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(71) Applicants: **VIROMETIX AG** [CH/CH]; Wagistrasse 14, CH-8952 Schlieren (CH). **SWISS TROPICAL AND PUBLIC HEALTH INSTITUTE** [CH/CH]; Socinstrasse 57, CH-4002 Basel (CH). **UNIVERSITÄT BASEL** [CH/CH]; Vizerektorat Forschung, Petersgraben 35, CH-4003 Basel (CH). **UNIVERSITÄT ZÜRICH** [CH/CH]; Prorektorat MNW, Rämistrasse 71, CH-8006 Zürich (CH).

(72) Inventors: **GHASPARIAN, Arin**; Salomon-Vögelin Strasse 62, CH-8038 Zürich (CH). **ZUNIGA, Armando**; Ottikerstrasse 38, CH-8006 Zürich (CH). **GEIB, Nina**; Neunbrunnenstrasse 161, CH-8050 Zürich (CH). **TAMBORINI, Marco**; Tramstrasse 87, CH-4132 Muttenz (CH). **JUD, Maja**; Schützenstrasse 13, CH-4103 Bottmingen (CH). **PLUSCHKE, Gerd**; Im Vogelsang 3, 79189 Bad Krozingen (DE). **MARRERO NODARSE, Aniebrys**; Bergacker 18, CH-8046 Zürich (CH). **ROBINSON, John Anthony**; Tobelstrasse 24, CH-8615 Wermatswil (CH).

(74) Agent: **LATSCHA SCHÖLLHORN PARTNER AG**; Austrasse 24, CH-4051 Basel (CH).

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(54) **Title**: PROLINE-RICH PEPTIDES PROTECTIVE AGAINST S. PNEUMONIAE

(57) **Abstract**: The invention relates to lipopeptides consisting of a peptide chain comprising a parallel coiled-coil domain, a proline-rich peptide antigen, and a lipid moiety, all covalently linked, which aggregate to synthetic virus-like particles. Proline-rich peptide antigens considered contain negatively and positively charged amino acid, and at least 15% of the amino acids are proline. Such synthetic virus-like particles carrying proline-rich antigens derived from pneumococcal proteins are useful as vaccines against infectious diseases caused by Gram-positive bacteria such as *Streptococcus pneumoniae*.

Proline-rich peptides protective against *S. pneumoniae*

Field of the invention

The invention relates to multimeric lipopeptides consisting of a peptide chain comprising a parallel coiled-coil domain, a proline-rich peptide antigen, and a lipid moiety, all covalently linked, which aggregate to synthetic virus-like particles. These synthetic virus-like particles carrying proline-rich antigens are useful as vaccines against infectious diseases caused by Gram-positive bacteria such as *Streptococcus pneumoniae*.

Background of the invention

Gram-positive bacteria, including *Streptococci* or *Staphylococci* are important pathogens and the etiological agent of a number of serious diseases including pneumonia, sepsis, meningitis, wound infections, endocarditis, acute rheumatic fever, neonatal sepsis or toxic shock syndrome. Therefore there is a need to develop vaccines against these pathogens. Vaccines are already available for some *S. pneumoniae* serotypes; these have shortcomings such as a highly complex manufacturing process.

S. pneumoniae is a highly diverse polysaccharide encapsulated alpha-hemolytic *Streptococcus* that frequently colonizes the human nasopharynx and can cause non-invasive pneumococcal diseases such as otitis media, sinusitis and non-bacteraemic pneumonia, and more severe invasive diseases such as bacteraemia/sepsis, meningitis and bacteraemic pneumonia, primarily among young children and the elderly.

The polysaccharide capsule is a major determinant of virulence during invasion and prevents C3b opsonisation and non-opsonic killing by neutrophils. Currently licensed vaccines contain capsular polysaccharide antigens formulated either alone in pneumococcal polysaccharide vaccines (PPSV) or conjugated to a carrier protein such as modified diphtheria toxin CRM 197 in pneumococcal conjugate vaccines (PCV). There are more than 90 different capsular serotypes in 40 serogroups.

Polysaccharide pneumococcal vaccines can provide serotype-specific protection but cross-protection is low even within the same serogroup. Serotype replacement has been observed after introduction of the conjugate vaccine Prevnar® 7 in the US in 2000. Among the emerging serotypes are also multi-drug resistant capsule-switch variants. Therefore

there is a need for next generation pneumococcal vaccines that target other antigens than the capsule.

One potential antigen for inclusion into a next generation pneumococcal vaccine is Pneumococcal Surface Protein A (PspA). PspA is a monomeric polymorphic cholin-binding protein and contains an N-terminal alpha-helical part, which forms an antiparallel coiled-coil with itself, a proline-rich region, which is sometimes interspersed by a relatively conserved non-proline block, and a C-terminal region containing multiple repeats of a choline binding domain.

The N-terminal region of PspA contains immunodominant epitopes. Recombinant proteins comprising this region and bacterial vectors expressing this region have shown protective potential in various models. For example, Langermann *et al.* have prepared recombinant Bacille Calmette-Guérin (rBCG) vectors expressing PspA. In order to be able to anchor the PspA in the bacterial membrane, a PspA-derived gene segment was fused to Mtb19 lipoprotein (see Langermann S. *et al.*, *J. Exp. Med.* **1994**, *180*, 2277-2286). There is a safety concern associated with the use of PspA as a vaccine antigen because the N-terminal region may resemble human myosin and thus immunization with an immunogen encompassing this region may lead to tissue cross-reactive antibodies. Therefore recent efforts have been made to use other regions of PspA as antigen. Another PspA region that may be suitable for inclusion into a next generation pneumococcal vaccine is the proline-rich region. Although the proline-rich region (PRR) of PspA is polymorphic, it contains several conserved motifs, including short amino acid motifs like PKP, PAPAP, PEKP, and a highly conserved non-proline block (NPB) that is present in some PspA molecules (Brooks-Walter, A. *et al.*, *Infect Immun* **1999**, *67*, 6533-6542; Hollingshead, S.K. *et al.*, *Infect Immun,* **2000**, *68*, 5889-5900; Daniels, C.C. *et al.*, *Infect Immun,* **2010**, *78*, 2163-2172.). Although the PRR does not contain immunodominant epitopes, antibodies against this part of PspA have been detected in children using an enzyme immunoassay (EIA) with a thioredoxin (Trx) fusion protein as antigen (Melin, M. *et al.,* *Vaccine* **2012**, *30*, 7157-7160). Because the NPB is highly conserved the authors hypothesize that antibodies to the PRR may cross-react with a majority of strains through their recognition of NPB epitopes.

The PRR of PspA has a small size (up to around 100 amino acids) and therefore may not be sufficiently immunogenic when used as an antigen alone. *Escherichia coli* Trx fusion proteins have been produced and their potential for protection has been demonstrated in a mouse model of intravenous infection (WO 2007/089866 and Daniels, C.C. *et al.*, *Infect*

Immun, **2010**, *78*, 2163-2172). However, Trx fusion proteins may not be suitable for human use as a vaccine because of a potential for the induction of immune responses to non-protective Trx epitopes and poor structural representation of native PR epitopes. Moreover NPB or proline-rich (PR) sequences may also contain non-protective epitopes, and hence it may be critical to concentrate immune responses to protective epitopes for efficacy.

Similar PR sequences can be found in other pneumococcal proteins, including the surface proteins PspC (also known as CbpA or Hic), and the PhtX proteins PhtA, PhtB, PhtD and PhtE, and proline-rich regions derived from such other pneumococcal proteins may be suitable for inclusion into a next generation pneumococcal vaccine, like proline-rich sequences from PspA.

Several immunogenic bacterial surface proteins from other Gram-positive bacteria contain proline-rich sequences that can likewise be targeted by vaccines against these pathogens. These proteins include surface proteins from other *Streptococci* such as the M6, SclA and SclB proteins of *S. pyogenes*, CBeta (bac) and BibA of *S. agalactiae* or the P1 adhesin of *S. mutans*, or proteins from *S. aureus*.

Synthetic bacterial lipopeptide analogs have received wide attention in vaccine research, both for their adjuvant effects and as carriers for peptide antigens (Ghielmetti M. *et al.*, *Immunobiology* **2005**, *210*, 211-215). Many lipopeptide constructs have been reported, in which a lipid with known adjuvant effects has been coupled to a peptide to generate self-adjuvanting vaccine candidates. Particularly well studied are tripalmitoyl-S-glyceryl cysteine (N-palmitoyl-S-(2,3-bis-(O-palmitoyloxy)-propyl)-cysteinyl- or Pam3Cys) and dipalmitoyl-S-glyceryl cysteine (2,3-bis-(O-palmitoyloxy)-propyl)-cysteinyl- or Pam2Cys). These lipid moieties are found in lipoprotein components of the inner and outer membranes of gram-negative bacteria. Patent application WO 98/07752 describes the use of lipopeptides for drug targeting purposes, wherein the peptide portion may be a collagen-like sequence capable of inducing triple helical structures. Patent application WO 2008/068017 describes synthetic virus-like particles comprising helical lipopeptide bundles and having a spherical or spheroidal structure with a lipid core and a peptidic outer surface. The peptide chain of the lipopeptides comprises a coiled-coil domain. The properties of the coiled-coil domain in the peptide chain of the lipopeptide building blocks determine the number of building blocks combining to form the synthetic virus-like particle.

Summary of the invention

The invention relates to lipopeptide building blocks consisting of (1) a peptide chain comprising a parallel coiled-coil domain which, as a self-standing lipid-free peptide, forms a parallel dimeric, trimeric or higher order oligomeric helical bundle, (2) a proline-rich (PR) peptide antigen comprising at least one negatively and at least one positively charged amino acid, and wherein at least 15% of the amino acids are proline, optionally linked to a further antigen, and (3) a lipid moiety comprising two or three long hydrocarbyl chains; wherein the peptide chain, the PR peptide antigen and the lipid moiety are covalently linked, either directly or through a linker. Preferably, the peptide chain comprising a parallel coiled coil is linked at one end to the PR peptide antigen and at the other end to the lipid moiety.

These lipopeptide building blocks aggregate to helical lipopeptide bundles and synthetic virus-like particles (SVLP). The presentation of the PR antigen on the SVLP surface enhances the immune response to PR epitopes.

Preferred are lipopeptide building blocks comprising PR peptide antigens derived from *Streptococci* and/or *Staphylococci,* more preferably from *Streptococcus pneumoniae.*

The invention further relates to processes of production of lipopeptide building blocks, helical lipopeptide bundles and synthetic virus-like particles; to the use of lipopeptide building blocks, helical lipopeptide bundles and synthetic virus-like particles carrying PR peptide antigens in the preparation of vaccines; and to methods of vaccination using such vaccines. The invention likewise relates to pharmaceutical preparations containing synthetic virus-like particles carrying PR antigens.

The compositions of the invention comprising PR peptide antigens derived from *Streptococci* and/or *Staphylococci,* in particular from *S. pneumoniae,* are useful for inducing immune responses against *S. pneumoniae* or other Gram-positive bacteria, and for the prevention or treatment of infectious diseases such as pneumococcal diseases caused by *S. pneumoniae*.

Brief description of the figures

Figure 1: IgG ELISA endpoint titers in sera from BALB/c mice immunized two times with lipopeptide **15** (closed circles and squares) or alum adjuvanted recombinant PspA (rPspA, closed triangles) and sera from non-immunized controls (open symbols). Symbols indicate endpoint titers sera from individual mice, lines indicate median values. Titers were measured against a peptide representing the proline-rich region (PR peptide) and recombinant PspA protein comprising the entire N-terminal alpha-helical part, the proline-rich region and the non-proline block (rPspA).

Figure 2: Survival times in days (D) post challenge of immunized and non-immunized BALB/c mice with increasing doses of *S. pneumoniae* serotype 1. The percent Survival (%S) is shown on the y-axis and immunogens and challenge doses (in CFU) are indicated on the right. Mice were immunized two times with lipopeptide **15** or rPspA + alum and then challenged intravenously.

Detailed description of the invention

The invention relates to lipopeptide building blocks consisting of
(1) a peptide chain (PC) comprising a parallel coiled-coil domain which, as a self-standing lipid-free peptide, forms a parallel dimeric, trimeric or higher order oligomeric helical bundle,
(2) a proline-rich (PR) peptide antigen, comprising at least one negatively and at least one positively charged amino acid, and wherein at least 15% of the amino acids are proline, optionally linked to a further antigen, and
(3) a lipid moiety (LM) comprising three or preferably two long hydrocarbyl chains, wherein the peptide chain, the PR peptide antigen and the lipid moiety are covalently linked, either directly or through a linker, in particular two different linkers.

Preferably, the peptide chain comprising a parallel coiled coil is linked at one end to the PR peptide antigen and at the other end to the lipid moiety.

The peptide chain (PC) comprises a parallel coiled-coil domain. Such coiled-coil domains will associate into a defined helical bundle, e.g. into a dimeric, trimeric, tetrameric, pentameric, hexameric or heptameric bundle. Parallel coiled-coil domains differ from antiparallel coiled-coils, wherein a monomeric peptide chain loops back to form a helical substructure by aligning two (or more) partial domains of the monomeric peptide in an

antiparallel alignment. The parallel coiled-coil domain may contain between 12 and 120 amino acid residues, preferably between 21 and 80 amino acid residues. Coiled-coil domains contain two or more consecutive repeat patterns (usually heptad repeats in which the seven structural positions are labeled *a-g*, with *a* and *d* denoting hydrophobic residues), which as self-standing lipid-free peptides possess the property of self-assembly into a parallel coiled-coil helical bundle (Lupas A.N., Gruber M.; The structure of alpha-helical coiled coils, *Adv. Protein Chem.*. **2005**, *70*, 37–78). The peptide chain must multimerize to form a parallel coiled-coil helical bundle of defined oligomerization state (e.g. dimer, trimer, tetramer, pentamer, hexamer or heptamer, in particular dimer, trimer, tetramer or pentamer). Preferred peptide sequences are non-human sequences to avoid the risk of autoimmune disorders when applied in the vaccination of humans.

The lipopeptide building block further comprises a proline-rich (PR) peptide antigen comprising at least one negatively and at least one positively charged amino acid. Charged amino acids considered herein are amino acids with side chains that are positively or negatively charged at physiological pH. Among the naturally occurring amino acids the most frequent positively charged amino acids considered here are lysine, arginine and histidine; the most frequent negatively charged amino acids are glutamic acid and aspartic acid. A peptide is considered “proline-rich” if at least 15% of the amino acids are proline. Preferred are proline-rich peptides comprising at least one glutamic acid residue and at least one lysine or arginine residue.

Preferred PR peptide antigens are derived from *Streptococci* and/or *Staphylococci,* e.g. from Gram-positive bacteria selected from the group consisting of *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus mutans*, *Streptococcus suis, Streptococcus equi, Streptococcus dysgalactiae, Peptostreptococcus magnus* and *Staphylococcus aureus.*

Preferably this PR peptide antigen is derived from proteins PspA and/or PspC or other proteins that are protective against pneumococcal infection, including PhtX proteins.

"Derived" means that the amino acid sequence or a substantial portion (i.e. 50% or more) of the amino acid sequence of the peptide antigen originates from one or more naturally occurring protein(s), whereas 0% up to 50% of the amino acid sequence is designed *de novo*. Included are also PR peptide antigens that comprise combinations of proline-rich sequences from different PspA/PspC molecules and/or from other proteins containing

proline-rich segments. “Proline-rich segment” means that at least 15% of the amino acids contained in the segment are proline.

Proline-rich (PR) peptide antigens of particular interest are derived from *S. pneumoniae* and are located immediately after the C-terminal end of the helical region of PspA or PspC and before the non-proline block (if the PspA or PspC sequence comprises a non-proline block) or the repeat region. Alternatively the PR peptide antigen is located in the central region of a pneumococcal polyhistidine triad protein (PhtX). Alternatively the PR peptide antigen is located in a region of PR proteins of Gram-positive bacteria selected from the group consisting of *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus mutans*, *Streptococcus suis, Streptococcus equi, Streptococcus dysgalactiae, Peptostreptococcus magnus* and *Staphylococcus aureus*, such as the central region of the P1 adhesin of *Streptococcus mutans*.

Protective PR peptide antigens are identified by sequencing PspA and/or PspC and/or other genes from clinical isolates, selecting a portion of the PR region, synthesizing the PR peptide, and conjugating it to a peptide chain (PC) to be incorporated or being part of a lipopeptide.

The efficacy of PR peptide antigens is tested by administering lipopeptide conjugates and synthetic virus-like particles obtained therefrom and observing the activity and efficacy in animal models of pneumococcal sepsis or other diseases caused by streptococcal infection.

The PR peptide antigen is optionally linked to a further antigen. Further antigens considered are other pneumococcal peptides or polysaccharides, in particular the peptides with an amino acid sequence SEQ ID NO: 113 to 119, and other antigens described below.

The PR peptide antigen is conjugated directly or through a linker, either at the N- or C-terminus of the PR peptide antigen, and is connected either to the N- or to the C-terminal of the peptide chain (PC) comprising the coiled coil domain, or optionally to an amino acid side chain. Alternatively the PR peptide antigen is conjugated to the peptide chain (PC) comprising the coiled coil domain through a side chain residue of the PR peptide antigen, such as a terminal or internal aspartic acid, glutamic acid, lysine, ornithine or cysteine side chain. Linkers considered are short peptides of 2 to 20 amino acids, hydroxyalkyl- or aminoalkyl-carboxylic acids, substituted or unsubstituted polyalkylenoxy glycols,

preferably containing one to twelve C_2 and/or C_3 alkylenoxy units, polyalkylenoxy glycol block co-polymers (e.g. pluronics), mono-, di-, tri- and oligosaccharides, which may comprise acetyl, glycerol-phosphate or other substituents at one or more positions, polysaccharides such as poly(sialylic acid) and derivatives (e.g. peptide conjugates) thereof, proteinogenic or non-proteinogenic amino acids, and C_1-C_8 saturated or unsaturated hydrocarbons, and may comprise one or more of the following functional groups: a disulphide bond, amine, amide, acetal, ester, ether, thioether, hydrazone, hydrazide, imine, oxime, urea, thiourea, carbonate, iminocarbonate, amidine, amide, imide, an alkyl succinimide, which may also be hydrolyzed to an amide, sulphonamide, sulfone, or a heterocyclic ring comprising one or more atoms selected from nitrogen and oxygen, preferably a triazole. Also considered are combinations of the aforementioned linkers, including those used in the Examples.

Any method used for conjugating peptides or other antigens to an antigen delivery system such as carrier protein, polymer, dendrimer, nanoparticle or virus-like particle, can be used to conjugate the PR peptide antigens to the peptide chain (PC) comprising the parallel coiled-coil domain. Such methods are well-known to those skilled in the art, see for example Hermanson, G.T, Bioconjugate Techniques, 2nd edition, Academic Press, 2008.

PR peptide antigens consist of 5-200 amino acids, preferably 8-80 amino acids. Multiple PR peptide conjugates can be used in combination in a vaccine formulation. PR peptide antigens can also be fused together in order to produce a longer artificial PR peptide. PR conjugates can also be combined with conjugates comprising other pneumococcal peptides or polysaccharides. Amino acids and derivatives thereof comprising a functional group (e.g. an amino-, halo-, hydrazino-, hydroxylamino- or sulfhydryl group) can be incorporated into the PR peptide in order to facilitate conjugation of the PR peptide and enhance stability.

The peptide chain (PC) may further comprise an amino acid sequence which includes one or more T-helper cell epitopes, and/or strings of polar residues that promote the solubility of the lipopeptide building block in water.

T-helper epitopes that may be incorporated into the peptide chain (PC) include those listed in Table 1 below, and variants thereof in which one, two, or three amino acids are replaced by other amino acids or are deleted.

Table 1

T-helper epitope	SEQ ID NO:	Sequence [a)]
TT830-843	1	QYIKANSKFIGITE
TT1064-1079	2	IREDNNITLKLDRCNN
TT1084-1099	3	VSIDKFRIFCKANPK
TT947-968	4	FNNFTVSFWLRVPKVSASHLET
TT1174-1189	5	LKFIIKRYTPNNEIDS
DTD271–290	6	PVFAGANYAAWAVNVAQVID
DTD321–340	7	VHHNTEEIVAQSIALSSLMV
DTD331–350	8	QSIALSSLMVAQAIPLVGEL
DTD351–370	9	VDIGFAAYNFVESIINLFQV
DTD411–430	10	QGESGHDIKITAENTPLPIA
DTD431–450	11	GVLLPTIPGKLDVNKSKTHI
TT632-651	12	TIDKISDVSTIVPYIGPALN
CTMOMP36-60	13	ALNIWDRFDVFCTLGATTGYLKGNS
TraT1	14	GLQGKIADAVKAKG
TraT2	15	GLAAGLVGMAADAMVEDVN
TraT3	16	STETGNQHHYQTRVVSNANK
HbcAg50-69	17	PHHTALRQAILCWGELMTLA
HbSAg19-33	18	FFLLTRILTIPQSLD
HA307-319	19	PKYVKQNTLKLAT
MA17-31	20	YSGPLKAEIAQRLEDV
MVF258-277	21	GILESRGIKARITHVDTESY
MVF288-302	22	LSEIKGVIVHRLEGV
CS.T3*	23	IEKKIAKMEKASSVFNVVNS
SM Th	24	KWFKTNAPNGVDEKIRI
PADRE1 [b)]	25	aKFVAAWTLKAAa
PADRE2 [b)]	26	aK-Chx-VAAWTLKAAa

[a)] References: SEQ ID NO: 1-5 and 17-20: *Eur. J. Immunol.* **2001,** *31,* 3816–3824; SEQ ID NO: 6-12: *JID* **2000**, *181,* 1001-1009; SEQ ID NO: 13-16, 21-22 and 24: US 5,759,551; SEQ ID NO: 23: *Nature* **1988**, *336*, 778-780; SEQ ID NO: 25-26: *Immunity* **1994**, *1*, 751–761.

[b)] „a“ denotes D-Ala and „Chx“ denotes cyclohexylalanine.

The total length of the peptide chain (PC) is preferably between 21 and 200 amino acid residues, more preferably between 21 and 120 amino acid residues.

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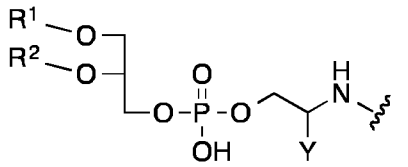
The lipid moiety (LM) contains a lipid anchor with two or three, preferably two, long hydrocarbyl chains and a structure combining these hydrocarbyl chains and connect it to the peptide chain (PC), either directly or via a linker. The lipid moiety can also be connected to the PR peptide again, which in turn is conjugated to the peptide chain comprising the parallel coiled-coil, however, connection of the lipid moiety to the peptide chain is preferred. Preferred lipid moieties are lipids containing two or three, preferably two extended hydrocarbyl chains.

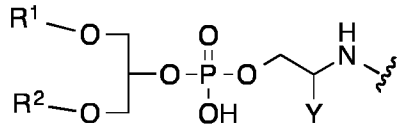
"Long hydrocarbyl" means a straight alkyl or alkenyl group of at least 7 carbon atoms, for example straight alkyl or alkenyl consisting of between 8 and 50 C atoms, preferably between 8 and 25 C atoms. Alkenyl has preferably one, two or three double bonds in the chain, each with E or Z geometry, as is customarily found in natural fatty acids and fatty alcohols. Also included in the definition of "long hydrocarbyl" is branched alkyl or alkenyl, for example alkyl bearing a methyl or ethyl substituent at the second or third carbon atom counted from the end of the chain, as e.g. as in 2-ethyl-hexyl.

“Lower alkyl” means alkyl with 1 to 7 carbon atoms (C_1-C_7), preferably 1 to 4 carbon atoms (C_1-C_4), such as methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl, iso-butyl or tert-butyl.

Particular preferred lipid moieties according to the invention are those of formula Z^1 to Z^8

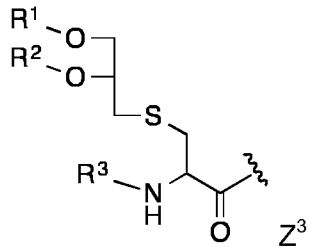


Z^1



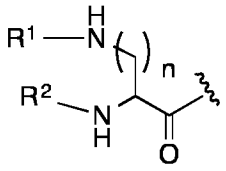
Z^2

wherein R^1 and R^2 are long hydrocarbyl or long hydrocarbyl-C=O and Y is H or COOH,

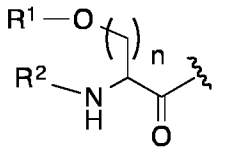


Z^3

wherein R^1, R^2 and R^3 are long hydrocarbyl or long hydrocarbyl-C=O or R^1 and R^2 are long hydrocarbyl or long hydrocarbyl-C=O and R^3 is H or acetyl or lower alkyl-C=O,

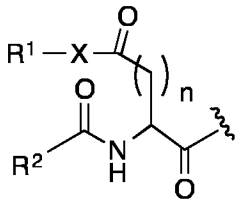


Z^4



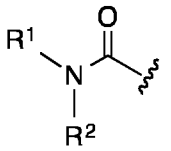
Z^5

wherein R^1 and R^2 are long hydrocarbyl or long hydrocarbyl-C=O and n is 1, 2, 3 or 4,

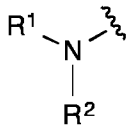


Z^6

wherein R^1 and R^2 are long hydrocarbyl, X is O or NH, and n is 1, 2, 3 or 4, or



Z^7



Z^8

wherein R^1 and R^2 are long hydrocarbyl.

The lipid moiety contains at least two long hydrocarbyl chains such as found in fatty acids, e.g. as in Z^1 to Z^8. One preferred lipid moiety is a phospholipid of various types, e.g. of formula Z^1 or Z^2, that possess either ester- or ether-linked extended alkyl or alkenyl chains, such as either enantiomer of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, or achiral analogues such as 1,3-dipalmitoyl-glycero-2-phosphoethanolamine. A preferred lipid moiety is a tri- or di-palmitoyl-S-glycerylcysteinyl residue (type Z^3) or lipid moieties of types Z^4 to Z^8. Most preferred are the lipid moieties described in the Examples.

The peptide chain (PC) is covalently linked to the lipid moiety (LM) at or near one terminus, i.e. the N terminus or the C terminus, preferably the N terminus. The lipid moiety may be directly attached as in

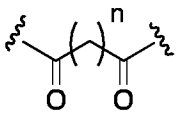
LM – PC (**1**)

or *via* a linker (L) as in

LM – L – PC (**2**).

If the peptide chain (PC) and the lipid moiety (LM) are directly linked, this is preferably accomplished through an amide bond between a lipid moiety carbonyl function and an amino function, e.g. the N terminal amino function, of the peptide chain (PC). Particular lipid moieties Z^1, Z^2 and Z^8 are preferably connected through an amide bond between their amine function and a carboxy function, e.g. the C terminal carboxy function, of the peptide chain (PC).

It will be apparent to those knowledgeable in this area, that a large variety of suitable linkers and coupling strategies exist, which include but are not limited to linkers based on dicarboxylic acid derivatives, linkers containing one or multiple ethylene glycol units, amino acid residues (including α-, β-, γ-, δ-amino acids), or sugar (carbohydrate) units, or containing heterocyclic rings. Particular linkers considered are linkers L^1 to L^{16}, wherein n is between 1 and 45 and m is between 1 and 45, for example wherein n is between 1 and 20 and m is between 1 and 20, shown with the connecting functional group C=O and/or X wherein X is O or NH: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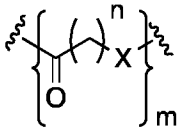
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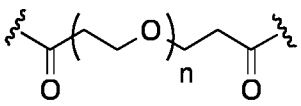
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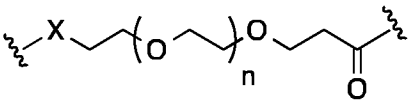
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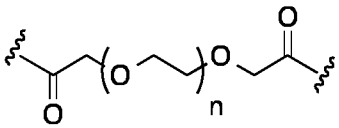
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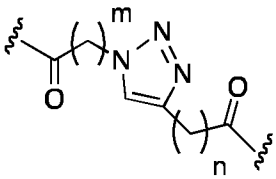
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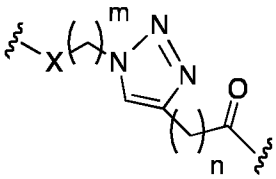
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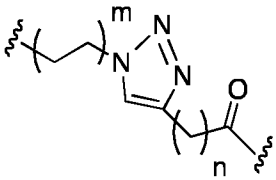
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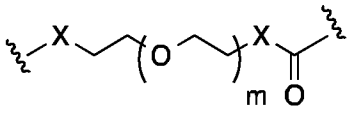
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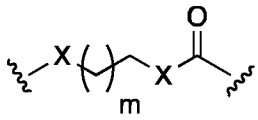
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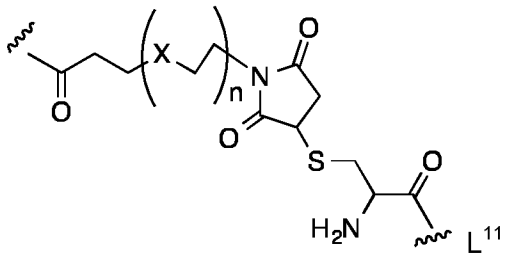
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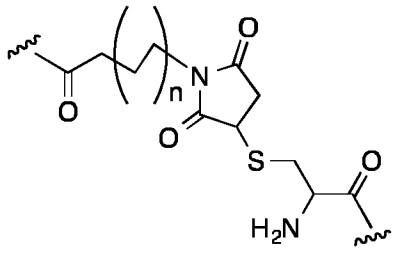
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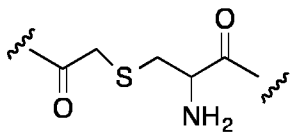
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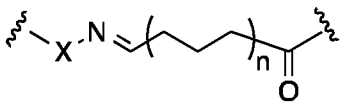
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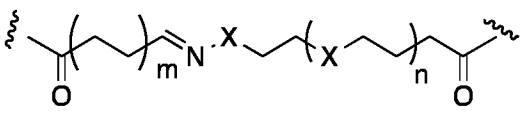
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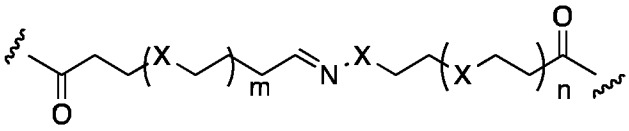

L16

Most preferred are the linkers described in the Examples.

Particular linkers L^1 to L^{16} may be connected to LM and PC as follows:

A carbonyl function shown for L^1 to L^{16} may be connected to an amino function of a suitable lipid moiety (LM) and/or an amino function, e.g. the N terminal amino function, of the peptide chain (PC) through an amide bond. Alternatively a carbonyl function shown for L^1 to L^{16} may be connected to a lipid moiety (LM) by replacement of the corresponding carbonyl function in particular lipid moieties Z^3 to Z^7.

A functional group X shown for L^1 to L^{16} (with the meaning NH or O) may be connected to a carbonyl function of a suitable lipid moiety (LM) and/or a carboxy function, e.g. the C terminal carboxy function, of the peptide chain (PC) through an amide bond (for X = NH) or through an ester bond (for X = O).

The terminal CH_2 group of L^8 may be connected to an amino function of a suitable lipid moiety (LM), an amino function, e.g. the N terminal amino function, of the peptide chain (PC), or a carbonyl function of a suitable lipid moiety (LM).

"Near one terminus" as understood in this connection means that the lipid moiety or the linker is bound to the first, second, third, fourth or fifth amino acid calculated from the N terminal or C terminal end, respectively, of the peptide. The lipid moiety may be attached, directly or through a linker, to the backbone of the peptide structure or to the side chain of one of these amino acids near to the terminus.

"Coiled-coil domains" are designed by careful selection of appropriate amino acid sequences that form a thermodynamically stable, alpha-helical, parallel bundle of helices by spontaneous self-association.

A coiled-coil domain includes peptides based on canonical tandem heptad sequence repeats that form right handed amphipathic α-helices, which then assemble to form helical bundles with left-handed supercoils. Also included are peptides built from non-canonical, non-heptad-based repeats that form coiled-coils that are not necessarily left-handed or even regular supercoils.

Canonical coiled-coils occur widely in naturally occurring biologically active peptides and proteins, and have also been designed *de novo*. A set of rules has been elucidated for designing coiled-coil peptides that adopt helical bundles of defined oligomerization state, topology and stability (e.g. dimer, trimer, tetramer, pentamer, hexamer or heptamer).

These rules allow designers to build a peptide sequence compatible with a given target structure. Most important, the sequences of canonical coiled-coil peptides contain a characteristic seven-residue motif, which is repeated typically 3-10 times. The positions within one heptad motif are traditionally denoted *abcdefg*, with mostly (but not exclusively) hydrophobic residues occurring at sites *a* and *d* and generally polar, helix-favoring residues elsewhere. Tandem heptad motifs along a peptide chain have an average separation between the *a* and *d* residues that allows them to fall on one face of the alpha-helix. When two or more helices pack together into a coiled-coil bundle the hydrophobic faces of the helices associate and wrap around each other in order to maximize contacts between hydrophobic surfaces. The type of residue that may occur at each position within a heptad repeat will influence the stability and oligomerization state of the helical bundle. In general, mostly hydrophobic residues (Ala, Ile, Leu, Met, Val), or aromatic hydrophobic side chains (Phe, Trp and Tyr), are used at the *a* and *d* sites. The remaining *b*, *c*, *e*, *f* and *g* sites tend to be more permissive than the *a* and *d* sites, though polar and helix-favoring residues (Ala, Glu, Lys and Gln) are favored. The choice of residues at the *a* and *d* sites can influence the oligomerization state of the coiled coil (i.e. dimer *vs*. trimer). Thus, dimers are favored when non-β-branched residues (e.g. Leu) occur at the *d* positions; at these sites β-branched residues (Val and Ile) disfavor dimers. On the other hand, in dimers β-branched residues (Ile, Val) are preferred at the *a* sites. Another rule is that *a* = *d* = Ile or Leu favors trimers, which is useful in designing coiled coils that specifically form parallel trimers. These and other design rules are discussed in more detail in Woolfson, D.N., Adv. Prot. Chem. **2005**, *70*, 79-112.

The heptad motif codes for amphipathic alpha-helices that oligomerize through their hydrophobic faces. The coiled-coil domain includes at least three tandem heptad repeat motifs. The upper number of heptad repeats in each chain will influence the stability of the helical bundle. It is limited mainly by the feasibility of chemical synthesis of long peptides, but sequences containing more than three heptad repeats (e.g. four, five, six, seven and eight heptad repeats) are preferred. Examples discussed below form trimeric alpha-helical coiled-coils, but the invention likewise concerns dimeric, tetrameric, pentameric, hexameric and heptameric coiled-coil domains.

Coiled-coil domains according to the invention may have longer repeat units, for example 11-residue repeats and 15-residue repeats such as are present in naturally occurring coiled-coils. Thus the helical bundles required for the formation of aggregate structures may also arise when using coiled-coil motifs with periodicities other than seven. Coiled coils with unusual periodicities are also possible. In many naturally occurring coiled-coils

the unbroken heptad repeat pattern may contain various discontinuities. Two common discontinuities are insertions of one residue into the heptad pattern, as well as insertions of three or four residues. For example, a one residue insertion is seen in the trimeric coiled coil of influenza hemagglutinin. Other naturally occurring coiled coils display a periodicity other than seven, for example, the regular periodicity of 11 residues (termed hendecads) found in the surface layer protein tetrabrachion of *Staphylothermus marinus*.

Other examples of coiled-coil peptide sequences occurring naturally in viral coat proteins are coiled-coil motifs forming trimeric helical bundles in the gp41 coat protein of HIV-1, and the F-glycoprotein of RSV. These coiled-coil domains are included in the definition of coiled-coil domain according to the invention.

The preferred coiled-coil peptides should contain between 3-8 tandemly linked heptad motifs. The heptad motifs within the coiled-coil may have identical sequences, or they may each have different sequences. In all cases, the seven positions of the seven amino acid residues within one heptad motif are designated with letters: *a b c d e f g*. The coiled-coil peptide, therefore, comprises an amino acid sequence having the positions $(abcdefg)_{3-8}$.

Preferred are coiled-coil peptide sequences containing between 3-8 tandemly linked heptad motifs, wherein positions *a* and *d* in each heptad motif (*abcdefg*) contain alpha-amino acids belonging to the Group 1 and/or to the Group 2 as defined hereinbelow. In addition, not more than *two* of all the *a* and *d* positions may be occupied by any amino acid residue belonging to the Group 3, and not more than *one* of all the *a* and *d* positions may be occupied by any amino acid residue belonging to the Group 4 or Group 5 or by glycine. In addition, in positions *b*, *c*, *e*, *f* and *g*, alpha-amino acids belonging to the Groups 3, 4 and 5 are preferred, but amino acids belonging to the Groups 1 and 2 are allowed, with the addition that not more than one of these positions within any one heptad motif may be glycine, but none may be proline.

Group 1 comprises alpha-amino acid residues with small to medium sized hydrophobic side chains. A hydrophobic residue refers to an amino acid side chain that is uncharged at physiological pH and that is repelled by aqueous solution. These side chains generally do not contain hydrogen bond donor groups, such as primary and secondary amides, primary and secondary amines and the corresponding protonated salts thereof, thiols, alcohols, ureas or thioureas. However, they may contain hydrogen bond acceptor groups such as ethers, thioethers, esters, tertiary amides, or tertiary amines. Genetically encoded amino acids in this group include alanine, isoleucine, leucine, methionine and valine.

Group 2 comprises amino acid residues with aromatic or heteroaromatic side chains. An aromatic amino acid residue refers to a hydrophobic amino acid having a side chain containing at least one ring having a conjugated aromatic π(pi)-electron system. In addition it may contain additional hydrophobic groups such as lower alkyl, aryl or halogen, hydrogen bond donor groups such as primary and secondary amines, and the corresponding protonated salts thereof, primary and secondary amides, alcohols, and hydrogen bond acceptor groups such as ethers, thioethers, esters, tertiary amides or tertiary amines. Genetically encoded aromatic amino acids include phenylalanine and tyrosine. A heteroaromatic amino acid residue refers to a hydrophobic amino acid having a side chain containing at least one ring having a conjugated aromatic π-system incorporating at least one heteroatom such as O, S and N. In addition such residues may contain hydrogen bond donor groups such as primary and secondary amides, primary and secondary amines and the corresponding protonated salts thereof, alcohols, and hydrogen bond acceptor groups such as ethers, thioethers, esters, tertiary amides or tertiary amines. Genetically encoded heteroaromatic amino acids include tryptophan and histidine.

Group 3 comprises amino acids containing side chains with polar non-charged residues. A polar non-charged residue refers to a hydrophilic side chain that is uncharged at physiological pH, but that is not repelled by aqueous solutions. Such side chains typically contain hydrogen bond donor groups such as primary and secondary amides, primary and secondary amines, thiols, and alcohols. These groups can form hydrogen bond networks with water molecules. In addition, they may also contain hydrogen bond acceptor groups such as ethers, thioethers, esters, tertiary amides, or tertiary amines. Genetically encoded polar non-charged amino acids include asparagine, cysteine, glutamine, serine and threonine.

Group 4 comprises amino acids containing side chains with polar cationic residues and acylated derivatives thereof, such as acylamino-derived residues and urea-derived residues. Polar cationic side chains refer to a basic side chain, which is protonated at physiological pH. Genetically encoded polar cationic amino acids include arginine, lysine and histidine. Citrulline is an example for a urea-derived amino acid residue.

Group 5 comprises amino acids containing side chains with polar anionic residues. Polar anionic refers to an acidic side chain, which is deprotonated at physiological pH.

Genetically encoded polar anionic amino acids include aspartic acid and glutamic acid. A particular polar cationic residue is $-(CH_2)_aCOOH$ wherein a is 1 to 4.

More preferred are coiled-coil peptide sequences containing between 3 to 8 tandemly linked heptad motifs, wherein each heptad motif (*abcdefg*) may have any one of the following sequences:
1xx1xxx (referring respectively to the positions *abcdefg*);
1xx2xxx (referring respectively to the positions *abcdefg*);
2xx1xxx (referring respectively to the positions *abcdefg*); or
2xx2xxx (referring respectively to the positions *abcdefg*);
wherein *1* is a genetically encoded amino acid from Group 1, *2* is a genetically encoded amino acid from Group 2, and wherein *x* is a genetically encoded amino acid from Groups 1, 2, 3, 4 or 5 or glycine.

Equally preferred are coiled-coil peptide sequences identified in naturally occurring peptides and proteins, but excluding those of human origin. These are, for example, coiled-coils identified in viral and bacterial proteins.

The invention also relates to synthetic virus-like particles carrying PR peptide antigens, and to a method of preparing such synthetic virus-like particles involving dissolving the lipopeptide building blocks in a suitable carrier, preferably an aqueous buffer system (e.g. buffered saline or unbuffered saline). The solvent may be removed after preparation of the synthetic virus-like particles, for example by lyophilization or spray drying.

The invention further relates to a method of eliciting an immune response wherein an immunogenically effective amount of a synthetic virus-like particle carrying PR peptide antigens as described herein is administered to an animal. Any animal can be used, although warm-blooded animals, especially humans are considered here the most.

The invention also relates to a vaccine (or likewise to any other pharmaceutical preparation or medicine) comprising as principal or further active ingredient one or more synthetic virus-like particles carrying PR peptide antigens, alone or in combination with a pharmaceutically acceptable carrier.

The vaccine may also comprise one or more adjuvants such as a mineral salt (e.g. aluminium hydroxide, aluminium phosphate, aluminium sulfate, calcium phosphate), monophosphoryl lipid A (MPL), plant extracts containing saponins (e.g. QS-21), imidazo-

quinolines (e.g. Imiquimod), muramyl dipeptides and tripeptides, lipopeptides, oil-in-water emulsions (e.g. Montanide ISA 720), cytokines (e.g. IL-2 or GM-CSF), mycobacterial and bacterial derivatives (e.g. Freund's complete adjuvant), BCG, nucleic acid derivatives (e.g. polyIC) and other adjuvants known to those skilled in the art.

Some components of the vaccine may also be encapsulated in or attached to bio-degradable polymers, which may for example be useful for controlled release, for example polylactic acid, poly-epsilon-caprolactone, polyhydroxybutyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates, and cross-linked or amphipathic block copolymers of hydrogels, or may be formulated in liposomes.

The vaccine is prepared in a manner known *per se*, for example by means of conventional dissolving and lyophilizing processes and/or may comprise excipients, for example preservatives, stabilizers, wetting agents, tonicity adjusting agents and/or emulsifiers, solubilizers, salts for regulating osmotic pressure and/or buffering substances to stabilize the pH.

The vaccine may be in liquid form or solid (e.g. lyophilized) form and can be sterilized by conventional, well-known sterilization techniques or sterile filtered. The resulting aqueous solution can be packaged for use as it is, or lyophilized, spray dried, or the solvent can be removed in another way. The solid form may be combined with a sterile diluent (e.g. water) prior to administration or may be administered as it is. Likewise the vaccine may comprise an emulsion, dispersion or suspension or any other form suitable for the intended route of administration.

The vaccine may be administered by any suitable enteral or parenteral route such as the intranasal, oral, sublingual, intramuscular, intradermal, transdermal, and subcutaneous or transcutaneous route. Other routes are known in the art that could also be employed.

A device may be used for administration such as conventional needles and syringes, micro needles, ballistic devices for administration of solids (e.g. as in WO 99/27961), patches (e.g. as in WO 98/20734), needle free injection systems (e.g. as in WO 01/054539), spray devices and the like, depending on the dose form and administration route. The device may be pre-filled or coated with the vaccine.

The vaccine comprises from approximately 0.05% to approximately 50% of the active ingredient in an appropriate dose form. Unit dose forms for parenteral administration are,

for example, ampoules, pre-filled syringes or vials, e.g. vials containing from about 0.0001 mg to about 0.75 g of the active ingredient in a dose volume between approximately 0.25 ml and 1.5 ml.

The dosage of the active ingredient depends upon the intended recipient (e.g. species), its age, weight, and individual condition, and the administration route. An optimal dosage for a particular active ingredient and a particular target population can be determined by standard studies involving observation of appropriate immune responses in subjects.

The amount of vaccine sufficient to confer immunity to pathogenic *pneumococci* or other Gram-positive bacteria is determined by methods well known to those skilled in the art. This quantity will be determined based upon the characteristics of the vaccine recipient and the desired level of immunity to a disease caused by infection with *pneumococci* or other Gram-positive bacteria.

The vaccine may be administered as a single dose or as two or more doses at adequately spaced time points. The vaccine may also be administered together with other vaccines. For example, the vaccine may be used in prime-boost regimens in combination with other vaccines.

The vaccine is used for prophylactic or therapeutic purposes, or both, for the prevention and/or treatment of bacteraemia and other diseases caused by *S. pneumoniae* infections, such as pneumonia, acute sinusitis, otitis media, meningitis, bacteraemia, septicemia, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis or brain abscesses, or carriage. The vaccine is likewise useful for prophylactic or therapeutic purposes, or both, for the prevention and/or treatment of diseases caused by Group A *Streptococci* or *S. mutans*, such as pharyngitis, pyoderma, rheumatic fever, glomerulonephritis or caries.

For example, the vaccine protects against bacteraemia, pneumonia and meningitis, or against invasive pneumococcal disease (IPD), an infection in which *S. pneumoniae* can be isolated from the blood or another normally sterile site, or against bacteraemia, pneumonia and otitis media. Alternatively the vaccine protects against pharyngitis, pyoderma, rheumatic fever and glomerulonephritis or caries.

The vaccine may be administered to different target populations, including populations that are naive or have failed to respond previously to infection or vaccination, elderly

persons suitably aged (e.g. over 65, 75 or 85 years old), adults at elevated risks such as people working in health institutions, young adults with a risk factor, immune-compromised persons or pediatric populations.

The invention furthermore relates to a method of making a vaccine comprising mixing synthetic virus-like particles carrying PR peptide antigens with synthetic virus-like particles carrying other antigens or antigens not carried by synthetic virus-like particles.

The invention also relates to antibodies to synthetic virus-like particles carrying protective PR peptide antigens, especially to antibodies to PR peptide antigens. Antibodies to PR peptide antigens can cross-react with a broad variety of genetically diverse strains and different capsular serotypes, and extend the median survival time in a model of passive transfer.

Protective antibodies to PR peptide antigens can have opsonophagocytic activity (OPA). Antibodies with OPA may be detected *in vitro* by a suitable assay, for example an opsonophagocytic killing assay (OPKA) using a cell line or whole blood from a donor. Protective antibodies can also mediate protection by other mechanisms that do not involve OPA.

The invention also relates to the use of protective antibodies to PR peptides as described herein for the manufacture of a pharmaceutical preparation or medicine for therapeutic and/or prophylactic purposes and also for the manufacture of a diagnostic kit.

Not all PR peptides may be equally protective as vaccine antigens. Protective PR peptides are preferred. They may be identified by using well-known methods such as challenge studies in a suitable animal model of streptococcal diseases. For example, in a pneumococcal sepsis model, mice may be immunized with synthetic virus-like particles carrying PR peptide antigens as described herein, and subsequently challenged intravenously with a lethal dose of *S. pneumoniae*. In this model mice immunized with synthetic virus-like particles carrying protective PR peptide antigens then have significantly longer survival time compared to that of immunized control mice.

PR peptide antigens can also be derived from other pathogenic streptococci such as *S. pyogenes*, *S. agalactiae*, *S. mutans*, *S. equi, S. suis*, *S. dysgalactiae, Peptostreptococcus magnus* or other pathogenic Gram-positive bacteria, such as

Staphylococcus aureus, and synthetic virus-like particles carrying these PR peptide antigens are likewise useful for vaccines against these bacteria.

Preferred PR peptide antigen sequences from *S. pneumoniae* PspA and PspC are collected in Table 2 below. Peptide antigen P3 is a synthetic construct combining two sequences.

Table 2

Name	SEQ ID NO:	Sequence
P1	27	PAPKPEQPAEQPKPAPAPQPAPAPKPEKT
P2	28	PKPEQPAPAPKPEQPAKPEKPA
P3	29	PAPKPEQPAEQPKPEQPAPAPKPEQPAKPEKP
P4	30	PAPKPEQPAEQPKPA
P5	31	PAPQPAPAPKPEKT
P6	32	QPAEQPKPAPAPQPAP
P7	33	PAPAPKPEQPAEQPKP
P8	34	PAPEAPAEQPKPAPAPQPAPAPKPEKPAEQPKPEKT
P9	35	PAEQPKPAPAPQPAPAPKPEKPAEQ
P10	36	PKPAPAPQPAPAPKPEKPAEQPKPEKT
P11	37	KAEKPAPAPQPEQPAPAPKT
P12	38	PAPAPQPEQPAPAPQPEQPAPAPKPEQPAPAPKPEQPTPA
P13	39	PAPAPQPEQPAPAPKPEQPAPAPKPEQPTPAPKPEQPTPAPKT
P14	40	PEQPAPAPKPEQPAPAPKPEQPTPAPKPEQPTPAPKT
P15	41	PKPEQPTPAPKPEQPTPAPKT
P16	42	PKPEQPAEQPKPAPAPQ
P17	43	PKPEQPAPAPKPEQPAKPEKPAEEPTQPEKPATPKT
P18	44	PKPEQPAKPEKPAEEPTQPEKPATPKT
P19	45	PAPAPQPAPAPKPAPAPQPEKPAEQPKAEKPA
P20	46	PETPAPAPKPETPAPAPEAPAPAPAPKPEQPAPAPKPEKSA
P21	47	PAPAPKPEQPAPAPKPEKSA
P22	48	PKPEQPAPAPKPEKSA
P23	49	KAEKPAPAPKPEQPVPAPKT
P24	50	PAPAPKPAPAPQPEKPAPAPAPKPEKSA
P25	51	PAPEQPTEPTQPEKPAEETPAPKPEKPAEQPKAEKT
P26	52	PAPKPEKPAEQPKAEKT
P27	53	PAPAPKPEQPAEQPKPAPAPQPEKPAEEPENPAPAP
P28	54	APAPKPETPAPAPEAPAPAPAPKPEQPAPAPKPEKS
P29	55	APAPETPAPEAPAEQPKPAPAPQPAPAPKPEKPAEQPKPEKT
P30	56	PAPEQPTEPTQPEKPAEETPAPKPEKPAEQPKAEKT
P31	57	APAPKPETPAPAPEAPAPAPAPKPEQPAPAPKPEKS
P32	58	APAPETPAPEAPAEQPKPAPAPQPAPAPKPEKPAEQPKPEKT
P33	59	APAPKPETPAPAPEAPAPAPAPKPEQPAPAPKPEKS
P34	60	APAPETPAPEAPAEQPKPAPAPQPAPAPKPEKPAEQPKPEKT
P35	61	APAPETPAPEAPAEQPKPAPAPQPAPAPKPEKPAEQPKAEKPA

P36	62	PQPEQPAPAPKPEQPAPAPKPEQPTPAPKPEHP
P37	63	PAPAPQPEQPAPAPQPEQPAPAPKPEQPAPAPKPE
P38	64	PAPQPEQPAPAPKPEQPAPAPKPEQPTPAPKPP
P39	65	PAPAPAPKPEQPAPAPAPKPEQPAPAPAPKPEQPA
P40	66	PAPAPKPEQPAPAPAPKPEQPAPAPAPKPEQPT
P41	67	PAPAPQPEQPAPAPKPEQPAPAPKPEQPTPAPKPE
P42	68	PAPAPKPEQPAEQPKPAPAPQPAPAPKPEKQ
P43	69	PAPAPQPEQPAPAPQPEQPAPAPKPEQPAPAPKPA
P44	70	PKPEQPTPAPKPEQPTPAPKPEQPTPAPKPEQPT
P45	71	PEKPAPAPEKPAPAPEKPAPA
P46	72	PAPKPAPAPKPAPAPAPKPEKPA
P47	73	PAPAPTPEAPAPAPKP
P48	74	PKPEQPAKPEKPAEEPTQPEKPA
P49	75	PAKPEKPAEEPTQPEKPA
P50	76	PAPAPKPEQPAKPEKPAEEPTQPEKPA
P51	77	PKPEQPAPAPNPEQPAKPEKPAEEPTQPEKPA
P52	78	PKPEQPAPAPAPKPEQPAPAPAPKPEQPA
P53	79	PKPEQPAPAPKPEQPAKPEKPAEEPTQPEKPA
P54	80	PKPEQPAPAPKPEQPAKPEKPAEEPTQPEKPA
P55	81	PAPAPQPEQPAPAPKPEQPAPAPKPEQPAPAPKPEQPA
P56	82	PAPAPKPEQPTPAPKPEQPTPAPKPEQPAPAPKPEQPAPAPKP
P57	83	PARALQPEQPAPAPKPEQPTPAPKPEQPTPAPKPEQPAPAPKP

In the preferred PR peptides of SEQ ID NO: 27 to 83, one, two or three amino acids may be replaced by other amino acids.

Alternative PR peptide antigen sequences from *S. pneumoniae* and from *S. pyogenes*, *S. agalactiae*, *S. mutans*, *S. suis, S. equi, S. dysgalactiae, Peptostreptococcus magnus* and *Staphylococcus aureus* proteins are collected in Table 3 below.

Table 3

Name	SEQ ID NO:	Sequence[a)]
P58	84	SRLEQPSLQPTPEPSPGPQPAPN
P59	85	RPEEPSPQPTPEPSPSPQPAPSNP
P60	86	HWVPDSRPEQPSPQSTPEPSPSPQPAPNPQPAPSNP
P61	87	PKSNQIGQPTLPNNSLATPSPSLPINPGTSHE
P62	88	PEVTPTPETPEQPGEKAPEKSPEVTPTPETPEQP
P63	89	PEVTPTPETPEQPGEKAPEK
P64	90	PEKSPEVTPTPETPEQP
P65	91	KAPEKSPEVTPTPEMP
P66	92	PGKPAPKTPEVPQKPDTAPHTPKTP
P67	93	KPSAPKAPEKAPAPKAPK
P68	94	PAPKAPKASEQSSNPKAPAPKSAP
P69	95	PGPAGPRGLQGPQGPRGDKGET
P70	96	PQAPSTPEKQPEVPESP
P71	97	PETPDAPSTPKDEPQAP
P72	98	PAPVEPSYEAEPTPPTRTPDQAEPNKPT
P73	99	PTYETEKPLEPAPVEPSYEAEPT
P74	100	KPTAPTKPTYETEKPLKPAPVAPNYEKEPT
P75	101	KPVVPEQPDEPGEIEPIP
P76	102	PEVPSEPETPTPPTPEVPAEPGKPVPPAK
P78	103	KYTPKKPNKPIYPEKPKDKTPPTKPDHS
P79	104	PEKPVEPSEPST
P80	105	KPVEPSEPSTPDVPSNPSNPSTPDVPSTPDVPSNPSTPEVPSNP
P81	106	PQVEPNVPDTPQEKPLT
P82	107	KPLTPLAPSEPSQPSIPETPLIPSEPSVPET
P83	108	PEVKPDVKPEAKPEAKPA
P84	109	KPEAKPEAKPA
P85	110	PDVKPEAKPEAKPDVKPEAK
P86	111	PETPDTPKIPELPQ
P87	112	PDTPQAPDTPHVPESPKTPE

[a)] SEQ ID NO of proteins from *S. pneumoniae*: 84-87; *S. pyogenes*: 88-92, 95; *S. equi*: 93-94, *S. suis*: 96-97; *S. mutans*: 98-100; *S. aureus*: 101-103; *Peptostreptococcus magnus*: 104; *S. dysgalactiae*: 105-107*; S. agalactiae*: 108-112.

In the preferred PR peptides of SEQ ID NO: 84 to 112, one, two or three amino acids may be replaced by other amino acids.

Additional sequences, which, when administered alone, offer limited protective potential, may be conjugated to PR peptide antigens, in particular sequences derived from regions of PspA or PspC, or other proteins, which do not comprise proline in every 3rd or 4th position and/or comprise less than 15% proline, in particular the sequences:

QQAEEDYARRSEEEYNRLPQQQPPKAEKP (non-proline block) (SEQ ID NO:113),
and
AEDQKEEDRRNYPTNTYKTLELEIAESDVEV (helical peptide from PspC)
(SEQ ID NO:114).

Other sequences that may be combined with PR peptide antigens include sequences derived from bacterial surface proteins that do not contain a proline rich region, including:

Sequences from StkP, preferably the C-terminal 79-82 amino acids,
SVAMPSYIGSSLEFTKNNLIQIVGIKEANIEVVEVTTAPAGSAEGMVVEQSPRAGEKVDLN
KTRVKISIYKPKTTSATP (SEQ ID NO:115),
and fragments thereof;

sequences from PsaA, preferably amino acids 250–309:
SLFVESSVDDRPMKTVSQDTNIPIYAQIFTDSIAEQGKEGDSYYSMMKYNLDKIAEGLAK
(SEQ ID NO:116);

sequences from cholesterol dependent cytolysins, such as the 4th domain of Ply, amino acids 360-471:
NGDLLLDHSGAYVAQYYITWNELSYDHQGKEVLTPKAWDRNGQDLTAHFTTSIPLKGNV
RNLSVKIRECTGLAWEWWRTVYEKTDLPLVRKRTISIWGTTLYPQVEDKVEND
(SEQ ID NO:117),
and fragments thereof;

streptococcal polyhistidine triad proteins, for example fragments from the C-terminal half of *S. pneumoniae* PhtD, e.g. the amino acids 680–770:
VEHPNERPHSDNGFGNASDHVRKNKVDQDSKPDEDKEHDEVSEPTHPESDEKENHAG
LNPSADNLYKPSTDTEETEEEAEDTTDEAEIPQV (SEQ ID NO:118),
or the amino acids 771–839:
ENSVINAKIADAEALLEKVTDPSIRQNAMETLTGLKSSLLLGTKDNNTISAEVDSLLALLKE
SQPAPIQ (SEQ ID NO:119),
or fragments thereof.

In the preferred sequences SEQ ID NO: 113–119, one, two or three amino acids may be replaced by other amino acids.

Further sequences include the sequences described in PCT/US2012/022127 or US 2005/0020813 A1. Alternative sequences include sequences described in EP 0280576 A2.

Additional antigens may be combined with synthetic virus-like particles carrying PR peptide antigens. For example, *S. pneumoniae* proteins identified in WO 98/18931, WO 98/18930, US 6,699,703, US 6,800,744, WO 97/43303, and WO 97/37026; Lyt family (LytX), Pht family (PhtX), Sp128, type 1 or type 2 pilus proteins, other streptococcal antigens such as those identified in WO 1993/005155, WO 2002/034771, WO 2002/083859, WO 2002/34771, WO 2003/093306, WO 2004/041157, or WO 2005/002619; or other antigens such as Sp101, Sp130, Sp125 or Sp133, may be combined with synthetic virus-like particles carrying pneumococcal PR peptide antigens.

Saccharide antigens may also be combined with synthetic virus-like particles carrying PR peptide antigens, such as capsular saccharides of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 14, 18C, 19A, 19F, 22F, 23F or 33F. Alternatively other saccharides may be combined with PR peptide antigens, such as saccharides derived from other *S. pneumoniae* serotypes, and/or saccharides from other Gram-positive bacteria (e.g. saccharides derived from *S. agalactiae*, *S. pyogenes, and/or S. aureus*).

Likewise proteins from other Gram-positive bacteria may be combined with synthetic virus-like particles carrying PR peptide antigens, e.g. one or more proteins from *S. pyogenes*, including M protein, fibronectin binding protein (Sfbl), Streptococcal heme-associated protein (Shp), or proteins identified in Streptolysin S (SagA), and/or one or more proteins from *S. aureus*, such as Alpha-toxin, Clumping factor A (ClfA), Collagen binding protein (CNA), Fibronectin-binding protein A (FbA), Extracellular Fibrinogen-binding Protein (Efb), Iron regulated surface determinant (Isd) proteins, Penicillin binding protein 2a (PBP2a), Serine Aspartate repeat proteins (Sdr) and/or binder of IgG (Sbi). Likewise also peptide antigens derived from such proteins may be combined with synthetic virus-like particles carrying PR peptide antigens.

Examples

Abbreviations:

Boc, t-butoxycarbonyl;
BSA, bovine serum albumin;
DIEA, diisopropylethylamine;
DMF, N,N-dimethylformamide;
EDT, ethanedithiol;
Fmoc, 9-fluorenylmethoxycarbonyl;
HATU, 2-(1H-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate;
HBTU, 2-[1H-benzotriazole-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate;
HOBt, N-hydroxybenzotriazole;
Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl;
NMP, N-methylpyrrolidone;
MBHA, methylbenzhydrylamine;
OD, optical density;
iPr_2O, diisopropylether;
PCR, polymerase chain reaction
PyBOP, (benzotriazol-1-yloxy)-tripyrrolidinophosphonium-hexafluorophosphate;
PEO6, N-Fmoc-21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid
r.t., room temperature;
RP-HPLC, reversed-phase high performance liquid chromatography;
TA, thioanisole;
TIS, triisopropylsilane;
Trt, trityl;
TFA, trifluoroacetic acid;
TFE, 2,2,2-trifluoroethanol;
t_R, retention time;
SD, standard deviation.

Example 1: Design and Synthesis of PR peptides

The proline-rich region of the PspA from a highly virulent serotype 1 clinical isolate SP1577 (Leimkugel et al., *JID*, **2005**, *192*, 192-199) was amplified and sequenced using two primers (LSM13 and SKH2) according to Hollingshead, Becker et al., *Infect Immun*, **2000**, *68*, 5889-5900.

LSM13: 5`-GCAAGCTTATGATATAGAAATTTGTAAC-3` (SEQ ID NO:120)
SKH2: 5`-CCACATACCGTTTTCTTGTTTCCAGCC-3` (SEQ ID NO:121)

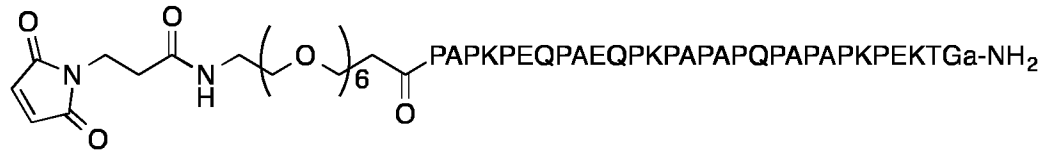
Amplification of the proline-rich region was carried out by PCR using the primers LSM13 and SKH2 and GoTaq Polymerase (PCR conditions: Annealing 48°C for 1 min, Elongation 72°C for 3 min, 30 cycles). The obtained fragments, which were around 1.2 kb in size, were isolated from the PCR reaction and sequenced using the primers LSM13 and SKH2. Around 1'100 bases of the pspA gene could be read. The translated nucleotide sequence is shown below. The proline-rich region, including non-proline block is shown in italics.

XXLGAGFVXX XPTXXXXXEA PVASQXKAEK DXDAXKRDAE NXKKALEEAK XXQKKYEDDQ KKTEEKXKKE KEASKEEQAA NLKYQQELVK YASEKDSVKK AKILKEVEEA EKEHKKKRAE FEKVRSEVIP SAEELKKTRQ KAEEAKAKEA ELIKKVEEAE KKVTEAKQKL DAERAKEVAL QAKIAELENE VYRLETELKG IDESDSEDYV KEGLRAPLQS ELDAKRTKLS TLEELSDKID ELDAEIAKLE KNVEYFKKTD AEQTEQYLAA AEKDLADKKA ELEKTEADLK KAVNEPEKPA EET*PAPAPKP EQPAEQPKPA PAPQPAPAPK PEKTDDQQAE EDYARRSEEE YNRLPQQQPP KAEKPAPAPK PEQPVPAPKT* GWKQENGMWC R (SEQ ID NO:122)

From this sequence the P1 PR Sequence (PAPKPEQPAEQPKPAPAPQPAPAPKPEKT, SEQ ID NO:27) was selected. P1 is located between the helical/coiled-coil region and the non-proline block of the SP1577 PspA.

In order to enable conjugation to SVLP lipopeptides, the following maleimidopeptides were designed and synthesized:

Maleimidopeptide **1**:



(**1**)

In maleimidopeptide **1** 3-maleimidopropionic acid is coupled to the N-terminus in P1 (SEQ ID NO:27) *via* an 21-amino-3,6,9,12,15,18-hexaoxaheneicosan-21-oic acid linker, and a

glycine is added to the C-terminus P1, followed by a D-alanine residue ("a") as the amide ("NH_2") in order to confer stability towards exoproteases.

The synthesis of maleimidopeptide **1** was carried out using Fmoc Solid Phase Peptide Synthesis (SPPS) methods as follows:

The peptide PAPKPEQPAEQPKPAPAPQPAPAPKPEKTGa (SEQ ID NO:27 extended by glycine-D-alanine) was assembled on an ABI 433A peptide synthesizer using Rink Amide MBHA resin (loading: 0.69 mml/g) (362 mg, 0.25 mmol) and standard Fmoc-SPPS protocols. The following amino acids were used (in the correct order): Fmoc-Ala-OH, Fmoc-D-Ala-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH and Fmoc-Thr(tBu)-OH. After assembly and removal of the N-terminal Fmoc protecting group, the resin was washed with *N*-methyl-2-pyrrolidone (NMP) and CH_2Cl_2. For coupling of the maleimide, a portion of the resin (ca. 0.1 mmol) was washed with DMF and a solution of Fmoc-PEO6-OH (115 mg, 0.2 mmol) PyBOP (104 mg, 0.2 mmol), HOBt (27 mg, 0.2 mmol) and DIEA (66 µl, 0.4 mmol) in 4.5 ml DMF was prepared, mixed for 30 seconds and added to the resin under argon. The mixture was shaken for 16 h. The resin was filtered and washed 4 x with DMF. The Fmoc group was then removed by treatment with 20% piperidine in DMF (6 x 2 min.). The resin was then washed again with DMF and a solution of 3-maleimidopropionic acid (34 mg, 0.2 mmol), PyBOP (104 mg, 0.2 mmol), HOBt (27 mg, 0.2 mmol) and DIEA (66 µl, 0.4 mmol) in DMF was prepared and added to the resin under argon. The resin was shaken for 3 h, filtered, washed sequentially 4 times with DMF, CH_2Cl_2 and MeOH, and dried over night *in vacuo* over KOH pellets. For cleavage of the peptide from the resin and removal of side-chain protecting groups, TFA/TIS/TA/phenol 85:5:5:5 (10 ml) was prepared and added to the dry resin under argon atmosphere. The resin was shaken for 3 h, filtered and the maleimidopeptide **1** was precipitated with iPr_2O, pre-chilled to -20°C (50 ml). The peptide was then washed 4 times with iPr_2O, air-dried over night and purified by RP-HPLC using a preparative C18 column (Agilent Zorbax SB300 PrepHT, 250 x 21.5 mm) and a linear gradient of 10 – 40% MeCN in H_2O (+ 0.1% TFA) in 16 min. and lyophilized to afford **1** as a white powder. The peptide was analyzed by analytical RP-HPLC using an Agilent XDB-C18 column (250 x 4.6 mm) and a linear gradient of 10 – 100% MeCN in H_2O (+ 0.1 % TFA) in 25 min: Purity > 97%; t_R = 8.53 min.

ESI-MS: MW calculated for $C_{163}H_{259}N_{41}O_{51}$: 3609.1 Da; MW found: 3609.7 (± 0.02%).

Maleimidopeptide **2**:

(**2**)

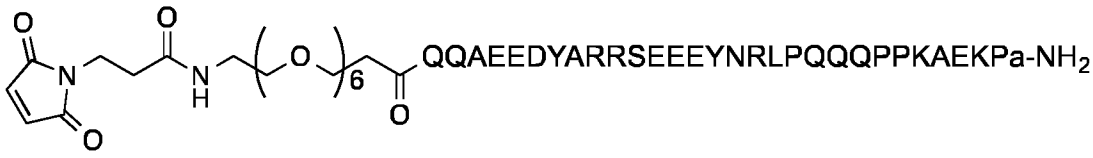
In this maleimidopeptide a glycine is added to the C-terminus in P1 (SEQ ID NO:27) and the maleimide is coupled to the glycine P1 *via* an amino ethyl spacer. The N-terminus is acetylated.

The peptide chain in maleimidopeptide **2** was assembled using Fmoc SPPS on an ABI 433A as described for **1**, except that 2-chlorotrityl resin preloaded with Fmoc-Gly-OH to a resin substitution level of 0.6 mmol/g (416 mg, 0.25 mmol) was used instead of Rink amide MBHA resin as the solid phase support. Following assembly and removal of the N-terminal Fmoc protecting group, the resin was acetylated by treatment with a solution of 0.5 M Ac_2O, 0.05 M HOBt and 0.136 M DIEA in NMP (10 ml) with shaking for 30 min. The resin was then washed 4 times with DMF, 4 times with CH_2Cl_2 and treated with TFE/CH_2Cl_2 2:8 (10 ml) with shaking under argon for 4 h to release the fully side chain protected peptide from the resin. The resin was filtered and washed twice with 10 ml TFE/CH_2Cl_2 2:8, the filtrate was concentrated and the protected peptide was precipitated with 4°C cold Et_2O and washed 4 times with Et_2O. The protected peptide was then dried *in vacuo* over night and stored at -20°C.

For coupling of the maleimide, a portion of the crude side-chain protected peptide (100 mg), HATU (15 mg, 39 µmol), HOAt (5 mg, 39 µmol) were dissolved in DMF (0.8 ml), DIEA (23 µl, 142 µmol) was added and the mixture was stirred for 1 min. A solution of *N*-(2-aminoethyl)maleimide TFA salt (150 mg, 60 µmol) in DMF (0.2 ml) was added and the mixture was stirred for 3 h under argon atmosphere. The DMF was then removed under reduced pressure. The side-chain protected peptide was suspended in 0.3 ml CH_2Cl_2, precipitated with 4°C cold Et_2O, washed 4 times with Et_2O and dried *in vacuo* over night.

The side-chain protecting groups were then removed and the peptide was precipitated and purified as described above for **1** and the final product **2** was analyzed by analytical RP-HPLC using an Agilent XDB-C18 column (250 x 4.6 mm) and a linear gradient of 10 – 100% MeCN in H_2O (+ 0.1 % TFA) in 25 min: Purity > 97%; t_R = 7.06 min. MALDI-TOF MS: MW calculated for $C_{146}H_{227}N_{39}O_{43}$: 3216.6 Da; MW found: 3215.7 Da (± 0.05%).

Maleimidopeptide **3**:



(**3**)

In maleimidopeptide **3**, 3-maleimidopropionic acid is coupled to the N-terminus of SEQ-ID NO:113 *via* an 21-amino-3,6,9,12,15,18-hexaoxaheneicosan-21-oic acid linker, and the C-terminus is capped with D-alanine ("a") and amidated. SEQ ID NO:113 corresponds to the non-proline block of the P1577 PspA.

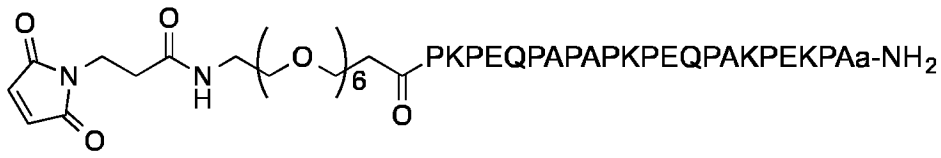
Maleimidopeptide **3** was synthesized and purified as described above for maleimidopeptide **1** and analyzed by analytical RP-HPLC using an Agilent XDB-C18 column (250 x 4.6 mm) and a linear gradient of 10 – 100% MeCN in H_2O (+ 0.1 % TFA) in 25 min: Purity > 97%; t_R = 5.31 min. MALDI-TOF MS: MW calculated for $C_{174}H_{272}N_{50}O_{63}$: 4072.3 Da; MW found: 4071.0 Da (± 0.05%).

Other PR sequences can be obtained by sequencing pspA or pspC genes, or, alternatively, may be accessed in public databases, such as UniProtKB. For example, the PspA sequence of serotype 19A isolate TCH8431 (UniProtKB accession no. D6ZPW2) is:

MNKKKMILTS LASVAILGAG FVTSQPTVVR AEESPVASQS KAEKDYDAAV KKSEAAKKHY EEAKKKAEDA QKKYDEDQKK TEAKAEKERK ASEKIAEATK EVQQAYLAYL QASNESQRKE ADKKIKEATQ RKDEAEAAFA TIRTTIVVPE PSELAETKKK AEEAKAEEKV AKRKYDYATL KLALAKKEVE AKELEIEKLQ YEISTLEQEV ATAQHQVDNL KKLLAGADPD DGTEVIEAKL KKGEAELNAK QAELAKKQTE LEKLLDSLDP EGKTQDELDK EAEEAELDKK ADELQNKVAD LEKEISNLEI LLGGADPEDD TAALQNKLAA KKAELAKKQT ELEKLLDSLD PEGKTQDELD KEAEEAELDK KADELQNKVA DLEKEISNLE ILLGGADSED DTAALQNKLA TKKAELEKTQ KELDAALNEL GPDGDEEET*P APAPQPEQPA PAPKPEQPAP APKPEQPAPA PKPEQPAPAP KPEQPAKPEK PAEEPTQPEK PATPKT*GWKQ ENGMWYFYNT DGSMATGWLQ NNGSWYYLNA NGSMATGWVK DGDTWYYLEA SGAMKASQWF KVSDKWYYVN SNGAMATGWL QYNGSWYYLN ANGDMATGWL QYNGSWYYLN ANGDMATGWA KVNGSWYYLN ANGAMATGWA KVNGSWYYLN ANGSMATGWV KDGDTWYYLE ASGAMKASQW FKVSDKWYYV NGLGALAVNT TVDGYKVNAN GEW (SEQ ID NO:123)

From this sequence the P2 sequence (PKPEQPAPAPKPEQPAKPEKPA, SEQ ID NO:28) was selected. P2 is located immediately after the helical/coiled-coil region of the TCH8431 PspA. In order to facilitate conjugation to SVLP lipopeptides the following maleimido-peptides were designed and synthesized:

Maleimidopeptide **4**



(**4**)

This maleimidopeptide comprises 3-maleimidopropionic acid coupled *via* an 21-amino-3,6,9,12,15,18-hexaoxaheneicosan-21-oic acid linker to the N-terminus of P2 (SEQ ID NO:28). The "a" denotes D-alanine. The C-terminus is amidated.

Maleimidopeptide **4** was synthesized and purified as described above for maleimidopeptide **1** and analyzed by analytical RP-HPLC using an Agilent XDB-C18 column (250 x 4.6 mm) and a linear gradient of 10 – 100% MeCN in H_2O (+ 0.1 % TFA) in 25 min: Purity > 97%; t_R = 5.21 min. MALDI-TOF MS: MW calculated for $C_{131}H_{210}N_{32}O_4$: 2889.3 Da, MW found: 2888.8 Da (± 0.05%).

Maleimidopeptide **5**

(**5**)

In this maleimidopeptide a glycine is added to the C-terminus of P2 (SEQ ID NO: 28), and the maleimide is coupled to the glycine *via* an amino ethyl spacer. The N-terminus is acetylated.

Maleimidopeptide **5** was synthesized and purified as described above for maleimidopeptide **2** and analyzed by analytical RP-HPLC using an Agilent XDB-C18 column (250 x 4.6 mm) and a linear gradient of 10 – 100% MeCN in H_2O (+ 0.1 % TFA) in 25 min: Purity > 97%; t_R = 6.31 min. ESI MS: MW calculated for $C_{113}H_{178}N_{30}O_{33}$: 2484.8 Da; MW found: 2483.2 Da (± 0.02%).

Maleimidopeptide **6**

Artificial PR sequences can be generated by fusing short PR sequences from two or more distinct PspA proteins. For example the sequence P3 (SEQ ID NO: 29) was designed by fusing the N-terminal residue PAPKPEQPAEQ (SEQ ID NO: 124) in P1 to P2 (SEQ ID NO: 28) and replacing the C-terminal Ala in P2 by a Gly residue. In order to enable conjugation the following maleimidopeptide was designed and synthesized:

6

(**6**)

Maleimidopeptide **6** was synthesized and purified as described above for maleimidopeptide **2** and analyzed by analytical RP-HPLC using an Agilent XDB-C18 column (250 x 4.6 mm) and a linear gradient of 10 – 100% MeCN in H_2O (+ 0.1 % TFA) in 25 min: Purity > 97%; t_R = 10.11 min. MALDI MS: MW calculated for $C_{164}H_{255}N_{45}O_{50}$: 3657.1 Da; MW found: 3654.9 Da (± 0.05%).

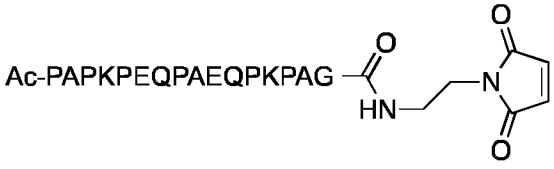
Further examples for PR peptide antigen are described below:

Maleidmidopeptide **7**

(**7**)

Maleimidopeptide **7** was synthesized and purified as described above for maleimidopeptide **2**. ESI MS: MW calculated for $C_{91}H_{137}N_{25}O_{27}$: 2012.0 Da; MW found: 2012.4 Da (± 0.05%).

Maleimidopeptide **8**



(**8**)

Maleimidopeptide **8** was synthesized and purified as described above for maleimidopeptide **2.** ESI-MS: MW calculated for $C_{81}H_{124}N_{22}O_{25}$: 1804.9 Da; MW found: 1805.4 Da (± 0.05%)

Maleimidopeptide **9**

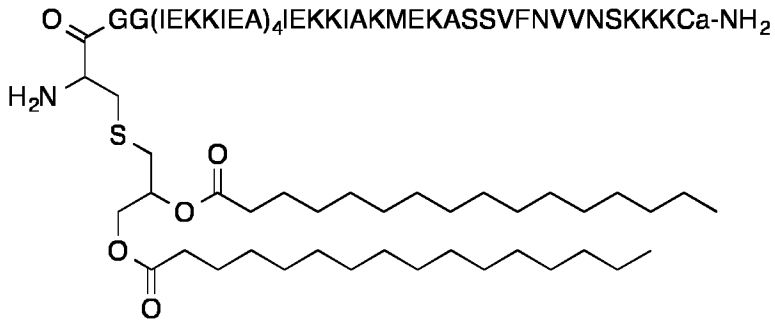
(**9**)

This maleimidopeptide is derived from the PR peptide of PhtD (P60, SEQ ID NO:86). The N-terminus is acetylated. The maleimidopeptide was synthesized and purified as described above for maleimidopeptide **2** and analyzed by analytical RP-HPLC using an Agilent XDB-C18 column (250 x 4.6 mm) and a linear gradient of 20 – 100% MeCN in H_2O (+ 0.1 % TFA) in 25 min: Purity > 97%; t_R = 3.41 min. ESI-MS: MW calculated for $C_{136}H_{201}N_{37}O_{48}$: 3120.4 Da; MW found: 3120.6 Da (± 0.05%).

Example 2: Conjugation of PR Peptide Antigens to Lipopeptides

In order to prepare lipopeptide conjugates for immunizations the following four lipopeptide building blocks were synthesized.

Lipopeptide Building Block **10**

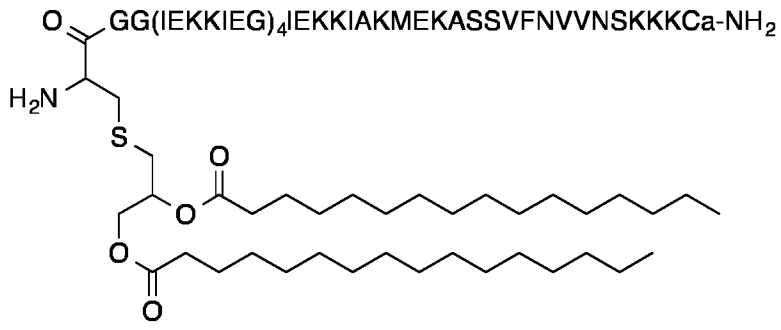


(**10**)

This lipopeptide corresponds to Example 13 in WO 2008/068017. The synthesis was carried out and the product was characterized as described in WO 2008/068017 and Ghasparian, Riedel et al., *Chembiochem*, **2011**, *12*, 100-109. Analytical RP-HPLC

(Interchrom UP5WC4-25QS, 25 to 100% MeCN in H_2O (+ 0.1% TFA) over 25 min.): Purity > 96%, t_R = 22.71 min. MALDI-TOF: MW calculated for $C_{312}H_{552}N_{74}O_{85}S_3$: 6796.4 Da; MW found: 6798.2 Da (± 0.05%).

Lipopeptide Building Block **11**

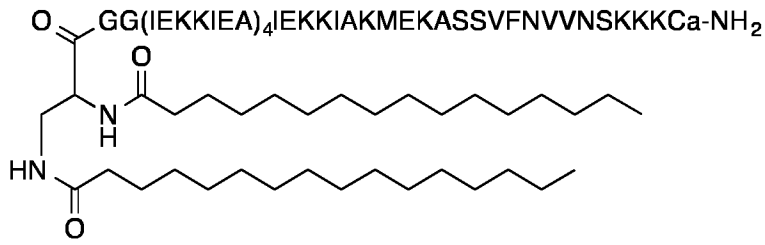


(**11**)

This lipopeptide building block contains a modified coiled-coil domain, which has Gly in the "*c*" positions of the heptad repeat *"defgabc"* IEKKIEG (SEQ ID NO:125).

The modified lipopeptide building block was synthesized and purified as described in WO 2008/068017. Analytical RP-HPLC (Interchrom UP5WC4-25QS, 25 to 100% MeCN in H_2O (+ 0.1% TFA) over 25 min.): Purity > 98%, t_R = 21.41 min. ESI-MS: MW calc. for $C_{308}H_{544}N_{74}O_{85}S_3$ 6740.3 Da; found 6741.7 Da.

Lipopeptide Building Block **12**



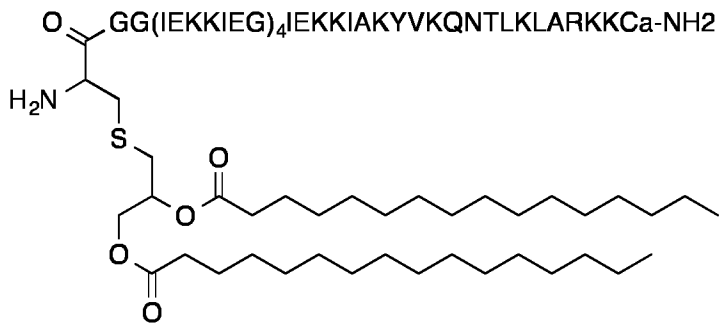
(**12**)

This lipopeptide building block contains a modified lipid *N,N'*-dipalmitoyl.-2,3-diamino-propionamide ("Pam_2Dap"). "a" denotes D-alanine.

The lipopeptide building block was synthesized and purified as described in WO 2008/068017, except that Pam2Dap was incorporated at the end of the synthesis instead of Pam2Cys. The lipopeptide was analyzed by analytical HPLC and MS. Analytical RP-HPLC (C4 column, A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 20 to

100% B in 25 min.): Purity: >95%. t_R = 22.1 min. ESI-MS: MW calc. 9594.5 Da, found 9596.17 Da.

Lipopeptide Building Block **13**



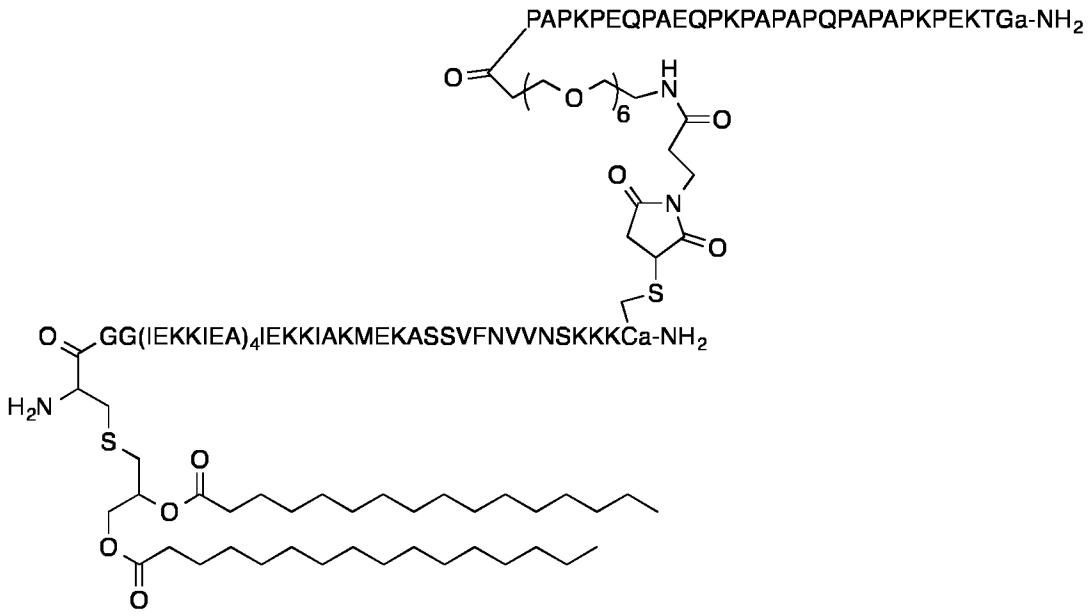
(**13**)

This lipopeptide building block contains a promiscuous T-helper epitope (KYVKQNTLKLARK, SEQ ID NO:126) derived from a HLA-DRB 101 restricted epitope from Influenza hemagglutinin residues 307-309 (SEQ ID NO:19) (Stern, LJ. et al. *Nature* **1994**, *368*, 215). "a" denotes D-alanine.

The lipopeptide building block was synthesized and purified essentially as described in WO 2008/068017 and analyzed by analytical HPLC and MS. Analytical RP-HPLC (C4 column, A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 20 to 100%B in 25 min.): Purity: >95%. t_R = 22.35 min. ESI-MS: MW calc. for $C_{302}H_{538}N_{74}O_{79}S_2$: 6530.0 Da; found 6530.4 Da (± 0.05%).

The following conjugates were synthesized:

Conjugate **14** (Maleimidopeptide **1** + Lipopeptide **10**)

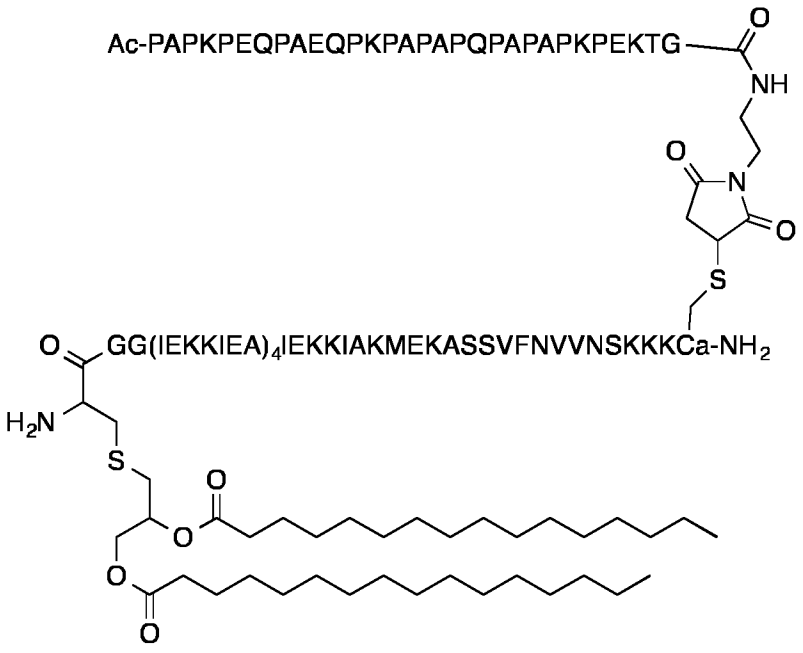


(**14**)

The conjugation of **1** to **10** was performed essentially as described in WO 2008/068017. To a solution of **10** (6.0 mg, 0.9 µmol) in H_2O/MeCN 1:1 (3 ml) was added a solution of **1** (4.8 mg, 1.3 µmol) in H_2O/MeCN 1:1 (2.4 ml). The pH was carefully adjusted and maintained at pH 6.5 – 7.0 with 0.1 NaOH, and the mixture was stirred for 2 h at room temperature. The conjugate was then purified by RP-HPLC using a C4 preparative column (Interchrom UP5WC4-25M, 250 x 10 mm) and a gradient of 50 to 100% MeCN in H_2O (+ 0.1% TFA) in 17 min. The conjugate **14** was analyzed by analytical RP-HPLC using an Interchrom UP5WC4-25QS column (250 x 4.6 mm) and a gradient of 20 to 100% MeCN in H_2O (+ 0.1 % TFA) in 25 min: Purity > 97%; t_R = 22.34 min. MALDI-TOF MS: MW calc. $C_{475}H_{811}N_{115}O_{136}S_3$: 10406.6 Da; found 10407.8 Da (± 0.1%).

The conjugate was suspended in PBS, equilibrated for 30 min., diluted to 0.5 mg/ml and analyzed by Dynamic Light Scattering (DLS) on a Wyatt DynaPro Titan instrument at 4°C, 25°C and 37°C using a laser intensity of 400'000 counts/s and an acquisition time of 10 s. The size distribution by regularization analysis was monomodal and the size dispersity was small. The mean hydrodynamic radius (R_h) was 12.0 nm, and % Pd value 12.3% at 25°C. Similar values for R_h and % Pd were obtained at other temperatures.

Conjugate **15** (Maleimidopeptide **2** + Lipopeptide **10**)

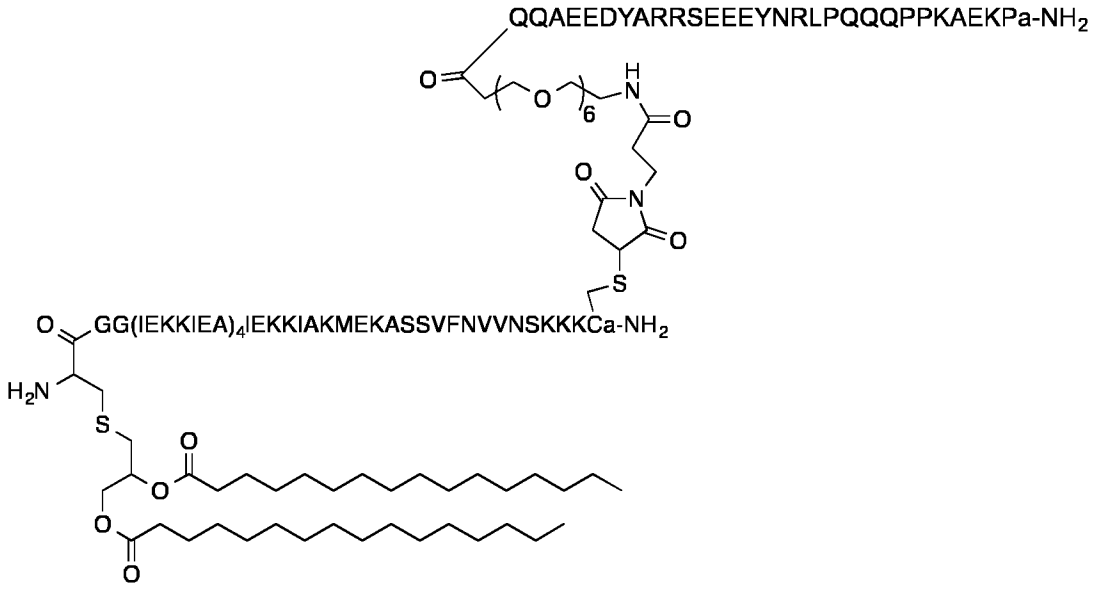


(**15**)

The conjugation of **2** to **10** and purification of the conjugate was performed essentially as described above for conjugate **14**. Product **15** was analyzed by analytical RP-HPLC using an Interchrom UP5WC4-25QS column (250 x 4.6 mm) and a gradient of 20 to 100% MeCN in H_2O (+ 0.1% TFA) in 25 min: Purity > 97%; t_R = 22.41 min. ESI-MS: MW calc. for $C_{458}H_{779}N_{113}O_{128}S_3$: 10012.9 Da; found 10011.1 Da (± 0.1%).

A suspension of conjugate **12** in PBS was prepared and analyzed using DLS as described above for **14**. R_h values were in the range of 13.2 -14.2 nm, and % Pd values in the range of 12.6 – 18.0% at 25°C.

Conjugate **16** (Maleimidopeptide **3** + Lipopeptide **10**)

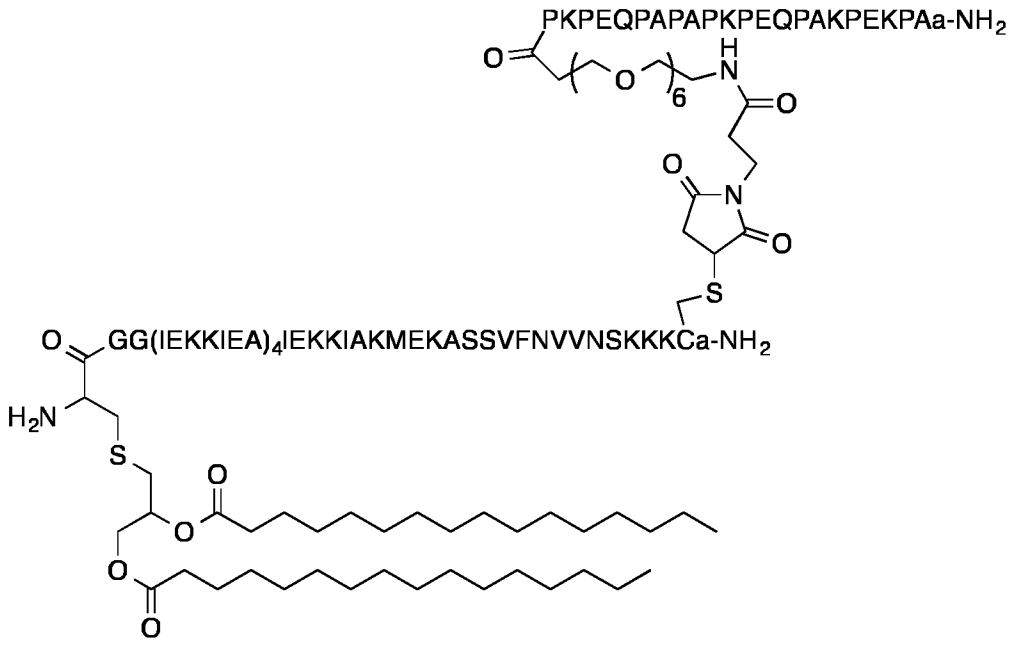


(**16**)

The conjugation of **3** to **10** and purification of the conjugate was performed essentially as described above for conjugate **14**. Product **16** was analyzed by analytical RP-HPLC using an Interchrom UP5WC4-25QS column (250 x 4.6 mm) and a gradient of 20 to 100% MeCN in H_2O (+ 0.1% TFA) in 25 min: Purity > 97%; t_R = 22.0 min. MALDI-TOF MS: MW calc. for $C_{475}H_{811}N_{115}O_{136}S_3$: 10869.8 Da; found 10'872.3 Da (± 0.1%).

A suspension of conjugate **13** in PBS was prepared and analyzed using DLS as described above for **14**. R_h values were in the range of 14.0 – 15.0 nm, and % Pd values in the range of 13.0 - 13.7%. DLS analysis of a mixture of Conjugate **14** and Conjugate **16** particles yielded an R_h of 12.3 - 13.3 nm and a % Pd values around 25-26%, indicating that mixing the particles did not alter the overall size distribution.

Conjugate **17** (Maleimidopeptide **4** + Lipopeptide **14**)

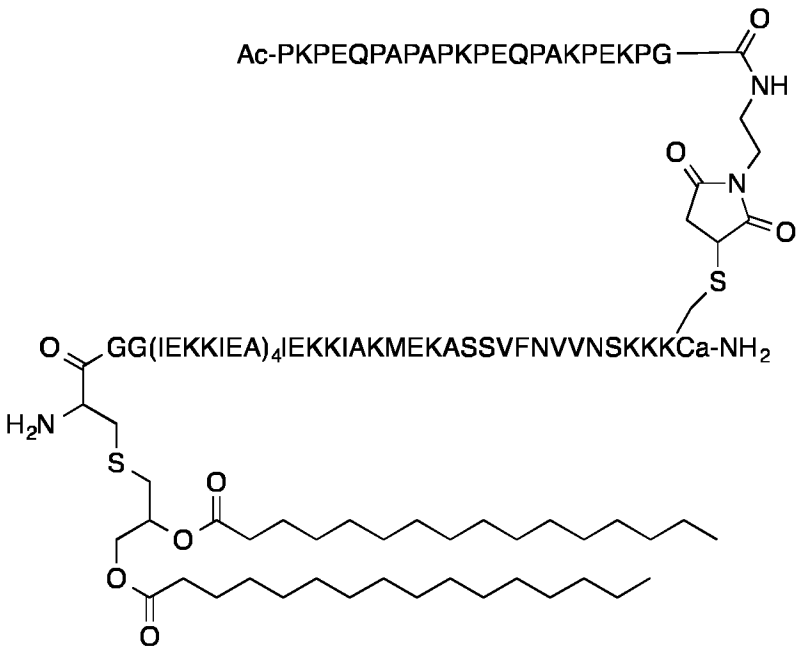


(**17**)

The conjugation of **4** to **10** and purification of the conjugate was performed essentially as described above for conjugate **17**. Product **17** was analyzed by reversed phase HPLC on a C4 analytical column (Interchrom, UP5WC4-25QS, 4.6 mm x 250 mm, 300 Å) and by MALDI-MS. Analytical RP-HPLC (C4 column, A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 20 to 100% B in 25 min.): Purity: >95%. t_R =18.8 min. MALDI-TOF MS: MW calc. for $C_{443}H_{762}N_{106}O_{126}S_3$: 9685.6 Da; found 9686.2 Da (± 0.1%).

A suspension of conjugate **17** in PBS was prepared and analyzed using DLS as described above for **14**. R_h values were in the range of 11.1 - 11.8 nm and % Pd values around 13%.

Conjugate **18** (Maleimidopeptide **5** + Lipopeptide **10**)

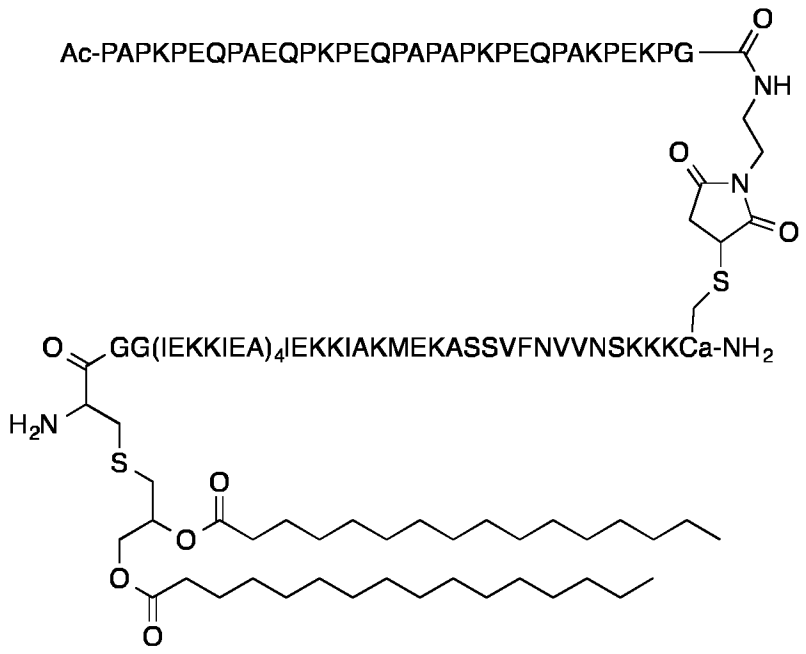


(**18**)

The conjugation of **5** to **10** and purification of the conjugate was performed essentially as described above for conjugate **18**. Product **18** was analyzed by reversed phase HPLC on a C4 analytical column (Interchrom, UP5WC4-25QS, 4.6 mm x 250 mm, 300 Å) and by MALDI-MS. Analytical RP-HPLC (C4 column, A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 20 to 100% B in 25 min.): Purity: >96%. t_R = 22.55 min. MALDI-TOF MS: MW calc. for $C_{425}H_{728}N_{104}O_{118}S_3$: 9279. 2 Da; found 9280.2 Da (± 0.1%).

A suspension of conjugate **18** in PBS was prepared and analyzed using DLS as described above for **14**. R_h values were in the range of 10.0 – 10.5 nm and % Pd values around 16%.

Conjugate **19** (Maleimidopeptide **6** + Lipopeptide **10**)

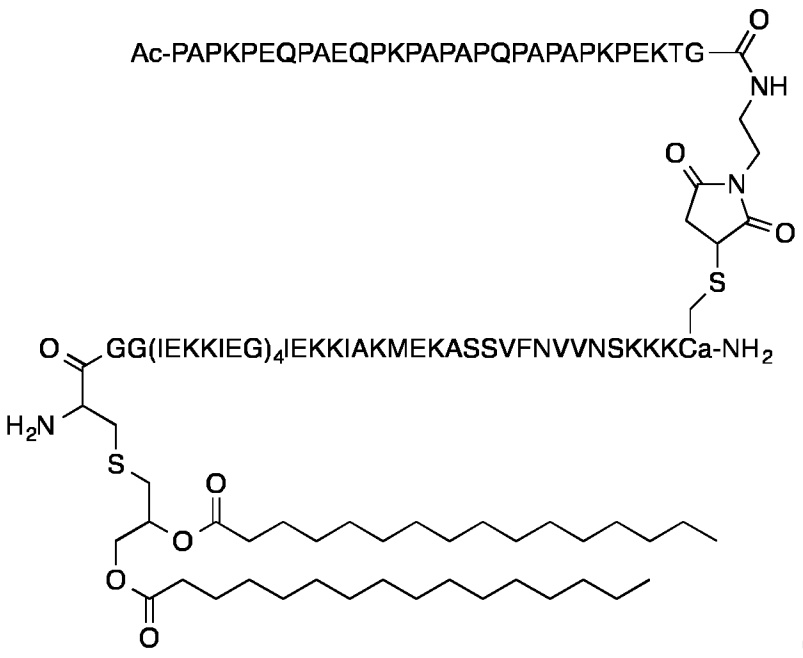


(**19**)

The conjugation of **6** to **10** and purification of the conjugate was performed essentially as described above for conjugate **14**. Product **19** was analyzed by reversed phase HPLC on a C4 analytical column (Interchrom, UP5WC4-25QS, 4.6 mm x 250 mm, 300 Å) and by MALDI-MS. Analytical RP-HPLC (C4 column, A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 20 to 100% B in 25 min.): Purity: >96%. t_R =22.34 min. MALDI-TOF MS: MW calc. for $C_{476}H_{807}N_{119}O_{135}S_3$: 10453.4 Da; found 10452.7 Da (± 0.1%).

A suspension of conjugate **17** in PBS was prepared and analyzed using DLS as described above for **19**. R_h values were in the range of 12.1 - 14.5 nm and % Pd values 12 – 20%.

Conjugate **20** (Maleimidopeptide **6** + Lipopeptide **11**)

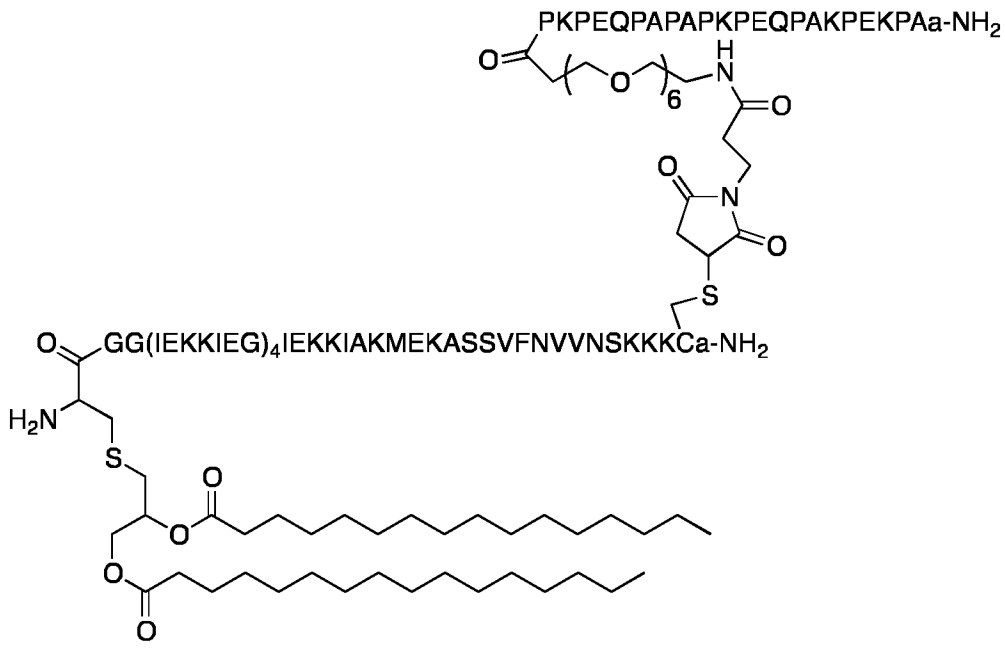


(**20**)

The conjugation of **6** to **11** and purification of the conjugate was performed essentially as described above for conjugate **14**. Product **20** was analyzed by reversed phase HPLC on a C4 analytical column (Interchrom, UP5WC4-25QS, 4.6 mm x 250 mm, 300 Å) and by MALDI-MS. Analytical RP-HPLC (C4 column, A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 20 to 100% B in 25 min.): Purity: >98%. t_R =22.34 min. ESI-MS: MW calc. for $C_{454}H_{773}N_{113}O_{129}S_3$ (succinimide ring hydrolysis): 9975.02 Da; found 9974.7 Da (± 0.01%).

A suspension of conjugate **20** in PBS was prepared and analyzed using DLS as described above for **14**. R_h values were in the range of 11.7 - 12.3 nm and % Pd values around 20%.

Conjugate **21** (Maleimidopeptide **4** + Lipopeptide **11**)

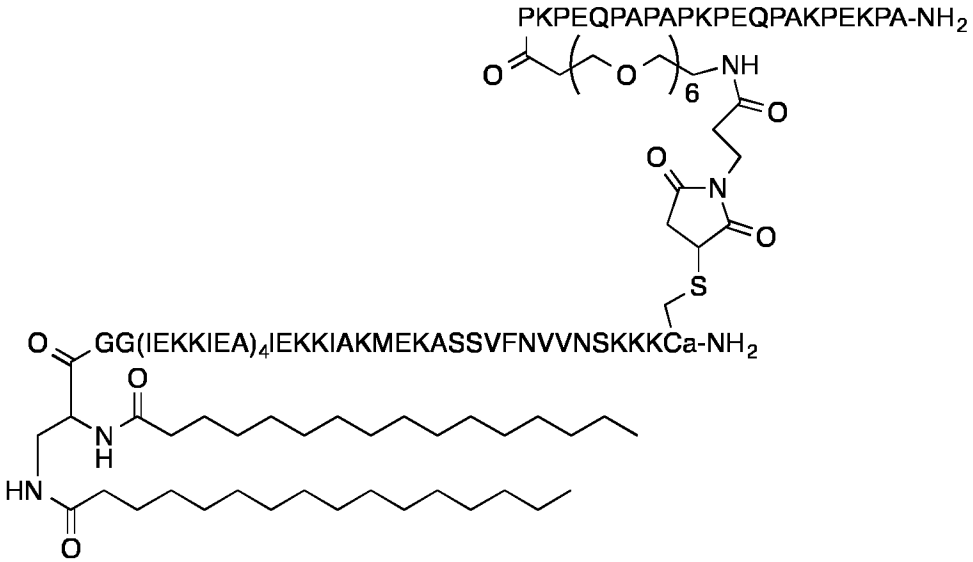


(**21**)

Maleimidopeptide **4** was conjugated to **11** and the conjugate was purified as described above for conjugate **14**. Analytical RP-HPLC (C4 column, A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 20 to 100% B in 25 min.): Purity: >95%. t_R = 17.78 min. MW calc. for $C_{439}H_{753}N_{105}O_{127}S_3$: 9630.5 Da; found 9631.2 Da.

DLS (0.5 mg/ml in PBS, 25°C): R_h = 10.7 nm; % Pd = 12 - 13%.

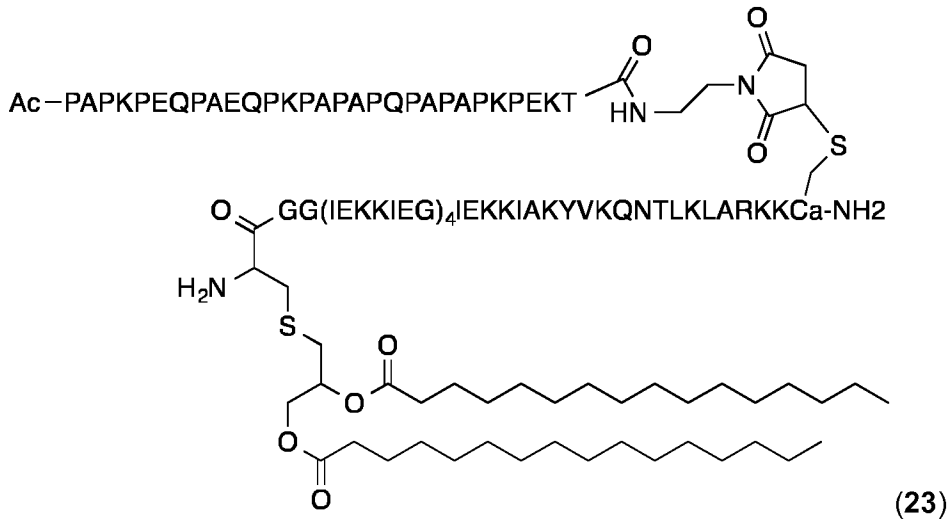
Conjugate **22** (Maleimidopeptide **6** + Lipopeptide **12**)



(**22**)

The maleimido peptide **4** was conjugated to **12** and the conjugate was purified as described above for conjugate **14**. Analytical RP-HPLC (C4 column, A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 20 to 100% B in 25 min.): Purity: >95%. t_R = 18.37 min. MALDI-TOF: MW calc. for $C_{440}H_{757}N_{107}O_{124}S_2$,: 9594.5 Da, found 9595.1 (± 0.1%) Da.
DLS measurements (0.5 mg in PBS, 25°) yielded R_h of 10.4 -11.1 nm and % Pd values of 10 - 12%.

Conjugate **23** (Maleimidopeptide **6** + Lipopeptide **13**)

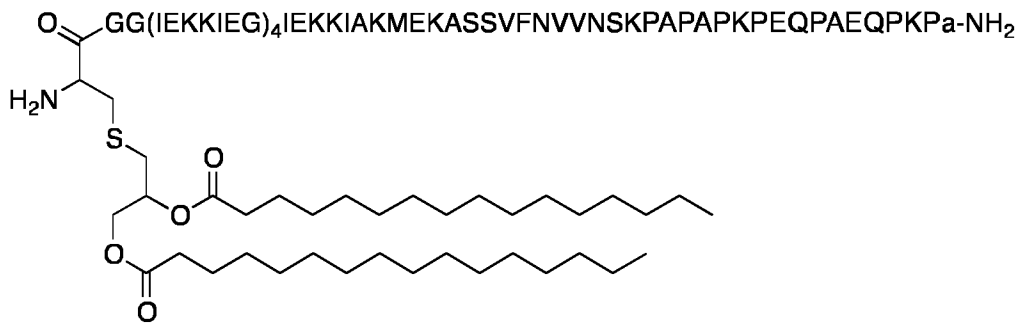


(**23**)

The maleimido peptide **6** was conjugated to **13** and the conjugate was purified as described above for conjugate **14**. Analytical RP-HPLC (C4 column, A = H_2O + 0.1 % TFA, B = MeCN + 0.1% TFA, 20 to 100% B in 25 min.): Purity: >95%. t_R = 18.25 min. MALDI-TOF: MW calc. for $C_{448}H_{765}N_{113}O_{123}S_2$ (succinimide ring hydrolysis): 9768.7 Da, found 9767.0 Da (± 0.1%).
DLS measurements (0.5 mg in PBS, 25°) yielded R_h of 9.9 -10.2 nm and %Pd values of 15 - 18%.

Additional Lipopeptides were prepared by fusing the N-terminus of the PR peptide antigen to the C-terminus of the lipopeptide building block. The following fusion lipopeptides were prepared.

Lipopeptide (**24**)

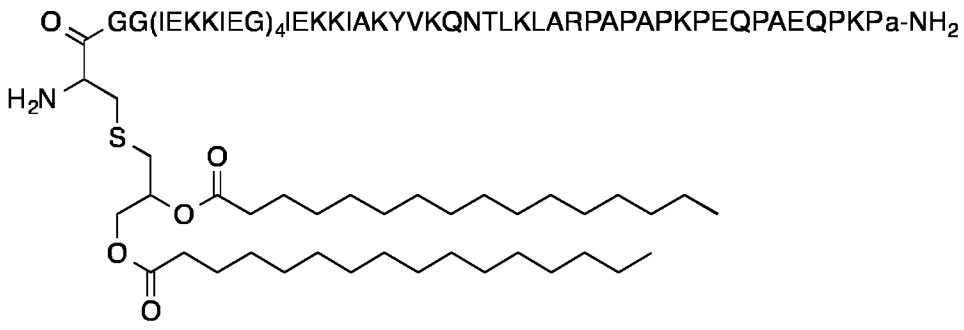


(**24**)

In this example the PR sequence P7, PAPAPKPEQPAEQPKP (SEQ ID NO: 33) was fused directly to the C-terminus of GG(IEKKIEG)$_4$IEKKIAKMEKASSVFNVVNSK (SEQ ID NO: 127) to yield the sequence GG(IEKKIEG)$_4$IEKKIAKMEKASSVFNVVNSKPAPAPKPEQPAEQPKP (SEQ ID NO: 128). The N-terminus was lipidated by addition of Pam2Cys and D-alanine ("a") was added to the C-terminus in lipopeptide **24.**

The fusion lipopeptide **24** was synthesized using conventional solid-phase peptide synthesis methods (W.C. Chan, P.D. White, Fmoc Solid Phase Peptide Synthesis: A Practical Approach, Oxford University Press, Oxford, UK, 2000) and purified as described above for lipopeptide **10**. MALDI-TOF MS: MW calc. for $C_{369}H_{633}N_{89}O_{104}S_2$: 8044.6 Da; found: 8044.6 Da (± 0.1%). Analytical RP-HPLC (Interchrom UP5WC4-25QS, 25 to 100% MeCN in H_2O (+ 0.1% TFA) over 25 min.): Purity > 98%, t_R = 20.48 min.
DLS (0.5 mg/ml in PBS, 25°C): R_h = 10.7 nm; % Pd = 12 - 13%.

Lipopeptide (**25**)



(**25**)

In this example the PR sequence P7, PAPAPKPEQPAEQPKP (SEQ ID NO: 33) was fused directly to the C-terminus of

GG(IEKKIEG)$_4$IEKKIAKYVKQNTLKLAR (SEQ ID NO: 129) to yield the sequence GG(IEKKIEG)$_4$IEKKIAKMEKASSVFNVVNSKPAPAPKPEQPAEQPKP (SEQ ID NO: 130). The N-terminus was lipidated by addition of Pam2Cys and D-alanine ("a") was added to the C-terminus in lipopeptide **25.**

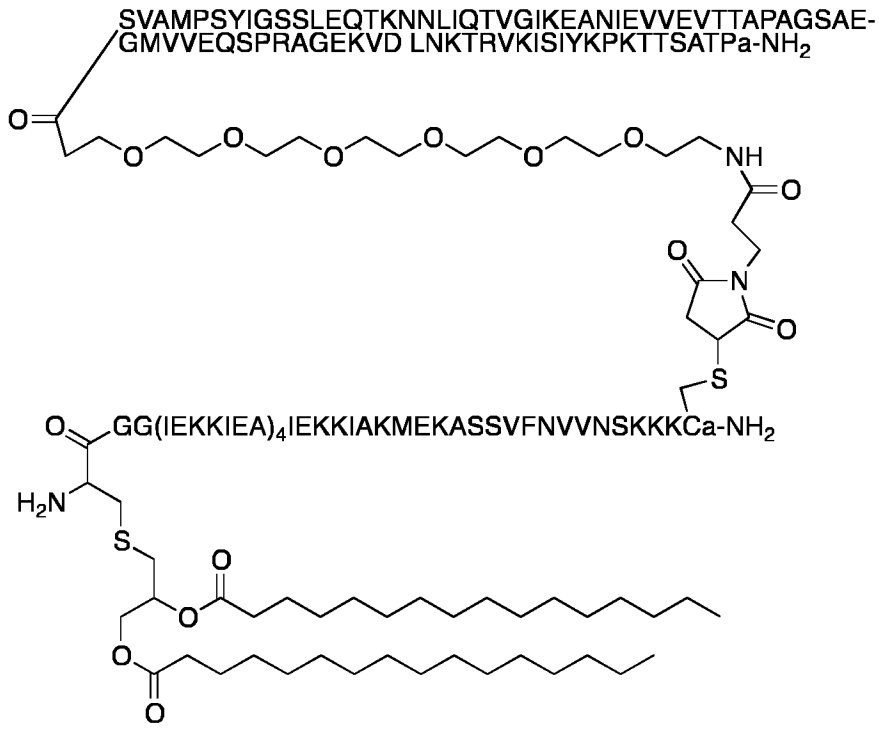
The fusion lipopeptide **25** was synthesized and purified as described above for **24**. ESI-MS: MW calc. for $C_{360}H_{639}N_{91}O_{99}S$: 7966.6 Da; found: 7967.0 Da (± 0.1%). Analytical RP-HPLC (Interchrom UP5WC4-25QS, 25 to 100% MeCN in H_2O (+ 0.1% TFA) over 25 min.): Purity > 98%, t_R = 21.41 min.

DLS (0.5 mg/ml in PBS, 25°C): R_h = 12.2 – 13.7 nm; % Pd = 10 - 15%.

Example 3: Preparation of Controls

The following control compounds were prepared for immunizations and challenge experiments.

Conjugate **26** (non PR conjugate)



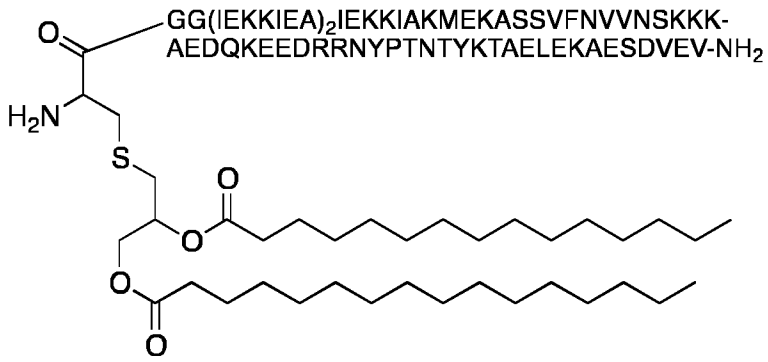
(**26**)

This conjugate contains the C-terminal part (StkP-C; PASTA + C-terminus) of StkP (SEQ ID NO: 115), except that 2 mutations (F594Q and I602T) were incorporated into the StkP

sequence to remove surface-exposed hydrophobic residues, resulting in sequence SVAMPSYIGSSLEQTKNNLIQTVGIKEANIEVVEVTTAPAGSAEGMVVEQSPRAGEKVDL NKTRVKISIYKPKTTSATP (SEQ ID NO: 131). The C-terminus was blocked with a-NH2, where "a" denotes D-alanine. This StkP peptide in the conjugate adopts a regular Penicillin-binding protein and Ser/Thr kinase Associated (PASTA) domain-structure by NMR.

The corresponding maleimidopeptide **27** (3-maleimidopropionyl)-21-amino-4,7,10,13,16,19-hexaoxaheneicosanoyl-(SEQ ID NO: 131)-a-NH_2) was synthesized and conjugated to **10** as described above for **14**. Conjugate **26** was analyzed by HPLC, MALDI-MS and DLS. Analytical RP-HPLC (Interchrom UP5WC4-25QS, 250 x 4.6 mm, A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 20 to 100% B in 25 min.): Purity: >95%. t_R =18.41 min. MALDI-TOF: MW calc. for $C_{700}H_{1201}N_{177}O_{216}S_5$: 15713.41Da; found 15715.4 Da (± 0.1%). DLS (0.5 mg/ml in PBS, 25°C): R_h = 16.3 nm, % Pd = 16.9%.

Lipopeptide **28**



(**28**)

In this lipopeptide the 31 amino acids at the C terminus correspond to amino acids 344 to 377 from PspC of TIGR4 (SEQ ID NO: 116), except that two mutations were incorporated into the antigen to improve solubility (L366A, I370K; TIGR4 PspC numbering), resulting in peptide AEDQKEEDRRNYPTNTYKTAELEKAESDVEV (SEQ ID NO: 132). The C-terminus was blocked with r-NH2, where "r" denotes D-arginine. This peptide is further coupled through a short linker (KKK) to the universal T-helper cell epitope CST.3* (SEQ ID NO: 23).

Lipopeptide **28** was synthesized and purified as described in WO 2008/068017, and was analyzed by HPLC, MALDI-MS and DLS. Analytical RP-HPLC (Interchrom UP5WC4-25QS, 250 x 4.6 mm, A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA, 20 to 100% B in 25

min.): Purity: >95%. t_R =18.41 min. MALDI-TOF: MW calc. for $C_{386}H_{654}N_{100}O_{121}S_2$: 8696.1 Da; found 8696.0 Da. DLS (0.5 mg/ml in PBS, 25°C): R_h = 7.9 nm; % Pd = 29%.

Recombinant PspA protein (rPspA)

The proline-rich region of the PspA from *S. pneumoniae* strain SP1577 was cloned and expressed as recombinant Trx fusion protein (rPspA). Cloning and expression of recombinant PR from the SP1577 strain was performed as described in WO 2007/089866. The purity and identity were confirmed by SDS-PAGE, dot-blot using anti-PspA antibodies and mass spectrometry. The sequence of the protein is:

MSDKIIHLTD DSFDTDVLKA DGAILVDFWA EWCGPCKMIA PILDEIADEY QGKLTVAKLN IDQNPGTAPK YGIRGIPTLL LFKNGEVAAT KVGALSKGQL KEFLDANLAG SGSGHMHHHH HHSSGLVPRG SGMKETAAAK FERQHMDSPD LGTDDDDKAM ADLKKAVNEP EKPAEET*PAP APKPEQPAEQ PKPAPAPQPA PAPKPEKTDD QQAEEDYARR SEEEYNRLPQ QQPPKAEKPA PAPKPEQPVP APKPEQPVPA PKT*GWKQE (SEQ ID NO: 133)

PspA proline-rich region including non-proline block are shown in italics.

Example 4: Mouse Immunization Studies

Conjugates were tested for immunogenicity against *S. pneumoniae* in mice. All experiments were performed in accordance with the Swiss rules and regulations for the protection of animal rights and have been approved by the responsible authorities.

For analysis of the antibody response outbred six to eight week-old female NMRI outbred mice (10 per group) were subcutaneously immunized two times in three-week intervals on days 0 and 21 with 0.1 ml of the formulations shown in Table 4. Control animals were immunized with PBS or rPspA + alum in saline. Blood was collected before the first and ten days after the second immunization and sera were analyzed using ELISA to determine titers of IgG antibodies to the proline-rich peptide, Western Blot to determine IgG to endogenous proteins and flow cytometry to determine surface binding of IgG to intact pneumococci.

Table 4. Formulations used for immunizations of mice

Formulation	Adjuvant	Concentration (mg/ml)[a)]
14 in PBS	None	0.2
15 in PBS	None	0.2
16 in PBS	None	0.2
17 in PBS	None	0.2
18 in PBS	None	0.2
19 in PBS	None	0.2
16 + **14** in PBS	None	0.2 + 0.2
rPspA in saline	Alum	0.2
PBS	None	-
[a)] Shown is the overall lipopeptide conjugate / protein concentration.		

For ELISA, MaxiSorp 96-well microtitre plates (Nunc, Fischer Scientific) were coated at 4°C overnight with 5 µl/ml solutions of PR peptides or rPspA in PBS, pH 7.2 (50 µl/well). The ELISA was performed essentially as described in WO 2008/068017, using goat anti-mouse IgG (γ-chain-specific) antibodies (Sigma, St. Louis, MO) and 1 mg/ml p-nitrophenyl phosphate (Sigma) for IgG detection. Endpoint titers were defined as the highest serum dilution for which the OD of the test sera was larger than the mean OD of PBS plus two SD.

For Western Blot analysis of immune sera, SP1577 was cultured in blood agar plates at 37°C, 5% CO_2 and total bacterial lysates were prepared. Lysates were separated by SDS-PAGE under reducing conditions and blotted onto nitrocellulose membranes. Blots were incubated with immune or pre-immune sera (1:500 in PBS) and developed using the ECL system. A PspA-specific monoclonal antibody was used as positive control.

For flow-cytometry analysis (FACS), SP1577 was cultured as described above, inactivated with formalin for 30 min., blocked with 5 mg/ml fatty-acid-free BSA in PBS and approximately 7 x 10^5 CFU were incubated with immune or pre-immune sera (1:100 in PBS) for 1 h at room temperature. Surface-bound IgG was detected using an Alexafluor 488-conjugated secondary antibody.

ELISA Geometric Mean Endpoint titers (GMT) ± one Standard Deviation of the Mean (SEM) and results from the Western Blot and FACS analyses (as positive vs. negative reactivity) are summarized in Table 5.

Table 5: IgG Response in mice after two immunizations

Immunogen	ELISA (GMT ± SEM)[a)]	Western Blot (Pos./Neg.)	FACS (Pos./Neg.)
14	26013 ± 30255	9/1	8/2
15	271227 ± 82014	10/0	10/0
16	65606 ± 13592	10/0	10/0
17	82066 ± 36672	8/2	6/4
18	10354 ± 6760	7/3	5/5
19	263069 ± 62452	7/3	7/3
14 + **16**[a)]	9027 ± 6150	10/0	10/0
14 + **16**[b)]	65606 ± 13592	10/0	10/0
rPspA + alum	212977 ± 65684	10/0	10/0
PBS	< 100	0/10	0/10

[a)] Pre-immune sera showed no significant reactivity in ELISA, Western Blots and FACS.
[b)] IgG response measured in ELISA against **14**
[c)] IgG response measured in ELISA against **16**

Although the antibody response was variable in the out-bred mice, all mice developed high titers of antigen-specific IgG as measured in ELISA and IgG in most of the immune sera bound to endogenous PspA and to intact SP1577 cells. No significant levels of antigen-specific IgG could be detected in pre-immune sera and in sera from PBS-immunized mice.

The cross-reactivity of the elicited IgG to was assessed using Western Blot and FACS and a panel of genetically diverse pneumococcal strains representing different PspA belonging to different clades from PspA families 1-3, which are defined in Hollingshead, Becker et al., *Infect Immun,* **2000**, *68*, 5889-5900, and different pneumococcal capsular serotypes, including serotypes that are not covered by the currently licensed pneumococcal conjugate vaccines.

Bacteria were cultured and Western Blot and FACS analyses were performed with sera from immunized mice as described above for SP1577. The results are summarized below in Table 6.

Table 6: Cross-reactivity of IgG in mouse sera with genetically diverse *Pneumococci*

Immunogen	Test [a)]	Strain /Serotype						
		SP1577 /1	SP920 /8	SP4408 /19A	SP1272 /2	SP1388 /4	SP1260 /4	SP716 /5
14	WBA	++	+	+	++	+	+	++
	FACS	++	+	++	++	+	++	++
15	WBA	++	++	++	++	+	++	++
	FACS	++	+	++	+	+	++	++
16	WBA	++	++	+	++	++	++	++
	FACS	++	++	++	++	++	++	++
17	WBA	++	-	-	+	++	-	-
	FACS	++	++	++	++	++	-	-
18	WBA	++	-	-	+	++	-	-
	FACS	++	++	++	++	++	-	-
19	WBA	+	-	-	+	++	-	+
	FACS	+	+	++	++	++	++	++
14 + 16	WBA	++	++	++	++	++	++	++
	FACS	++	++	++	++	++	++	++
rPspA + alum	WBA	++	++	++	++	++	++	++
	FACS	++	++	++	++	++	++	++

[a)] Sera after two immunizations were used. Pre-immune sera showed no reactivity in the assays. ++ = strong band/signal; + = weak band/signal; - = no binding could be detected.

The results obtained with the genetically diverse panel indicates that immunization with PR peptide antigens elicited broadly cross-reactive IgG, although some PR peptide antigen constructs elicited more broadly cross-reactive IgG than others.

For challenge studies NMRI outbred mice were immunized with different formulations as described above. For comparison of the immunogenicity additional animals were immunized with rPspA + alum, conjugate **44** or lipopeptide **46**. The mice were bled 10 days after the final immunization, sera were analyzed by ELISA as described above in order to determine seroconversion. The mice were then challenged intravenously (iv) *via* the tail vein with 100 times of a pre-determined lethal dose (100 x LD_{100}) *pneumococci*, which were rendered highly virulent by passage in mice prior to the challenge. Bacteria were either passaged by intraperitoneal (ip) or intravenous (iv) injection. After challenge, the health status of the mice was monitored over 14 days. Moribund animals were

euthanized and the time to moribund was recorded. The survival and time to moribund of the animals immunized with lipopeptides or the protein were compared to that of animals immunized with PBS alone.

Results obtained with strain SP1577 are summarized in below in Table 7. The SP1577 strain is highly virulent in mice. Unprotected mice died or became moribund within the first 12-24 h after challenge. Protection, therefore, indicates high efficacy.

Table 7: Protection of NMRI mice from lethal challenge with strain SP1577

Formulation	Passage	Days to moribund		P-value for survival[b)]
		Test	Control (PBS)	
14 in PBS	ip	1, 2, 3 x > 14	5 x 1	0.0143
14 in PBS	iv	2 x 1, 5, 2 x > 14	5 x 1	0.0495
15 in PBS	ip	1, 2, 3, 2 x > 14	5 x 1	0.0143
15 in PBS	iv	3 x 2, 2 x > 14	5 x 1	0.0027
16 + **14** in PBS	ip	1, 1.5, 4, 2 x > 14	5 x 1	0.0143
16 + **14** in PBS	iv	3 x 2, 6, 14	5 x 1	0.0027
18 in PBS	ip	1, 1.5, 3 x > 14	5 x 1	0.0143
19 in PBS	ip	2 x 2, 3 x > 14	5 x 1	0.0027
17+26+28 in PBS	ip	2 x 1, 3 x > 14	5 x 1	0.0495
rPspA + alum	ip	3 x 1, 2 x >14	5 x 1	NS
rPspA + alum	iv	3 x 1, 5, >14	5 x 1	NS
16 in PBS	iv	5 x 1	5 x 1	NS
26 in PBS	ip	5 x 1	5 x 1	NS
28 in PBS	ip	4 x 1, 1 x 3	5 x 1	NS

[b)] Shown is the P-value for the survival distribution compared to the distribution for mice immunized with PBS by Logrank test. NS = non-significant (P > 0.05).

Mice immunized with PR peptide antigens conjugated to SVLPs were partially protected and the median survival time was prolonged compared to that for PBS immunized mice and mice immunized with rPspA. The survival distributions obtained for mice immunized with SVLPs or rPspA were compared with that for mice immunized with PBS alone (negative control) by the Log-rank test. The results for mice immunized with PR conjugates, alone or in combination with other antigens, were significantly different to those for PBS immunized mice. The P-values were in the range of 0.0027-0.0143. The results for mice immunized with rPspA were not significantly different (P = 0.1336)

compared to that of PBS immunized mice, although some mice were protected. The difference may become more significant for larger groups. Results for StkP and PspC-derived conjugates were not significant from PBS, indicating that these antigens are not protective in this model.

For passive immunization/challenge experiments to determine if antibodies mediate the protection, monoclonal antibodies were generated in mice. In one experiment BALB/c mice were immunized three times with conjugate **17.** Seven B cell hybridoma lines producing antigen-specific monoclonal IgG antibodies (mAbs) were generated from spleen cells of one mouse and tested for cross-reactivity with different pneumococcal strains by FACS analysis. One mAb (5H8) bound to a broad range of strains (clinical isolates) representing different PspA clades and capsular serotypes. Other mAbs bound to the PR antigen in ELISA but not to intact bacteria. Epitope mapping indicated that mAb 5H8 recognized an epitope in the C-terminal part of the PR peptide, which occurs in a broad variety of different PspA sequences, whereas the other 6 mAbs recognized other epitopes, which are less frequently found in different PspA sequences. For the challenge 0.1 to 0.5 mg of purified 5H8 or other mAbs was administered to groups of 5 NMRI mice by iv injection. Animals passively immunized with an StkP-derived mAb (1A7) and naïve animals were used as controls. After sufficient time for equilibration the mice were challenged iv with passaged *pneumococci* and the health status and time to moribund was monitored as described above. Results obtained with strain SP1577 are shown in Table 8 below.

Table 8: Passive protection of NMRI mice from lethal challenge with strain SP1577

Dose (mg/ml)	mAb	Days to moribund		P-value for survival[a)]
		Test	Control	
0.5	5H8	1.5, 2, 3, 2 x >14	5 x 1	0.0027
0.1	5H8	2 x 1, 1 x 1.5, > 14	5 x 1	0.0495
0.5	3H5	5 x 1	5 x 1	NS
0.5	1H9	5 x 1	5 x 1	NS
0.5	1A7	5 x 1	5 x 1	NS

[a)] Shown is the P-value for the survival distribution compared to the distribution for naïve mice by Logrank test. NS = non-significant (P > 0.05).

Only mAb 5H8 gave significantly different results after passive immunization, compared to naïve mice. The protection was dose dependent. MAbs 3H5 and 1H9 did not show a

significant effect in this model of passive protection. Sequencing revealed that the PspA of strain SP1577 contains only the 5H8 epitope but not the epitopes of the other two PR-derived antibodies. No protection was also seen for the StkP-derived mAb 1A7 in this model.

The results demonstrate that immunization with SVLP-forming lipopeptides carrying proline-rich peptide antigens elicits highly *S. pneumoniae* cross-reactive antibodies in mice when administered alone or in combination with other antigens, without co-administration of an adjuvant.

Example 5: Rabbit Immunization Studies

In order to characterize the antibody response in non-rodents, New Zealand White rabbits were sc immunized three times on days 0, 28 and 56 with different concentrations of conjugates **17**, **18** or **19** in 0.4 ml PBS, without or with an adjuvant (R848) (Table 9). Blood samples were taken on days 0, 14, 38 and 66 to determine seroconversion.

Table 9: Formulations used for immunizations of rabbits

Group	Formulation	Adjuvant	Concentration (mg/ml)[a)]
1	**17** in PBS	None	0.35
2	**22** in PBS	None	0.35
3	**21** in PBS	None	0.1
4	**21** in PBS	None	0.025
5	**21** in PBS	R848	0.025
[a)] Shown is the lipopeptide concentration.			

Pre-immune sera and sera after the third immunization were analyzed by Western Blot and FACS using rabbit-IgG-specific secondary antibodies and various pneumococcal isolates as described above for the mice. The development of the IgG response was also analyzed by ELISA using a monoclonal anti-rabbit IgG (γ-chain specific) alkaline phosphatase antibody with 4-nitrophenyl-phosphate for IgG detection, essentially as described above. Results are shown in Table 10. All immunized rabbits developed IgG binding to endogenously expressed PspA and PspA expressed on intact pneumococci in response to the immunization. Pre-immune sera showed no significant reactivity in these assays. High titers of PR peptide-specific IgG antibodies were detected in the immune

sera from immunized rabbits, even after administration of low doses of the conjugate without an adjuvant.

Table 10: Development of the IgG response In NZW rabbits (N = 3)

Group	ELISA (GMT ± SEM)[a)]			Western Blot (Pos./Neg.)[b)]	FACS (Pos./Neg.)[b)]
	1st imm.	2nd imm.	3rd imm.		
1	4.01 ± 0.22	4.92 ± 0.15	4.66 ± 0.16	3/0	3/0
2	2.96 ± 0.05	4.13 ± 0.19	4.15 ± 0.08	3/0	3/0
3	< 2.3	4.47 ± 0.05	4.67 ± 0.16	3/0	3/0
4	< 2.3	2.84 ± 0.25	3.79 ± 0.34	3/0	3/0
5	< 2.3	3.70 ± 0.38	4.56 ± 0.08	3/0	3/0

[a)] Shown are log10 Geometric Mean Titers (GMT) ± one Standard Error of the Mean (SEM) after one (1st imm.), two (2nd imm.) or three (3rd imm.) immunizations.
[b)] Results for strain SP1577 are shown.

The results demonstrate that immunization with SVLP-forming lipopeptides carrying proline-rich peptide antigens elicits broadly cross-reactive antibodies in non-rodents also when administered without an adjuvant.

Example 6: Comparison of antigen-specific antibody responses in BALB/c mice

The antigen-specific antibody response elicited by conjugate **15** SVLPs was compared to that elicited with recombinant PspA protein. Six to eight week-old female BALB/c mice (18 per group) were subcutaneously immunized two times in three-week intervals on days 0 and 21 with 0.1 ml of conjugate **15** in PBS or rPspA + alum in saline prepared as described above in Example 5. Blood was collected ten days after the second immunization.

In order to determine the antigen specificity of the antibody response, ELISA were performed as described in Example 5, using PR peptide SEQ ID NO:27 as the coating antigen for the measurement of PR-specific antibodies and rPspA as the coating antigen for the measurement of total anti-PspA antibodies (i.e. antibodies to N-terminal epitopes, NPB and PR). The results are shown in Figure 1. Sera from non-immunized mice showed no cross-reactivity with either antigen in ELISA.

As expected rPspA was highly immunogenic (anti-PspA IgG GMT ± SEM = 81'969 ± 28'068) but failed to elicit significant levels of anti-PR antibodies (anti-PspA IgG GMT ± SEM = 213 ± 218). This was not due to a failure of antibodies raised against rPspA to bind to the PR peptide, since antibodies raised against conjugate **15** SVLPs recognized both, the PR peptide (anti-PspA IgG GMT ± SEM = 27'583 ± 6'204) and the rPspA antigen (20'919 ± 3'444) in ELISA. It is, therefore, likely that the majority of rPspA-elicited antibodies bind to epitopes in the N-terminal alpha helical part.

In order to determine whether PspA lacking the N-terminal alpha helical would elicit more PR-specific antibodies, mice were immunized with a truncated recombinant PspA-Trx fusion protein (rPspA-delta-N-term; contains PR and NPB). Surprisingly also the truncated protein failed to elicit significant levels of anti-PR antibodies: The GMT ± SEM was 128'496 ± 28'481 for the protein and 213 ± 1'094 for the PR antigen. This was significantly lower than for conjugate **15** SVLP-immunized control animals (GMT ± SEM of 32'776 ± 6974 for the protein and 47'679 ± 23'383 for the PR peptide). Together these results indicate that SVLPs elicit significantly higher levels of PR-specific antibodies than recombinant PspA.

Example 7: Protection from increasing challenge doses

In order to determine the protection of immunized BALB/c mice from increasing challenge doses, mice were immunized with conjugate **15** or rPspA as described above in Example 6, challenged intravenously with increasing doses of serotype 1 bacteria ranging from 10^2 to 10^6 CFU and monitored for survival. Standardized pneumococcal serotype 1 bacterial inocula were prepared as described in Aaberge I.S. *et al.*, *Microbial Pathogenesis*, **1995**, 18, 141-152. LD_{50} in non-immunized mice were verified by intravenously injecting BALB/c mice increasing doses of bacteria and monitoring for survival.

The protection of immunized mice was dependent on the bacterial challenge dose and the type of immunogen. At low challenge doses the protection was comparable for animals immunized with rPspA or lipopeptide building block **15** SVLPs (See Figure 2). At higher challenge doses (100–10'000 x LD_{50}), the mice immunized with conjugate **15** SVLPs were better protected than those immunized with rPspA. Together these results indicate that the anti-PR antibodies elicited by SVLPs protect over a wider range of bacterial challenge doses than antibodies raised against rPspA.

Claims

1. Lipopeptide building block consisting of
(1) a peptide chain comprising a parallel coiled-coil domain which, as a self-standing lipid-free peptide, forms a parallel dimeric, trimeric or higher order oligomeric helical bundle,
(2) a proline-rich peptide antigen comprising at least one negatively and at least one positively charged amino acid, and wherein at least 15% of the amino acids are proline, optionally linked to a further antigen, and
(3) a lipid moiety comprising two or three long hydrocarbyl chains,
wherein the peptide chain, the proline-rich peptide antigen and the lipid moiety are covalently linked, either directly or through a linker.

2. Lipopeptide building block according to claim 1 wherein the peptide chain comprises between 21 and 200 amino acid residues.

3. Lipopeptide building block according to claim 1 or 2 wherein the peptide chain comprises a coiled-coil domain consisting of three to eight heptad motifs.

4. Lipopeptide building block according to claim 3 wherein in the coiled-coil domain positions *a* and *d* in each heptad motif (*abcdefg*) comprise alpha-amino acids with small to medium-sized hydrophobic side chains and/or aromatic or heteroaromatic side chains, in zero, one or two of all the *a* and *d* positions an amino acid with a polar non-charged residue and in zero or one of all the *a* and *d* positions an amino acid with a polar cationic residue or an acylated derivative thereof, or with a polar anionic residue, or glycine.

5. Lipopeptide building block according to claim 4 wherein
alpha-amino acids with small to medium-sized hydrophobic side chain are alanine, isoleucine, leucine, methionine and valine;
alpha-amino acids with aromatic or heteroaromatic side chain are phenylalanine, tyrosine, tryptophan and histidine;
alpha-amino acids with polar non-charged residue are asparagine, cysteine, glutamine, serine and threonine;
alpha-amino acids with polar cationic residue are arginine, lysine and histidine; and
alpha-amino acids with polar anionic residue are aspartic acid and glutamic acid.

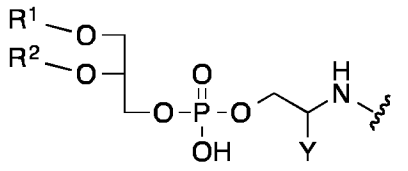
6. Lipopeptide building block according to any one of claims 1 to 5 wherein the proline-rich peptide antigen comprises at least one glutamic acid residue and at least one lysine or arginine residue.

7. Lipopeptide building block according to any one of claims 1 to 6 wherein the proline-rich peptide antigen is derived from proteins of *Streptococci* and/or *Staphylococci*.

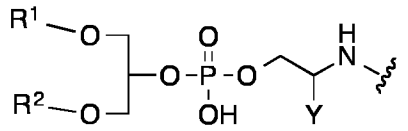
8. Lipopeptide building block according to any one of claims 1 to 6 wherein the proline-rich peptide antigen is derived from proteins PspA and/or PspC.

9. Lipopeptide building block according to any one of claims 1 to 8 wherein the proline-rich peptide antigen comprises a peptide of SEQ ID NO:27 to 112, and such peptides in which one, two or three amino acids are replaced by other amino acids.

10. Lipopeptide building block according to any one of claims 1 to 9 wherein the lipid moiety is one of types Z^1 to Z^8

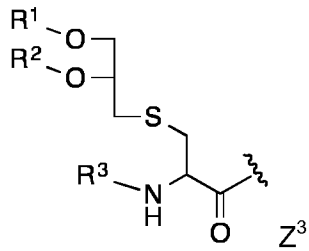


Z^1



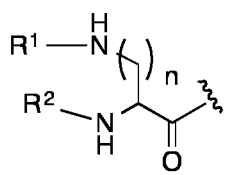
Z^2

wherein R^1 and R^2 are long hydrocarbyl or long hydrocarbyl-C=O and Y is H or COOH,

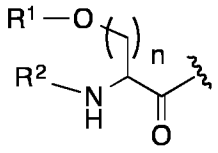


Z^3

wherein R^1, R^2 and R^3 are long hydrocarbyl or long hydrocarbyl-C=O or R^1 and R^2 are long hydrocarbyl or long hydrocarbyl-C=O and R^3 is H or acetyl or lower alkyl-C=O,

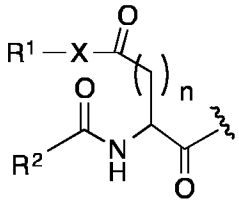


Z^4



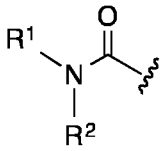
Z^5

wherein R^1 and R^2 are long hydrocarbyl or long hydrocarbyl-C=O and n is 1, 2, 3 or 4,

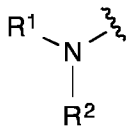


Z^6

wherein R^1 and R^2 are long hydrocarbyl, X is O or NH, and n is 1, 2, 3 or 4, or



Z^7



Z^8

wherein R^1 and R^2 are long hydrocarbyl.

and wherein long hydrocarbyl is straight or branched alkyl or alkenyl consisting of between 8 and 25 carbon atoms and optionally one, two or three double bonds in the chain.

11. Lipopeptide building block according to claim 10 wherein the lipid moiety is di-palmitoyl-S-glycerylcysteinyl of formula **Z**^{3}, wherein R^1 and R^2 are palmitoyl and R^3 is H or acetyl.

12. Lipopeptide building block according to any one of claims 1 to 11 wherein the peptide chain comprising a parallel coiled coil is linked at one end to the PR peptide antigen and at the other end to the lipid moiety.

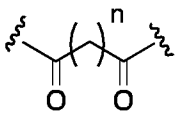
13. Lipopeptide building block according to claim 12 wherein the peptide chain PC is covalently linked to the lipid moiety LM at or near one terminus of the peptide chain either directly as in

LM – PC (**1**)

or *via* a linker (L) as in

LM – L – PC (**2**)

wherein linker L is selected from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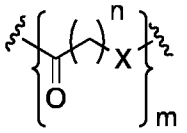
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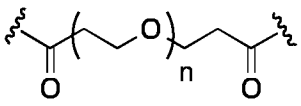
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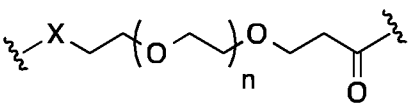
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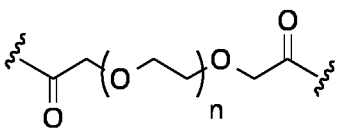
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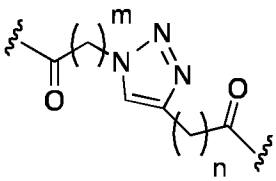
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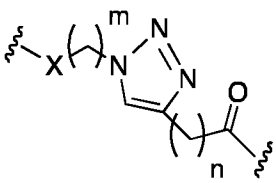
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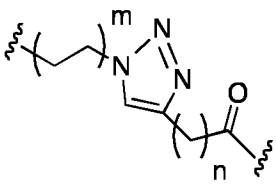
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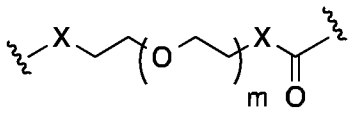
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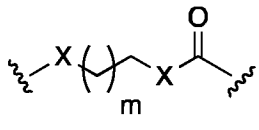
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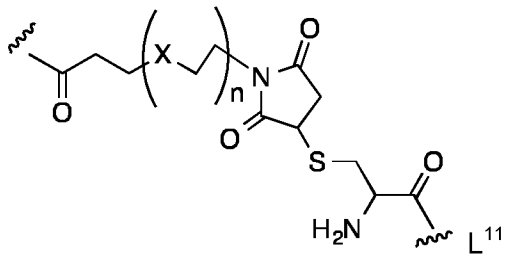
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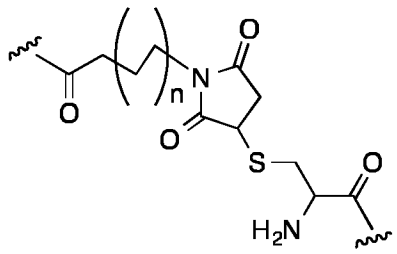
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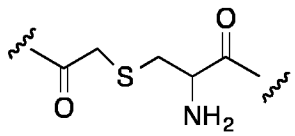
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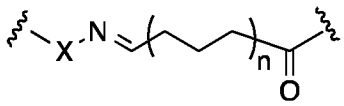
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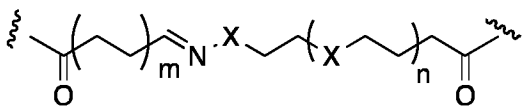
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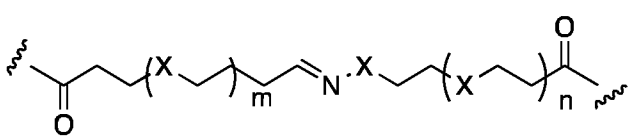

L16

wherein X is O or NH, m is between 1 and 45 and n is between 1 and 45.

14. Synthetic virus-like particles consisting of helical lipopeptide bundles comprising two, three, four, five, six or seven lipopeptide building blocks according to any one of claims 1 to 13.

15. A vaccine comprising a synthetic virus-like particle according to claim 14.

16. A method of vaccination against a disease caused by Gram-negative bacteria wherein an immunogenically effective amount of a synthetic virus-like particle according to claim 14 is administered to a patient in need thereof.

Fig. 1

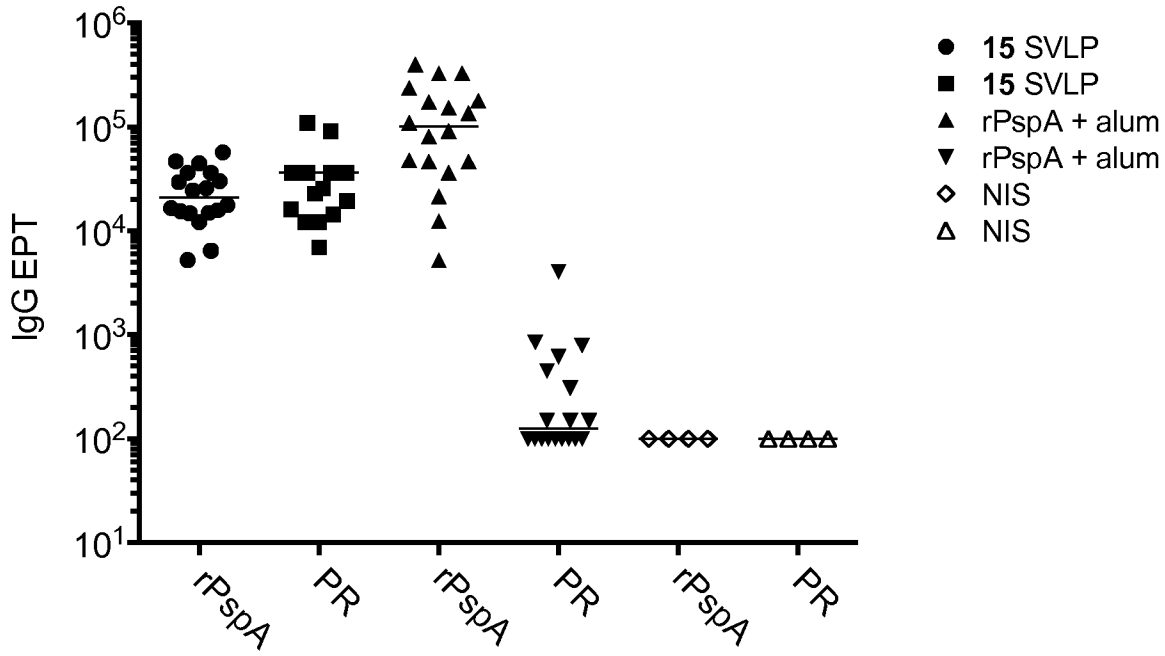
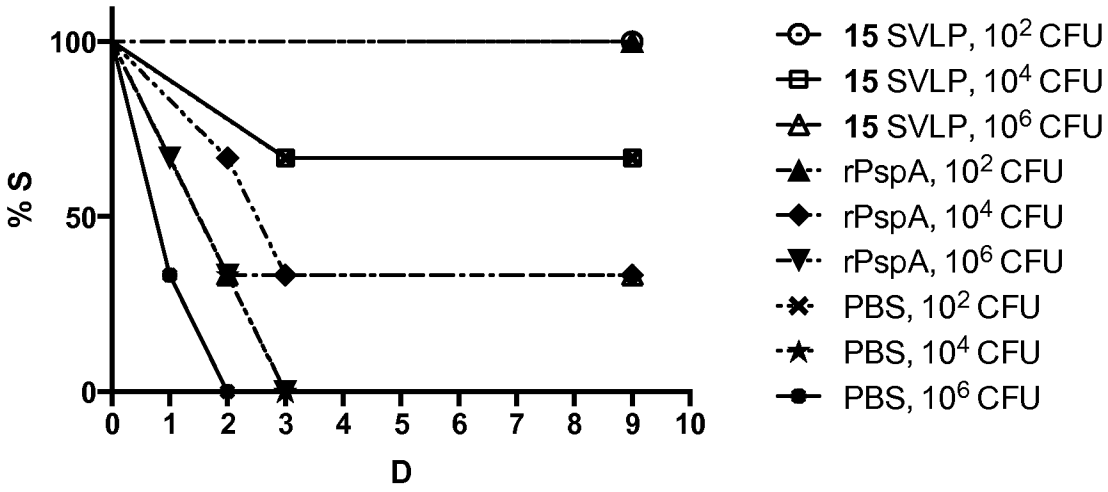

IgG EPT
10^6
10^5
10^4
10^3
10^2
10^1
rPspA
PR
rPspA
PR
rPspA
PR
15 SVLP
15 SVLP
rPspA + alum
rPspA + alum
NIS
NIS

Fig. 2


% S
100
50
0
0 1 2 3 4 5 6 7 8 9 10
D
15 SVLP, 10^2 CFU
15 SVLP, 10^4 CFU
15 SVLP, 10^6 CFU
rPspA, 10^2 CFU
rPspA, 10^4 CFU
rPspA, 10^6 CFU
PBS, 10^2 CFU
PBS, 10^4 CFU
PBS, 10^6 CFU

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/076313

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/315 A61K39/09 A61K39/085
ADD. A61K39/00

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 C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LANGERMANN S ET AL: "PROTECTIVE HUMORAL RESPONSE AGAINST PNEUMOCOCCAL INFECTION IN MICE ELICITED BY RECOMBINANT BACILLE CALMETTE-GUERIN VACCINES EXPRESSING PNEUMOCOCCAL SURFACE PROTEIN A", THE JOURNAL OF EXPERIMENTAL MEDICINE, ROCKEFELLER UNIVERSITY PRESS, US, vol. 180, no. 6, 1 December 1994 (1994-12-01), pages 2277-2286, XP000918247, ISSN: 0022-1007, DOI: 10.1084/JEM.180.6.2277	1-9
Y	the whole document	1-16

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

[X] See patent family annex.

* Special categories of cited documents :

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"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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 February 2015	17/02/2015

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

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	YOTHER J ET AL: "STRUCTURAL PROPERTIES AND EVOLUTIONARY RELATIONSHIPS OF PSPA, A SURFACE PROTEIN OF STREPTOCOCCUS PNEUMONIAE, AS REVEALED BY SEQUENCE ANALYSIS", JOURNAL OF BACTERIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, DC; US, vol. 174, no. 2, 1 January 1992 (1992-01-01), pages 601-609, XP000600024, ISSN: 0021-9193 the whole document	1-16
X	WO 2012/100233 A1 (GENOCEA BIOSCIENCES INC [US]; CHILDRENS MEDICAL CENTER [US]; PATH VACC) 26 July 2012 (2012-07-26) cited in the application	1-9
Y	the whole document page 54, line 22 - page 55, line 15; claims; examples	1-16
X	WO 96/40290 A1 (CONNAUGHT LAB [US]) 19 December 1996 (1996-12-19)	1-9
Y	the whole document claims; examples 3,5	1-16
Y	WO 2008/068017 A1 (UNIV ZUERICH PROREKTORAT FORSC [CH]; UNIV ZUERICH [CH]; BOATO FRANCESC) 12 June 2008 (2008-06-12) cited in the application the whole document	1-16
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Y	MERIT MELIN ET AL: "Development of cross-reactive antibodies to the proline-rich region of pneumococcal surface protein A in children", VACCINE, vol. 30, no. 50, 13 October 2012 (2012-10-13), - 1 November 2012 (2012-11-01), pages 7157-7160, XP055115762, ISSN: 0264-410X, DOI: 10.1016/j.vaccine.2012.10.004 the whole document	1-16
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A	BERNDT P ET AL: "SYNTHETIC LIPIDATION OF PEPTIDES AND AMINO ACIDS: MONOLAYER STRUCTURE AND PROPERTIES", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, ACS PUBLICATIONS, US, vol. 117, no. 37, 20 September 1995 (1995-09-20), pages 9515-9522, XP002030763, ISSN: 0002-7863, DOI: 10.1021/JA00142A019 the whole document	1-16
Y	RAJNI SHARMA ET AL: "Synthetic Virus-Like Particles Target Dendritic Cell Lipid Rafts for Rapid Endocytosis Primarily but Not Exclusively by Macropinocytosis", PLOS ONE, vol. 7, no. 8, 14 August 2012 (2012-08-14) , page e43248, XP55167520, DOI: 10.1371/journal.pone.0043248 the whole document	1-16

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International application No
PCT/EP2014/076313

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TINA RIEDEL ET AL: "Synthetic Virus-Like Particles and Conformationally Constrained Peptidomimetics in Vaccine Design", CHEMBIOCHEM, vol. 12, no. 18, 11 November 2011 (2011-11-11), pages 2829-2836, XP55167514, ISSN: 1439-4227, DOI: 10.1002/cbic.201100586 the whole document -----	1-16

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/076313

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