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(54) **SYNTHETIC PULMONARY SURFACTANT PEPTIDES**

SYNTHETISCHE LUNGENSURFACTANT PEPTIDE

PEPTIDES TENSIOACTIFS PULMONAIRES SYNTHÉTIQUES

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Description**TECHNICAL FIELD**

5 [0001] The present invention is directed to peptides analogues of the native surfactant protein SP-B and their use in the preparation of formulations for the prophylaxis and/or treatment of respiratory distress syndrome (RDS) and other respiratory disorders.

BACKGROUND OF THE INVENTION

10 [0002] The human lung is composed of a large number of small air sacs, called alveoli, in which gases are exchanged between the blood and the air spaces of the lungs. In healthy individuals, this exchange is mediated by the presence of a protein-containing surfactant complex that prevents the lungs from collapsing at the end of expiration.

15 [0003] Lung surfactant complex is composed primarily of lipid and contains minor amounts of various proteins. An absence of adequate levels of this complex results in malfunction of the lung. This syndrome is called Respiratory Distress Syndrome (RDS) and it commonly affects preterm infants.

[0004] Said syndrome is effectively treated with modified natural surfactant preparations extracted from animal lungs.

20 [0005] The main constituents of these surfactant preparations are phospholipids, such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine commonly known as dipalmitoyl-phosphatidylcholine (DPPC), phosphatidylglycerol (PG) and surfactant hydrophobic proteins B and C (SP-B and SP-C) that are known to have the capacity to effect the surfactant-like activity of said preparations.

[0006] Due to the drawbacks of the surfactant preparations from animal tissues, such as the complication of the production and sterilization processes and possible induction of immune reactions, synthetic surfactants mimicking the composition of the modified natural surfactants have been developed.

25 [0007] Said synthetic surfactants are known as reconstituted surfactants.

[0008] However the development of clinically active reconstituted surfactants turned out to be complicated as the isolation of significant amounts of hydrophobic SP-B and SP-C proteins from natural sources is both expensive and labor intensive.

30 [0009] Likewise, production of these proteins by recombinant DNA techniques requires substantial effort in terms of design and achieving optimal host/vector expression systems. In addition, considerable effort is required to develop effective isolation strategies to separate and purify the expressed protein of interest from the unwanted material.

[0010] In particular the SP-B protein is characterised by high molecular weight, extreme hydrophobicity and a large number of cysteine residues which markedly complicates its commercial production via isolation from natural materials or its expression via recombinant DNA strategies.

35 [0011] Therefore the medical community has a need for simple, easy-to-prepare, synthetic analogues of the protein SP-B able of mimicking all the properties of the native protein.

[0012] More particularly there is a need of synthetic analogues of the protein SP-B that, when admixed with synthetic analogues of the native protein SP-C and with a lipid carrier, give rise to reconstituted surfactant preparations able of efficaciously maintaining alveolar patency at the end of expiration.

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SUMMARY OF THE INVENTION

[0013] The present invention is directed to a polypeptide of sequence represented by the general formula (I).

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$$X\Delta LCRALIKR-SEQ-PQLVCRLVLR\Phi\Sigma_n \quad (I)$$

wherein

50 \times is an amino acid residue independently selected from the group consisting of C, A, G, K, R, D and E;

Δ is an amino acid residue selected from the group consisting of W, L, nL and I;

SEQ is an amino acid sequence selected from the group consisting of FNRYLT and YNGK;

Φ is an amino acid residue independently selected from the group consisting of C, A, G, K, R, D and E;

Σ is an amino acid residue selected from the group consisting of S, G and A

n is 0 or 1.

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[0014] The invention also includes the pharmaceutically acceptable salts of said polypeptides and their blocked N- and/or C-terminus derivatives, e.g *via* acetylation and amidation.

[0015] In another embodiment, the present invention provides a reconstituted pulmonary surfactant comprising a lipid

carrier admixed with a polypeptide of general formula (I) and pharmaceutical formulations thereof.

[0016] Finally the invention is directed to the use of the polypeptides of general formula (I) for the preparation of a reconstituted surfactant for the prophylaxis and/or treatment of respiratory distress syndrome and other respiratory disorders.

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FIGURE

[0017] Figure shows the amino acid sequence of human protein SP-B.

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DEFINITIONS

[0018] The respiratory function after *in vivo* treatment with the exogenous surfactant preparations is determined by measuring two parameter: i) the tidal volume which is an index of the lung compliance and ii) the lung gas volume which is an index of the alveolar air expansion or patency at the end of expiration, and hence of the capability of forming a stable phospholipidic film in the alveoli at the end of expiration.

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[0019] The protein SP-B, which has also been defined as "SP18", is a 17k Da dimeric protein wherein the monomeric chain is a 79 residue polypeptide and has three intrachain disulfide linkages, linking Cys8 to Cys77, Cys11 to Cys71, and Cys35 to Cys46. In its native form, the human SP-B subunit exists as a disulfide-linked homodimer having an interchain disulfide linkage at Cys 48. The sequence of the monomeric chain of human SP-B is reported in Figure.

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[0020] The term "reconstituted surfactant", as used herein, means a lipid carrier to which polypeptide analogues of the surfactant proteins, made through recombinant technology or synthetic methods have been added.

[0021] The term "lipid carrier" means a mixture of phospholipids and optionally further lipid components, for example neutral lipids such as triacylglycerols, free fatty acids and/or cholesterol.

25

[0022] The terms "polypeptide" and "peptide" are used interchangeably herein to designate a linear series of no more than about 60 amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues.

[0023] The term "polypeptide analogues of the native surfactant protein SP-B", includes peptides having an amino acid sequence in which, compared to the native proteins, one or more amino acids are missing or have been replaced by other amino acids so long as the polypeptides, in a mixture with a lipid carrier, show pulmonary surfactant activity.

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[0024] The term "polypeptides analogues of the native surfactant protein SP-C", includes polypeptides having an amino acid sequence in which, compared to the native proteins, one or more amino acids are missing or have been replaced by other amino acids, so long as the polypeptides, in a mixture with a lipid carrier, show pulmonary surfactant activity.

[0025] Polypeptides displaying conservative substitutions are those where one amino acid residue is replaced by another, biologically similar residue. Examples of conservative substitutions include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another such as between arginine and lysine, or between glutamic and aspartic acids.

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[0026] The term "conservative substitution" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that the resulting polypeptide also displays the required surfactant activity.

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[0027] Moreover, the term "conservative substitution" includes substitutions different from the previous ones, provided that the resulting polypeptide maintains the secondary structure as well as the required surfactant activity of the parent polypeptide.

[0028] The amino acid sequences are shown according to the three-letter code with the amino acid which carries the free amino group at the left end (amino terminus) and the amino acid which carries the free carboxyl group at the right end (carboxy terminus).

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[0029] All the amino acid residues identified herein are in the natural L-configuration and the sequences identified herein are reported according to standard abbreviations for amino acid residues as shown in the following Table of Correspondence.

50

TABLE OF CORRESPONDENCE

AMINO ACID	SYMBOL	
	One-letter	Three-letter
Glycine	G	Gly
L-proline	P	Pro
L-isoleucine	I	Ile
L-leucine	L	Leu

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(continued)

	AMINO ACID	SYMBOL	
		One-letter	Three-letter
5	L-tyrosine	. Y	Tyr
	L-cysteine	C	Cys
	L-tryptophane	W	Trp
	L-alanine	A	Ala
10	L-lysine	K	Lys
	L-arginine	R	Arg
	L-glutamine	Q	Glu
	L-methionine	M	Met
	L-serine	S	Ser
15	L-valine	V	Val
	L-asparagine	N	Asn
	L-aspartic acid	D	Asp
	L-glutamic acid	E	Gln
	L-histidine	H	His
20	L-threonine	T	Thr
	L-phenylalanine	F	Phe
	L-nor-leucine	-	nLeu
	L-ornithine	-	Orn

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DETAILED DESCRIPTION OF THE INVENTION

[0030] The present invention is directed to a polypeptide having the sequence represented by the general formula (I).

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wherein

× is an amino acid residue independently selected from the group consisting of C, A, G, K, R, D and E;

Δ is an amino acid residue selected from the group consisting of W, L, nL and I;

35

SEQ is an amino acid sequence selected from the group consisting of FNRYLT and YNGK;

Φ is an amino acid residue independently selected from the group consisting of C, A, G, K, R, D and E;

Σ is an amino acid residue selected from the group consisting of S, G and A;

n is 0 or 1.

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[0031] The polypeptides of the invention include the N-terminal sequence 8-17 and the C-terminal sequence 67-78 of SP-B protein linked through an amino acid sequence able of forming a turn motif selected from the group consisting of FNRYLT and YNGK.

[0032] Said polypeptides also include modifications such as those encompassed by the general formula (I) wherein some of the amino acid residues of the sequences of native SP-B protein have been replaced and/or are missing.

45

[0033] Otherwise they may further include additional amino acid residues at the amino- or carboxy-terminal end. Said modifications may serve to enhance expression of the polypeptide or may serve as a "linker" sequence, but preferably do not decrease or otherwise interfere with the biological activity of a polypeptide of the present invention.

[0034] The invention also includes the pharmaceutically acceptable salts of said polypeptides and their blocked N- and/or C-terminus derivatives, e.g. *via* acetylation and amidation.

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[0035] Pharmaceutically acceptable salts include for example, salts of hydrochloric acid, acetic acid, and trifluoroacetic acid.

[0036] In one embodiment, the polypeptides of general formula (I) may be in the form of disulfide linked molecule wherein the intramolecular disulfide linkage is between the Cys residue at position 4 and the one in the C-terminal part.

[0037] A first group of preferred polypeptides of general formula (I) is that in which

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X and Φ are both C;

Δ is as defined above;

SEQ is FNRYLT;

n is 0.

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[0038] Advantageously said polypeptides may be in the form of disulfide linked molecule wherein the intramolecular disulfide linkage is between the two Cys residues at position 1 and 27 and/or between the two Cys residues at position 4 and 21.

[0039] A second group of preferred polypeptides of general formula (I) is that in which

5
X and Φ are both C;
 Δ is as defined above;
SEQ is YNGK;
n is 0 or 1.

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[0040] Advantageously, said polypeptides may be in the form of disulfide linked molecule wherein the intramolecular disulfide linkage is between the two Cys residues at position 1 and 25 and/or between the two Cys residues at position 4 and 19.

[0041] A third group of preferred polypeptides of general formula (I) is that in which

15
X is A or G;
 Δ is as defined above;
SEQ is FNRYLT;
n is 0 or 1.
20 Φ is A or G

[0042] Advantageously, said polypeptides may be in the form of cyclic molecule wherein the intramolecular linkage is between the Ala or the Gly residues at positions 1 and 27. More advantageously a disulfide linkage is also present between the two Cys residues at positions 4 and 21.

25 **[0043]** A fourth group of preferred polypeptides compounds of general formula (I) is that in which

30
X is A or G;
 Δ is as defined above;
SEQ is YNGK;
n is 0 or 1.
 Φ is A or G.

[0044] Advantageously, said polypeptides may be in the form of cyclic molecule wherein the intramolecular linkage is between the Ala or the Gly residues at positions 1 and 25. More advantageously, a disulfide linkage is also present between the two Cys residues at positions 4 and 19.

35 **[0045]** A fifth group of preferred polypeptides of general formula (I) is that in which

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X is K or R;
 Δ is as defined above;
SEQ is FNRYLT;
n is 0 or 1;
 Φ is D or E.

45 **[0046]** Advantageously, said polypeptides may be in the form of cyclic molecule wherein the linkage is between the K or R residue at position 1 and the D or E residue at position 27. The linkage may be in form of amide covalent bond between the free amino group of the K or R residue and the free carboxylic group of the D or E residue, otherwise the linkage may be under the form of salt bridge.

[0047] More advantageously, a disulfide linkage is also present between the two Cys residues at positions 4 and 21.

[0048] A sixth group of preferred polypeptides compounds of general formula (I) is that in which

50
X is K or R;
 Δ is as defined above;
SEQ is YNGK;
n is 0 or 1;
55 Φ is D or E.

[0049] Advantageously, said polypeptides may be in the form of cyclic molecule wherein the linkage is between the K or R residue at position 1 and the D or E residue at position 25. The linkage may be in form of amide covalent bond

between the free amino group of the K or R residue and the free carboxylic group of the D or E residue, otherwise the linkage may be under the form of salt bridge.

[0050] More advantageously, a disulfide linkage is also present between the two Cys residues at positions 4 and 19.

[0051] A seventh group of preferred polypeptides of general formula (I) is that in which

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X is D or E;
 Δ is as defined above;
 SEQ is FNRYLT;
 n is 0 or 1;
 Φ is K or R.

[0052] Advantageously, said polypeptides may be in the form of cyclic molecule wherein the linkage is between the D or E residue at position 1 and the K or R residue at position 27. The linkage may be in form of amide covalent bond between the free amino group of the K or R residue and the free carboxylic group of the D or E residue, otherwise the linkage may be under the form of salt bridge.

15

[0053] More advantageously, a disulfide linkage is also present between the two Cys residues at positions 4 and 21.

[0054] A eighth group of preferred polypeptides compounds of general formula (I) is that in which

20

X is D or E;
 Δ is as defined above;
 SEQ is YNGK;
 n is 0 or 1;
 Φ is K or R.

[0055] Advantageously, said polypeptides may be in the form of cyclic molecule wherein the linkage is between the D or E residue at position 1 and the K or R residue at position 25. The linkage may be in form of amide covalent bond between the free amino group of the K or R residue and the free carboxylic group of the D or E residue, otherwise the linkage may be under the form of salt bridge.

25

[0056] More advantageously a disulfide linkage is also present between the two Cys residues in positions 4 and 19.

[0057] Preferred polypeptides encompassed by general formula (I) are reported below

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CLLCRALIKRFNRYLTPQLVCRLVLRC (Ia)

CLLCRALIKRYNGKPQLVCRLVLRC (Ib)

35

CWLCRALIKRFNRYLTPQLVCRLVLRC (Ic)

CWLCRALIKRYNGKPQLVCRLVLRC (Id)

40

ALLCRALIKRFNRYLTPQLVCRLVLRRAA (Ie)

ALLCRALIKRYNGKPQLVCRLVLRRAA (If)

45

GLLCRALIKRFNRYLTPQLVCRLVLRGG (Ig)

GLLCRALIKRYNGKPQLVCRLVLRGG (Ih)

[0058] The polypeptides (Ic) and (Id) in the form of disulfide linked molecules wherein the intramolecular disulfide linkages are between the Cys residues are hereinafter indicated as ox-(Ic) and ox-(Id) polypeptides.

50

[0059] The polypeptides of the invention, when admixed with a lipid carrier comprising phospholipids, form a reconstituted surfactant able of reducing the surface tension to values near zero.

[0060] We have also found that, in a model of RDS wherein the immature newborn were treated without applying a positive end expiratory pressure (PEEP), the polypeptides of general formula (I), when admixed with particular synthetic analogues of the protein SP-C and with a suitable lipid carrier, improve the respiratory function as expressed by the tidal volumes to an extent comparable with that achieved after administration of a modified natural surfactant.

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[0061] Moreover, said reconstituted surfactant preparation turned out to improve the lung gas volume which is an index of the alveolar patency at the end of expiration.

[0062] The polypeptides of general formula (I) may be synthesized by any techniques that are known to those skilled

in the polypeptide art.

[0063] Many of the techniques available may be found in J.M. Steward and J.D. Young, "Solid Phase Peptide Synthesis", W.H. Freeman Co., San Francisco, 1969, and J. Meienhofer, Hormonal Proteins and Peptides", Vol.2, p. 46, Academic Press (New York), 1983 for solid phase peptide synthesis, and E. Schroder and K. Kubke, "The Peptides", Vol. 1, Academic Press (New York), 1965 for classical solution synthesis. The polypeptides of the invention may also be prepared using the solid-phase synthetic technique initially described by Merrifield, in J. Am. Chem. Soc. 85: 2149-2154 (1963). Other polypeptide synthesis techniques may be found, for example, in M. Bodanszky et al., Peptide Synthesis, John Wiley & Sons, 2d Ed., (1976) as well as in other reference works known to those skilled in the art.

[0064] Appropriate protective groups for use in such syntheses will be found in the above texts as well as in J.F.W. McOmie, Protective Groups in Organic Chemistry, Plenum Press, New York, NY (1973).

[0065] In general, these methods comprise the sequential addition of one or more amino acid residues or suitably protected amino acid residues to a growing peptide chain.

[0066] Using a solid phase synthesis as exemplary, the protected or derivatized amino acid is attached to an inert solid support through its unprotected carboxyl or amino group. The protecting group of the amino or carboxyl group is then selectively removed and the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected is admixed and reacted under conditions suitable for forming the amide linkage with the residue already attached to the solid support. The protecting group of the amino or carboxyl group is then removed from this newly added amino acid residue, and the next amino acid (suitably protected) is then added, and so forth.

[0067] After all the desired amino acids have been linked in the proper sequence, any remaining terminal and side group protecting groups (and solid support) are removed sequentially or concurrently, to give rise to the final polypeptide.

[0068] The raw polypeptide is generally purified by HPLC and isolated by lyophilisation.

[0069] The polypeptide may be obtained in the form of pharmaceutically acceptable salt. Optionally the obtained salt may be converted in another type of salt using a column packed with a suitable ion exchange resin according to a procedure well-known to a skilled person.

[0070] The polypeptides of the invention may also be prepared using recombinant nucleic acid methodologies well known in the art.

[0071] Therefore the DNA sequences coding the polypeptides of the invention, the recombinant expression vectors capable of expressing them and methods thereof are included in the present invention.

[0072] A DNA sequence coding for a polypeptide of this invention may be synthesized by chemical techniques well known to the skilled person. The DNA segment may then be ligated into an expression vector, and a host transformed therewith may be used to produce the polypeptide.

[0073] By chemically synthesizing the coding sequence, any desired modifications may be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence.

[0074] The recombinant expression vectors capable of expressing a subject polypeptide and methods of their use for producing the polypeptides of general formula (I) are included in the present invention.

[0075] Also included in the present invention are ribonucleic acid (RNA) equivalents of the above described DNA segments.

[0076] An important and well known feature of the genetic code is its redundancy. That is, for most of the amino acids used to make proteins, more than one coding nucleotide triplet (codon) can code for or designate a particular amino acid residue. Therefore, a number of different nucleotide sequences may code for a particular amino acid residue sequence. Such nucleotide sequences are considered functionally equivalent since they may result in the production of the same amino acid residue sequence in all organisms.

[0077] The polypeptides of general formula (I) may be admixed with a pharmaceutically acceptable lipid carrier to form a reconstituted surfactant.

[0078] The weight ratios between the polypeptide and the lipid carrier is advantageously in the range of about 1:5 to about 1:5000, preferably about 1:10 to about 1:2000, and more preferably about 1:50 to about 1:1000. In a more preferred embodiment, the polypeptide:lipid carrier weight ratio is in the range of about 1:5 to about 1:1000, preferably about 1:7 to about 1:500, and more preferably about 1:10 to about 100.

[0079] Advantageously, the lipid carrier comprises the phospholipids that are contained in natural pulmonary surfactant preparations, for example phosphatidylcholines (PC) such as dipalmitoylphosphatidylcholine (DPPC) and palmitoyl-oleoylphosphatidylcholine (POPC), and phosphatidylglycerols (PG), such as palmitoyl-oleoylphosphatidylglycerol (POPG) and dipalmitoylphosphatidylglycerol (DPPG).

[0080] Other phospholipids which can be advantageously used are phosphatidylinositols (PI), phosphatidylethanolamines (PE), phosphatidylserines and sphingomyelins (SM).

[0081] In a particular embodiment, the lipid carrier may comprise further components, for example neutral lipids such as triacylglycerols, free fatty acids and/or cholesterol.

[0082] The reconstituted surfactant may comprise one or more polypeptides of general formula (I) or may further comprise a synthetic peptide analogue of the native surfactant protein SP-C such as those disclosed in WO 95/32992,

WO 00/47623, and WO 03/097695.

[0083] Reconstituted surfactants comprising a polypeptide of the invention may be prepared by mixing a solution or a suspension of said polypeptide, optionally a solution or a suspension of another peptide and the lipid carrier, then by subsequently drying the mixture.

5 **[0084]** Otherwise they may be prepared by lyophilisation or spray-drying according to methods known in the art.

[0085] The administration of said reconstituted surfactant preparation may be carried out in a manner known to the person skilled in the art, preferably by intratracheal installation (infusion or bolus). Otherwise the administration may be carried out by aerosolization or by nebulisation.

10 **[0086]** The present invention also concerns pharmaceutical compositions including the reconstituted surfactant comprising the polypeptides of the invention.

[0087] Said compositions are advantageously administered in the form of a solution, dispersion, suspension or dry powder. Preferably said compositions comprise the reconstituted surfactant dissolved or suspended in a suitable solvent or resuspension medium.

15 **[0088]** Preferably said pharmaceutical compositions are supplied as suspension in a buffered physiological saline aqueous solution in single-use glass vials. Advantageously the reconstituted surfactant concentration (expressed as phospholipid content) is in the range of from about 2 to about 160 mg of surfactant per ml, preferably between 10 and 100 mg/ml, more preferably between 20 and 80 mg/ml.

[0089] Said compositions may further comprise electrolytes, such as calcium, magnesium and/or sodium salts (for example calcium chloride or sodium chloride).

20 **[0090]** The compositions in the form of aqueous suspension may also be administered by nebulisation.

[0091] If it is to be used in aerosol administration, the reconstituted surfactant is supplied in finely divided form along with a propellant. Useful propellants are typically gases at ambient conditions that are condensed under pressure such as hydrofluoroalkanes.

25 **[0092]** The aerosol is packaged in a container equipped with a suitable valve so that the ingredients may be maintained under pressure until released.

[0093] The pharmaceutical compositions obtained with the polypeptides of the invention are suitable for the treatment or prophylaxis of respiratory distress syndrome (RDS) in prematurely born babies or in the treatment or prophylaxis of other diseases related to a surfactant-deficiency or dysfunction including RDS in adults (ARDS), meconium aspiration syndrome (MAS), and bronchopulmonary dysplasia (BPD).

30 **[0094]** They may also be useful for the treatment or prophylaxis of other respiratory disorders such as pneumonia, bronchitis, COPD (chronic obstructive pulmonary disease), asthma, and cystic fibrosis as well as for the treatment of serous otitis media (glue ear).

[0095] The following example illustrates the invention in more details.

35 Example 1 - Synthesis and purification of the polypeptide ox-(Ic)

[0096] The polypeptide ox-(Ic) was prepared by standard SPPS (Solid Phase Peptide Synthesis) methods based on Fmoc chemistry, according to the Scheme 1 reported below.

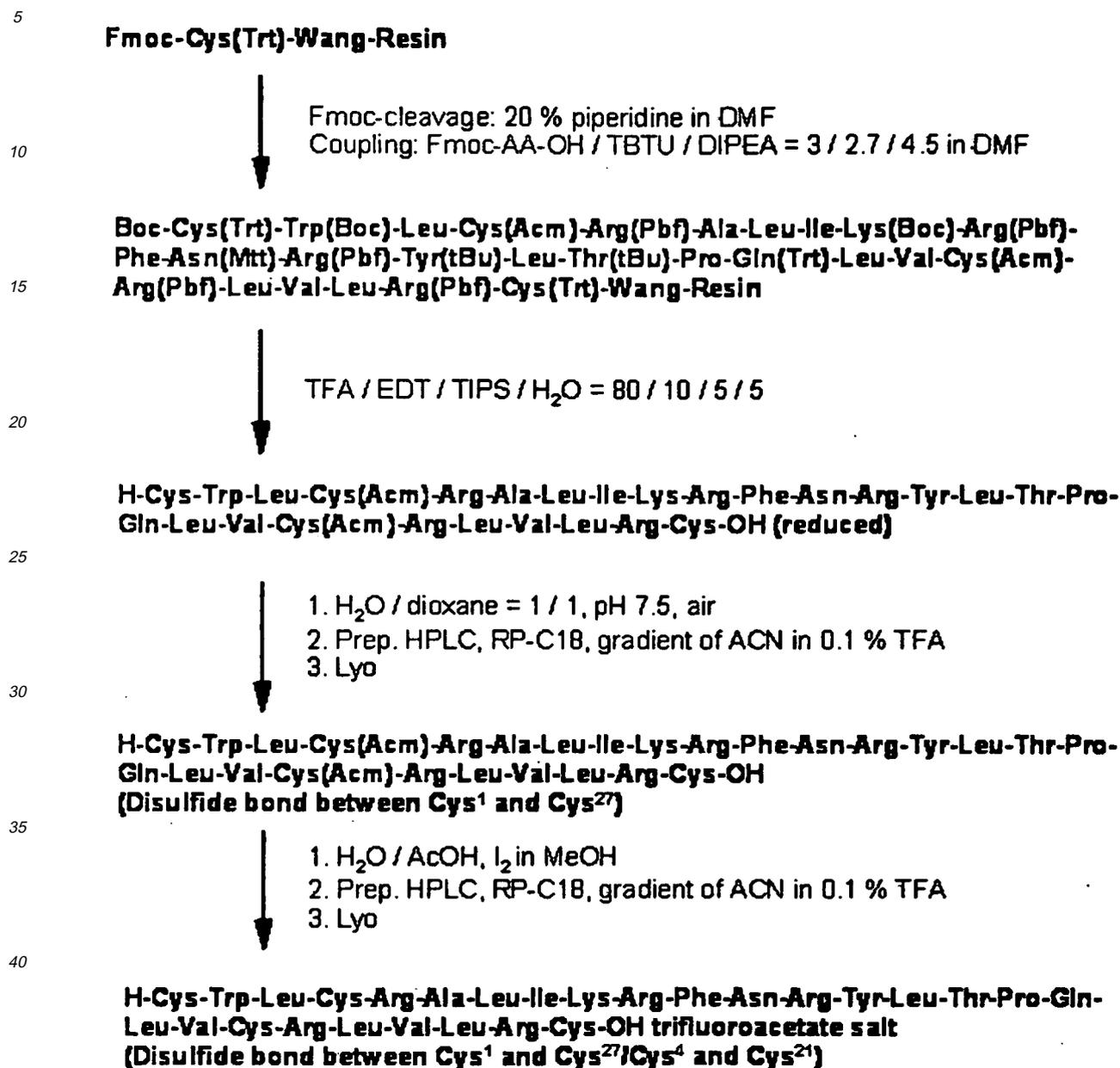
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Scheme 1



[0097] After the solid phase synthesis, the crude peptide without disulfide bridges was cleaved from the resin by standard methods, then the disulfide bridges were formed selectively by two different oxidation steps.

[0098] To selectively form the disulfide bridges, a defined protection pattern for the lateral groups of the four cysteines was chosen during the synthesis. In the process showed in the Scheme 1, Cys¹ and Cys²⁷ were introduced as Fmoc-Cys(Trt)-OH during SPPS, whereas Cys⁴ and Cys²¹ were coupled as the corresponding Acm-derivatives.

[0099] The first disulfide bridge was formed by air oxidation and the peptide having one bridge was purified by HPLC systems and isolated by lyophilisation. The second disulfide bridge was formed by iodine oxidation, and the final peptide was purified by HPLC system and isolated by lyophilisation.

[0100] The purity of the final polypeptide was checked by reversed phase high performance liquid chromatography (HPLC), using a C₁₈ column and turned out to be 92%.

Its molecular mass was determined by ESI-MS.

Yield: 1.73 g (2.4%).

Example 2 - Synthesis and purification of the polypeptide ox-(Id)

[0101] The polypeptide ox-(Id) was prepared in a similar way according to the Scheme 2 reported below.

Scheme 2

Fmoc-Cys(Trt)-Wang-Resin

↓ Fmoc-cleavage: 20 % piperidine in DMF
Coupling: Fmoc-AA-OH / TBTU / DIPEA = 3 / 2.7 / 4.5 in DMF

Boc-Cys(Trt)-Trp(Boc)-Leu-Cys(Acm)-Arg(Pbf)-Ala-Leu-Ile-Lys(Boc)-Arg(Pbf)-Tyr(tBu)-Asn(Trt)-Gly-Lys(Boc)-Pro-Gln(Trt)-Leu-Val-Cys(Acm)-Arg(Pbf)-Leu-Val-Leu-Arg(Pbf)-Cys(Trt)-Wang-Resin

↓ TFA / EDT / TIPS / H₂O = 80 / 10 / 5 / 5

H-Cys-Trp-Leu-Cys(Acm)-Arg-Ala-Leu-Ile-Lys-Arg-Tyr-Asn-Gly-Lys-Pro-Gln-Leu-Val-Cys(Acm)-Arg-Leu-Val-Leu-Arg-Cys-OH (reduced)

↓ 1. H₂O / dioxane = 1 / 1, pH 7.5, air
2. Prep. HPLC, RP-C18, gradient of ACN in 0.1 % TFA
3. Lyo

H-Cys-Trp-Leu-Cys(Acm)-Arg-Ala-Leu-Ile-Lys-Arg-Tyr-Asn-Gly-Lys-Pro-Gln-Leu-Val-Cys(Acm)-Arg-Leu-Val-Leu-Arg-Cys-OH (Disulfide bond between Cys¹ and Cys²⁵)

↓ 1. H₂O / AcOH, I₂ in MeOH
2. Prep. HPLC, RP-C18, gradient of ACN in H₂O containing NaClO₄
3. Prep. HPLC, RP-C18, gradient of ACN in 0.1 % TFA
4. Lyo

H-Cys-Trp-Leu-Cys-Arg-Ala-Leu-Ile-Lys-Arg-Tyr-Asn-Gly-Lys-Pro-Gln-Leu-Val-Cys-Arg-Leu-Val-Leu-Arg-Cys-OH trifluoroacetate salt (Disulfide bond between Cys¹ and Cys²⁵/Cys⁴ and Cys¹⁹)

[0102] The introduction the disulfide bridges between Cys¹ and Cys²⁷ and between Cys⁴ and Cys²¹, the purification and the isolation were carried out as reported in the Example 1.

[0103] The purity of the final polypeptide was checked by reversed phase high performance liquid chromatography (HPLC), using a C₁₈ column and turned out to be 86%.

Its molecular mass was determined by ESI-MS.

Yield: 0.81 g (2.4%).

Legend**[0104]**

5	AA-OH	Amino acid
	ACN	Acetonitrile
	AcOEt	Ethyl acetate
	AcOH	Acetic acid
	Acm	Acetamidomethyl
10	Boc	t-Butyloxycarbonyl
	n-BuOH	n-Butanol
	tBu	t-Butyl
	DIPEA	N,N-Diisopropylethylamine
	DMF	N,N-Dimethylformamide
15	EDT	1,2-Ethanedithiol
	ESI-MS	Electrospray ionization-mass spectrometry
	Fmoc	9-Fluorenylmethyloxycarbonyl
	HPLC	High-performance liquid chromatography
	Lyo	lyophilisation
20	Mtt	Methyltrityl
	PBF	2,2,4,6,7-Pentamethyl-2,3-dihydro-benzofuran-5-sulfonyl
	SPPS	Solid phase peptide synthesis
	TBTU	2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
	TFA	Trifluoroacetic acid
25	TIPS	Triisopropylsilyl
	Trt	Trityl

SEQUENCE LISTING

30 **[0105]**

<110> Chiesi Farmaceutici Spa

<120> SYNTHETIC PULMONARY SURFACTANT PEPTIDES

35

<130> 1849eur

<160> 8

40

<170> PatentIn version 3.3

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<211> 27

<212> PRT

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<213> artificial sequence

<220>

<223> synthetic peptide

50

<400> 1

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55

Pro Gln Leu Val Cys Arg Leu Val Leu Arg Cys
20 25

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Leu Val Cys Arg Leu Val Leu Arg Cys
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Pro Gln Leu Val Cys Arg Leu Val Leu Arg Cys
20 25

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<213> artificial sequence

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<220>
<223> synthetic peptide

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Cys Trp Leu Cys Arg Ala Leu Ile Lys Arg Tyr Asn Gly Lys Pro Gln
1 5 10 15

55

Leu Val Cys Arg Leu Val Leu Arg Cys
20 25

<210> 5
<211> 28

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CWLCRALIKRFNRYLTPQLVCRLVLRRC (lc)

CWLCRALIKRYNGKPQLVCRLVLRRC (ld)

5 ALLCRALIKRFNRYLTPQLVCRLVLRRAA (le)

ALLCRALIKRYNGKPQLVCRLVLRRAA (lf)

10 GLLCRALIKRFNRYLTPQLVCRLVLRGG (lg)

GLLCRALIKRYNGKPQLVCRLVLRGG (lh)

13. The polypeptide of claim 12 which is the polypeptide having the sequence (lc)

15 CWLCRALIKRFNRYLTPQLVCRLVLRRC

14. The polypeptide of claim 13 in the form of disulfide linked molecule wherein the intramolecular disulfide linkage is between the two Cys residues at positions 1 and 27 and/or between the two Cys residues at positions 4 and 21.

20 15. The polypeptide of claim 12 which is the polypeptide having the sequence (ld)

CWLCRALIKRYNGKPQLVCRLVLRRC

25 16. The polypeptide of claim 13 in the form of disulfide linked molecule wherein the intramolecular disulfide linkage is between the two Cys residues at positions 1 and 25 and/or between the two Cys residues at positions 4 and 19.

17. A reconstituted surfactant which comprises a polypeptide according to any one of claims 1 to 16 in admixture with a lipid carrier.

30 18. A reconstituted surfactant according to claim 17 further comprising a synthetic peptide analogue of the native surfactant protein SP-C.

19. A reconstituted surfactant according to claim 17 or 18 wherein the lipid carried comprises a mixture of phospholipids.

35 20. Use of a polypeptide of claims 1 to 16 for the manufacture of a medicament for the prophylaxis and/or treatment of respiratory distress syndrome (RDS) in prematurely born babies.

40 21. Use of a polypeptide of claims 1 to 16 for the manufacture of a medicament for the prophylaxis and/or treatment of a disease related to a surfactant-deficiency or dysfunction selected from the group consisting of RDS in adults (ARDS) meconium aspiration syndrome (MAS), and bronchopulmonary dysplasia (BPD).

Patentansprüche

45 1. Polypeptid mit der durch die allgemeine Formel (I) dargestellten Sequenz

$X\Delta LCRALIKR-SEQ-PQLVCRLVLR\Phi\Sigma_n$ (I)

worin

50 X ein Aminosäurerest ist, der unabhängig ausgewählt ist aus der Gruppe bestehend aus C, A, G, K, R, D und E;

Δ ein Aminosäurerest ist, der ausgewählt ist aus der Gruppe bestehend aus W, L, nL und I;

SEQ eine Aminosäuresequenz ist, die ausgewählt ist aus der Gruppe bestehend aus FLTRYLT und YNGK;

Φ ein Aminosäurerest ist, der unabhängig ausgewählt ist aus der Gruppe bestehend aus C, A, G, K, R, D und E;

55 Σ ein Aminosäurerest ist, der ausgewählt ist aus der Gruppe bestehend aus S, G und A;

n eine ganze Zahl mit einem Wert von 0 oder 1 ist.

2. Polypeptid nach Anspruch 1, worin X und Φ beide C sind, SEQ FNRYLT ist und n 0 ist.

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18. Rekonstituiertes Tensid gemäß Anspruch 17, des Weiteren umfassend ein synthetisches Peptidanalogue des nativen Tensidproteins SP-C.
19. Rekonstituiertes Tensid gemäß Anspruch 17 oder 18, wobei der Lipidträger ein Phospholipidgemisch umfasst.
20. Verwendung eines Polypeptids nach Anspruch 1 bis 16 zur Herstellung eines Medikaments zur Prophylaxe und/oder Behandlung des Atemnotsyndroms (Respiratory Distress Syndrom, RDS) bei früh geborenen Babys.
21. Verwendung eines Polypeptids nach Anspruch 1 bis 16 zur Herstellung eines Medikaments zur Prophylaxe und/oder Behandlung einer Erkrankung, die bedingt ist durch eine Tensiddefizienz oder Dysfunktion, ausgewählt aus der Gruppe, bestehend aus Atemnotsyndrom (RDS) bei Erwachsenen (ARDS), Mekoniumaspirationssyndrom (MAS) und bronchopulmonärer Dysplasie (BPD).

Revendications

1. Polypeptide ayant la séquence représentée par la formule générale (I)



où

X est un résidu d'acide aminé choisi indépendamment parmi le groupe consistant en C, A, G, K, R, D et E;

Δ est un résidu d'acide aminé choisi parmi le groupe consistant en W, L, nL et I;

SEQ est une séquence d'acides aminés choisis parmi le groupe consistant en FNRYLT et YNGK;

Φ est un résidu d'acide aminé choisi indépendamment parmi le groupe consistant en C, A, G, K, R, D et E

Σ est un résidu d'acide aminé choisi parmi le groupe consistant en S, G et A n est un entier ayant une valeur égale à 0 ou 1.

2. Polypeptide selon la revendication 1, où X et Φ représentent tous les deux C, SEQ représente FNRYLT, et n est égal à 0.
3. Polypeptide selon la revendication 2, sous la forme d'une molécule à pont disulfure où la liaison disulfure intramoléculaire est entre les deux résidus Cys aux positions 1 et 27 et/ou entre les deux résidus Cys aux positions 4 et 21.
4. Polypeptide selon la revendication 1, où X et Φ représentent tous les deux C, SEQ représente YNGK, et n est égal à 0.
5. Polypeptide selon la revendication 4, sous la forme d'une molécule à pont disulfure où la liaison disulfure intramoléculaire est entre les deux résidus Cys aux positions 1 et 25 et/ou entre les deux résidus Cys aux positions 4 et 19.
6. Polypeptide selon la revendication 1, où X et Φ représentent tous les deux A ou G, et SEQ représente FNRYLT.
7. Polypeptide selon la revendication 1, où X et Φ représentent tous les deux A ou G, et SEQ représente YNGK.
8. Polypeptide selon la revendication 1, où X représente K ou R, SEQ représente FNRYLT, et Φ représente D ou E.
9. Polypeptide selon la revendication 1, où X représente K ou R, SEQ représente YNGK, et Φ représente D ou E.
10. Polypeptide selon la revendication 1, où X représente D ou E, SEQ représente FNRYLT, et Φ représente K ou R.
11. Polypeptide selon la revendication 1, où X représente D ou E, SEQ représente YNGK, et Φ représente K ou R.
12. Polypeptide selon la revendication 1 choisi parmi le groupe consistant en



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CWLCRALIKRFNRYLTPQLVCRLVLRC (lc)

CWLCRALIKRYNGKPQLVGRLVLRC (ld)

5 ALLCRALTKRFNRYLTPQLVCRLVLRAA (le)

ALLCRALIKRYNGKPQLVCRLVLRAA (lf)

10 GLLCRALIKRFNRYLTPQLVCRLVLRGG (lg)

GLLCRALIKRYNGKPQLVCRLVLRGG (lh).

13. Polypeptide selon la revendication 12 qui est le polypeptide ayant la séquence (lc)

15 CWLCRALIKRPNRYLTPQLVCRLVLRC.

14. Polypeptide selon la revendication 13, sous la forme d'une molécule à pont disulfure où la liaison disulfure intramoléculaire est entre les deux résidus Cys aux positions 1 et 27 et/ou entre les deux résidus Cys aux positions 4 et 21.

20 15. Polypeptide selon la revendication 12 qui est le polypeptide ayant la séquence (ld)

CWLCRALIKRYNGKPQLVCRLVLRC.

25 16. Polypeptide selon la revendication 13, sous la forme d'une molécule à pont disulfure où la liaison disulfure intramoléculaire est entre les deux résidus Cys aux positions 1 et 25 et/ou entre les deux résidus Cys aux positions 4 et 19.

17. Tensioactif reconstitué qui comprend un polypeptide selon l'une quelconque des revendications 1 à 16 en mélange avec un support lipidique.

30 18. Tensioactif reconstitué selon la revendication 17 comprenant en outre une analogue peptidique de synthèse de la protéine tensioactive native SP-C.

35 19. Tensioactif reconstitué selon la revendication 17 ou 18, où le support lipidique comprend un mélange de phospholipides.

20. Utilisation d'un polypeptide selon la revendication 1 à 16 pour la fabrication d'un médicament pour la prophylaxie et/ou le traitement du syndrome de détresse respiratoire (RDS) chez les bébés à naissance prématurée.

40 21. Utilisation d'un polypeptide selon la revendication 1 à 16 pour la fabrication d'un médicament pour la prophylaxie et/ou le traitement d'une maladie associée à une déficience ou dysfonction de tensioactif choisie parmi le groupe consistant en RDS chez l'adulte (ARDS), syndrome d'aspiration méconiale (MAS), et dysplasie broncho-pulmonaire (BPD).

45

50

55

Figure

Phe	Pro	Ile	Pro	Leu	Pro	Tyr	Cys	Trp	Leu	Cys	Arg	Ala	Leu	Ile	Lys
1				5					10					15	
Arg	Ile	Gln	Ala	Met	Ile	Pro	Lys	Gly	Ala	Leu	Ala	Val	Ala	Val	Ala
			20					25					30		
Gln	Val	Cys	Arg	Val	Val	Pro	Leu	Val	Ala	Gly	Gly	Ile	Cys	Gln	Cys
		35					40					45			
Leu	Ala	Glu	Arg	Tyr	Ser	Val	Ile	Leu	Leu	Asp	Thr	Leu	Leu	Gly	Arg
	50					55					60				
Met	Leu	Pro	Gln	Leu	Val	Cys	Arg	Leu	Val	Leu	Arg	Cys	Ser	Met	
65					70					75					

REFERENCES CITED IN THE DESCRIPTION

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