17-(Cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxy-6-hydrazidomorphinans (10–12). To a solution containing 14 (50 mg, 0.14 mmol) and fatty acid (0.15 mmol) in dimethylformamide (5 mL) was added DMAP (1.7 mg, 0.15 mmol) and DCC (37 mg, 0.18 mmol). After 3 h at 23 °C, the mixture was filtered, and the filtrate was chromatographed on a silica gel column using ethyl acetate–hexane (60:30). The common feature of the ^{1}H NMR for the β-hydrazide derivatives was the doublet corresponding to C5-H at δ 4.5 (J(5,6) = 7.43–7.62 Hz).

Binding Assay. Membrane Preparation. Male mice (20–25 g) were decapitated and the brains minus the cerebellum were removed and frozen immediately on dry ice. They were weighed and homogenized in 10 volumes of 0.32 M sucrose in a Duall tissue grinder (Kontes) homogenizer with the pestle driven by a motor at about 2000 rpm. The homogenate was centrifuged at 1000g in 16-mL polypropylene tubes in a Superspeed centrifuge for 10 min. The supernatant was centrifuged for 30 min at 17000g. The pellet obtained was osmotically shocked with three volumes of ice-cold distilled water and centrifuged for 20 min at 12000g. The supernatant was carefully decanted and diluted 1:1 with pH 7.4 Hepes buffer (NaCl, 118 mM; KCl, 4.8 mM; $CaCl_2$, 2.5 mM; $MgCl_2$, 1.2 mM; Hepes, 25 mM) and centrifuged for 30 min at 40000g. This final pellet was resuspended in Hepes buffer, diluted to four volumes of the initial wet weight, and either used immediately or saved at −80 °C for further experiments.

Binding Procedures. Membrane homogenate (0.4 mL) and 0.025 mL of unlabeled ligand (50 nM) in distilled water were added to a 1.5-mL polypropylene microfuge tube (Kontes), incubated for 30 min at 25 °C, and centrifuged in a Fisher Microcentrifuge at 11 000 rpm for 5 min. The capped tubes then were inverted and spun at 800g for 30 s on a rotating wheel to separate the pellet and supernatant. For each wash cycle, the membrane was resuspended in 30 μL of fresh Hepes buffer with the aid of a special pestle (Kontes), diluted with an additional 1 mL of buffer, incubated for 10 min at 37 °C, and recentrifuged. To estimate the remaining apparent receptor occupancy by the ligand, the washed sample was resuspended in a mixture of 200 μL of buffer containing [^{3}H]dihydromorphine (67 Ci/mmol, final concentration 2 nM) and 25 μL of either buffer alone or buffer with unlabeled naltrexone (final concentration 1 μM). This mixture was incubated for 20 min at 25 °C and centrifuged, and the supernatant was removed as described above. The tips of the tubes containing the membrane pellet were cut off and transferred to a scintillation vial. The pellet was emulsified in Biofluor and the radioactivity counted with a Beckman LS 6800 liquid scintillation counter at an efficiency of about 50%. The apparent receptor occupancy after each wash cycle was determined from the change in the specific binding of [^{3}H]dihydromorphine to membranes treated with fatty acyl ligand as compared to specific binding of membranes in a control experiment without ligand present. Specific binding is defined as the difference in cpm for membranes incubated with [^{3}H]dihydromorphine in the presence and in the absence of naltrexone.

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Registry No. **4a**, 134031-51-9; **4b**, 134031-59-7; **5a**, 134107-40-7; **5b**, 117250-89-2; **6a**, 134031-52-0; **6b**, 134031-60-0; **7a**, 134031-53-1; **7b**, 134054-98-1; **8a**, 134031-54-2; **8b**, 134107-41-8; **9a**, 134031-55-3; **9b**, 134107-42-9; **10**, 134031-56-4; **11**, 134031-57-5; **12**, 134031-58-6; **13a**, 84774-95-8; **13b**, 67025-97-2; **14**, 124206-70-8; acetic acid, 64-19-7; oleic acid, 112-80-1; linolenic acid, 463-40-1; stearic acid, 57-11-4; palmitic acid, 57-10-3; linoleic acid, 60-33-3.

Lipopeptides Containing 2-(Palmitoylamino)-6,7-bis(palmitoyloxy)heptanoic Acid: Synthesis, Stereospecific Stimulation of B-Lymphocytes and Macrophages, and Adjuvanticity in Vivo and in Vitro

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Lipopeptides, carrying the N-terminal lipoamino acid 2-(palmitoylamino)-6,7-bis(palmitoyloxy)heptanoic acid (Pam_3Adh-OH, **1**), were obtained by solid-phase synthesis and by synthesis in solution. 2-Amino-6,7-dihydroxyheptanoic acid (Adh) can be regarded as a methylene analogue of *S*-glycerylcysteine, the N-terminal amino acid of lipoprotein from the outer cell membrane of *Escherichia coli* (a methylene group substitutes for the sulfur atom). The lipopeptides Pam_3Adh-Ser-Ser-Asn-Ala **2a–d**, in which the four possible stereoisomers of Pam_3Adh-OH (2*S*,6*S*)-**1** (**1a**), (2*S*,6*R*)-**1** (**1b**), (2*R*,6*S*)-**1** (**1c**), and (2*R*,6*R*)-**1** (**1d**) are linked to the naturally occurring sequence Ser-Ser-Asn-Ala of the N-terminus of lipoprotein, and also Pam_3Adh-Ser-$(Lys)_4$ ((2*S*,6*S*)-**3**), with a peptide part rendering the molecule water soluble, were capable of stimulating murine splenocytes polyclonally in vitro, as determined in a proliferation assay and in a hemolytic plaque assay against trinitrophenylated sheep erythrocytes. The diastereomers (2*S*,6*S*)-**2** and (2*R*,6*S*)-**2** with *S*-configurated C-6 were more active than the diastereomers (2*S*,6*R*)-**2** and (2*R*,6*R*)-**2** with *R*-configurated C-6; a change of the configuration at C-2 had less effect on the stimulatory activity. (2*S*,6*S*)-**2** and (2*S*,6*S*)-**3** are potent immunoadjuvants. A significantly enhanced primary immune response against trinitrophenylated sheep erythrocytes was obtained in vitro at lipopeptide concentrations of about 5 μg/mL and an immunization dose of 10^7 sheep erythrocytes/mL. Balb/c mice, which were immunized with a mixture of ovalbumin and (2*S*,6*S*)-**2** or (2*S*,6*S*)-**3**, respectively, had a substantially higher antiovalbumin titer 28 days after immunization than mice which had received ovalbumin, (2*S*,6*S*)-**2** or (2*S*,6*S*)-**3** alone. Finally, the novel lipopeptides constitute potent macrophage activators: (2*S*,6*S*)-**3** was able to induce tumor cytotoxicity against the tumor cell line L929 in bone marrow derived macrophages.

Synthetic lipopeptides derived from the N-terminus of lipoprotein from the outer cell membrane of *Escherichia coli* are macrophage activators[1,2] and B-lymphocyte stimulants.[3–5] Conjugates in which these lipopeptides are covalently coupled to antigens or mixtures of lipopeptides and antigens induce antigen-specific antibodies in vivo and

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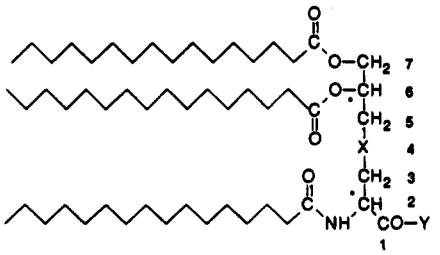
Scheme I



X = S, Y = OH:
N-palmitoyl-*S*-[2,3-bis(palmitoyloxy)propyl]-(R)-cysteine (Pam_3Cys-OH)
X = CH_2, Y = OH:
2-palmitoylamino-6,7-bis(palmitoyloxy)heptanoic acid (Pam_3Adh-OH); **1a**, 2*S*,6*S*; **1b**, 2*S*,6*R*; **1c**, 2*R*,6*S*; **1d**, 2*R*,6*R*
X = CH_2, Y = Ser-Ser-Asn-Ala: **2a**, 2*S*,6*S*; **2b**, 2*S*,6*R*; **2c**, 2*R*,6*S*; **2d**, 2*R*,6*R*
X = CH_2, Y = Ser-$(Lys)_4$: **3**, 2*S*,6*S*

in vitro.[6-11] Recently a novel low molecular mass synthetic vaccine against foot-and-mouth disease was developed, which contained tripalmitoyl-*S*-glycerylcysteinylserylserine as built-in adjuvant linked to an α-helical B- and T-cell epitope of the virus protein VP1.[12] It could also be shown that lipopeptide vaccines carrying killer cell epitopes can effectively prime virus specific cytotoxic T-lymphocytes in vivo.[13] The essential part of these immunologically active synthetic lipopeptides is the lipoamino acid *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)propyl]-(*R*)-cysteine (Pam_3Cys-OH). It can be obtained in a six-step synthesis as a mixture of diastereomers or diastereomerically pure.[3,14]

In order to determine the molecular requirements for biological activity of lipopeptide analogues of bacterial lipoprotein, we have synthesized and compared many *S*-glycerylcysteinyl peptides so far. We could show that the capability of polyclonal activation of murine splenocytes by lipopeptides depends on the molecular structure as follows: (i) the minimal structure for biological activity is the lipodipeptide Pam_3Cys-Ser,[15] the lipoamino acid Pam_3Cys-OH is almost inactive;[4] (ii) many variations of the peptide part (sequence, length) are possible without loss of activity;[5,15,16] however, side chain protecting groups reduce activity;[15] (iii) the differences in activity of lipopeptides differing in the length of the acyl chains (C-8 to C-18) attached to the *S*-glycerylcysteinyl moiety are only marginal (Metzger, J. et al., unpublished results); (iv) lipopeptides with less (Wiesmüller, K.-H. et al., unpublished results) or more[17] than three acyl chains have reduced or no activity; (v) lipopeptides with *R* configuration at the asymmetric carbon atom of the glyceryl part are more active than those with *S* configuration.[18]

Here, we describe the synthesis of lipopeptides containing 2-(palmitoylamino)-6,7-bis(palmitoyloxy)heptanoic acid (Pam_3Adh-OH, **1**), in which a CH_2 group substitutes for the sulfur atom in the tripalmitoyl-*S*-glycerylcysteinyl residue (Scheme I). The four possible stereoisomers of this novel lipoamino acid (2*S*,6*S*)-**1** (**1a**), (2*S*,6*R*)-**1** (**1b**), (2*R*,6*S*)-**1** (**1c**), and (2*R*,6*R*)-**1** (**1d**) were prepared by stereoselective synthesis[19] and were linked to the tetrapeptide Ser-Ser-Asn-Ala, the natural N-terminal sequence of lipoprotein. Comparison of the biological activity of the four resultant diastereomers **2a–d** should reveal a possible stereospecifity, which is important for understanding the molecular requirements for activation by these lipopeptides. As mentioned above, investigations showed that the respective tripalmitoyl-*S*-glycerylcysteinyl peptide Pam_3Cys-Ser-Ser-Asn-Ala with *R* configuration at the asymmetric carbon atom of the *S*-glyceryl part are more active than those with *S* configuration.[18] As *S*-alkylcysteinyl compounds have a rather high tendency to racemize at C_α under coupling conditions (Metzger, J. et al., unpublished results), it is difficult to obtain the four possible diastereomeric *S*-glycerylcysteinyl peptides in pure form. This problem was not encountered with the methylene analogues, a fact that makes them superior in studies of stereoselective activation.

As the lipohexapeptide Pam_3Cys-Ser-$(Lys)_4$ had already proven to be one of the most biologically active and interesting lipopeptides investigated by us so far,[5,10,20] we also synthesized the corresponding water-soluble methylene analogue $Pam_3CysAdh$-Ser-$(Lys)_4$ **3** with 2*S*,6*S* configuration. The four basic lysine residues in the peptide part of (2*S*,6*S*)-**3** are responsible for its amphiphilicity and solubility in water. Water solubility facilitates the handling of lipopeptides in biological tests. **2a–d** can only be solubilized in aqueous solutions by ultrasound sonication, whereas **3** has a water solubility of ca. 0.5 mg/mL (without sonication). The adjuvanticity of the Pam_3Adh peptides **2a–d** and (2*S*,6*S*)-**3** and their capability of stereoselectively activating B-lymphocytes and macrophages were investigated and compared with corresponding Pam_3Cys peptides.

Synthesis of the Lipopeptide Immunoadjuvants

Lipopeptides **2a–d**, consisting of one of the four possible stereoisomers of the N-terminal lipoamino acid 2-(palmitoylamino)-6,7-bis(palmitoyloxy)heptanoic acid **1a–d** and a peptide part identical with the natural sequence of the N-terminus of lipoprotein from *E. coli*, were syn-

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thesized. The synthesis of **1a–d** was published recently.[19] **2a–d** are analogues of the synthetic lipopentapeptide Pam_3Cys-Ser-Ser-Asn-Ala (TPP, tripalmitoylpentapeptide) which was found to have stimulating properties comparable to natural lipoprotein.[3,4]

The tetrapeptide Ser-Ser-Asn-Ala was built up on an acid-labile *p*-benzalkoxybenzyl alcohol polystyrene resin cross-linked with 1% divinylbenzene by using N_α-Fmoc-(*S*)-amino acids with *O-tert*-butyl side chain protection for serine. The synthesis cycles were performed on a fully automatic peptide synthesizer. The respective stereoisomer of the lipoamino acid **1a–d** was coupled with *N,N'*-dicyclohexylcarbodiimide/1-hydroxybenzotriazol (DCC/HOBt) to the resin-bound N_α-deprotected tetrapeptide. After 6 h a ninhydrin test of the resin indicated the absence of free amino groups and the completeness of the coupling. Coupling yields were high, which shows that the three fatty acids of the lipoamino acid do not cause any sterical hindrance during coupling. The lipopentapeptides **2a–d** were cleaved from the resin with thioanisol/trifluoroacetic acid (1:9) and isolated by precipitation from chloroform/methanol (1:1).

Since Pam_3Cys-Ser-$(Lys)_4$ had proven to be a potent water-soluble immunoadjuvant[5,10] and an activator of macrophages[1,2] and human neutrophils[20] we also synthesized the corresponding methylene analogue (2*S*,6*S*)-**3** of this lipohexapeptide. The pentapeptide Z-Ser(tBu)-$[Lys(Boc)]_4$-OtBu **4** was built up by using N_α-benzyloxycarbonyl amino acids and Boc/tBu side chain protection. After preparation of H-$[Lys(Boc)]_4$-OtBu,[21] Z-Ser(tBu)-OH was coupled to this tetrapeptide ester by using the DCC/HOBt method. The Z group of **4** was removed by hydrogenation on palladium/charcoal. The fully protected lipopeptide **6** was obtained in high yield by DCC/HOBt coupling of the lipoamino acid (2*S*,6*S*)-**1** to the pentapeptide ester H-Ser(tBu)-$[Lys(Boc)]_4$-OtBu **5**. Finally blocking groups were removed in thioanisol/trifluoroacetic acid (1:9). Precipitation from acetone yielded pure lipohexapeptide (2*S*,6*S*)-**3**.

Lipopeptides **2a–d** and (2*S*,6*S*)-**3** tend to aggregate in aqueous solutions. This tendency and the poor solubility of **2a–d** in commonly used solvents restrict the possibilities of purifying these lipopeptides by adsorption or size-exclusion chromatography; because of solubility reasons and a strong interaction of the fatty acid chains with reversed-phase material, reversed-phase HPLC, which is standard for the purification of peptides, cannot be used for most lipopeptides. However, precipitation with suitable solvents or solvent mixtures yields pure compounds, because impurities (coupling reagents, free peptides, which are not conjugated to the lipoamino acid, etc.) are generally more soluble than the lipopeptides and remain in the mother liquor. The purity of lipopeptides **2a–d** and **3** was checked by ion-spray mass spectrometry (cf. Figure 2), which allows detection of impurities down to 1% (Metzger, J.; Jung, G., unpublished results). Besides ion-spray mass spectrometry, the synthetic protected and unprotected lipopeptides and peptide segments were characterized by elemental and amino acid analysis, thin-layer chromatography and field-desorption mass spectrometry (Figure 1). On the analogy of the corresponding Pam_3Cys residue,[2] Pam_3Adh forms a heterogeneous mixture after acidic hydrolysis with 6 N HCl (Metzger, J. et al., unpublished results). These products could not be quantified by gas chromatography.

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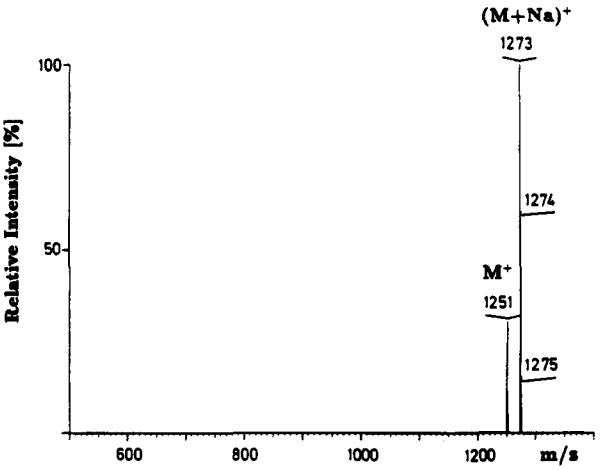

Figure 1. Field-desorption mass spectrum of (2*S*,6*S*)-Pam_3Adh-Ser-Ser-Asn-Ala (**2d**; calculated molecular mass 1251.8). Almost identical spectra were obtained from the other diastereomers **2a–c**.

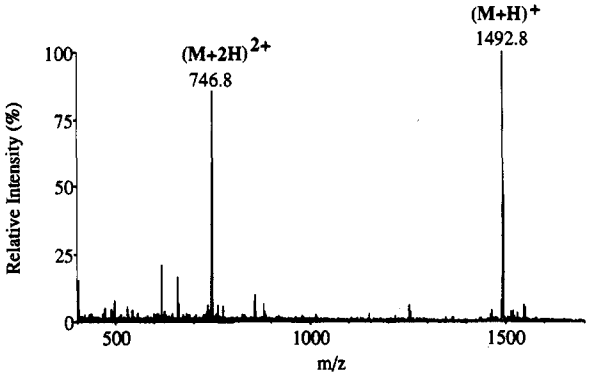

Figure 2. Ion-spray mass spectrum of (2*S*,6*S*)-Pam_3Adh-Ser-$(Lys)_4$ (**3**; calculated molecular mass 1492.2). The spectrum shows the singly charged $(M + H)^+$ and the doubly charged $(M + 2H)^{2+}$ quasimolecular peak of the lipohexapeptide.

Biological Tests

Polyclonal Activation of B-Lymphocytes. Tripalmitoyl-*S*-glycerylcysteinyl peptides are polyclonal activators of B-lymphocytes.[3-5] The intention of this study was to investigate the effect of a replacement of the sulfur atom in the Pam_3Cys moiety of these lipopeptides by a methylene group on the biological activity and the degree of stereospecificity of the activation. We synthesized the "methylene" analogues **2a–d** with a peptide part identical with the natural sequence of the N-terminus of lipoprotein (Ser-Ser-Asn-Ala) and also the analogue **3** with a serine residue in neighborhood to the lipoamino acid Pam_3Adh (the minimum structure for biological activity of corresponding Pam_3Cys peptides is Pam_3Cys-Ser[15]) and additional four lysine residues, which were introduced to render the lipopeptide water soluble. These lipopeptides were chosen for this study, because the corresponding Pam_3Cys peptides were among the most immunologically active lipopeptides we have investigated so far: Pam_3Cys-Ser-Ser-Asn-Ala (TPP) has a stimulatory activity comparable to native lipoprotein,[3,4] and Pam_3Cys-Ser-$(Lys)_4$ is a potent water-soluble, nontoxic immunoadjuvant, which can replace Freunds complete adjuvant.[5,10] The four possible diastereomers of Pam_3Adh-Ser-Ser-Asn-Ala **2a–d**, with defined configurations at the two asymmetric carbon atoms C-2 and C-6 of the lipoamino acid Pam_3Adh, were compared with each other in the in vitro proliferation test (Figure 3). When comparing biological activities of diastereomers of Pam_3Cys and Pam_3Adh peptides it has to

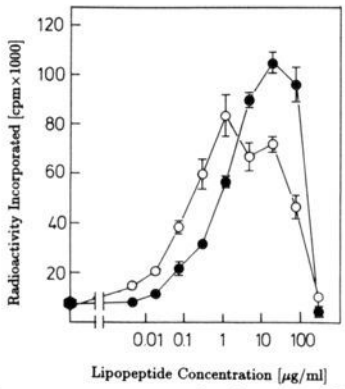

Figure 3. Dose–response curve for incorporation of [^{3}H]thymidine in Balb/c splenocytes after stimulation with the amphiphilic lipohexapeptide (2*R*,6*R*)-Pam$_3$Cys-Ser-(Lys)$_4$ (○) and its corresponding methylene analogue (2*S*,6*S*)-**3** (●). 3.3×10^6 cells/mL were cultured for 48 h in the presence of varying lipopeptide concentrations. The values for the incorporated radioactivity (counts per minute, cpm) represent mean values of triplicate determinations.

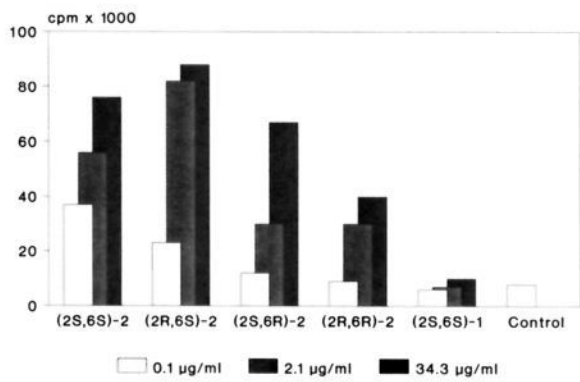

Figure 4. Determination of [^{3}H]thymidine incorporation in Balb/c splenocytes after stimulation with lipopeptides. 3.3×10^6 cells/mL were cultured for 48 h in the presence of varying lipopeptide concentrations (0.1, 2.1, and 34.3 μg/mL). The values for the incorporated radioactivity (counts per minute, cpm) represent mean values of triplicate determinations (control is buffer solution in the absence of lipopeptides).

be taken into account, that according to the *RS* nomenclature of Cahn, Ingold, and Prelog (priority: sulfur > oxygen) (2*S*,6*S*)-Pam$_3$Adh has the same spatial arrangement of its substituents at the asymmetric carbon atoms C-2 and C-6 as the sulfur-containing (2*R*,6*R*)-Pam$_3$Cys.

The five novel lipopeptides **2a–d** and (2*S*,6*S*)-**3** stimulated the proliferation of Balb/c lymphocytes as shown by incorporation of [^{3}H]thymidine into the DNA of murine splenocytes (Figures 3 and 4). A comparison between the mitogenic activity of (2*S*,6*S*)-**3** and the corresponding Pam$_3$Cys peptide Pam$_3$Cys-Ser-(Lys)$_4$ is shown in Figure 3. Both lipopeptides are potent water-soluble B-lymphocyte activators. Significant stimulating effects caused by (2*S*,6*S*)-**3** start at lipopeptide concentrations of 0.1 μg/mL. In comparison to the corresponding Pam$_3$Cys peptide optimal stimulation is attained at a ca. 30-fold higher concentration (ca. 30 μg/mL; see Figure 4) and stimulation at low lipopeptide concentrations is somewhat less pronounced. However, the activity is still high and comparable with activities of other Pam$_3$Cys peptides we have tested so far. Comparable results were also obtained with the methylene analogues **2a–d** in comparison with Pam$_3$Cys-Ser-Ser-Asn-Ala (data not shown).

Interestingly, the four diastereomers **2a–d** activated B-lymphocytes to a different degree: the diastereomers

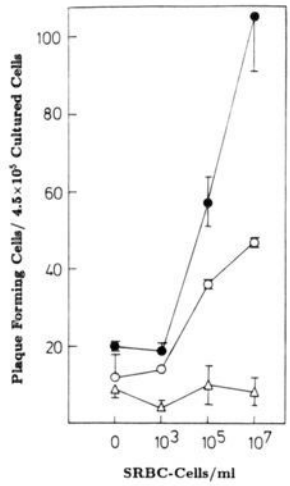

Figure 5. Adjuvanticity of (2*S*,6*S*)-**2** (**2a**) in vitro: enhancement of the primary antibody response against TNP-SRBC by **2a**. Balb/c splenocytes (4.5×10^6) were cultured in the presence of different TNP-SRBC concentrations with no (△), 5 μg/mL (●), or 30 μg/mL **2a** (○). Direct plaque forming cells were established on day 5 of culture. Each value represents the mean of triplicate experiments ± standard deviation.

2a and **2c** with *S* configuration at C-6 of the N-terminal lipoamino acid showed higher activities in the in vitro proliferation test than **2b** and **2d** with *R* configuration at C-6 (Figure 4). These results are in agreement with results previously obtained with Pam$_3$Cys peptides with different configurations at C-6 of the *S*-glyceryl part: 2*R*,6*R* diastereomers of Pam$_3$Cys peptides (which have the same spatial arrangement of their substituents at C-2 and C-6 as (2*S*,6*S*)-methylene analogues; see above) and synthetic vaccines prepared from the 2*R*,6*R* diastereomers were found to be stronger activators than the 2*R*,6*S* diastereomers with *S* configurated *S*-glyceryl C-2.[18] Especially diastereomer (2*S*,6*S*)-**2** (**2a**) was highly active already at a low concentration of 0.1 μg/mL. At higher concentrations (<30 μg/mL) the differences in activity between the four diastereomers are less pronounced, but they are still obvious (Figure 4). Optimal stimulation for all four diastereomers is achieved at concentrations of about 30 μg/mL. It is interesting that the configuration at the α-C atom of the lipoamino acid seems to play a more minor role in the stimulation mechanism of murine splenocytes than the configuration at C-6; in the case of Pam$_3$Cys peptides racemization at C_α of the Pam$_3$Cys residue, which was mentioned above, should therefore have not too much impact on the biological activity of the resulting lipopeptides as long as the *R* configuration at C-6 is retained.

Like Pam$_3$Cys-OH, the lipoamino acid Pam$_3$Adh-OH (not linked to a peptide) is only a marginal B-lymphocyte activator (Figure 4). The four diastereomers of the lipoamino acid **1a–d** exhibit splenocyte stimulation starting at concentrations of 100 μg/mL; here maximal stimulation was found in 2*S*,6*S* configuration (data not shown).

Adjuvanticity. The in vitro adjuvanticity was studied with the (2*S*,6*S*)-**2** (**2a**), the diastereomer, which had the highest activity in the in vitro proliferation test. The in vitro primary immune response against trinitrophenylated sheep erythrocytes could be significantly enhanced by addition of **2a** to the antigen (Figure 5). A particularly pronounced effect was obtained at concentrations of about 5 μg/mL and an immunization dose of 10^7 sheep erythrocytes/mL.

In vivo adjuvanticity of **2a** was tested with ovalbumin as antigen. Ovalbumin alone, **2a** alone, or **2a** admixed to

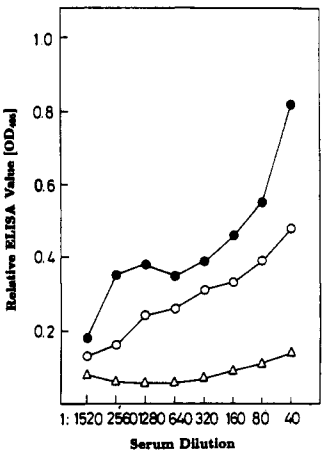

Figure 6. Adjuvanticity of (2*S*,6*S*)-2 (**2a**) in vivo. Groups of five Balb/c mice (age 6–8 weeks) were immunized twice (day 1, day 14) with 100 μg of ovalbumin (open circles), with 100 μg of **2a** (open triangles), or with 100 μg of ovalbumin in combination with 100 μg of **2a** (closed circles) and bled on day 28, on which the sera of each group were pooled. Sera were tested for their ovalbumin specific Ig content by ELISA. The data represent mean values obtained from triplicate experiments.

ovalbumin were injected twice into Balb/c mice. After 28 days the ovalbumin-specific antibody titers of the mice were determined. The ovalbumin-specific immune response could be markedly enhanced by addition of **2a** to the antigen (Figure 6).

The activation of macrophages by the novel lipopeptides was tested as previously described for TPP.[2] Comparable with the results obtained for TPP and other analogues, **2a**-induced tumor cytotoxicity in bone marrow derived macrophages against the fibroblast tumor cell line L929. At a lipopeptide concentration of 50 μg/mL and an effector target cell ratio of 10:1, 92% tumor cell lysis was observed. The corresponding values for the lipohexapeptide Pam_3Cys-Ser-$(Lys)_4$ were 98.4% and for LPS from *Salmonella abortus equi* 68.6%.

Our results show that lipopeptides containing the three-chain lipoamino acid 2-(palmitoylamino)-6,7-bis(palmitoyloxy)heptanoic acid like the corresponding tripalmitoyl-*S*-glycerylcysteinyl peptides are potent B-cell and macrophage stimulants and act as immunoadjuvants in combination with antigens. Obviously, the cysteine sulfur atom in Pam_3Cys conjugates can be replaced by CH_2 without significant loss of biological activity. Furthermore, it could be shown that a stereospecific activation of B-lymphocytes can be observed: lipopeptides containing the lipoamino acid with *S* configuration at the asymmetric carbon atom C-6 are more active than those with *R* configuration, the configuration at C-2 is of less influence on the activation. From these results it may be concluded, that for recognition of *S*-glycerylcysteinyl peptides the *S*-glyceryl part of the molecule seems to be more important than the cysteinyl part.

Although Pam_3Adh peptides have slightly reduced biological activities, they are superior to Pam_3Cys peptides with regard to their stability during certain synthesis strategies (e.g. oxidation, hydrogenation on Pd/C), their lower racemization tendency and the storability of the adjuvants and vaccines based on this principle. At present, we are investigating the possibilities of using Pam_3Adh peptides as built-in adjuvants in synthetic low molecular mass peptide vaccines.

Experimental Section

Abbreviations are as follows: ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; FD-MS, field-desorption mass spectroscopy; NEM, *N*-ethylmorpholine; Pam_3Adh-OH, 2-(palmitoylamino)-6,7-bis(palmitoyloxy)heptanoic acid; Pam_3Cys-OH, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-propyl]-(*R*)-cysteine; PBS, phosphate-buffered saline; SRBC, sheep red blood cells; TNP, 2,4,6-trinitrophenyl; TPP, tripalmitoylpentapeptide (Pam_3Cys-Ser-Ser-Asn-Ala).

N_α-Fmoc-(*S*)-amino acids and *p*-benzalkoxybenzyl alcohol polystyrene resin cross-linked with 1% divinylbenzene were obtained from Novabiochem (Läufelfingen, Switzerland). The synthesis of the four stereoisomers of 2-(palmitoylamino)-6,7-bis(palmitoyloxy)heptanoic acid (Pam_3Adh-OH) was published recently.[19]

R_F values were determined on preformed plates 60 F_{254}, 5 × 10 cm (Merck, Darmstadt, FRG) and the following solvent systems (v/v): I, chloroform/methanol/water (65:25:4); II, chloroform/methanol/acetic acid (90:10:1); III, ethyl acetate saturated with water; IV, 1-butanol/acetic acid/water (2:1:1); V, chloroform/methanol (8:2). For detection ninhydrin, chlorine/4,4′-bis(dimethylamino)diphenylmethane (TDM) and water were used. Amino acid analysis: The lipopeptides were hydrolyzed with 6 N HCl in the presence of phenol for 18 h at 110 °C. The hydrolysate was extracted twice with chloroform to remove palmitic acid. The amino acids of the hydrolysate were converted into the *N*-(trifluoroacetyl) amino acid *n*-propyl esters and their quantity and optical purity determined gas chromatographically on Chirasil-Val capillary columns[22] (Fa. Grom, Ammerbuch; FRG) by means of enantiomer labeling.[23] Pam_3Adh was not quantified by gas chromatographical amino acid analysis (see above).

Ion-spray mass spectra were recorded on a triple quadrupole mass spectrometer API III equipped with an ion spray source (Sciex, Toronto, Canada). Field-desorption mass spectra were recorded with the mass spectrometer MAT 711A (Varian Bremen, FRG) at the ion source temperature of 50 °C.

Synthesis of 2a–d. 1. Synthesis of the Resin-Bound Tetrapeptide Ser-Ser-Asn-Ala. The protected tetrapeptide Fmoc-Ser(tBu)-Ser(tBu)-Asn-Ala-resin was built up on a *p*-benzalkoxybenzyl alcohol polystyrene copolymer cross-linked with 1% divinylbenzene (1 g, 0.26 mmol/g free OH groups) by using the N_α-Fmoc amino acids Fmoc-Ala-OH, Fmoc-Asn-OH, and Fmoc-Ser(tBu)-OH (1 mmol per each coupling); the synthesis and washing steps were performed with the fully automatic peptide synthesizer ABI 430 A from Applied Biosystems (Pfungstadt, FRG). The Fmoc group was removed with 20% piperidine/dimethylformamide in 15 min. *N*,*N*′-diisopropylcarbodiimide/1-hydroxybenzotriazol (1:1; 1 mmol each) was used for coupling. The reaction cycles did not include capping steps.

2. Coupling of the Lipoamino Acid. The respective stereoisomer (**a–d**) of the lipoamino acid 1 (67 mg; 75 μmol) was activated with 1-hydroxybenzotriazol (10.5 mg; 75 μmol) and *N*,*N*′-dicyclohexylcarbodiimide (15.5 mg; 75 μmol) in CH_2Cl_2/dimethylformamide (9:1; 3 mL). After 15 min the mixture was added to the resin-bound N-terminally deprotected tetrapeptide (150 mg; 37.5 μmol) and shaken for 6 h. The resin was washed three times with dimethylformamide and CH_2Cl_2 and dried in vacuo.

3. Cleavage of the Lipopeptides 2a–d from the Resin. The resin-bound lipopeptide was suspended in thioanisol/trifluoroacetic acid (1:9) and filtered off after 1 h. The resin was washed three times with CH_2Cl_2. The filtrates were combined and evaporated to dryness. By precipitation from chloroform/methanol (1:5) lipopeptides **2a–d** were obtained. They were lyophilized from *tert*-butyl alcohol. Yields (referring to the amount of resin): 2*S*,6*S*, 45 mg (96%); 2*S*,6*R*, 44 mg (94%); 2*R*,6*S*, 46 mg (98%); 2*R*,6*R*, 42 mg (90%). $C_{68}H_{126}N_6O_{14}$ (1251.8). For all four stereoisomers, R_F (I) = 0.39 (in comparison to the analogous Pam_3Cys compound TPP: R_F (I) = 0.40). FD-MS of **2a–d**: *m*/*z* M^+ 1251, $(M + Na)^+$ 1274 (Figure 1). Amino acid analysis (gas chromatographical determination on Chirasil-Val[14] (Fa. Grom, FRG) by enantiomer labeling[15]): (**2a**) Ser 1.97, Asx 1.04, Ala 1.00;

(22) Frank, H.; Nicholson, G.; Bayer, E. J. *Chromatogr. Sci.* **1977**, *15*, 174.
(23) Frank, H.; Nicholson, G. J.; Bayer, E. *J. Chromat.* **1978**, *167*, 187.

(2b) Ser 2.04, Asx 1.05, Ala 1.00; (2c) Ser 2.03, Asx 1.07, Ala 1.00; (2d) Ser 1.98, Asx 1.05, Ala 1.00.

Synthesis of (2*S*,6*S*)-3. (1) *N*-(Benzyloxycarbonyl)-*O*-*tert*-butyl-(*S*)-seryl-tris[N_ϵ-(*tert*-butyloxycarbonyl)-(*S*)-lysyl]-N_ϵ-(*tert*-butyloxycarbonyl)-(*S*)-lysine *tert*-Butyl Ester (4; Z-Ser(tBu)-[Lys(Boc)]$_4$-OtBu). Z-Ser(tBu)-OH (85 mg; 0.29 mmol) was dissolved in dimethylformamide/dichloromethane (1:1; 1 mL) and activated with 1-hydroxybenzotriazol (40 mg; 0.29 mmol) and *N*,*N*'-dicyclohexylcarbodiimide (59.7 mg, 0.29 mmol) for 30 min at 0 °C. H-[Lys(Boc)]$_4$-OtBu[21] (0.22 g; 0.22 mmol) and NEM (30 μL) were added and the mixture was stirred for 4 h. A few drops of acetic acid were added, and the solvent was removed. The residue was chromatographed on Sephadex LH-20 ($CHCl_3$/methanol 1:1; column 80 × 2.5 cm). The fractions containing the product (chlorine/TDM positive) were combined, the solvent was removed, and the residue was lyophilized from *tert*-butyl alcohol. The colorless pentapeptide ester is soluble in chloroform and methanol: yield 0.2 g (72%); ion-spray mass spectrum, *m*/*z* 1266 $(M + H)^+$; R_F (I) = 0.92; R_F (II) = 0.76; R_F (III) = 0.63; R_F (V) = 0.88. Anal. ($C_{63}H_{109}N_9O_{17}$).

(2) *O*-*tert*-Butyl-(*S*)-seryl-tris[N_ϵ-(*tert*-butyloxycarbonyl)-(*S*)-lysyl]-N_ϵ-(*tert*-butyloxycarbonyl)-(*S*)-lysine *tert*-Butyl Ester (5; H-Ser(tBu)-[Lys(Boc)]$_4$-OtBu). 4 (0.15 g; 0.12 mmol) was hydrogenated on Pd/C in methanol/acetic acid (9:1). After 90 min the catalyst was filtered off and the solvent was removed. The residue was lyophilized from *tert*-butyl alcohol. The pentapeptide ester was obtained as colorless powder: yield 0.11 g (78%); ion-spray mass spectrum, *m*/*z* 1132 $(M + H)^+$; R_F (I) = 0.80; R_F (II) = 0.35; R_F (IV) = 0.93; R_F (V) = 0.77.

(3) 2-(Palmitoylamino)-6,7-bis(palmitoyloxy)-(2*S*,6*S*)-heptanoyl-*O*-*tert*-butyl-(*S*)-seryl-tris[N_ϵ-*tert*-(butyloxycarbonyl)-(*S*)-lysyl]-N_ϵ-(*tert*-butyloxycarbonyl)-(*S*)-lysine *tert*-Butyl Ester (6; Pam$_3$Adh-Ser(tBu)-[Lys(Boc)]$_4$-OtBu). (2*S*,6*S*)-1 (11 mg; 12.3 μmol) was dissolved in CH_2Cl_2/dimethylformamide (1:1; 0.5 mL) and activated with 1-hydroxybenzotriazol (2 mg; 12.3 μmol) and *N*,*N*'-dicyclohexylcarbodiimide (2.5 mg; 12.3 μmol) for 15 min at 15 °C. A solution of 4 (15 mg; 13.3 μmol) in dimethylformamide (300 μL) and NEM (5 μL) were added. The mixture was stirred for 4 h. The solvent was removed and the residue chromatographed on Sephadex LH-20 ($CHCl_3$/methanol 1:1; column 45 × 1.5 cm). The first eluated fraction (Cl_2/TDM positive, ninhydrin positive, because the N_ϵ-Boc groups are not stable at 110 °C on the TLC plate) contained the protected lipopeptide. After evaporation to dryness the residue was lyophilized from *tert*-butyl alcohol: yield 22 mg (89% referring to 1); R_F (II) = 0.82. FD-MS, *m*/*z* 2027 $(M + Na)^+$. $C_{110}H_{206}N_{10}O_{21}$ 2004.9.

(4) 2-(Palmitoylamino)-6,7-bis(palmitoyloxy)-(2*S*,6*S*)-heptanoyl-(*S*)-seryl-tris((*S*)-lysyl)-(*S*)-lysine Tris(trifluoroacetate) ((2*S*,6*S*)-3; Pam$_3$Adh-Ser-(Lys)$_4$-OH ×3 CF$_3$COOH). 5 (22 mg; 11.0 μmol) was dissolved in 10% thioanisol/trifluoroacetic acid (1 mL). The acid was removed in vacuo after 1 h. The residue was dissolved in acetone. The lipopeptide was precipitated at 0 °C, filtered off, and lyophilized from *tert*-butyl alcohol: yield 19 mg (94%); ion-spray mass spectrum, *m*/*z* 1493 $(M + H)^+$, 747 $(M + 2H)^{2+}$ (Figure 2); amino acid analysis Ser 1.1 (1); Lys 4.00 (4); no racemization detectable; R_F (IV) = 0.46 (in comparison to the analogous Pam$_3$Cys compound, R_F (IV) = 0.48).

Biological Tests. Lymphocytes and Incorporation of [^{3}H]Thymidine. Balb/c mice, 6–10 weeks old, from the Bundesforschungsanstadt für Viruskrankheiten der Tiere, Tübingen, FRG, or from the Institut für Immunbiologie, Freiburg, FRG, were sacrificed by cervical dislocation. Spleens were removed immediately and the splenic lymphocytes prepared as described previously.[24] Thymidine incorporation experiments were performed in flat-bottom Falcon 3040 microtiter plates (Falcon Plastics, Los Angeles, CA). Lymphocytes (3.3×10^6 mL^{-1}) were cultured for 48 h in 0.18-mL aliquots in serum-free medium (Iscove's modified DMEM complete, kindly donated by Behring-Werke, Marburg, FRG). Before harvesting, cells were pulsed for 24 h with 0.625 μCi/well of [^{3}H]thymidine (Amersham, Braunschweig, FRG; specific activity 5 Ci/mmol). The cells were harvested with an automatic cell harvester (Skatron AS, Lier, Norway), collected on glass fiber filters (Skatron), and measured by scintillation. Assays were done in triplicate.

Macrophage Activation. Macrophage activation to tumor cytotoxicity was performed by using Balb/c bone marrow derived macrophages as described previously by us.[2]

Immunological Methods. Hemolytic Plaque Assay. The development of immunoglobulin-secreting cells was measured by using a TNP-SRBC hemolytic plaque assay.[25] Splenocytes with a cell density of 4.5×10^6 cells/mL were cultured in Falcon 2045 culture tubes in 1-mL aliquots in the presence of varying doses of antigen (TNP-SRBC) and lipopeptides. After 4 days, the cell suspension was washed once in MEM and appropriately diluted in MEM containing additional glucose (0.45% w/v). A 100-μL sample of this suspension, together with 20 μL of guinea pig complement (Serva, Heidelberg, FRG) and 20 μL of a suspension of TNP-SRBC, were quickly added to 300 μL of 0.5% agarose in medium at 45 °C. The suspensions were mixed and immediately plated on disposable petri dishes (Falcon Plastics, Los Angeles, CA). The dishes were kept in a humid atmosphere at 37 °C for 3 h, after which plaques were scored by using a plaque viewer (Tecnomara, Zürich, Switzerland).

ELISA. The amount of specific antibodies in the serum was determined by ELISA.[26] Antiovalbumin antibodies were detected after coupling ovalbumin (100 μg/mL) to microtiter plates. Subsequently, the wells of flat-bottom microtiter plates (Dynatech M 129B, Denkendorf, FRG) were incubated with serum dilutions (50 μL, 3 h, 37 °C). Next, 50 μL of peroxidase-labeled goat anti mouse Ig from Nordic Immunology (Tilburg, Netherlands) diluted 1:2000 in 1% BSA in PBS was added to each well, and the plates were left for 2 h at room temperature. The substrate ABTS (100 μL/well, Sigma, München, FRG) was then applied for the enzyme reaction (30 min). Each step described above, subsequent to the fixing of ovalbumin, was followed by copious washing with PBS. Photometrical measurement of the enzyme reaction was performed at 405 nm by a SLT-Easy Reader (SLT, Overath, FRG). To exclude BSA-specific antibodies from the determinations, sera were adsorbed 3 h at 37 °C on BSA coupled plates.

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Registry No. 1a, 132970-52-6; 1b, 132970-54-8; 1c, 132970-53-7; 1d, 132970-55-9; 2a, 133933-84-3; 2b, 134001-84-6; 2c, 134001-85-7; 2d, 134001-86-8; 3 (free base), 133933-85-4; 3·3TFA, 134001-87-9; 4, 133933-86-5; 5, 133933-87-6; 6, 133933-88-7; Fmoc-Ala-OH, 35661-39-3; Fmoc-Asn-OH, 71989-16-7; Fmoc-Ser(*t*-Bu)-OH, 71989-33-8; Z-Ser(*t*-Bu)-OH, 1676-75-1; H-[Lys(BOC)]$_4$-OBu-*t*, 62146-39-8.

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