

The active modulator of lipophilicity in the form of dimethyl sulfone for use as a transition promoter of active substances, the composition containing it, and the method of its production

The object of the invention is an active modulator of lipophilicity in the form of dimethyl sulfone (other names: methylsulfonylmethane, DMSO₂, CAS No.: 67-71-0) for use as a transition promoter of active substances, a composition containing it and a method of manufacturing it.

Conotoxins are small peptides derived from cone snail venom that have evolved to capture prey and defend against predators. They represent a structurally and functionally very diverse class of bioactive molecules that are highly selective for different ligand and voltage-gated ion channel subtypes. For example, the μ -CnIIIC conopeptide from the cone cochlea *Conus consors* exhibits myorelaxant activity through specific blockade of skeletal muscle Nav (Nav1.4) channels. This property can be exploited in cosmetics for daily anti-wrinkle therapy (Green B.R., Bulaj G., Norton R.S. (2014). *Structure and function of μ -conotoxins, peptide-based sodium channel blockers with anesthetic activity. Future Medicinal Chemistry* 6(15); 1677-1698. doi:10.4155/FMC.14.107 Markgraf R., Leipold E., et al. (2012). *Mechanism and molecular basis of the sodium channel subtype specificity of μ -conopeptide CnIIIC. British Journal of Pharmacology* 167; 576-586. doi:10.1111/j.1476-5381.2012.02004.x).

Cosmetics applied through the skin are the main group of anti-ageing preparations. The transdermal administration of a substance to produce a systemic effect has a number of important advantages, among them: it avoids the first-pass effect (hepatic metabolism), it eliminates the potential breakdown of the therapeutic substance in the gastrointestinal tract, it eliminates adverse effects of the substance on the gastrointestinal tract, it eliminates interactions of the therapeutic substance with food and other orally administered drugs, it allows the therapeutic effect to be obtained after absorption of lower doses, the rate of absorption of the therapeutic substance depends on the rate of its release (applies to transdermal therapeutic systems), allows a reduction in the frequency of application of drugs with a short biological half-life, which is particularly important in the treatment of chronic diseases (applies to transdermal therapeutic systems) (Cal K, Stefanowska J. *Methods to increase the permeation of therapeutic substances through the skin. Farm Pol.* 2010, 66(7): 514-520).

The epidermis, particularly the outer stratum corneum (SC), protects the skin from water loss and provides an airtight barrier for other molecules, including active substances and irritants, from penetrating the skin. It is known from research that the higher the molecular weight and the number of polar groups in the active molecule, the higher the degree of hydrophilicity increases. This relationship can be approximately written by the logP partition coefficient (or clogP if this parameter has been determined theoretically). Active molecules with logP < 0 are assumed to exhibit a hydrophilic character, which hinders their solubility in non-polar hydrophobic organic solvents. The epidermis shows hydrophobic properties, which hinders its penetration by hydrophilic active molecules.

It is estimated that only 1-2% of the active substance applied to the skin is able to penetrate into the deeper layers of the skin, where it shows biological activity. This is due to the compact structure of the SC, which resembles a brick wall with hydrophobic areas consisting of lipids and hydrophilic areas consisting of water and hydrated corneocytes. To date, many methods and techniques have been developed to improve the penetration of active substances through the epidermis. These include a special application system (e.g. micro-puncturing), encapsulation of the active substance in a lipophilic envelope (e.g. microbubbles) or the use of low-molecular-weight organic compounds, so-called transition promoters. The mechanism of action of transition promoters is not precisely known, with some postulating the formation of special channels that temporarily facilitate the passage of the active substance through the SC. In the case of promoters based on saturated fatty acids, however, it is postulated that they temporarily increase the permeability of SC lipids.

In cosmetic preparations on the market, the transition promoters used are oils, e.g. cholesterol succinate or commercially available mixtures such as Olifeel Skin by Roelmi or GPS-P and GPS-M by Innovi. The principle of such promoters is to increase fluidisation, the loosening of the skin layers. They bind to fatty residues on the epidermis - a passive rather than bioactive action. Similar dissolves into similar, so the fat promoter binds to the fatty area on the skin. This makes it more mobile and there is greater passive diffusion. In contrast, these promoters only act on fatty compounds. Other examples of transition promoters include, for example, alcohols (ethanol), glycols (propylene glycol), unsaturated fatty acids (oleic acid), terpenes (menthol), azone, sulphoxides (dimethylsulphoxide), surfactants, surfactants (*Cal K, Stefanowska J. Methods to increase the permeation of medicinal substances through the skin. Farm Pol, 2010, 66(7): 514-520*).

The aim of the invention is to provide a new effective transition promoter for water-soluble active substances as well as fat-soluble active substances.

The essence of the invention is an active lipophilicity modulator in the form of dimethyl sulfone (MSM) for use as a transition promoter for high-molecular-weight active substances with molecular weight (M_w) > 500 g/mol (Da, daltons).

Advantageously, the active substance is chosen from the group comprising peptides.

Advantageously, the active substance is a conopeptide with the amino acid sequence Sequence No. 2 or Sequence No. 4.

Another essence according to the invention is a cosmetic composition for transdermal administration containing an active substance and a transition promoter, characterised in that the active substance is encapsulated by a transition promoter, which is an active lipophilicity modulator according to the invention, while the active substance is a compound or mixture of organic compounds with $M_w > 500$ Da.

Advantageously, the composition according to the invention comprises at least one cosmetically acceptable excipient, advantageously selected from the group comprising an emulsifier, a homogenizer, an emollient, a thickener, distilled water, a preservative or a combination thereof

Advantageously, the composition according to the invention is an oil-in-water (o/w) or water-in-oil (w/o) emulsion.

Advantageously, the %wag. of active substance to transition promoter is 1:1 - 1:50.

Advantageously, the composition contains at least one cosmetically acceptable excipient.

Advantageously, the active substance is a conotoxin, advantageously a μ -conotoxin.

Advantageously, the μ -conotoxin is a conopeptide with the amino acid sequence Seq. no. 2.

Advantageously, the μ -conotoxin is a conopeptide with the amino acid sequence of Sequence No. 4. or a genetic modification thereof

Advantageously, the composition according to the invention comprises at least one additional active ingredient.

A further embodiment according to the invention is a method for producing a composition according to the invention, characterised in that it is carried out by either hot emulsification or cold emulsification, hot emulsification comprising the following steps:

- a) mixing of dimethyl sulfone and diluent in a reaction vessel;
- b) bringing the mixture to 70-85 °C;
- c) stirring the mixture at 70-85 °C; the onset of homogenisation depends on the stages of phase combination;
- d) cooling of the mixture to 20-40 °C, preferably 30 °C;
- e) addition of a high-molecular-weight active substance with $M_w > 500$ Da and homogenisation of the mixture, preferably for 1-3 min;

Whereas cold emulsification involves the following steps:

- a) mixing of the dimethyl sulfone with the diluent in a reaction vessel and dissolving at 20-25 °C
- b) bringing the mixture to a temperature of 15 - 35 °C, preferably to ambient temperature or 25 °C;

- c) mixing of the mixture at a temperature of 15 - 80 °C, preferably 25 °C; the onset of homogenisation depends on the stages of phase combination.
- d) addition of a high-molecular-weight active substance with $M_w > 500$ Da and homogenisation of the mixture, preferably for 1-3 min.

Advantageously in at least one of the steps a-e) of hot emulsification, at least one additional active ingredient and/or at least one cosmetically acceptable excipient is added to the reaction vessel, which is advantageously selected from the group consisting of an emulsifier, a homogenizer, an emollient, a thickener, distilled water, a preservative or a combination thereof.

Advantageously in at least one of the steps a-d) of cold emulsification, at least one additional active ingredient and/or at least one cosmetically acceptable excipient is added to the reaction vessel, which is advantageously selected from the group consisting of an emulsifier, a homogenizer, an emollient, a thickener, distilled water, a preservative or a combination thereof.

The invention in question provides the following benefits:

The active modulator of lipophilicity in the form of dimethyl sulfone (MSM, CAS No. 67-71-0) as a transition promoter improves the permeability of high-molecular-weight active substances ($M_w > 500$ Da) through the stratum corneum (SC), particularly hydrophilic polypeptides, by creating a unique biocompatible nano-shell stabilized by strong hydrogen and non-covalent bonds, showing a dual hydrophilic or hydrophobic character. Compared to the commonly used technology for encapsulating active substances in lipid envelopes (microbubbles), the nano-shell has a much smaller size, depending on the mass and geometry of the active substance.

Dimethyl sulfone due to its favourable physicochemical properties such as low molecular weight ($M_w = 94.1$ Da), high boiling point ($T = 238^\circ\text{C}$, does not evaporate from the skin surface), high solubility in water (37g/100g of water), high biocompatibility (no known toxicity to living organisms), ability to form strong and directional hydrogen interactions (through the negatively polarised fragment SO_2) with cations, water molecules and polar groups (m. among others $-\text{OH}$, $-\text{NH}_2$, $-\text{P}(=\text{O})\text{R}$), and non-covalent bonds with anions and lipophilic groups (through the highly positively polarised methyl groups $-\text{CH}_3$).

Due to its dual nature, the dimethyl sulfone nanoshell in which the active substance is contained can be either hydrophilic (SO_2 groups outside the shell) or lipophilic (methyl groups outside the shell). As a result, this allows for optimal and highly efficient transport of the active substance through the hydrophobic and hydrophilic areas of the SC. In addition, the dimethyl sulfone molecules have a high affinity for the water molecule, so that some of them can bind intercellular water, which further benefits the transport of the active substance through the SC.

After fulfilling their purpose, dimethylsulfone molecules are further absorbed through the skin into the body, from which they are safely excreted unchanged. Furthermore, recent studies indicate that dimethylsulfone exhibits antibacterial and anti-inflammatory properties on the skin. These features add to the current technology of transdermal delivery systems for active ingredients (including drugs) and are highly desirable in therapeutic and (dermo)cosmetic products.

The invention is illustrated in the performance examples and the drawing wherein:

fig. 1 shows the electrophoretic separation of *E. coli* S4B proteins transformed with a plasmid containing the TRX+TIHA gene in a 15% SDS-PAGE polyacrylamide gel, where 1 is the Full-Range Rainbow Amersham standard: 225; 150; 102; 76; 52; 38; 31; 24; 17; 12 kDa; 2 is the *E. coli* S4B lysate. coli S4B/pDM/TRX+TIHA, mass approximately 15 kDa from culture at 25 °C; 3 is bacterial pellet after sonication from culture at 25 °C; 4 is supernatant after sonication from culture at 25 °C; 5 is bacterial lysate of *E. coli* S4B/pDM/TRX+TIHA from culture at 24 °C, 6 is bacterial precipitate after sonication from culture at 24 °C, 7 is supernatant after sonication from culture at 24 °C, 8 is bacterial lysate of *E. coli* S4B/pDM/TRX+TIHA from culture at 30 °C, 9 is bacterial precipitate after sonication from culture at 30 °C, 10 is supernatant after sonication from culture at 30 °C;

fig. 2 shows the electrophoretic separation of *E. coli* S4B proteins transformed with a plasmid containing the TRX+TIHA gene in a 15% SDS-PAGE polyacrylamide gel, where 1 is the Full-Range Rainbow Amersham standard: 225; 150; 102; 76; 52; 38; 31; 24; 17; 12 kDa, 2 is the *E. coli* S4B lysate. coli S4B/pDM/TRX+TIHA; mass approximately 15 kDa from culture at 37 °C, 3 is bacterial pellet after sonication from culture at 37 °C, 4 is supernatant after sonication from culture at 37 °C, 5 is lysate of *E. coli* S4B/pDM/TRX+TIHA lysate from a 30 °C culture, 6 is bacterial precipitate after sonication from a 30 °C culture, 7 is supernatant after sonication from a 30 °C culture, 8 is *E. coli* S4B/pDM/TRX+TIHA bacterial lysate from an 18 °C culture, 9 is bacterial precipitate after sonication from an 18 °C culture, 10 is supernatant after sonication from an 18 °C culture;

fig. 3 shows a chromatogram of *E. coli* transformed as in example 1;

fig. 4 shows the electrophoretic separation of *E. coli* proteins transformed with a plasmid containing the TRX+TIHA gene cultured at 30°C in 15% SDS-PAGE, where 1 is *E. coli* S4B/pDM/TRX+TIHA bacterial lysate, mass ca. 14.4 kDa, 2 is the supernatant after sonication, 3 is the bacterial proteins not captured on the Ni-NTA bed, 4 is the proteins after elution buffer elution, 5 is the Full-Range Rainbow Amersham standard: 225; 150; 102; 76; 52; 38; 31; 24; 17; 12 kDa, 6 is the sample after dialysis after 48 h (TRX::TIHA fusion protein);

fig. 5 shows the electrophoretic separation of proteins after BrCN digestion of the fusion protein TRX::TIHA in a 15% SDS-PAGE polyacrylamide gel, where 1 indicates TRX+TIHA undigested, 2 indicates 0.1M HCl digestion after 24 hr. at +4°C BrCN 100:1, 3 indicates 0.3M HCl digestion after 24

hr at +4°C BrCN 100:1, 4 indicates Full-Range Rainbow Amersham standard: 225; 150; 102; 76; 52; 38; 31; 24; 17; 12 kDa, 5 indicates 0.1M HCl digestion after 3 hr. at +24°C BrCN 100:1, 6 indicates 0.1M HCl digestion after 6h at +24°C BrCN 100:1, 7 indicates 0.1M HCl digestion after 24h at +24°C BrCN 100:1, 8 indicates 0.3M HCl digestion after 3h at +24°C BrCN 100:1. at +24°C BrCN 100:1, 9 means 0.3M HCl etching after 6 hrs at +24°C BrCN 100:1, 10 means 0.3M HCl etching after 24 hrs at +24°C BrCN 100:1;

fig. 6 shows a spectrum of the synthetic conotoxin TIIIA without disulfide bridges;

fig. 7 shows the spectra of a reaction mixture enriched with a synthetic fully reduced peptide (C), where peak A represents the signal of low-mass organic compounds, peak B represents TIIIA with disulphide bridges, peak C represents synthetic doped TIIIA without disulphide bridges, peak D represents a carrier protein of 12.85 kDa and an undigested construct of 15.395 kDa;

fig. 8 shows an RP-HPLC chromatogram in the preparatory phase, where the signal at 19.1 min corresponds to TIIIA, the signals from 21 min to 50 min correspond to some digestion of the targets and/or purification of the HIS tag, the peaks between 50 and 56 min are the 12.8 kDa carrier protein, and 57-63 min are the undigested construct;

fig. 9 shows an excerpt from the chromatogram of fig. 10, with particular reference to the magnification to 17-22 min retention time, where the signal from 18.12 to 20.00 min corresponds to 3 mg TIIIA;

fig. 10 shows the results of patch-clamp measurements of conotoxin activity on an oocyte model expressing human sodium channels Nav 1.4 for the conotoxin TIIIA (Toxin A), the conotoxin SIIIA (Toxin B), the conotoxin TIIAlaMut (Toxin C), the standard CnIIIC (Toxin D), the conotoxin TIIIA extracted from the composition according to the invention (Toxin E), where the left column shows individual ion channel responses of Nav 1.4 as an example of the observed inhibitory effect, while the right column shows the effect of the test substance on the conductance curve from channel voltage and channel accessibility - two important parameters of Nav channel function that are usually evaluated;

fig. 11 shows the concentration-response curve for TIIIA toxin, TIIAlaMut toxin and the standard, commercially available CnIIIC toxin; where the effects were measured on the human Nav1.4 ion channel, and the data were fitted to a dose-response curve (Hill equation assuming a single binding site), with fitting performed using Graphpad Prism 8, with fixed parameters: Top = 0, Bottom = 99, Hill N = -1. Error bars represent S.E.M.

The invention is presented in worked examples in which all the tests and experimental procedures described below were carried out using commercially available test kits, reagents and apparatus, following the recommendations of the manufacturers of the kits, reagents and apparatus used, unless

expressly indicated otherwise. All test parameters were measured using standard, well-known methods used in the field to which the present invention belongs.

However, the following examples of implementation serve to illustrate the solution in question and should not constitute a limitation of the scope of patent protection.

Example 1.

Design of a bacterial *E. coli* expression system with a pDM/TRX+TIIIA construct

In order to obtain a recombinant soluble TIIIA conotoxin protein a genetic construct was designed, which is a construct encoding a TRX::TIIIA fusion protein, comprising the TIIIA conotoxin gene and the gene encoding the leader protein thioredoxin (TRX). The TRX protein provides reducing conditions to facilitate the correct folding of proteins having disulphide bridges, moreover, it also often leads to the expression of recombinant proteins in a soluble form, which in this case significantly shortens the purification process of the recombinant protein. In this study, the nucleotide sequence of the thioredoxin TRX gene was modified using a point mutagenesis reaction, whereby the amino acid methionine (M) at position 37 was removed and replaced with lysine (K). This modification was aimed at obtaining the correct protein fragments after digestion with bromocyanine (BrCN), whose cut site is methionine. The nucleotide sequence of the TRX::TIIIA fusion gene was (Sequence No. 1) optimized for bacterial codon usage (codon usage). With that said, the above construct is under the control of a constitutive promoter (e.g. deoP1P2), which allows continuous synthesis of the recombinant protein in bacterial cells and does not require induction for gene expression.

To enable purification of the protein on a NiNTA bed chromatography column, a sequence encoding 6 histidines (6His) and a short linker spanning the amino acids serine-glycine-serine (SGS) was added to the 5' end of the construct. The TRX::TIIIA fusion protein gene was inserted into an expression vector, which in this implementation example is a pDM expression vector digested with NdeI/XbaI restriction enzymes. However, other vectors, such as pDMR, can be used.

The nucleotide sequence of the cloned genes was confirmed, and an *E. coli* S4B production strain (being either a derivative of *E. coli* strain DH10B or any of JM109, HB101, CSH50R, DH10B) containing the pDM/TRX+TIIIA construct (a single molecule of TIIIA conotoxin fused to thioredoxin) was constructed to produce a soluble peptide.

The procedure was performed analogously for a construct encoding a TRX::TIIIAAlaMut fusion protein with nucleotide sequence Seq. no. 2.

Example 2.

The method for producing μ -conotoxin TIIIA in *E. coli* with an expression vector containing the construct according to Example 1 comprises the following steps:

Step (a) *transformation of an E. coli cell with an expression vector containing the construct according to Example 1*

In this performance example, a prokaryotic pDM/TRX+THIA expression vector containing a construct with nucleotide sequence Sequence No. 1 encoding a TRX::THIA fusion protein with amino acid sequence Sequence No. 2 was introduced by electroporation into an *E. coli* S4B expression strain. With this example of execution, the construct is under the control of the constitutive promoter deoP1P2, which allows continuous synthesis of the recombinant protein in bacterial cells and does not require induction for gene expression.

Step (b) *expression of TRX::THIA fusion protein in a transformed E. coli strain*

In order to verify that the protein obtained is in soluble form and to select culture conditions leading to the highest possible expression of the gene encoding the recombinant fusion protein TRX::THIA, material from a single colony of a transformed *E. coli* strain was inoculated with liquid LB medium supplemented with tetracycline (100 µg/ml) and cultured at various temperatures of 18°C, 25°C, 30°C, 37°C until an optical density of OD₆₀₀≈1.0 was achieved. The results are shown in figs. 1–2.

The analysis showed that the recombinant protein is obtained in soluble form, while the highest expression of the gene encoding the TRX::THIA fusion protein is obtained at 30 °C.

Subsequently, for optimal expression of the THIA conotoxin, laboratory-scale transformed *E. coli* strains were cultured in LB medium (10 g/l Bacto Tryptone, 5 g/l yeast extract, 10 g/l NaCl) with the addition of the selection marker tetracycline (100 µg/ml), at 30°C, at 150 rpm, for 18 hours./min, for 18 h, achieving an OD of 3.2 to 3.5. Cultures were inoculated with an appropriate volume of culture material (stock) stored at -70°C (500 µl stock per 500 ml LB medium). Stocks were prepared in a 1:1 ratio of bacterial culture (OD₆₀₀≈0.6) and 20% glycerol. Bacterial material is deposited in the Submitter's strain bank.

Step (c) *isolation and purification of TRX::THIA fusion protein*

After 18 h, the culture prepared in step (c) and cultured at 30 °C was centrifuged (8,000 rpm) and the centrifuged biomass from 1 L of culture was suspended in 50 ml of prepared dissolution buffer (50 mM TRIS-HCl pH 7.8; 300 mM NaCl), sonicated for 1 h 15 min, centrifuged twice for 15 min at 12,000 rpm. The supernatant was applied to a column previously equilibrated with calibration buffer. The bed was then washed with wash buffer, the recombinant TRX::THIA protein was eluted with elution buffer.

The flow rate when applying the sample was 1.0 ml/min, the column was washed at a flow rate of 1.5 ml and elution was carried out at a flow rate of 2.0 ml/min.

The following buffers were used for protein purification:

- Calibration buffer
 - 50mM phosphate buffer pH-7.8, 500mM NaCl, 10mM imidazole;
- Wash buffer
 - 50mM phosphate buffer pH-7.8, 500mM NaCl, 20mM imidazole;
- Elution buffer
 - 50mM phosphate buffer pH-7.8, 500mM NaCl, 150mM imidazole;

Fractions were collected at 5 ml each. Separation was performed on a Bio-Rad Duo Flow System instrument using a column from the same company. The concentration of recombinant TRX::THIA protein was determined using the Bradford method: 10 µl sample, 990 µl buffer 20 mM Tris-HCl pH 7.6, 1 ml Bradford reagent. Absorbance was read from a spectrophotometer at 595 nm. The result of the LPLC chromatographic separation from a 1 L bacterial culture expressing TRX::THIA protein on a Ni-NTA SuperFlow affinity bed is shown in fig. 3.

Step (d) *disulfide bridge folding by treatment of purified TRX::THIA fusion protein with glutathione GSH/GSSG and dialysis in buffer*

After chromatographic separation, approximately 30-35 ml of eluate with a protein concentration of approximately 1.0 mg/ml was obtained from 1 L of culture. Up to 4 mM of reduced glutathione (GSH) and up to 1 mM of oxidised glutathione (GSSG) were added to the collected fractions after elution for correct disulphide bridge assembly. The sample thus prepared was dialysed in buffer (50 mM TRIS-HCl buffer pH 7.8, 10% glycerol). Dialysis was carried out for 48 hours at 4°C, and the buffer was changed after 24 hours.

The composition of the obtained fractions was assessed by polyacrylamide gel electrophoresis (SDS-PAGE). Protein electrophoresis under denaturing SDS - PAGE conditions was used to analyse the purity of the resulting recombinant TRX::THIA protein obtained in the prokaryotic system. The results obtained are shown in fig. 4.

Step (e) *digestion of TRX::THIA fusion protein with complex disulfide bridges by bromocyanate*

Bromocyanine (BrCN) digestion of proteins occurs in an acidic environment. The digestion reaction is highly specific, BrCN reacts with the sulphur of the side chain of the amino acid methionine (Met) residue causing the peptide bond to be broken on the carboxyl moiety of methionine, which is one of the rarest amino acids in proteins.

In order to digest the recombinant conotoxin from the leader protein thioredoxin, it was decided to digest BrCN given in a molar excess of 100:1 (100 moles of BrCN per 1 Met residue) in acidic medium. The nucleotide sequence of the TRX::TIIIA fusion protein contains two methionines followed by a BrCN cutting reaction.

The amount of BrCN required for the reaction per methionine residue was calculated from the molar mass of the fusion protein:

Fusion protein:	TRX::TIIIA
molar mass:	15403.67 g/mol
BrCN excess relative to methionine residues:	100:1

For 1 mg of fusion protein, 1.375 mg of BrCN should be taken. This amount is contained in 2.60 μ L of a 5 M solution of BrCN in CH₃CN (d=1.093).

TRX::TIIIA fusion protein at 1.0 mg/ml, obtained after chromatographic separation and 48 h dialysis, was digested with BrCN with different concentrations of HCl, at +4°C, and at room temperature in the dark with stirring. Samples digested at room temperature were taken after 3, 6 and 24 hours, in order to determine the most efficient digestion time was carried out.

Samples after digestion were submitted for LC-MS (Liquid Chromatography-Mass Spectrometry).

Protein electrophoretic separation after BrCN digestion of the TRX::TIIIA fusion protein in a 15% SDS-PAGE polyacrylamide gel (fig. 5) showed that the most optimal digestion conditions for the TRX::TIIIA fusion protein at a BrCN molar excess of 100:1 in acidic medium were 0.1 M HCl, three hours at room temperature, in the dark with stirring.

Step (f) purification of the digested TIIIA peptide

In this performance example, purification of the digested TIIIA peptide was carried out by RP-HPLC. After BrCN digestion, mainly two molecules are obtained from the TRX::TIIIA fusion protein with a molecular weight (MW) of 15 395 Da:

- TIIIA peptide with a molecular weight of 2426.836 Da,
- TRX leader protein with a molecular weight of 12,855 Da.

Other non-specific products occur in variable proportions.

In order to test the properties of the fully reduced peptide, without disulphide bridges, a linear peptide identical to the conotoxin sequence of TIIIA (Seq. No. 5) was synthesised. The linear peptide is more hydrophobic than the peptide containing disulphide bridges and also has a later retention time compared to the correctly folded peptide, as shown in figs. 6-7.

For the quality control (QC) shown in figs. 6-7, an analytical/semi-preparative HPLC kit (Waters) was used.

Separation parameters:

- C18 column 250x10mm, 100 Å, 5 µm
- Buffer A - 0.1% TFA in water
- buffer B 90% MeCN, 0.1% TFA, water
- MeCN gradient 5-100% B,
- flow rate 2 ml/min, 5-100 B buffer in 55 minutes.

For the purification process of the TIIIA peptide, shown in figs. 10-11, a preparative HPLC system (Knauer) was used.

Separation parameters:

- C18 column 250 x 21.2 mm, 100 Å, 5 µm
- Buffer A - 0.1% TFA in water
- buffer B 90% MeCN, 0.1% TFA, water
- MeCN gradient 5-100% B,
- flow 20 ml/min, buffer 5-100 B in 70 minutes.

One of the following two procedures can be used for cleaning:

Procedure I

The BrCN digestion mixture was divided into 25 ml aliquots in 50 ml conical vials, frozen in liquid nitrogen and lyophilised at -80°C, vacuum -3 mBar. The freeze-drying time depends on the total volume of solvent to be removed and varies from 10 to 30 h. The dry residue after evaporation of 25 ml of reaction mixtures was dissolved in 2 ml of 0.1% TFA, 5% MeCN, centrifuged at 15 kG for 20 min at room temperature. Samples were injected into the HPLC, using a 2 ml loop for semi-preparative separation and a 5 ml loop for preparative separation. Samples were separated in a MeCN A-B gradient, 5-100% in the presence of 0.1% TFA. The fraction containing TIIIA was collected, frozen in liquid nitrogen and lyophilised. The dry sample was weighed.

Separation on a preparative system results in the detection of more and different signals, due to the considerable amount of material applied. However, this does not affect the correct separation of the TIIIA conotoxin.

Due to the different column size and gradient length, separation of the undigested construct and carrier proteins occurs. Correct transfer of the method to a larger scale (>100 mg) requires the introduction of additional preparatory steps.

Procedure II

Due to the unfavourable ratio (1:6) of the TIIIA peptide (2.4 kDa) to the weight of the total TRX::TIIIA construct (15.3 kDa) and the resulting second TRX product (12.8 kDa) after BrCN digestion, and due to the limited capacity of the HPLC columns, it was advantageous to remove the undigested protein and second product before the actual HPLC separation. A 10g cassette with a C18 bed designed for FPLC chromatography was used to bind the peptides and proteins present in the mixture after digestion. Salts and other small molecules were removed during the washing step with 2% MeCN, 0.1% TFA. The crude fraction containing TIIIA was eluted with 30% MeCN, 0.1% TFA, frozen in liquid nitrogen and lyophilised, followed by an HPLC protocol for final purification. The C18 cartridge was washed with 90% MeCN 0.1% TFA, followed by 80% MeOH, 0.1%. The fractions were checked for the presence of the TIIIA peptide and again subjected to the purification procedure. The results are shown in figs. 8-9.

The procedure was performed analogously for a vector containing a construct with nucleotide sequence Sequence No. 3 encoding a TRX::TIIIA_{AlaMut} fusion protein with amino acid sequence Sequence No. 4.

Example 3.

Comparison of the effects of dimethylsulfone (MSM) with known transition promoters

The effectiveness of dimethylsulfone (MSM) as a transition promoter for high-molecular-weight active substances with $M_w > 500$ Da was investigated against μ -conotoxin according to Example 1 (hereafter the active molecule). The following transition promoters known from the state of the art were selected for comparative analysis:

- Dimethyl isosorbide - Transition promoter A;
- Urea - transition promoter B;
- Salicylic acid - promoter of C transition.

Results:

Dimethyl isosorbide - Transition promoter A

Mechanism of action

Result

Overview

Proposal

urea - promoter of the B transition

Mechanism of action: Urea as a promoter can form strong hydrogen bonds. NH_2 groups from urea only form hydrogen bonds e.g. with $-\text{OH}$ and $>\text{C}=\text{O}$ groups from a carboxylic acid or amide, only that the formation of such bonds makes the whole urea molecule highly polar and not fat soluble.

Result

Overview

Proposal

salicylic acid - C-transfer promoter

Mechanism of action: can act by exfoliating the skin (peeling increases skin permeability - exfoliation of the skin increases diffusion). The effect is only achieved in large quantities, so it can cause excessive exfoliation and irritation - not suitable for our purposes.

Result

Overview

Proposal

dimethylsulfone (MSM) transition promoter according to the invention

Mechanism of action: MSM has two S=O groups that allow hydrogen bonding and two methyl groups (CH₃-) that can form non-covalent bonds. By forming this hydrogen bond, through the S=O group with the corresponding groups in the active molecule, a highly polarised state is created on the outside, but a lipophilic area (i.e. CH₃ groups) is formed, which facilitates the absorption of the encapsulated active molecule through the first layer of the skin (epidermis). The degree of lipophilic and hydrophilic areas in the skin varies depending on the skin layer and skin site. The promoter can form a bond once through S=O (in which case we have lipophilic groups on the outside), but also when we have a hydrophilic area on the skin, it can form bonds non-covalently through CH₃ groups (in which case we have polar S=O groups on the outside). Thus, MSM acts as an active modulator of lipophilicity depending on the varying lipo/hydrophilic conditions in the skin layers through which it penetrates.

Result

Overview

Proposal

Example 4.

In this example of implementation, the composition according to the invention is a white, thick composition with a pH of 4.65 ± 0.02 (measured at 25.0 ± 0.2 °C), which can be formulated into a cream.

The qualitative and quantitative composition of the composition is shown in Table 1 below.

Table 1. Example qualitative and quantitative composition of a composition according to the invention in the form of a cream containing a transition promoter according to the invention.

Component	INCI / COSING	Function	PHASE	Quantity	
				(g)	% by weight
Water	Water	Thinner	A	330,50	53,87
Dimethyl sulphone (MSM)	Dimethyl sulphone	Transition promoter	A	29,80	4,86
Arglileline peptide solution C	Water (i) acetylhexapeptide-8 (i) caprylyl glycol	Active ingredient	A	30,20	4,92
Propanediol	Propanediol		A	19,01	3,10
Arbutin	Arbutin	Active ingredient	A	12,10	1,97
Inulin	Inulin	Active ingredient	A	3,08	0,50
Xanthan Gum	Xanthan gum	Viscosity modifier	A	1,87	0,30
Hyaluronic acid	Hyaluronic acid	Humectant	A	0,66	0,11
Sweet almond oil	Sweet almond oil	Emollient	B	36,90	6,01
Squalane (Neossance Squalane)	Squalane	Emollient	B	20,30	3,31
Evening primrose oil (Lipovol™ Evening Primrose)	Evening primrose oil	Emollient	B	18,80	3,06
Argan oil	Argan oil	Emollient	B	16,90	2,75
Caprylic/ Capric Triglyceride	Caprylic/capric triglycerides	Emollient	B	18,00	2,93
Tego Care PBS 6 MB	Polyglyceryl-6 Stearate (i) Polyglyceryl-6 Behenate	Emulsifier	B	18,50	3,02
Glycerine monostearate	Glycerine monostearate	Emulsion stabiliser	B	12,00	1,96
Cetyl alcohol	Cetyl alcohol	Emulsion stabiliser	B	6,00	0,98
Sculptessence™	Water (i) glycerine (i) Linum Usitatissimum (linseed) seed extract	Active ingredient	C	18,20	2,97

SKINectura NOF	Glycerine (i) Water (i) Anigozanthos Flavidus extract	Active ingredient	C	6,80	1,11
Lys'lastine™ V	water (i) butylene glycol (i) extract of Peucedanum Graveolens (dill) (i) pentylene glycol (i) xanthan gum	Active ingredient	C	5,90	0,96
Naturaboost H	1,2-Hexanediol	Preservative	C	6,00	0,98
Conotoxin in buffer (2.97mg/2mL)	µConotoxin in buffer (2.97mg/2mL) Almondade buffer 0,1M	Active ingredient	C	2,00	0,33
Total				613,5 2	100, 00

Example 5.

In this example implementation, the composition according to the invention is in the form of a cream and the qualitative and quantitative composition of the composition is shown in Table 2 below.

Table 2 Example of qualitative and quantitative composition of a composition according to the invention in the form of a cream containing a transition promoter according to the invention.

Phase	Trade name	Content [%]
IA	Water	47,5 (may change depending on amount of conotoxin buffer)
	Dimethyl sulphone (MSM)	5
	Prebulin AGA	0,5
	Naturaboost H	1
IB	Propanediol	3
	Xanthan Gum	0,2
II	Sweet almond oil	6

Phase	Trade name	Content [%]
	Squalane (Neossance Squalane)	3
	Argan oil	3
	Caprylic/ Capric Triglyceride	3
	Tego Care PBS 6 MB	3
	Glycerine monostearate	2
	Cetyl alcohol	1
	Evening primrose oil (Lipovol™ Evening Primrose)	3
III	Demin water.	1
IV	Hyaluronic acid	0,1
	Sculpcessence™	3
V	SKINectura NOF	1
VI	Lys'lastine™ V	1
VII	Argireline peptide solution C	5
VIII	Demin water.	5
	Arbutin	2
IX	Conotoxin	5 ml/ 1 kg weight
X	Citric acid 20%	0,2

The composition shown in Table 2 was prepared using the method according to the invention comprising the following steps:

Add phase IA ingredients to the main mixer. Turn on heating to 75-80°C, stirring on - 25 rpm. Homogenise 1 min, 1800 rpm.

2. Prepare phase IB in an auxiliary vessel. Mix thoroughly. Add to main mixer. Mixing on - 25 rpm. Homogenise 1 min, 1800 rpm.

3. Weigh the phase II ingredients into the task mixer. Heat to 75-80°C. Stir until evenly dissolved.

4. once both phases have reached a temperature of 75-80°C, add phase II to the main mixer. Mixing on 35 rpm during blending. Homogenisation 2500 rpm for 6 min. 5. stirring on 20 rpm, for about 15-20 min.

6 Start cooling to 40°C (2°C/min) with continuous stirring (40 rpm), activate 0.5 bar vacuum. 7. prepare phases III and VIII in auxiliary vessels. If there are problems dissolving phase III F, heat to 40-45°C.

8. Switch on 35°C cooling.

9. at 35°C add phase III. Mixing 35 rpm, for about 5 min.

10. at 35°C add phase IV. Stir 35 rpm, for approximately 5 min.

11. at 35°C add phase V. Mixing 35 rpm, for about 5 min.

12. At 35°C add phase VI. Mixing 35 rpm, for about 5 min.

13. At 35°C add phase VII. Stir 35 rpm, for about 5 min.

14. at 35°C, add phase VIII. Stir 35 rpm, for about 5 min.

15. at 35°C add phase IX. Stir 35 rpm, for approximately 5 min.

Measure pH before adding phase X. Add phase X components gradually to adjust pH (4.3 - 5.7).

17. Homogenisation 2 min 2000 rpm, stirring 30 rpm, 10 min.

Example 6.

In vitro determination of recombinant μ -conotoxin activity on oocytes by the patch-clamp method

In this example implementation, the activity of μ -conotoxins according to the invention with amino acid sequence Sequence No. 2 and Sequence No. 4 was determined against the known μ -conotoxins SIHA and CnIIIC. In particular, it was investigated whether the new recombinant conotoxins TIIIA and TIIAlaMut have sodium current blocking properties similar to the commercially available conotoxin CnIIIC. A summary of the samples tested is shown in Table 3.

Table 3. Summary of samples subjected to the activity test with molecular masses and effective concentrations.

Name of sample to be measured	Sample content	Molecular weight (Da)	Sample mass [mg].	Effective concentration [μ M].
Toxin A	TIIIA	2433	0,15	0,7-1

Toxin B	SIHA	2232	0,5	no data available
Toxin C	THIAIaMut	2375	0,62	no data available
Toxin D	CnIIIC	2376	0,1	0,5-1
Toxin E	THIA conotoxin extract from cream with (12.5 g)	2433	equivalent to 0,07 mg conotoxin	0,7-1

The activity of recombinant conotoxins was determined by the patch-clamp method on Nav1.4 ion channels expressed on *Xenopus* oocytes. The ion channel expression system on *Xenopus* oocytes is ideal for the electrophysiological characterisation of voltage-dependent ion channels due to the relatively low background of endogenous channels and the large oocyte cell size. The patch-clamp method is the standard in electrophysiology. Its concept is to carry out a measurement on a very small patch of the cell membrane, the so-called patch. An electrode placed inside a thin glass pipette, the tip of which has a diameter of about 1 μm , is used for this purpose. The inside of the micropipette is filled with a solution with a composition similar to extracellular fluid, as the measurement takes place on the outside of the cell. During the experiment, the tip of the micropipette is in direct contact with the membrane surface, creating a stable contact, both mechanically and electrically. Under these conditions, the measuring electrode records the currents flowing through the section of membrane bounded by the pipette tip. The number of ion channels present on such a patch is small enough that it is possible to distinguish currents that flow through individual channels.

Methodology:

In the first step, mRNA encoding Nav1.4 ion channels was introduced into the oocytes by microinjection. Preparation of oocytes for microinjection: 5-15 ml of oocytes were collected from *Xenopus* ovaries, then divided into small clusters (about 10-20 oocytes). An enzymatic solution was then used to dissociate the oocytes (30-90 min at room temperature) until the vesicles of some oocytes were broken down. The cells were then washed with ND96 solution at least 5 times to remove residual enzyme. Under the microscope, healthy oocytes at stages V and VI (>0.8 mm in diameter) were selected. Microinjection was performed on the day of collection. Approximately 100 oocytes were used for testing each time. Selected oocytes were then transferred to ND96 solution and injected with approximately 50 μl portions of cRNA solution (automatic oocyte injector). The cultures were then incubated at 18°C for 2-7 days, depending on the desired level of ion channel expression. Oocytes prepared in this way with Nav1.4 ion channels expressed on them were used to measure ion channel conductance.

Responses were measured at room temperature 1-6 days after cRNA injection and recorded at -70 mV using a software amplifier. Recombinant conotoxins SIIIA, TIIIA, TIIIAAlaMut and synthetic standard CnIIIC at 1 μ M were used to activate the Nav1.4 ion channel, respectively. Rapid and reproducible solution exchange (<300 ms) was achieved with a 50 μ l funnel-shaped oocyte chamber combined with a rapid vertical flow of solution fed through a collector mounted directly above the oocyte. Agonist pulses were applied for 2 s at 4-min intervals. Voltage-current curves obtained on the same cells before and after administration of the selected compound to the extracellular environment were then compared.

Results:

The results of the analysis are shown in figs. 10-11 and in Table 4.

Table 4. Toxin concentration causing 50% inhibition (IC₅₀) and 99% inhibition (IC₉₉) of the sodium ion current generated by the human Nav1.4 channel.

	[IC] ₅₀	[IC] ₉₉
TIIIA	9.7 μ M	0.97 mM
TIIIAAlaMut	3.8 μ M	0.38 mM
CnIIIC pattern	33.6 nM	3.3 μ M

The studies performed indicate that the recombinant conotoxins TIIIA, SIIIA, TIIIAAlaMut at a concentration of 1 μ M reduce the amplitude of sodium currents from voltage-gated sodium channels in oocytes. The results confirm the activity of all recombinant conotoxins (figs. 10-11). The IC₅₀ value, i.e. the concentration required to block 50% of the Nav1.4 ion channel for the recombinant conotoxins TIIIA and TIIIAAlaMut are at 1 μ M. The level of ion channel blocking for the TIIIAAlaMut conotoxin is approximately three times higher than for the TIIIA conotoxin fig. 11.

The activity of TIIIA conotoxin after extraction from the final product was also confirmed. The results confirm the activity of TIIIA conotoxin extracted from the final product at a higher level than pure TIIIA conotoxin. This is probably related to the fact that the extraction process used the addition of TFA acid, residues of which were present in the extract. According to literature data, conotoxins show higher activity in an acidic environment. From the results obtained, it can be concluded that the final formulation of the cosmetic does not reduce the biological activity of the conotoxin TIIIA.

Example 7.

Confirmation of the absence of toxicity of conotoxin encapsulated by the MSM transition promoter.

Sequence list:

Sequence number (No. Sequ.): 1

Type of sequence: nucleotide sequence

Length: 423

Description: nucleotide sequence of the TRX+TIIIA construct according to the invention

Sequence:

ATGCATCACCATCATCACCATTCTGGTTCTTCTGACAAAATCATCCACCTGACCGACGACT
CTTTCGACACCGACGTTCTGAAAGCTGACGGTGCTATCCTGGTTGACTTCTGGGCTGAATG
GTGCGGTCCGTGCAAAAAGATCGCTCCGATCCTGGACGAAATCGCTGACGAATACCAGG
GTAAACTGACCGTTGCTAAACTGAACATCGACCAGAACCCGGGTACCGCTCCGAAATACG
GTATCCGTGGTATCCCGACCCTGCTGCTGTTCAAAAACGGTGAAGTTGCTGCAACCAAAG
TTGGTGACTGTCTAAAGGTCAGCTGAAAGAATTCCTGGACGCTAACCTGGCTATGCGTC
ATGGCTGCTGCAAAGGCCCGAAAGGCTGCAGCAGCCGTGAATGCCGTCCGCAGCATTGCT
GC

Sequence number (No. Sequ.): 2

Type of sequence: amino acid sequence

Length: 141

Description: amino acid sequence of the recombinant protein encoded by the TRX+TIIIA construct according to the invention

Sequence:

MHHHHHHS GSSDKIIHLTDDSFDTDLKADGAILVDFWAEWCGPCKKIAPILDEIADEYQGKL
TVAKLNIDQNP GTAPKYGIRGIPTLLL FKNGEVAATKVGALSKGQLKEFLDANLAMRHGCCK
GPKGCS SRECRPQHCC

Sequence No.: 3

Type of sequence: nucleotide sequence

Length: 423

Description: nucleotide sequence of the construct according to the invention in TRX+TIIIA_{AlaMut} variant

Sequence:

ATGCATCACCATCATCACCATTCTGGTTCTTCTGACAAAATCATCCACCTGACCGACGACT
CTTTCGACACCGACGTTCTGAAAGCTGACGGTGCTATCCTGGTTGACTTCTGGGCTGAATG
GTGCGGTCCGTGCAAAAAGATCGCTCCGATCCTGGACGAAATCGCTGACGAATACCAGG
GTAAACTGACCGTTGCTAAACTGAACATCGACCAGAACCCGGGTACCGCTCCGAAATACG
GTATCCGTGGTATCCCGACCCTGCTGCTGTTCAAAAACGGTGAAGTTGCTGCAACCAAAG
TTGGTGACTGTCTAAAGGTCAGCTGAAAGAATTCCTGGACGCTAACCTGGCTATGCGTC
ATGGCTGCTGCAAAGGCCCGAAAGGCTGCAGCAGCCGTGctTGCCGTCCGCAGCATTGCTG
C

Sequence No.: 4

Type of sequence: amino acid sequence

Length: 141

Description: amino acid sequence of the recombinant protein encoded by the construct according to the invention in TRX+TIIIAIaMut variant

Sequence:

MHHHHHHSGSSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKKIAPILDEIADEYQGKL
TVAKLNIDQNP GTAPKYGIRGIPTLLL FKNGEVAATKVGALSKGQLKEFLDANLAMRHGCCCK
GPKGCSSRACRPQHCC

Sequence no. (Sequ. no.):5

Type of sequence: amino acid sequence

Length: 22

Description: linear peptide identical to the conotoxin sequence of TIIIA

Sequence: RHGCCCKPKGCSRECRPQHCC

Patent claims

1. An active lipophilicity modulator in the form of dimethylsulfone (MSM) for use as a transition promoter for high-molecular-weight active substances with $M_w > 500$ Da.
2. The active lipophilicity modulator according to claim. 1, **characterised in that** the active substance is selected from the group comprising peptides.
3. The active modulator according to claim. 1 or 2, **characterised in that** the active substance is a conopeptide with the amino acid sequence of Sequence No. 2 or Sequence No. 4.
4. A cosmetic composition for transdermal administration comprising an active substance and a transition promoter, **characterised in that** the active substance is encapsulated by a transition promoter which is an active lipophilicity modulator as defined in claims. 1-3, while the active substance is a high molecular weight active substance with $M_w > 500$ Da.
5. The composition according to claim. 4, **characterised in that it** comprises at least one cosmetically acceptable excipient, advantageously selected from the group consisting of an emulsifier, a homogeniser, an emollient, a thickener, distilled water, a preservative or a combination thereof.
6. The composition according to claim. 4 or 5, **characterised in that** it is an oil-in-water (o/w) or water-in-oil (w/o) emulsion.
7. The composition according to any of the preceding claims. 4 to 6, **characterised in that** the ratio %wag. of the active substance to the transition promoter is 1:1 - 1:50.
8. The composition according to any of the preceding claims. 4 to 7, **characterised in that it** comprises at least one cosmetically acceptable excipient.
9. The composition according to any of the preceding claims. 4 to 8, **characterised in that** the active substance is a conotoxin, preferably μ -conotoxin.
10. The composition according to claim. 9, **characterised in that** the μ -conotoxin is a conopeptide with the amino acid sequence of Seq. no. 2.
11. The composition according to claim. 9, **characterised in that** the μ -conotoxin is a conopeptide with the amino acid sequence of Sequence No. 4. or a genetic modification thereof.
12. The composition according to any of the preceding claims. 4 to 11, **characterised in that** it contains at least one additional active ingredient.
13. A method of manufacturing a composition as defined in any of the preceding claims. 4 to 12, **characterised in that** it is carried out by either hot emulsification or cold emulsification, the hot emulsification comprising the following steps:

- a) mixing of dimethylsulfone and diluent in a reaction vessel;
- b) bringing the mixture to 70-85 °C;
- c) stirring the mixture at 70-85 °C; the onset of homogenisation depends on the stages of phase combination;
- d) cooling of the mixture to 20-40 °C, preferably 30 °C;
- e) addition of a high-molecular-weight active substance with $M_w > 500$ Da and homogenisation of the mixture, preferably for 1-3 min;

whereas cold emulsification involves the following steps:

- a) mixing of the dimethylsulfone with the diluent in a reaction vessel and dissolving at 20-25 °C
- b) bringing the mixture to a temperature of 15 - 35 °C, preferably to ambient temperature or 25 °C;
- c) mixing of the mixture at a temperature of 15 - 80 °C, preferably 25 °C; the onset of homogenisation depends on the stages of phase combination.
- d) addition of a high-molecular-weight active substance with $M_w > 500$ Da and homogenisation of the mixture, preferably for 1-3 min.

14. The method according to claim. 13, **characterised in that** in at least one of the steps a-e) of hot emulsification at least one additional active ingredient and/or at least one cosmetically acceptable excipient is added to the reaction vessel, advantageously selected from the group consisting of an emulsifier, a homogeniser, an emollient, a thickener, distilled water, a preservative or a combination thereof.
15. The method according to claim. 13, **characterised in that** in at least one of the steps a-d) of cold emulsification at least one additional active ingredient and/or at least one cosmetically acceptable excipient is added to the reaction vessel, advantageously selected from the group consisting of an emulsifier, a homogeniser, an emollient, a thickener, distilled water, a preservative or a combination thereof.