. 96, 605-612 (1962)) or S-Acetylmercaptoessigsäure-N-hydroxysuccinimidester (R. Julian et al, Anal. Biochem. 132, 68-73 (1983).

- 5 One reagent, which allows the linking of a SH-reactive group to a tyrosins of proteins or peptides, is described in Duncan et al., J. Immunol. Methods 80, 137-140 (1985). These references are incorporated in this application.

[00075] In yet other embodiments, the „residue W“ is coupled to the
10 neurotoxic component via a peptide bond. In one embodiment the „residue W“ is a oligo- or polypeptide. In another embodiment, said peptides are further modified e.g. ubiquitinated, biotinylated, with added protease recognition sites. In a further embodiment polyglycine or oligoglycine are comprised. Wherein said peptides consist of up to 50 natural or non-natural
15 amino acids.

[00076] In one embodiment the „residue W“ is attached via a covalent chemical bond. In another embodiment the „residue W“s is attached via any chemical bond comprising covalent bond, polar covalent bond, ionic bond,
20 coordinate covalent bond, bent bonds, 3c-2e and 3c-4e bonds, one- and three-electron bonds, aromatic bond, metallic bond, intermolecular bonding, permanent dipole to permanent dipole bonding, hydrogen bond, instantaneous dipole to induced dipole (van der Waals) bonding and/or cation-pi interaction.

25

[00077] The negative moiety B is negative charged at physiological pH and comprises in some embodiments at least one member selected from the group of carboxyl ($R-COO^-$), phosphate ($R-PO_4^{3-}$) and sulfonate ($R-SO_2O^-$).

30 [00078] In another embodiment the negative moiety B comprises at least one member selected from the group of carboxylate group, carboxyl group, sulfon acid group, sulfonate group, sulfonyl group, sulfhydryl group, thiole group, phosphate group, carboxylic acid group, a carboxylate, a nitrate, a phosphate, carboxylic acid comprising glutaric acid, oleic acid, methanoic
35 acid, ethanoic acid, propanoic acid, butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, dodecanoic acid, hexadecanoic acid, octadecanoic acid, aspartic acid, 2-propanoic acid, a medium to long-chain saturated and unsaturated

5 monocarboxylic acid with even number of carbons, docosaheptaenoic acid, eicosapentaenoic acid, amino acid, keto acid, pyruvic acid, acetoacetic acid, aromatic carboxylic acid, benzoic acid, salicylic acid, dicarboxylic acid, aldaric acid, oxalic acid, malonic acid, malic acid, succinic acid, glutaric acid, adipic acid, tricarboxylic acid, citric acid, alpha hydroxy acid and lactic acid.

10

[00079] In one embodiment a negative charge is introduced by an organic compound, which possesses at least two COO^- groups at physiological pH.

[00080] In another embodiment the „residue W“ comprises a chelating
15 group, which possess at least two negative charged COO^- groups at physiological pH.

[00081] In one embodiment the „residue W“ is a an aminoacetic acid residue, acetic acid residue or a multivalent carboxylic acid selected from at
20 least one member of the group comprising a citric acid residue, a nitrilotriacetic acid group, diaminoethan-tetraacetic acid, NTA, EDTA, DTTA, DTPA or TETA.

[00082] Furthermore, in another embodiment, a counterion is bound to the
25 multivalent carboxylic acid, wherein the counterion is selected from the group comprising any ion of the metals Zinc (Zn), Manganese (Mn), Magnesium (Mg), Calcium (Ca), Copper (Cu), Nickel (Ni), Iron (Fe), Cobalt (Co).

[00083] In yet another embodiment the counterion comprises a lanthanide,
30 e.g. Europium (Eu), Terbium (Tb), Samarium (Sm) or Dysprosium (Dy).

[00084] In one embodiment the negative charged moiety at physiological pH decreases the antigenicity of the polypeptide.

35 [00085] Another example for a “residue W” is in one embodiment an Eu-linking reagent (figure 1 & 2) which is used to attach the organic residue N1-benzyl-DTTA chalted with Eu^{3+} via an isothiocyanate linker to the neurotoxic component.

5

[00086] In some embodiments the „residue W“ comprises an isothiocyanate reagent. In one embodiment this isothiocyanate reagent is N¹-(p-isothiocyanatobenzyl)-diethylenetriamine-N¹-N²-N³-N³-tetraacetic acid (DTTA). The isothiocyanate-group reacts with the primary aliphatic amino group on the neurotoxic component at alkaline pH to form a stable, covalent thiourea bond.

[00087] In another embodiment “residue W” comprises a member selected from the group consisting of the following entities: europium, radioactive atom, fluorophore, fluorescence quencher, affinity tag, crosslinking agent, nucleic acid cleaving reagent, spin link, chromophore, NT, peptide or amino acid which may optionally be modified, nucleotide, nucleoside, nucleic acid which may optionally be modified, carbohydrate, lipid, transfection reagent, intercalating agent, nanoparticle and bead.

20

[00088] The spacer molecule X within “residue W” comprises any saturated or unsaturated, branched or unbranched, substituted or unsubstituted, linear or circular organic compound.

25 [00089] In some embodiments X comprises a member selected from the group consisting of an alkyl, a C2-C18 alkenyl, a C2-C18 alkynyl, a C3-C18 allyl, a C5-C14 aryl, a C5-C14 heteroaryl, a C5-C14 heterocyclyl, a C2-C18 acetyl, a C1-C18 alkoxyl, a C1-C18 acyl, a C1-C18 amino alkyl, a carbohydrate, a fatty acid group, a mono- or polyene(oxide), a sulfonyl group, 30 a phenyl ring, a chelating group, a polyalcohol, a peptide, an anhydride and a combination of two or more thereof.

[00090] In another embodiment X comprises a member selected from the group consisting of a saturated or unsaturated, branched or unbranched member selected from the group consisting of alkyl wherein the number of C-atoms is ≥ 2 , alkenyl wherein the number of C-atoms is ≥ 2 , alkynyl wherein the number of C-atoms is ≥ 2 , allyl wherein the number of C-atoms is ≥ 3 , aryl wherein the number of C-atoms is ≥ 5 , heteroaryl wherein the number of

35

- 5 C-atoms is ≥ 5 , heterocyclyl wherein the number of C-atoms is ≥ 5 , acetyl wherein the number of C-atoms is ≥ 2 , alkoxyl wherein the number of C-atoms is ≥ 1 , acyl wherein the number of C-atoms is ≥ 1 , amino alkyl wherein the number of C-atoms is ≥ 1 and a combination of two or more thereof.
- 10 [00091] In some embodiments X comprises an anhydride. In one embodiment said anhydride is glutaric acid anhydride. In another embodiment said anhydride is an anhydride derived from any carboxylic acid comprising oleic acid, methanoic acid (formic acid), ethanoic acid (acetic acid), propanoic acid (propionic acid), butanoic acid (butyric acid), pentanoic acid (valeric acid), hexanoic acid (caproic acid), heptanoic acid (enanthic acid), octanoic acid (caprylic acid), nonanoic acid (pelargonic acid), decanoic acid (capric acid), dodecanoic acid (lauric acid), hexadecanoic acid (palmitic acid), octadecanoic acid (stearic acid), glutaric acid, aspartic acid, 2-propanoic acid (acrylic acid), any fatty acid (medium to long-chain saturated and unsaturated monocarboxylic acids, with even or uneven number of carbons (e.g. docosahexaenoic acid, eicosapentaenoic acid), amino acids, keto acids, pyruvic acid, acetoacetic acid, aromatic carboxylic acids (e.g. benzoic acid, salicylic acid), dicarboxylic acids (e.g. aldaric acid, oxalic acid, malonic acid, malic acid, succinic acid, glutaric acid, adipic acid), tricarboxylic acids (e.g. citric acid), alpha hydroxy acids (e.g. lactic acid). In a further embodiment said anhydrid is represented by the general formula $R_1\text{-CO-O-CO-R}_2$, wherein R represents any organic residue in one embodiment $R_1=R_2$, in another embodiment $R_1\neq R_2$.
- 20
- 25
- 30 [00092] In another embodiment X comprises an ester of the general formula $R_1\text{-CX-O-R}_2$, wherein R_1 and R_2 are organic residues and $X = \text{O}$ or S . Examples for such esters are ethylacetate or allyl thiopropionate.
- [00093] In some embodiments X comprises one or more member(s)
- 35 selected from the group consisting of acetoxyl group, , alkoxy group, amidate group, aminoacetal group, amino group, ammonium group, aryloxy group, azo group, phenyl group, benzyl group, carbonyl group, ethyl group, halogenide group, hydroxyl group, imid group, imine group, ketene group,

5 methylene group, methyl group, nitro group, oxime group, oxo group, phenyl group, vinyl ether group, vinyl group, C2-C18 alkyl, C2-C18 alkenyl, C2-C18 alkynyl, C2-C18 allyl, C5-C14 aryl, C1-C18 acetyl, C1-C18 alkoxy and/or C1-C18 acyl, C5-C14 heteroaryl, C5-C14 heterocyclyl, C2-C18 acetyl, C1-C18 amino alkyl, saturated or unsaturated, branched or unbranched member of
 10 the group of a carbohydrate, a fatty acid group, a mono- or polyene(oxide) group, a carboxylic acid derivative, a chelating group, a polyalcohol, an ether, a haloalkane, an acyl halide, an alcohol, a ketone, an acetaldehyde, a carbonate ester, an ester, an amide, an amine, an imine, an azide, a cyanate, a nitrile, a nitrooxy, a nitroso compound, a pyridyl, a sulfide, a thiol,
 15 a thioether, mono- and poly-ethylene(oxide), mono- and poly-propylene(oxide), diethylene(oxide), triethylene(oxide), tetraethylene(oxide), branched ethylene- or propylene(oxide), fluoroalkane, methyl isocyanate, phosphodiester, chloroalkane, bromoalkane, iodoalkane, acetyl chloride, sulfonyl chloride, and/or phosphodiester, glycerine, glycol, sorbitol, phenyl-
 20 R1, -CH₂-CH₂-R1, -CH=CH-R1, or -C≡C-R1 in alpha position to the nitrogen of the isothiocyano group, and/or N1-(p-isothiocyanatobenzyl)-diethylenetriamine-N1,N2,N3,N3-tetraacetic acid (DTTA) and a combination of two or more thereof, wherein said group(s) may be substituted or unsubstituted.

25

[00094] In one embodiment the general formula of the polypeptide of the invention is: NT-[NH-[C=X]-B]_n, wherein X = O or S, and B represents a negative charged moiety at physiological pH.

30 [00095] The term "aryl" represents phenyl or naphthyl, wherein the phenyl or naphthyl group is optionally substituted by one or more substituents, which may be the same or different, selected independently from halogen, trifluoromethyl, trifluoromethoxy, C1-6 alkyl, hydroxy C1-6 alkyl, C2-6 alkenyl, C1-6 alkoxy, amino, hydroxy, nitro, cyano, cyanomethyl, C1-6
 35 alkoxycarbonyl, C1-6 alkylcarbonyloxy, C1-6 alkylamino, di-(C1-6 alkyl) amino, C1-6 alkyl-carbonylamino, aminocarbonyl, N-C1-6 alkylaminocarbonyl, di-N,N-C1-6 alkylaminocarbonyl, pyrrolidinyl, piperidinyl,

- 5 morpholinyl, and piperazinyl, cycloC3-12 alkyl or optionally C1-6 alkylenedioxy.

[00096] The term "heteroaryl" represents an aromatic 5-6 membered ring containing from one to four heteroatoms selected from oxygen, sulfur and
10 nitrogen, or a bicyclic group comprising a 5-6 membered ring containing from one to four heteroatoms selected from oxygen, sulfur and nitrogen fused with a benzene ring or a 5-6 membered ring containing from one to four heteroatoms selected from oxygen, sulfur and nitrogen, wherein the heteroaryl group may be optionally substituted by one or more substituents,
15 which may be the same or different, selected independently from halogen, trifluoromethyl, trifluoromethoxy, C1-6 alkyl, hydroxyC1-6 alkyl, C2-6 alkenyl, C1-6 alkoxy, amino, hydroxy, nitro, cyano, C1-6 alkoxycarbonyl, C1-6 alkoxycarbonyloxy, C1-6 alkylamino, and di-(C1-6 alkyl)amino, C1-6 alkylcarbonylamino, aminocarbonyl, N-C1-6 alkylaminocarbonyl, di-N,N-C1-6
20 alkyl-aminocarbonyl, pyrrolidinyl, piperidinyl, morpholinyl, cyclo C3-12 alkyl, C1-6 alkylenedioxy and aryl. Representative heteroaryl groups include furanyl, thienyl, pyrrolyl, oxazolyl, isoxazolyl, isothiazolyl, oxadiazolyl, triazolyl, thiadiazolyl, thiazolyl, imidazolyl, oxadiazolyl, tetrazolyl, pyridinyl, pyrimidinyl, pyridazinyl, pyrazinyl, triazinyl, pyrazolyl, benzofuryl,
25 benzothienyl, indolyl, indoliziny, isoindolyl, indolinyl, indazolyl, benzimidazolyl, benzoxazolyl, benzothiazolyl, quinolinyl, quinazolinyl, quinoxalinyl, cinnolinyl, naphthyridinyl, and isoquinolinyl. Examples are pyridyl, pyrimidyl, thienyl, furyl and others.

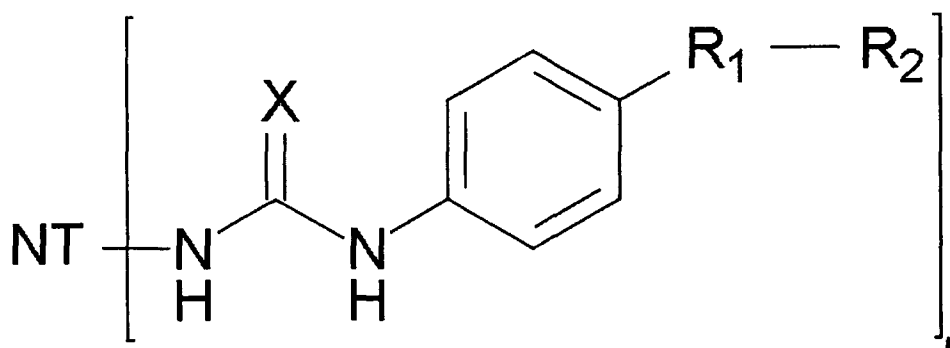
30 [00097] The term "heterocyclyl" represents a saturated or unsaturated non-aromatic 3 to 12 membered ring comprising one to four heteroatoms selected from oxygen, sulfur and nitrogen, and a saturated or unsaturated non-aromatic bicyclic ring system having 3 to 12 members comprising one to six heteroatoms selected from oxygen, sulfur and nitrogen, wherein the
35 heterocyclic ring or ring system is optionally substituted by one or more substituents selected independently from a halogen, trifluoromethyl, C1-6 alkyl, C2-6 alkenyl, C1-6 alkoxy, amino, hydroxy, nitro, cyano, C1-6 alkoxycarbonyl, C1-6 alkylamino, di-C1-6 alkylamino, pyrrolidinyl, piperidinyl,

5 morpholinyl, pyridinyl, and aryl; examples of such heterocyclyl groups include piperidinyl, morpholinyl, thiomorpholinyl, imidazolidinyl, pyrazolidinyl, pyrrolidinyl, or piperazinyl, wherein the heterocyclic ring or ring system is linked to the group to which it is attached optionally via nitrogen or a carbon atom.

10

[00098] The term oligoethylene(oxide) describes structurally defined ethylene(oxide) groups with a dispersity of 1. Examples can be chosen from a group consisting of tetraethylene(oxide), pentaethylene(oxide), hexaethylene(oxide), heptaethylene(oxide), oktaethylene(oxide),
 15 enneaethylene(oxide), dekaethylene(oxide) up to hekatonethylene(oxide).

[00099] In a still further embodiment, the polypeptide of the invention is represented by the following formulas:



20

Formula II

wherein

NT represents a neurotoxic component or fragment thereof, which still is
 25 capable of cleaving proteins of the SNARE complex and to bind to the native HC domain receptor as well as to translocate the light chain;

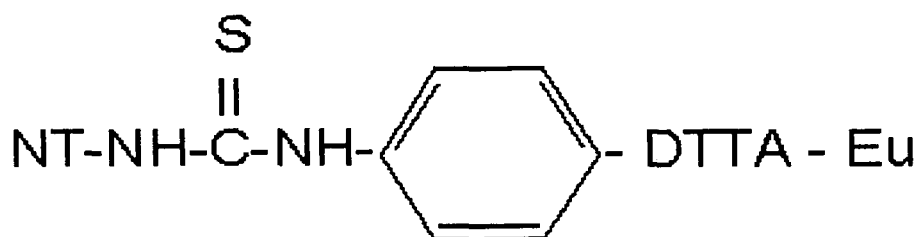
X represents S and O;

R₁ represents NTA, DTTA, EDTA, DTPA or TETA;

R₂ represents a counterion;

- 5 n represents an integer of 1 up to 3000,
m represents an integer of 0, 1 or 2, wherein, if m represents 2 the
corresponding N carries a positive charge.

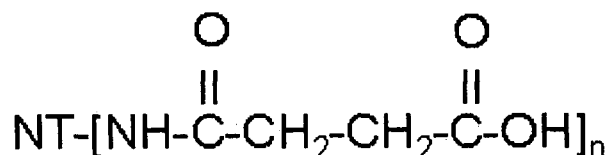
or



10

Formula III

or



Formula IV

15

[000100] The term “immunogenicity” is defined herein as the property enabling a substance to provoke an immune response, or the degree to which a substance possesses this property. Therefore the term “immunogenicity” encompasses the term “antigenicity” but encompasses also other traits of immune modulation comprising any of the adaptive and/or innate immune system, e.g. stimulation of leukocytes (e.g. macrophages, neutrophils, dendritic cells, eosinophils, basophils, mast cells, natural killer cells), stimulation of B-cells, stimulation of T-cells, activation of the complement system, activation via Toll-like receptors, etc. Accordingly the

20

25 “immunogenicity” can be measured by any test suitable to indicate a provoked immune response.

[000101] “Natural” amino acids are defined herein as the “proteinogenic” aminoacids alanine, cysteine, aspartic acid, glutamic acid, phenylalanine,

- 5 glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan and tyrosine.

[000102] "Non-natural amino acids" are defined herein as any amino-acid, which does not belong to the "natural" amino acids as defined above.

10 examples for such "non-natural", "un-natural" or "non-proteinogenic" amino acids are carnitine, GABA, L-dopa, hydroxyproline, selenomethionine, ornithine, homoserine, canavanine and pyrrolysine. further examples are alanine derivatives, alicyclic amino acids, arginine derivatives, aromatic amino acids, asparagine derivatives, aspartic acid derivatives, beta-amino

15 acids, cysteine derivatives, DAB (2,4-diaminobutyric acid), DAP (2,3-diaminopropionic acid), glutamic acid derivatives, glutamine derivatives, glycine derivatives, histidine derivatives, homo-amino acids, isoleucine derivatives, leucine derivatives, linear core amino acids, lysine derivatives, N-methyl amino acids, norleucine, norvaline, ornithine, penicillamine,

20 phenylalanine derivatives, phenylglycine derivatives, proline derivatives, pyroglutamine derivatives, serine derivatives, threonine derivatives, tryptophan derivatives, tyrosine derivatives or valine derivatives.

[000103] Another embodiment comprises the method for producing a

25 polypeptide, comprising the steps of:

- A. translating or synthesizing the neurotoxic component or fragment of the neurotoxic component;
- B. exposing said neurotoxic component or fragment of the neurotoxic component in a buffer to a reactive compound like
- 30 e.g. an activated ester, anhydride, aldehyde with e.g. an reducing agent, azido-/alkine, isothiocyanate; and
- C. purifying a modified neurotoxic component or neurotoxic component fragment from the buffer via known methods, which contains at least one or more „residue W"s.

5[000104] In one embodiment the polypeptide is translated in a eukaryotic or prokaryotic cell.

[000105] In another embodiment the polypeptide obtainable by the methods mentioned herein are used for the preparation of a pharmaceutical
10 composition for

- (a) treating a disease or condition which can be ameliorated by temporarily reducing muscle activity;
 - (b) treating a disease or condition which can be ameliorated by temporarily reducing glandular secretion;
 - 15 (c) temporarily reducing muscle activity during an operation or in support of wound healing; and/or
- for diagnosing any disease or condition of (a) to (c).

[000106] The invention also discloses in one embodiment a composition,
20 comprising one of the before mentioned polypeptides or a polypeptide obtainable by one of the before mentioned methods. In one embodiment said composition is a pharmaceutical composition or a cosmetic composition.

[000107] The invention also discloses in one embodiment the use of the said
25 composition for cosmetic treatment.

[000108] Furthermore, the invention discloses said polypeptide or a polypeptide obtainable by said methods for the use in treatment of

- (a) a disease or condition which can be ameliorated by
30 temporarily reducing muscle activity;
- (b) a disease or condition which can be ameliorated by temporarily reducing glandular secretion;
- (c) a disease or condition during an operation or in support of wound healing which can be ameliorated
35 by temporarily reducing muscle activity.

5[000109] Furthermore, In another embodiment the use of one of the before mentioned polypeptides or a polypeptide obtainable by one of the before mentioned methods is disclosed for the preparation of a pharmaceutical composition for

- 10
- (a) treating a disease or condition which can be ameliorated by temporarily reducing muscle activity;
 - (b) treating a disease or condition which can be ameliorated by temporarily reducing glandular secretion;
 - (c) temporarily reducing muscle activity during an operation or in support of wound healing.

15

[000110] In another embodiment, the polypeptide of the invention is part of a diagnostic assay, e.g. a phrenic nerve-hemidiaphragm-assay, which allows to diagnose e.g. antibody levels in a patient blood-serum.

20[000111] Treatments offered by the present invention may be directed at any of the following indications, most of which are described in detail in *Dressler D (2000) (Botulinum Toxin Therapy. Thieme Verlag, Stuttgart, New York)* for botulinum toxin:

- 25
- dystonia
 - cranial dystonia
 - blepharospasm
 - oromandibular dystonia
 - jaw opening type
 - jaw closing type
 - bruxism
 - Meige syndrome
 - lingual dystonia
 - apraxia of eyelid opening
 - 30
 - cervical dystonia
 - antecollis
 - retrocollis
 - laterocollis
 - torticollis
 - 35
 - pharyngeal dystonia
 - 40
 - laryngeal dystonia

- 5
 - spasmodic dysphonia/adductor type
 - spasmodic dysphonia/abductor type
 - spasmodic dyspnea
- limb dystonia
 - arm dystonia
 - 10
 - task specific dystonia
 - writer's cramp
 - musician's cramps
 - golfer's cramp
 - leg dystonia
 - 15
 - thigh adduction, thigh abduction
 - knee flexion, knee extension
 - ankle flexion, ankle extension
 - equinovarus deformity
 - foot dystonia
 - 20
 - striatal toe
 - toe flexion
 - toe extension
 - axial dystonia
 - 25
 - pisa syndrome
 - belly dancer dystonia
 - segmental dystonia
 - hemidystonia
 - generalised dystonia
 - dystonia in lubag
 - 30
 - dystonia in corticobasal degeneration
 - dystonia in lubag
 - tardive dystonia
 - dystonia in spinocerebellar ataxia
 - dystonia in Parkinson's disease
 - 35
 - dystonia in Huntington's disease
 - dystonia in Hallervorden Spatz disease
 - dopa-induced dyskinesias/dopa-induced dystonia
 - tardive dyskinesias/tardive dystonia
 - paroxysmal dyskinesias/dystonias
 - 40
 - kinesigenic
 - non-kinesigenic
 - action-induced
 - palatal myoclonus
 - myoclonus

- 5
 - myokymia
 - rigidity
 - benign muscle cramps
 - hereditary chin trembling
 - paradoxical jaw muscle activity
- 10
 - hemimasticatory spasms
 - hypertrophic branchial myopathy
 - masseteric hypertrophy
 - tibialis anterior hypertrophy
 - nystagmus
- 15
 - oscillopsia
 - hyperhidrosis
 - supranuclear gaze palsy
 - epilepsy partialis continua
 - planning of spasmodic torticollis operation
- 20
 - abductor vocal cord paralysis
 - recalcitrant mutational dysphonia
 - upper oesophageal sphincter dysfunction
 - vocal fold granuloma
 - stuttering
- 25
 - Gilles de la Tourette syndrome
 - middle ear myoclonus
 - protective larynx closure
 - postlaryngectomy speech failure
 - protective ptosis
- 30
 - entropion
 - sphincter Oddi dysfunction
 - pseudoachalasia
 - nonachalasia oesophageal motor disorders
 - vaginismus
- 35
 - postoperative immobilisation
 - tremor
 - genito-urinary diseases
 - bladder dysfunction

- 5
 - overactive bladder
 - urinary incontinence
 - urinary retention
 - spastic bladder
- 10
 - gastro-intestinal diseases
 - detrusor sphincter dyssynergia
 - bladder sphincter spasm
 - hemifacial spasm
 - reinnervation dyskinesias
 - cosmetic use
 - 15
 - crow's feet
 - frowning
 - facial asymmetries
 - mentalis dimples
 - frontal lines
 - 20
 - masseter lift
 - platysma
 - smoker's lines
 - marionette lines
 - stiff person syndrome
 - 25
 - tetanus
 - prostate diseases
 - prostate hyperplasia
 - prostate cancer
 - adipositas treatment
 - 30
 - infantile cerebral palsy
 - strabismus
 - mixed
 - paralytic
 - concomitant
 - 35
 - after retinal detachment surgery
 - after cataract surgery
 - in aphakia
 - myositic strabismus
 - myopathic strabismus
 - 40
 - dissociated vertical deviation
 - as an adjunct to strabismus surgery
 - esotropia
 - exotropia
 - achalasia
 - 45
 - anal fissures

- 5
 - exocrine gland hyperactivity
 - Frey syndrome
 - Crocodile Tears syndrome
 - hyperhidrosis
 - axillar
- 10
 - palmar
 - plantar
 - rhinorrhea
 - relative hypersalivation
 - in stroke
- 15
 - in parkinson's
 - in amyotrophic lateral sclerosis
 - spastic conditions
 - in encephalitis and myelitis
 - autoimmune processes
 - multiple sclerosis
 - transverse myelitis
 - Devic syndrome
 - viral infections
 - bacterial infections
 - parasitic infections
 - fungal infections
 - in hereditary spastic paraparesis
 - postapoplectic syndrome
 - hemispheric infarction
 - brainstem infarction
 - myelon infarction
 - in central nervous system trauma
 - hemispheric lesions
 - brainstem lesions
 - myelon lesion
 - in central nervous system hemorrhage
 - intracerebral hemorrhage
 - subarachnoidal hemorrhage
 - subdural hemorrhage
 - intraspinal hemorrhage
 - in neoplasias
 - hemispheric tumors
 - brainstem tumors
 - myelon tumors
- 20
 - in encephalitis and myelitis
 - autoimmune processes
 - multiple sclerosis
 - transverse myelitis
 - Devic syndrome
 - viral infections
 - bacterial infections
 - parasitic infections
 - fungal infections
 - in hereditary spastic paraparesis
 - postapoplectic syndrome
 - hemispheric infarction
 - brainstem infarction
 - myelon infarction
 - in central nervous system trauma
 - hemispheric lesions
 - brainstem lesions
 - myelon lesion
 - in central nervous system hemorrhage
 - intracerebral hemorrhage
 - subarachnoidal hemorrhage
 - subdural hemorrhage
 - intraspinal hemorrhage
 - in neoplasias
 - hemispheric tumors
 - brainstem tumors
 - myelon tumors
- 25
 - parasitic infections
 - fungal infections
- 30
 - hemispheric infarction
 - brainstem infarction
 - myelon infarction
- 35
 - hemispheric lesions
 - brainstem lesions
 - myelon lesion
- 40
 - intracerebral hemorrhage
 - subarachnoidal hemorrhage
 - subdural hemorrhage
 - intraspinal hemorrhage
- 45
 - headache
 - migraine
 - tension headache
 - sinus headache
 - chronic headache

- 5 ▪ and/or hair loss.

[000112] The polypeptide of the invention referred to herein above, may be part of a composition or a pharmaceutical composition. This pharmaceutical composition to be used herein may comprise botulinum toxin, e.g. in the form of the polypeptide of the invention as the sole active component or together
10 with complex proteins of BoNT, and may contain additional pharmaceutically active components e.g. a hyaluronic acid or a polyvinylpyrrolidone or a polyethleneglycol, such composition being optionally pH stabilized by a suitable pH buffer, in particular by a sodium acetate buffer, and/or a
15 cryoprotectant polyalcohol.

[000113] In one embodiment, the polypeptide of the invention has a biological activity of 10 to 500 LD₅₀ units per ng of the polypeptide of the invention, as determined in a mouse LD₅₀ assay. In another embodiment, the polypeptide
20 of the invention has a biological activity of about 150 LD₅₀ units per nanogram. Generally, the pharmaceutical composition of the present invention comprises polypeptide of the invention in a quantity of about 6 pg to about 30 ng.

25 [000114] A "pharmaceutical composition" is a formulation in which an active ingredient for use as a medicament or a diagnostic is contained or comprised. Such pharmaceutical composition may be suitable for diagnostic or therapeutic administration (i.e. by intramuscular or subcutaneous injection) to a human patient.

30

[000115] A pharmaceutical composition comprising the neurotoxic component of botulinum toxin type A in isolated form is commercially available in Germany from Merz Pharmaceuticals GmbH under the trademark Xeomin®. The production of the neurotoxic component of botulinum toxin type A and B
35 are described, for example, in the international patent applications WO 00/74703 and WO 2006/133818. Said pharmaceutical compositions may be adapted to the polypeptide of the invention.

5[000116] In one embodiment, said composition comprises the polypeptide of the invention derived from botulinum toxin type A. Said composition is a reconstituted solution of the polypeptide of the invention of botulinum toxin. In another embodiment the composition further comprises sucrose or human serum albumin or both, in still another embodiment the ratio of human serum
10 albumin to sucrose is about 1:5. In another embodiment, said human serum albumin is recombinant human serum albumin. Alternatively, said composition is free of mammalian derived proteins such as human serum albumin. Any such solution may provide sufficient stability to the polypeptide of the invention by replacing serum albumin with other non-proteinaceous
15 stabilizers (infra).

[000117] With regard to the composition and dosing of the medicament on the basis of botulinum toxin, and in regard to the composition, dosing and frequency of administration of the medicament on the basis of the neurotoxic
20 component of botulinum toxin, reference is made to PCT/EP2007/005754.

[000118] The pharmaceutical composition may be lyophilized or vacuum dried, reconstituted, or may prevail in solution. When reconstituted, in one embodiment the reconstituted solution is prepared adding sterile
25 physiological saline (0.9% NaCl).

[000119] Such composition may comprise additional excipients. The term "excipient" refers to a substance present in a pharmaceutical composition other than the active pharmaceutical ingredient present in the pharmaceutical
30 composition. An excipient can be a buffer, carrier, antiadherent, analgesic, binder, disintegrant, filler, diluent, preservative, vehicle, cyclodextrin and/or bulking agent such as albumin, gelatin, collagen, sodium chloride, preservative, cryoprotectant and/or stabilizer.

35[000120] A "pH buffer" refers to a chemical substance being capable to adjust the pH value of a composition, solution and the like to a certain value or to a certain pH range. In one embodiment this pH range can be between pH 5 to pH 8, in another embodiment pH 7 to pH 8, in yet another embodiment 7.2 to

5 7.6, and in yet a further embodiment a pH of 7.4. In another embodiment the pharmaceutical composition has a pH of between about 4 and 7.5 when reconstituted or upon injection, in yet another embodiment about pH 6.8 and pH 7.6 and in a further embodiment between pH 7.4 and pH 7.6.

10 [000121] The pH ranges mentioned above are only typical examples and the actual pH may include any interval between the numerical values given above. Suitable buffers which are in accordance with the teaching of the present invention are e.g. sodium-phosphate buffer, sodium-acetate buffer, TRIS buffer or any buffer, which is suitable to buffer within the above pH-
15 ranges.

[000122] In one embodiment the composition also contains a 1-100 mM, in another embodiment 10 mM sodium acetate buffer.

20 [000123] "Stabilizing", "stabilizes" or "stabilization" means that the active ingredient, i.e., the polypeptide of the invention in a reconstituted or aqueous solution pharmaceutical composition has greater than about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and up to about 100% of the toxicity that the biologically active polypeptide of the invention had prior to being incorporated
25 into the pharmaceutical composition.

[000124] Examples of such stabilizers are gelatin or albumin, in one embodiment of human origin or obtained from a recombinant source. Proteins from non-human or non-animal sources are also included. The
30 stabilizers may be modified by chemical means or by recombinant genetics. In one embodiment of the present invention, it is envisaged to use alcohols, e.g., inositol or mannitol, as cryoprotectant excipients to stabilize proteins during lyophilization.

35 [000125] In another embodiment of the present invention, the stabilizer may be a non proteinaceous stabilizing agent comprising a hyaluronic acid or a polyvinylpyrrolidone (Kollidon®), hydroxyethyl starch, alginate or a polyethylene glycol or any combination thereof, such composition being

5 optionally pH stabilized by a suitable pH buffer, in particular by a sodium acetate buffer, or a cryoprotectant or both. Said composition may comprise in addition to the mentioned stabilizers water and at least one polyalcohol, such as mannitol or sorbitol or mixtures thereof. It may also comprise mono-, di- or higher polysaccharides, such as glucose, sucrose or fructose. Such
10 composition is considered to be a safer composition possessing remarkable stability.

[000126] The hyaluronic acid in the instant pharmaceutical composition is in one embodiment combined with the instant polypeptide of the invention in a
15 quantity of 0.1 to 10 mg, especially 1 mg hyaluronic acid per ml in a 200 U/ml botulinum toxin solution.

[000127] The polyvinylpyrrolidone (Kollidon®) when present in the instant composition, is combined with the instant polypeptide of the invention in such
20 a quantity to provide a reconstituted solution comprising 10 to 500 mg, especially 100 mg polyvinylpyrrolidone per ml in a 200 U/ml solution of the polypeptide of the invention. In another embodiment reconstitution is carried out in up to 8 ml solution. This results in concentrations of down to 12.5 mg polyvinylpyrrolidone per ml in a 25 U/ml solution of the polypeptide of the
25 invention.

[000128] The polyethyleneglycol in the instant pharmaceutical composition is in one embodiment combined with the instant polypeptide of the invention in a quantity of 10 to 500 mg, especially 100 mg polyethyleneglycol per ml in a
30 200 U/ml botulinum toxin solution. In another embodiment, the subject solution also contains a 1-100 mM, in yet another embodiment 10 mM sodium acetate buffer.

[000129] In a further embodiment said polypeptide is part of a composition
35 comprising a member selected from the group of a peptide or amino acid which may optionally be modified, a complex protein found in the the wild-type neurotoxin, a nucleotide, a nucleoside, a nucleic acid which may

- 5 optionally be modified, a carbohydrate, a lipid, a transfection reagent, a intercalating agent, a nanoparticle and a bead.

[000130] The pharmaceutical composition in accordance with the present invention in one embodiment retains its potency substantially unchanged for
10 a six months, one year, a two years, a three years and/or a four years period when stored at a temperature between about +8°C and about -20°C. Additionally, the indicated pharmaceutical compositions may have a potency or percent recovery of between about 20% and about 100% upon reconstitution.

15

[000131] "Cryoprotectant" refers to excipients which result in an active ingredient, i.e., a polypeptide of the invention in a reconstituted or aqueous solution pharmaceutical composition that has greater than about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and up to about 100% of the toxicity that
20 the biologically active polypeptide of the invention had prior to being freeze-dried in the pharmaceutical composition.

[000132] In another embodiment, the composition may contain a polyhydroxy compound, e.g. a polyalcohol as cryoprotectant. Examples of polyalcohols
25 that might be used include, e.g., inositol, mannitol and other non-reducing alcohols. Some embodiments of the composition do not comprise a proteinaceous stabilizer, or do not contain trehalose or maltotriose or lactose or sucrose or related sugar or carbohydrate compounds which are sometimes used as cryoprotectants.

30

[000133] The terms "preservative" and "preservatives" refer to a substance or a group of substances, respectively, which prevent the growth or survival of microorganisms, insects, bacteria or other contaminating organisms within said composition. Preservatives also prevent said composition from
35 undesired chemical changes. Preservatives which can be used in the scope of this patent are all preservatives of the state of the art known to the skilled person. Examples of preservatives that might be used include, inter alia, e.g. benzylic alcohol, benzoic acid, benzalkonium chloride, calcium propionate,

5 sodium nitrate, sodium nitrite, sulphites (sulfur dioxide, sodium bisulfite, potassium hydrogen sulfite, etc.), disodium EDTA, formaldehyde, glutaraldehyde, diatomaceous earth, ethanol, methyl chlorisothiazolinone, butylated hydroxyanisole and/or butylated hydroxytoluene.

10 [000134] The term "analgesic" relates to analgesic drugs that act in various ways on the peripheral and central nervous systems and includes inter alia Paracetamol[®] (acetaminophen), the nonsteroidal anti-inflammatory drugs (NSAIDs) such as the salicylates, narcotic drugs such as morphine, synthetic drugs with narcotic properties such as Tramadol[®], and various others. Also
15 included is any compound with a local analgesic effect such as e.g. lidocaine, benzylic alcohol, benzoic acid and others.

[000135] In one embodiment the analgesic is part of the composition, in another embodiment, the analgesic is administered before, during or after the
20 treatment with the chemodenevating agent.

[000136] In another embodiment, the polypeptide of this invention may be in the form a a lyophilisate.

25 [000137] The term "lyophilization" is used herein for a treatment of a solution containing the polypeptide, wherein this solution is frozen and dried until only the solid components of the composition are left over. The freeze-dried product of this treatment is therefore defined in this document as "lyophilisate".

30

[000138] In this document the term "reconstitution" and "reconstituted solution" are defined herein as the process of solubilization of said freeze-dried composition of the polypeptide of the invention. This can be done by adding the appropriate amount of sterile water, e.g. if all necessary
35 components are already contained in the lyophilisate. Or, if this is not the case, it can be done e.g. by adding a sterile saline-solution alone or if applicable with the addition of components comprising e.g. a pH buffer, excipient, cryoprotectant, preservative, analgesic stabilizer or any

5 combination thereof. The saline of before mentioned "saline-solution" is a salt-solution, more preferably being a sodium-chloride (NaCl) solution, still more preferably being an isotonic sodium-chloride solution (i.e. a sodium-chloride concentration of 0,9%). The solubilization is carried out in such a manner that the final "reconstitution" is directly or indirectly, i.e. for example
10 after dilution, administrable to the patient. In one embodiment the polypeptide of the invention is reconstituted in isotonic media. In another embodiment in isotonic saline. In yet another embodiment, said saline is sterile saline.

[000139] The pharmaceutical composition comprising the botulinum toxin is
15 administered, in one embodiment several times, in an effective amount for improving the patient's condition.

[000140] Typically, the dose administered to the patient will be up to about 1000 units, but in general should not exceed 400 units per patient. In one
20 embodiment the range lies between about 80 to about 400 units. These values are in one embodiment valid for adult patients. For children, the respective doses range from 25 to 800 and in another embodiment from 50 to 400 units.

25 [000141] While the above ranges relate to the maximum total doses, the dose range per muscle is in one embodiment within 3 to 6 units/kg body weight (b.w.), for small muscles 0,5-2 U/kg b.w., in another embodiment 0,1-1 U/kg b.w. Generally doses should not exceed 50 U per injection site and 100 U per muscle.

30

[000142] In one embodiment of the present invention the effective amount of botulinum toxin administered exceeds 500 U of the polypeptide of the invention in adults or exceeds 15 U/kg body weight in children.

35 [000143] As to the frequency of dosing, the re-injection interval depend greatly on the persistency of the modified neurotoxin. Thus, according to the present invention the medicament to be administered is re-administered in intervals of between 3 and 6 months, in another embodiment the

5 medicament is re-administered in intervals of between 2 weeks and less than 3 months. However, depending on the modifications of the neurotoxin, in other embodiments treatments of more than 6 months up to 12 months or treatments in time periods shorter than 2 weeks are envisaged.

10 [000144] With regard to the composition and dosing of the medicament on the basis of botulinum toxin, and in regard to the composition, dosing and frequency of administration of the medicament on the basis of the neurotoxic component of botulinum toxin, US 60/817 756 is incorporated herein by reference.

15

[000145] In one embodiment said composition comprises only botulinum toxin as an active component, in another embodiment further active components e.g. analgesics, said muscle activating agent, etc. are part of the composition.

20

[000146] While the above stated values are to be understood as a general guideline for administering the medicament as used within the present invention, it is, however, ultimately the physician who is responsible for the treatment who decides on both the quantity of toxin administered and the
25 frequency of its administration.

[000147] The medicament on the basis of botulinum toxin can be injected directly into the affected muscles. In order to find the appropriate injection site, several means exist which help the physician in order to find the same.
30 Within the present invention, all methods for finding the best injection site are applicable, such as injection guided by electromyography (EMG), injection guided by palpation, injection guided by CT/MRI, as well as injection guided by sonography (ultra-sound). Among those methods, the latter is in one embodiment the method of choice when treating children. With respect to
35 further details regarding the injection guided by sonography, we refer to Berweck "Sonography-guided injection of botulinum toxin A in children with cerebral palsy", *Neuropediatric* 2002 (33), 221-223.

5[000148] The term "injection" is defined herein as any process, which allows the person skilled in the art to administer the active agent to the target site by penetrating the skin. An incomplete number of examples for "injections" are subcutaneous, intra-muscular, intra-venous, intra-theal, intra-arterial, etc.

10[000149] The terms "chemodenervating agent" / "chemodenervation" are used herein in the broad sense given in the introductory part of this application.

[000150] In another embodiment, the polypeptide of the invention is part of a
15 diagnostic assay, e.g. a phrenic nerve-hemidiaphragm-assay, which allows to diagnose e.g. antibody levels in a patient blood-serum. Examples for such assays can be found in e.g. Göschel H, Wohlfarth K, Frevert J, Dengler R, Bigalke H. "*Botulinum A toxin therapy: neutralizing and nonneutralizing antibodies--therapeutic consequences.*" Exp Neurol. 1997 Sep;147(1):96-
20 102.

[000151] It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. It must be noted that, as used in the specification and the appended
25 claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

[000152] The present invention is now further exemplified by way of the non-limited examples recited herein under.

30

[000153] Literature:

[000154] 1) „The Protein Protocols Handbook (Methods in Molecular Biology)", John M. Walker, Humana Press; Volume: 2 (February 2002),
35 ISBN-10: 0896039404, ISBN-13: 978-0896039407

5[000155] 2) "Protein Purification Methods: A Practical Approach", E. L. V. Harris (Ed.), S. Angal (Ed.), Oxford University Press (December 1989), ISBN-10: 019963002X, ISBN-13: 978-0199630028

[000156] 3) "Protein Purification: Principles and Practice", Robert K. Scopes,
10 Verlag: Springer, Berlin; Auflage: 3 Sub (Januar 1994), ISBN-10: 0387940723, ISBN-13: 978-0387940724

5

EXAMPLES AND DRAWINGS**[000157] A: NT-modification via reaction with Isothiocyanate Reagents**

[000158] The „residue W“ is N¹-(p-isothiocyanatobenzyl)-diethylenetriamine-
10 N¹,N²,N³,N³-tetraacetic acid (DTTA). The isothiocyanate-group reacts with primary aliphatic amino group on the NT at alkaline pH to form a stable, covalent thiourea bond (Figures 1 & 2).

[000159] MATERIALS

- 15
- BoNT/A neurotoxic component
 - Organic residue ITC-benzyl-DTTA
 - Linking buffer: 100 mM^oNa₂CO₃ (pH 9.3)
 - Dialysis system for changing buffers for NTs

20 [000160] **LINKING PROCEDURE**

- Dialysis of BoNT/A solution against linking buffer
- Addition of dialyzed NT solution to the „residue W“ and incubation overnight at +4°C.
- Separation of the linked NT from unreacted reagent by dialysis against
25 50 mmol/L TRIS-HCl (pH 7.8) containing 0.9 % NaCl

[000161] A-1: Detailed Reaction Procedure

[000162] The DTTA group forms a stable complex with Eu³⁺ and the isothiocyanate-group reacts with primary aliphatic amino group on the NT at
30 alkaline pH to form a stable, covalent thiourea bond (Figure 2). The high water solubility and the stability of the chelate, in addition to the mild coupling conditions of the isothiocyanate reaction, enable easy linking of NTs. In order to use a simple gel filtration for fractionation of linked NTs and separation of NTs from free Eu-chelates, the molecular weight of the NT needs to be at
35 least 5000. The thermodynamic stability of the chelate allows long-term storage of linked NTs and the kinetic stability allows use of the linked reagents in assays in contact with e.g. serum samples. The linked NT as

5 such is practically non-fluorescent. Consecutively to the immunoreactions and appropriate washing steps, however, Eu^{3+} is efficiently released from the chelate within a few minutes by the low pH of the Enhancement Solution. Free Eu^{3+} rapidly forms a new highly fluorescent chelate with the components of the Enhancement Solution. The fluorescence is then
10 measured with the time-resolved fluorometer.

[000163] **A-2: Conditions for NT Reaction**

[000164] The reaction depends upon the nature and concentration of the NT to be linked, the temperature and pH of the reaction, reaction time and the
15 intended final linking yield. The NTs to be linked must be in a buffer that does not contain any amines or sodium azide. The recommended conditions for linking are the following: 0.1 mol/L sodium carbonate pH 9 – 9.3, +4°C and overnight reaction.

20 [000165] REACTION PROCEDURE

[000166] The NT or peptide to be linked must not be stabilized with a NT (e.g. bovine serum albumin [BSA], casein or gelatin).

25 [000167] **Pretreatment:** If the buffer including the NT or peptide to be linked contains primary amines (e.g. TRIS, ammonium ions), sulfhydryl groups (e.g. mercaptoethanol) or sodium azide, a pretreatment step is necessary. The above mentioned compounds interfere with linking. Suitable methods for removing interfering compounds include gel filtration (e.g. NAP
30 and PD-10 columns by Amersham Pharmacia Biotech), dialysis and reverse phase HPLC (RP-HPLC).

[000168] **Concentrating NT:** When linking 1 mg of NT, the NT concentration should be approximately 4 mg/mL; correspondingly with 0.5
35 mg NT the concentration should be approximately 2 mg/mL. If the NT is too dilute (less than 1 mg/mL) a concentration step is necessary. Suitable concentrators are e.g. Centricon and Centriprep concentrators.

5

[000169] **Linking:** Open the Eu- reagent vial carefully. Add 250 μ L of the NT in the linking buffer (max. 1 mg) to the reagent vial. Mix gently to dissolve the reagent and incubate overnight at +4°C.

10 [000170] **Purification:** Separation of the linked NT from unreacted chelate is performed by gel filtration. Elution buffer should be TRIS-HCl based, e.g. 50 mmol/L TRIS-HCl (pH 7.8) containing 0.9 % NaCl and 0.05 % sodium azide (TSA buffer). NTs with a molecular weight over 100 000 can be purified using Superdex 200 column or a combination of Sephadex G-50
15 (e.g. 1 x 10 cm) layered on Sepharose 6B (e.g. 1 x 30 - 40 cm). NTs with a MW in the range of 30 000 - 100 000 are best purified using Superdex 75 or Sephadex G-50. Sephadex G-50 is suitable also for purification of NTs with a MW between 15 000 and 30 000. In gel filtration the collected fraction size should be about 1 mL. The gel filtration eluate can be monitored by UV-
20 absorbance at 280 nm. The first peak contains the linked NT and the second peak unreacted chelate. The Eu concentration of the fractions can be measured by making a 1:1000 – 1:10000 dilution in DELFIA Enhancement Solution (supplied with the kit). The dilutions should be mixed gently and let stand for about 2 minutes before measuring in a time-resolved fluorometer.
25 The fractions from the first peak with the highest Eu counts are pooled and characterized. Collecting of fractions should be stopped at least two fractions before the signal of unreacted chelate starts to rise. When linking only a small amount of antibody (< 0.5 mg) the purification can be done with a PD-10 column by applying the reaction mixture in the equilibrated column and
30 collecting 0.5 mL fractions. The fractions from the first peak with the highest Eu counts should be pooled and characterized.

[000171] Recommended columns for purification of NTs after linking with Eu-N1 ITC chelate:

- 35
- Superdex 200
 - Sephadex G-50 / Sepharose 6B

5[000172] There should be dedicated columns for each lanthanide (europium, terbium, samarium, dysprosium) used for reaction. After purification, the gel filtration column should be decontaminated by washing with 10 mmol/L phthalate buffer (pH 4.1) containing 0.01 % DTPA. Before each run, it is advisable to saturate and further purify the gel filtration column on the
 10 previous day by applying concentrated BSA solution of high purity (e.g. 0.3 mL 7.5 % BSA; supplied with the kit). After adding the BSA the column should be equilibrated overnight. RP-HPLC columns can be washed using the phthalate buffer described above.

15[000173] **A-3: Characterization of the NTs**

[000174] **Eu³⁺ Content:** The exact concentration of Eu³⁺ after dilution with DELFIA Enhancement Solution (1:10 000 - 1:100 000), is calculated by measuring the fluorescence in microtitration strip wells (200 µL/well; in triplicates) and comparing to the fluorescence of 1 nmol/L Eu-standard (stock
 20 standard diluted 1:100 in Enhancement Solution) (1 nmol/L Eu in Enhancement Solution in a clear 96-well plate, 200 µL per well, gives about 1 000 000 cps when measured in 1234 DELFIA Research Fluorometer or 1420 VICTOR Multilabel Counter).

25[000175] **NT Content:** NT concentration in the pooled fractions can be measured with appropriate methods, e.g. Lowry's method, or it can be calculated from the NT absorbance at 280 nm. The molar absorptivity of reacted Eu-N1 ITC chelate is 8000 at 280 nm (1 µmol/L reacted chelate gives an absorbance of 0.008 at 280 nm).

30

[000176] **Calculations:** The following equations can be used for IgG. They are valid when the linking yield is < 20 Eu³⁺/IgG. 1.34 is used for absorptivity value (for 1 mg/mL) of IgG, and 160 000 for MW. Eu-counts x dilution factor
 Eu³⁺ (µmol/L) = 1000 x counts of 1 nmol/L Eu³⁺ Abs(280) - 0.008 x Eu³⁺
 35 (µmol/L) NT (mg/mL) = 1.34 NT (mg/mL) x 1 000 000 NT (µmol/L) = 160 000 (g/L) Eu³⁺ (µmol/L) Yield (Eu³⁺/IgG) = NT (µmol/L) 100 x NT(mg/mL) x volume of pooled fractions (mL) Recovery (%) = NT added (mg)

5

[000177] **Filtration:** To remove particles and possible aggregates the linked NT should be filtered through a 0.22 µm low NT binding membrane.

[000178] **Storage of linked compounds:** To ensure stability, the linked NTs
10 should be stored at a high concentration and in the absence of chelators or competing metals in the buffer. The stability can be increased by adding purified BSA (supplied with the kit) to a final concentration of 0.1 %. Temperature during storage is determined by the stability of the NT. Suitable temperatures are e.g. +4°C, -20°C and -70°C. In cases where bovine
15 albumin cannot be used as a carrier NT, the linked NTs could be stored as such, if the NT concentration is over 100 µg/mL. If other NTs are used as carriers, these need to be purified from any heavy metal contaminations prior to addition. The carrier used also needs to be free from chelating agents.

20 [000179] **B: Anionic NT-modification**

[000180] **Anionic NT:** NT-derivatives with pI lower than 4,2 can be achieved by chemical modification of NT with glutaric acid anhydride (figure 3).

25

[000181] **Protocol for the anionization of NT:** To a solution of BSA (7.5 µmol NT) in 50 mL 0.1 mM phosphate buffer, pH 8.2 is added glutaric acid anhydride (0.44 mmol) dissolved in 2 mL dioxan. The reaction mixture is stirred at RT for 2 hours and the modified NT is purified by ultrafiltration and
30 ice-dried to give 50 mg red powder. MALDI-TOF mass spectrum: A broad peak with maximum. Yield 92%.

[000182] **C: Characterization of Accessible Primary Amino Groups**

[000183] **Quantification of the number of accessible primary amino
35 groups at the NT surface via the reaction with an UV probe:** The number of amino group is analyzed using the specific reaction of primary amines with 2,4,6-trinitrobenzen 1-sulfonic acid (TNBS). A method for quantification of ε-

5 amino groups of native proteins is described in the literature. This method is based on the absorbance of the obtained TNP-protein derivatives at 335 nm and using a standard curve generated by the difference in absorbance observed with L-lysine and L-glutamic acid after their reaction with TNBS reagent (figure 6a). The method works well for native non-linked NT. But
10 when a cationized or dye-linked species are involved, the relationship between absorbance and concentration of the amino groups is no longer linear in the concentration interval. The tendency of the obtained results is correct. The absorbance rises with the degree of cationization, but the obtained number of amino-groups per molecule is higher than the reality.

15

[000184] Therefore, the relationships between the absorbance at 335 nm and the concentration of the NT in the analyzed sample is investigated first. This allows to find an interval where this relation is linear and to draw a new calibration curve. Another difficult point is the influence of the chromophore.
20 The linking of BSA does not lead to a significant change in the absorption of the conjugate at 335 nm (figure 6b).

[000185] **Analysis of the secondary structure of modified proteins via circular dichroism:** In order to assess whether the secondary structure of
25 the protein is changed via the introduction of functional groups at the protein surface, circular dichroism measurements are performed. Circular Dichroism (CD) relies on differential absorption of left and right circularly polarized radiation by chromophores which either possess intrinsic chirality or are placed in a chiral environment. CD has been used extensively to give useful
30 information about the protein's structure, the structural changes during conjugation, and ligand binding (Kelly, S.M.; Price, N.C. *Curr. Protein and Peptide Sci.* **2000**, *1*, 349-384).

[000186] Proteins have a number of chromophores (e.g. tryptophan
35 substituents), which can give rise to CD signals. In the far UV region (240-80 nm), which corresponds mainly to peptide bond absorption (intense π - π^* transition at about 190 nm, and a broad n - π^* absorption centered around 210 nm), the CD spectra can be quantitatively analyzed to give the content of

5 regular secondary structure (α -helix and β -sheet, respectively). This is possible because the different forms of the regular secondary structure in proteins give rise to distinct CD spectra. Our experiment showed that the conversion of carboxyl side-groups of NT to primary amines via treatment with ethylene diamine, does not affect the secondary structure of NT. The CD
10 spectra of NT and cNT are superimposable, suggesting that the cationization of the NT is not triggering any major modification in the secondary structure.

[000187] **Matrix-assisted time of flight spectrometry:** The study of NT with gel electrophoresis confirmed that the molecular weight of the native NT,
15 estimated by this method, does not differ from the molecular weight of the modified one. In order to investigate the molecular weight more precisely, the products were analyzed by MALDI-TOF spectrometry. Different matrices were tested and it turned out that applying sinapinic acid as matrix gave the best results with sharp signals of a good intensity. The resulting spectra
20 confirmed the results obtained by isoelectric focussing but also clearly showed how the average molecular weight increases with progress of the cationization reaction.

[000188] **Fluorescence Correlation Spectroscopy:** In principle, the
25 increase of the number of functional groups has an impact on the hydrodynamic radius of the NT. Therefore, the hydrodynamic radius of the modified NT macromolecules was studied by applying fluorescence correlation spectroscopy (FCS). The results of this analysis combined with the molecular weight determined by MALDI – TOF are summarized in Table
30 2. As expected, an increase of the number of charges results in lower diffusion coefficients and higher hydrodynamic radii, respectively.

[000189] **D: In vitro Characterization of BoNT/A - Europium Complex**

35 [000190] HDA Test (Toxinactivity)

- 89 min time of paralysis at 1ng Eu-Toxin / 4ml
- approx. 72min at 0,6ng NT201 / 4ml (reference 1)
- approx. 55 min at 2 ng NT101 pro 4 ml (reference 2)

5

[000191] NT determination (Bradford protein test)

- 17,07 µg/ml NT (in 1,7ml)

[000192] Europium determination via TRF (Time-resolved fluorometry)

- 10
- 1,50 nmol/ml Europium $\Rightarrow$ 13,1 Europium-Atoms per NT-molecule

[000193] Binding of NT-Eu to botulinum toxin-Antibodies

- Coating of microtiterplates with NT
- Blocking of unspecific bindingsites
- 15 • Incubation with antibodies at different concentrations (Botulismus Antitoxin, Chiron-Behring)
- Washing
- Incubation with NT-Eu (strength of marking: 13,1 or respectively 2,1 Mol Eu per Mol NT)
- 20 • Washing
- Fluorescence analysis (figure 7)

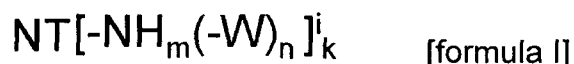
[000194] Reduced antibody binding by factor of ≥ 50 upper limit of detection with weakly marked NT (NT-2.1Eu) is obtained at approx. 100 mU/ml of
25 Antibodies (followed by a Hook-effect). With strongly marked NT (NT-13.1Eu) the fluorescence signal stays significantly below the corresponding signals of weakly marked NT and does not show any Hook-effect up to 100 000 mU/ml of Antibodies. At the same fluorescence signal approx. 6 times more NT-(2,1)Eu than NT-(13,1)Eu is bound to the antibodies because of the stronger
30 marking (figure 7).

5

CLAIMS

1. A modified polypeptide wherein the polypeptide is represented by formula I:

10



wherein

15

NT represents a biologically active neurotoxic component or fragment thereof, which is still capable of cleaving proteins of the SNARE complex and of binding to the native HC domain receptor as well as to translocate the light chain;

NH_m represents an amino group of NT;

W represents a residue comprising at least one moiety that is negative charged at physiological pH;

20

m represents an integer of 0, 1 or 2;

n represents an integer of 1, 2 or 3 and

$(m + n) = 2$ or 3;

k represents an integer of 1 to 1000 wherein for $k > 1$, each set of m, W and n can be equal or different from each other;

25

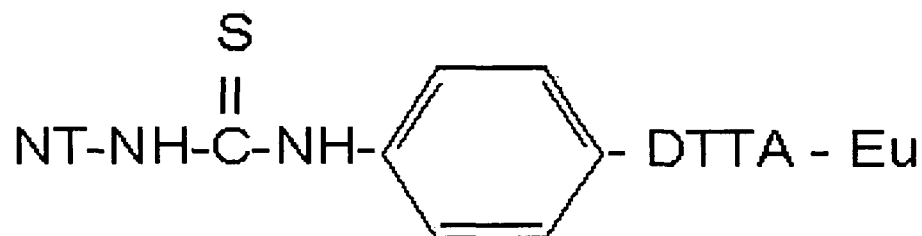
i represents a positive charge if $(m + n)$ is 3, wherein if $(m+n)$ is 2 then i represents no charge.

30

2. The polypeptide of claim 1, wherein said negative charged moiety is selected from the group consisting of a carboxylate, a sulfonate, a phosphate and a combination of two or more thereof.
3. The polypeptide of claim 1 or 2, wherein said negative charged moiety is derived from a multivalent carboxylic acid selected from the group

5 consisting of citric acid, NTA, EDTA, DTTA, DTPA, TETA and derivatives thereof.

4. The polypeptide according to claim 3 represented by formula III:



10 Formula III

5. The polypeptide of any of the preceding claims, wherein a counterion is bound to said negative charged moiety.

15 6. The polypeptide of claim 4, wherein the counterion is selected from the group comprising any ion of the metals Zinc (Zn), Manganese (Mn), Magnesium (Mg), Calcium (Ca), Copper (Cu), Nickel (Ni), Iron (Fe), Cobalt (Co), Europium (Eu), Samarium (Sm), Dysprosium (Dy) and Terbium (Tb).

20

7. The polypeptide of any one of the preceding claims, wherein the NH_m-moiety is part of a surface or solvent exposed lysine residue of said neurotoxic component.

25 8. The polypeptide of any one of the preceding claims, wherein said neurotoxic component is at least 30% identical to

(a) BoNT/A - SEQ ID No. P10845;
gi|399133|sp|P10845.4|BXA1_CLOBO[399133].

(b) BoNT/B - SEQ ID No. P10844;
gi|399134|sp|P10844.3|BXB_CLOBO[399134]

30

(c) BoNT/C1 - SEQ ID No. P18640;
gi|115185|sp|P18640.2|BXC1_CLOBO[115185]

- 5 (d) BoNT/D – SEQ ID No. P19321;
gi|115188|sp|P19321.1|BXD_CLOBO[115188]
- (e) BoNT/E - SEQ ID No. Q00496;
gi|399135|sp|Q00496.2|BXE_CLOBO[399135]
- 10 (f) BoNT/F - SEQ ID No. P30996;
gi|399137|sp|P30996.1|BXF_CLOBO[399137]
- (g) BoNT/G - SEQ ID No. Q60393;
gi|2499920|sp|Q60393.2|BXG_CLOBO[2499920]
- (h) Neurotoxic component of neurotoxin type E; *Clostridium*
butyricum - SEQ ID No. P30995;
15 gi|399136|sp|P30995.2|BXE_CLOBU[399136]
- (i) TeNT; *Clostridium tetani* - SEQ ID No. P04958;
gi|135624|sp|P04958.2|TETX_CLOTE[135624]
- (j) Neurotoxic component of neurotoxin type F; *Clostridium*
baratii - SEQ ID No. Q45851;
20 gi|75346848|sp|Q45851|Q45851_9CLOT[75346848]
9. The polypeptide of any one of the preceding claims, wherein said
fragment is the light or heavy chain of a clostridial neurotoxic
component.
- 25 10. The polypeptide of any one of the preceding claims, wherein said
clostridial neurotoxic component is obtainable from *C. botulinum*, *C.*
butyricum, *C. baratii* and *C. tetani*.
- 30 11. The polypeptide of claim 10, wherein said *C. botulinum* is of serotype A,
B, C, D, E, F or G.
12. A method for preparing the polypeptide of any one of the preceding
claims, wherein the modification resulting in the formation of the
35 compound of formula I is the result of a chemical reaction comprising a

5 condensation reaction, a Michael-type reaction, native chemical ligation, sulfonation, glycosylation or phosphorylation.

13. The method of claim 12, comprising the steps of:

- 10 (a) translating or synthesizing the polypeptide or polypeptide fragment according to any one of claims 1 to 10;
- (b) exposing said polypeptide or polypeptide fragment in a buffer to a reactive compound; and
- 15 (c) purifying the modified polypeptide from the buffer, which contains at least one or more moieties as defined as W in claim 1.

14. A composition, comprising a polypeptide according to any one of claims 1 to 11 or the polypeptide obtainable by the method of any one of claims 12 or 13.

20

15. Use of the polypeptide according to any one of claims 1 to 11, or the polypeptide obtainable by the method according to claim 12 or 13, or the composition of claim 14 for cosmetic treatment.

25 16. The polypeptide according to any one of claims 1 to 11, or the polypeptide obtainable by the method according to claim 12 or 13, or the composition of claim 14 for the use in treatment of

- (a) a disease or condition which can be ameliorated by temporarily reducing muscle activity;
- 30 (b) a disease or condition which can be ameliorated by temporarily reducing glandular secretion;
- (c) a disease or condition during an operation or in support of wound healing which can be ameliorated by temporarily reducing muscle activity.

35

- 55 -

- 5 17. Use of a polypeptide according to any one of claims 1 to 11, or the polypeptide obtainable by the method according to claim 12 or 13 for the preparation of a pharmaceutical composition for
- (a) treating a disease or condition which can be ameliorated by temporarily reducing muscle activity;
- 10 (b) treating a disease or condition which can be ameliorated by temporarily reducing glandular secretion;
- (c) temporarily reducing muscle activity during an operation or in support of wound healing.

15

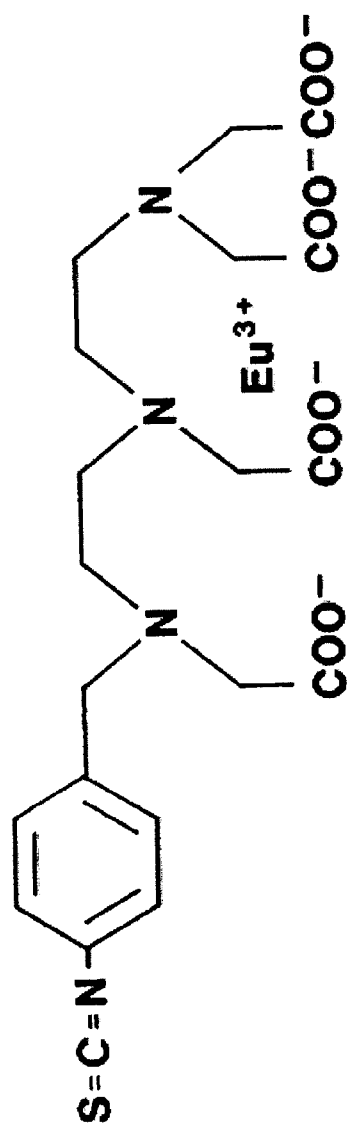


Figure 1

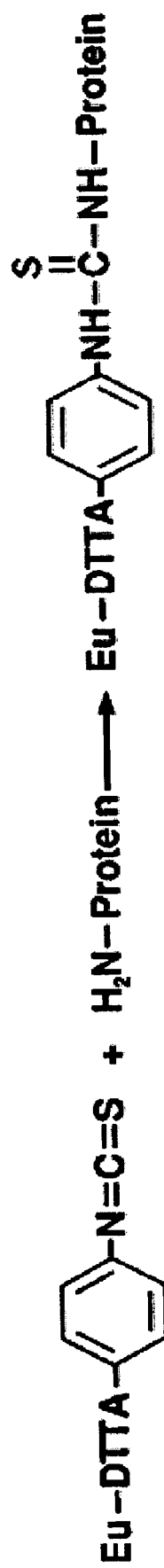


Figure 2

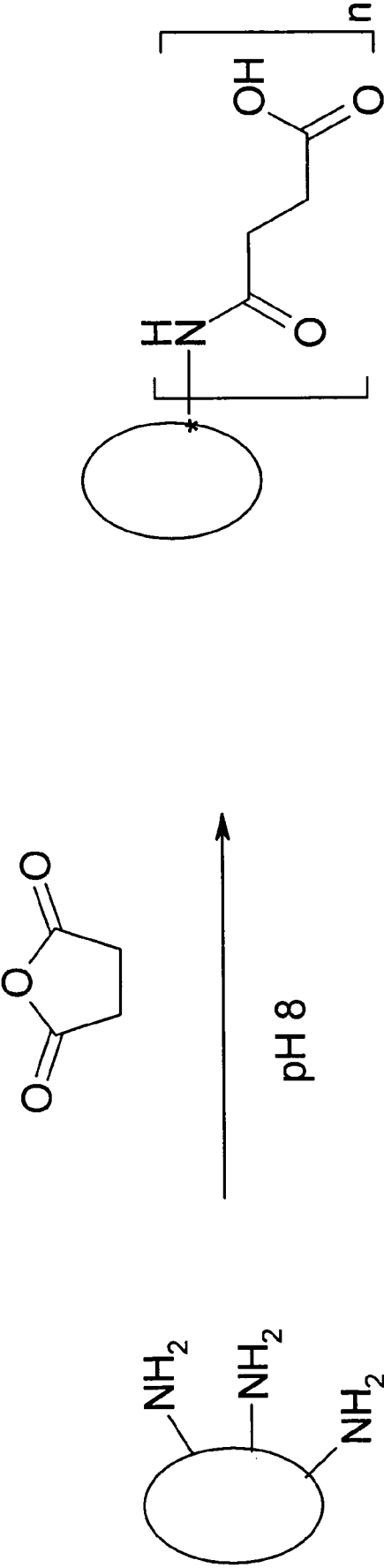


Figure 3

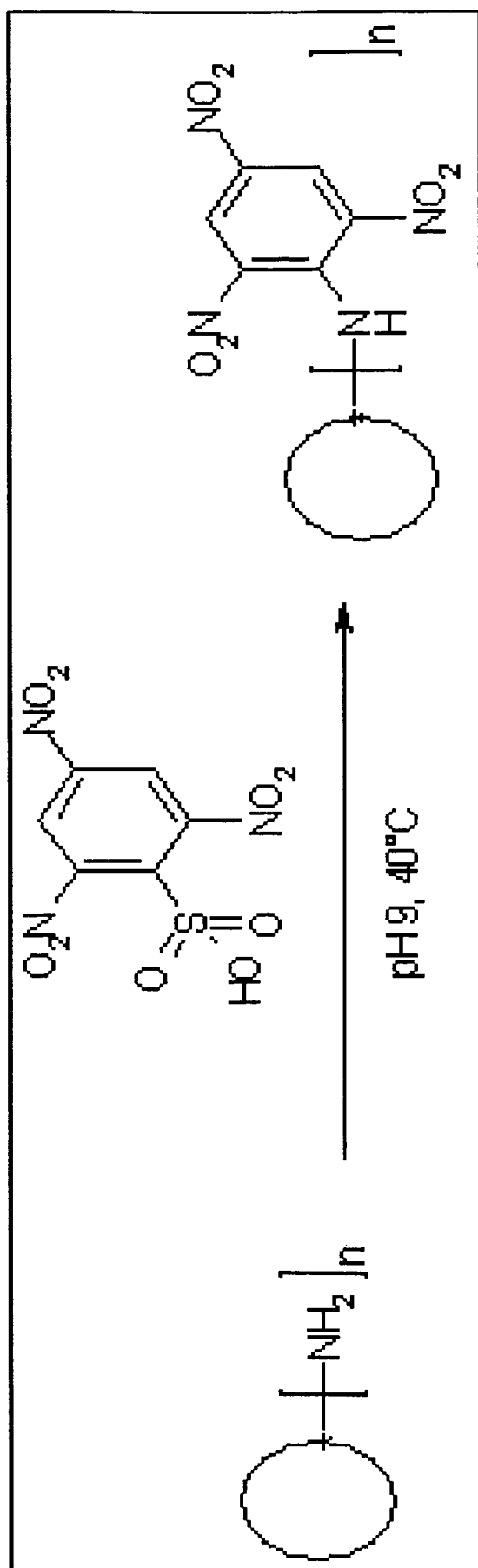


Figure 4a

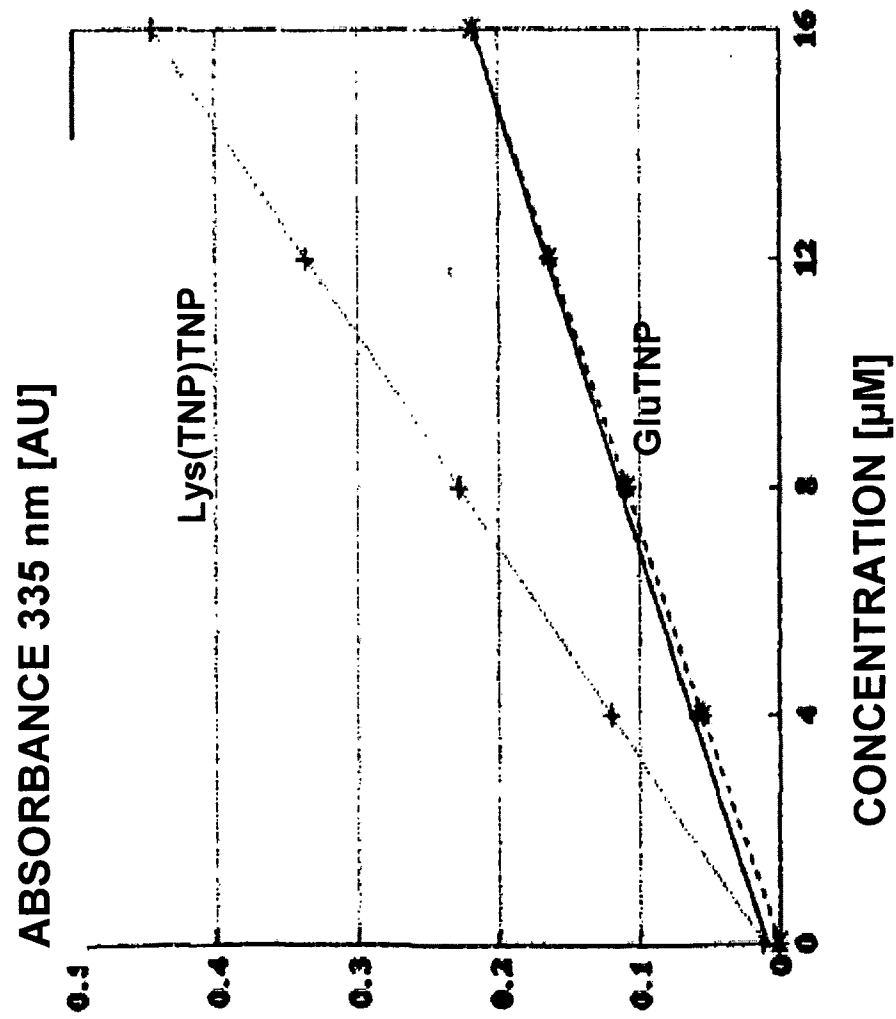


Figure 4b

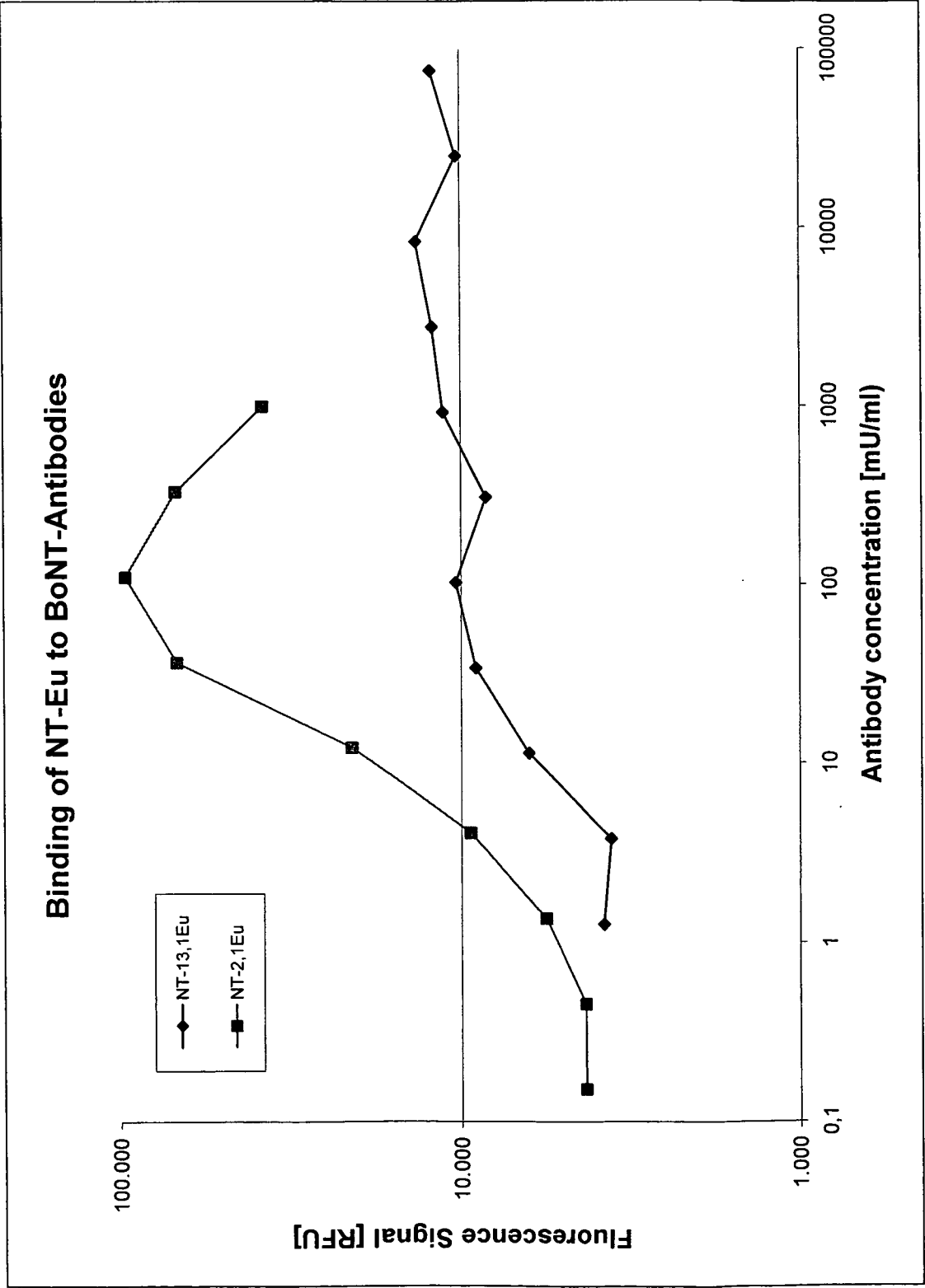


Figure 5

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/006273

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K1/107 C07K1/13 C07K14/33 A61K38/16 A61K38/48
A61K47/48

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99/17806 A (SPEYWOOD LAB LTD [GB]; MICROBIOLOGICAL RES AUTHORITY [GB]; DUGGAN MICH) 15 April 1999 (1999-04-15) claims; examples	1-17
A	CHADDOCK J A ET AL: "INHIBITION OF VESICULAR SECRETION IN BOTH NEURONAL AND NONNEURONAL CELLS BY A RETARGETED ENDOPEPTIDASE DERIVATIVE OF CLOSTRIDIUM BOTULINUM NEUROTOXIN TYPE A" INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, vol. 68, no. 5, 1 May 2000 (2000-05-01), pages 2587-2593, XP001010268 ISSN: 0019-9567 Materials and Methods; Results;	1-17



Further documents are listed in the continuation of Box C.



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

27 April 2010

Date of mailing of the international search report

12/05/2010

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Vogt, Titus

INTERNATIONAL SEARCH REPORT

International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DUGGAN M J ET AL: "Inhibition of release of neurotransmitters from rat dorsal root ganglia by a novel conjugate of a Clostridium botulinum toxin A endopeptidase fragment and Erythrina cristagalli lectin" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOCHEMICAL BIOLOGISTS, BIRMINGHAM,; US, vol. 277, no. 38, 20 September 2002 (2002-09-20), pages 34846-34852, XP002266555 ISSN: 0021-9258 Experimental Procedures; Results; -----	1-17
A	WO 96/33273 A (SPEYWOOD LAB LTD [GB]; MICROBIOLOGICAL RES AUTHORITY [GB]; FOSTER KEIT) 24 October 1996 (1996-10-24) claims; examples -----	1-17
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/006273

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/32599 A1 (UNIV CALIFORNIA [US]) 12 September 1997 (1997-09-12) cited in the application claims -----	1,2,5, 7-17
X	US 2003/165541 A1 (DONOVAN STEPHEN [US]) 4 September 2003 (2003-09-04) claims -----	1,2,5, 9-17
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2009/006273

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

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

a. (means)



on paper



in electronic form

b. (time)



in the international application as filed



together with the international application in electronic form



subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in 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/EP2009/006273

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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Information on patent family members

International application No

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