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Synthetic *S*-(2,3-Dihydroxypropyl)-cysteinyl Peptides Derived from the N-terminus of the Cytochrome Subunit of the Photoreaction Centre of *Rhodopseudomonas viridis* Enhance Murine Splenocyte Proliferation

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Abstract: Various lipopeptides representing the N-terminal part of the cytochrome subunit of the photosynthetic reaction centre from the purple bacterium *Rhodopseudomonas virdis* were prepared by solid-phase peptide synthesis. These lipopeptides consisted of a *S*-[2,3-dihydroxypropyl]-cysteinyl (Dhc) residue N-terminally coupled to the nonapeptide FEPPPATTT. Different numbers of palmitoyl (Pam) chains were attached to Dhc via ester and/or amide bonds. The lipopeptide Dhc(Pam)$_2$-FEPPPATTT containing two ester-bonded palmitoyl residues and a free N-terminus was a potent polyclonal activator of murine (BALB/c) spleen cells at subnanomolar concentrations. The lipopeptide Pam-Dhc(Pam)$_2$-FEPPPATTT containing three palmitoyl residues, the two-chain lipopeptide Pam-Dhc(Pam)-FEPPPATTT containing one amide- and one ester-bonded palmitoyl residue, and the N-terminally elongated lipopeptide SLVAG-Dhc(Pam)$_2$-FEPPPATTT were less active. The nonapeptide FEPPPATTT and the decapeptide Dhc-FEPPPATTT were only marginal splenocyte activators, even at concentrations as high as 1 μM. Thus, lipopeptide Dhc(Pam)$_2$-FEPPPATTT constitutes the first potent splenocyte stimulating Dhc-lipopeptide described so far that contains only two fatty acid residues.

Keywords: Immunomodulators; lipopeptides; peptide synthesis; *S*-[2,3-dihydroxypropyl]-cysteine; splenocyte activators

Abbreviations

CTL, cytotoxic T-lymphocytes; Dhc, *S*-[2(*RS*), 3-dihydroxypropyl]-(*R*)-cysteine; Dhc(Pam)$_2$, *S*-[2(*RS*), 3-bis(palmitoyloxy)propyl]-(*R*)-cysteine; DIC, *N,N'*-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; ES-MS, electrospray mass spectrometry; HONB, *N*-hydroxy-5-norbornene-2,3-dicarboxylic acid imide; Pam, palmitoyl; Pam$_3$Cys, *N*-palmitoyl-S-[2(*RS*), 3-bis(palmitoyloxy)propyl]-(*R*)-cysteine (= Pam-Dhc(Pam)$_2$-OH); Pam-Dhc(Pam), *N*-palmitoyl - S - [2(*RS*)-hydroxy,3 - (palmitoyloxy)propyl] - (*R*) - cysteine; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoroborat.

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INTRODUCTION

The mature cytochrome subunit of the photosynthetic reaction centre from the purple bacterium *Rhodopseudomonas viridis* is a lipoprotein which contains two covalently bonded fatty acids at its N-terminus which are linked to the protein *via* the hydroxy groups of *S*-(2,3-dihydroxypropyl)-cysteine (Dhc) [1]. This post-translationally modified cysteine residue is also found in other structural and functional lipoproteins of both Gram-negative and Gram-positive bacteria (for a review see [2]). The cytochrome subunit, however, has been the only native Dhc-

containing lipoprotein with a free N-terminal amino group described so far.

Synthetic lipopeptides containing tripalmitoylated Dhc ('Pam_3Cys'), which are derived from the lipoprotein from the outer membrane of *Escherichia coli*, are potent B-lymphocyte and macrophage activators [3–5]. Conjugates consisting of lipopeptides and T-helper cell and cytotoxic T-cell (CTL) epitopes from viral or bacterial proteins constitute fully synthetic, low-molecular mass vaccines with incorporated adjuvant principle [6–8] which are capable of *in vivo* priming of virus specific CTLs [9, 10]. The N-terminus of the cytochrome subunit resembles these lipopeptides, however, it lacks the N-terminal fatty acid. In the following we describe the synthesis of the N-terminus of the cytochrome subunit and further analogues **2–6**, which differ in the number of palmitoyl residues bonded to their N-terminal Dhc. We investigated the influence of the number of palmitoyl chains in these synthetic partial structures of the cytochrome subunit on the ability to enhance murine spleen cell proliferation.

MATERIALS AND METHODS

Chemicals

1-Hydroxybenzotriazol, diisopropylethylamine, *N*-hydroxy-5-norbornene-2,3-dicarboxylic acid imide, *N,N'*-diisopropylcarbodiimide and dimethylformamide were purchased from *Fluka*, Buchs. Fmoc-Ala, Fmoc-Gly, Fmoc-Glu(*t*Bu), Fmoc-Leu, Fmoc-Phe, Fmoc-Pro, Fmoc-Ser(*t*Bu), Fmoc-Thr(*t*Bu), Fmoc-Val, Fmoc-Thr(*t*Bu)-*p*-benzalkoxy-benzylalcohol-polystyrene (PS)-1%-divinylbenzene (DVB) and 2-(1H-benzotriazol-1-yl)- 1, 1, 3, 3-tetramethyluroniumtetrafluoroborate were obtained from *Novabiochem*, Läufelfingen. N_α-Fluorenylmethoxycarbonyl-*S*-[2(*RS*),3-dihydroxypropyl]-(*R*)-cysteine, N_α-palmitoyl-*S*-[2(*RS*),3-bis(palmitoyloxy)propyl]-(*R*)-cysteine and N_α-fluorenylmethoxycarbonyl-*S*-[2(*RS*), 3-bis (palmitoyloxy)-propyl]-(*R*)-cysteine were synthesized as previously described [3, 11] (the latter two compounds are commercially available from *Novabiochem*, Läufelfingen).

Synthesis of Dhc-derivatives

The syntheses of the Dhc-derivatives N_α-fluorenyl-methoxycarbonyl-*S*-(2(*RS*),3-dihydroxypropyl)-(*R*)-cysteine (Fmoc-Dhc-OH), N_α-fluorenyl methoxycarbonyl-*S*-[2(*RS*), 3-bis(palmitoyloxy)propyl]-(*R*)-cysteine (Fmoc-Dhc$(Pam)_2$-OH) and N_α-palmitoyl-*S*-[2(*RS*),3-bis(palmitoyloxy)propyl]-(*R*)-cysteine (Pam-Dhc$(Pam)_2$-OH = 'Pam_3Cys-OH') have been previously described [3, 11].

N-Palmitoyl-*S*-(2(RS)-hydroxy-3-(palmitoyloxy) propyl-(*R*)-cysteine (Pam-Dhc(Pam)-OH)

The two-chain lipoamino acid Pam-Dhc(Pam)-OH used for the preparation of **3** was obtained by treatment of the *tert*-butyl ester Pam-Dhc(Pam)-O*t*Bu with trifluoroacetic acid. The latter compound was obtained by the reaction of palmitic acid 2,3-epoxy-1-propyl ester with *N*-palmitoyl-cysteine-*tert*-butylester similar to the procedure applied for the preparation of Fmoc-Dhc$(Pam)_2$-O*t*Bu [11].

N,N'-dipalmitoyl-cystine di-*tert*-butyl ester (1 g, 1.21 mmol; [3]) was dissolved in dichloromethane (5.5 ml). Methanol (4 ml) and Zn granulate (550 mg) were added. Under vigorous stirring a mixture consisting of conc. HCl, conc. sulphuric acid and methanol (7:1:100; v:v:v; 6 ml) were added. After 20 min palmitic acid 2,3-epoxy-1-propyl ester (1.62 g, 4.84 mmol [12]) was added. After 48 h at 45°C the reaction mixture was evaporated to dryness. Purification by precipitation from methanol/acetic acid/water (5:1:1; v:v:v) and from light petroleum (b.p. 30–50°C) at −20°C followed by lyophilization from *tert*-butylalcohol afforded Pam-Dhc(Pam)-O*t*Bu as a colourless powder. Yield: 1.73 g (78%). R_F = 0.63 (chloroform). $C_{42}H_{81}NO_6S$ 728.2 (ES-MS $[M+H]^+$ 729).

The *tert*-butyl ester (0.5 g, 0.69 mmol) was dissolved in trifluoroacetic acid, and the mixture was stirred for 1.5 h. The acid was evaporated *in vacuo*, and the residue was recrystallized from methanol at − 20°C. The lipoamino acid was lyophilized from *tert*-butylalcohol. Yield: 0.39 g (85%). R_F = 0.15 (chloroform). $C_{38}H_{73}NO_6S$ 672.1 (ES-MS $[M+H]^+$ 673).

Peptide and Lipopeptide Synthesis

The side chain-protected nonapeptide Phe-Glu(*t*Bu)-Pro-Pro-Pro-Ala-Thr(*t*Bu)-Thr(*t*Bu)-Thr(*t*Bu) was built up with an automated peptide synthesizer (ABI 430A; *Applied Biosystems*, Weiterstadt, Germany) using the fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl(*t*Bu) strategy and starting with Fmoc-Thr(*t*Bu)-*p*-benzylalkoxy-benzylalcohol-PS-1%-DVB resin (loading 0.6 mmol/g; 500 mg). Fmoc was removed with 20% piperidine in DMF (2 × 15 min). A threefold excess of the respective Fmoc–amino acid

was activated with TBTU (1 eq.), HOBt (1 eq.) and DIPEA (1.5 eq.). The lipoamino acids Pam_3Cys-OH, Pam-Dhc(Pam)-OH and Fmoc-Dhc-OH were coupled in double excess to the resin-bonded N_α-deprotected nonapeptides with DIC/HONB for 12 h in DMF [11, 13]. For removal of the Fmoc group from the lipoamino acid under mild conditions a mixture of morpholine/piperidine (1:1, v:v) in DMF (2 × 30 min; 4°C) was used instead of neat piperidine [14]. A part of the resin-bonded lipopeptide **4** was elongated with the sequence SLVAG by using Fmoc/*t*Bu strategy as described above.

Free peptides and lipopeptides **1–6** (Table I) were obtained by treatment of the resins with trifluoroacetic acid containing 5% thioanisole and 5% thiocresol for 1 h. Peptides **1** and **2** were precipitated from ether and lyophilized from water. The lipopeptides **3–6** were dissolved in a small volume of dichloromethane, precipitated repeatedly by addition of acetone in an ice bath and lyophilized from *tert*-butylalcohol.

Thin-Layer Chromatography and HPLC

R_F values were determined on preformed silicagel plates 60 F_{254}, 5 × 10 cm (*Merck*, Darmstadt, Germany) and the solvent systems 1-butanol/acetic acid/water (2:1:1) and chloroform, respectively. RP-HPLC of **4** and **5** was performed with a *Waters* 600 multisolvent delivery system, combined with a *Waters* 7112 WISP autoinjector and a C-8 column (Nucleosil C-8; 125 × 4.6 mm; 5 μm; *Grom*, Herrenberg, Germany). **4** and **5** were dissolved in methanol (1 mg/ml) and eluted using a linear gradient from 20 to 60% B in 30 min (*A* = 0.1% trifluoroacetic acid in water, B = acetonitrile).

Amino Acid Analysis

The peptides and lipopeptides were hydrolysed with 4N methanesulphonic acid for 18 h at 110°C. Under these conditions Dhc remains intact and can be quantified, whereas hydrolysis with 6N HCl partially converts Dhc into *S*-[2-chloro-3-hydroxypropyl]-(*R*)-cysteine and *S*-[2,3-dichloropropyl]-(*R*)-cysteine [15, 16]. After being at room temperature the hydrolysates were neutralized with an equal volume of 3.8N NaOH and – in the case of **3–6** – extracted twice with hexane to remove palmitic acid. This solution was diluted appropriately with water and then directly used for amino acid analysis based on ion exchange chromatography (*Biotronik* ASA LC 6000E, Maintal, Germany). The amino acid compositions determined for **1–6** were in good agreement with the expected compositions (see Table II).

Sequence Analysis

Automated Edman degradation was performed in a pulse-liquid protein sequencer 477A equipped with an on-line phenylthiohydantoin (PTH)-amino acid analyser 120A (*Applied Biosystems*, Weiterstadt, Germany). All reagents and solvents were from Applied Biosystems. A glass-fiber filter activated with trifluoroacetic acid was coated with BioBrene Plus (1 mg) prior to sequencing. Sequencing was carried out using the standard programs BEGIN-1 and NORMAL-1 (*Applied Biosystems*).

Table I Synthetic Cytochrome Subunit Analogues R_1NH-$CH(CH_2$-S-CH_2-$CHOR_2$-$CH_2OR_3)$-CO-(FEPPPATTT)

	FA[a]	R_1	R_2	R_3	Yield [mg]	[%][b]	R_F[c]	RMM[d]
1	0	–	–	–	8.2	85	–	960.1
2	0	H	H	H	6.3	82	–	1137.3
3	2	Pam	H	Pam	6.7	70	0.38	1614.1
4	2	H	Pam	Pam	20.2	67	0.60	1614.1
5	2	SLVAG	Pam	Pam	10.4	62	0.57	2041.6
6	3	Pam	Pam	Pam	4.2	83	0.73	1852.5

[a] Number of fatty acid chains.
[b] Obtained from 10–50 mg resin; calculation of theoretical yield is based on initial loading of resin.
[c] *n*-Butanol/acetic acid/water 2:1:1; detection with chlorine/4,4'-methylenebis(*N*,*N*-dimethylaniline) (TDM) and with ninhydrin (for detection of **4** and **5**).
[d] Calculated relative molecular mass; all masses were confirmed by electrospray mass spectrometry with an accuracy of ± 0.02%.

Table II Amino Acid Analyses of the Synthetic Cytochrome Subunit Analogues **1–6** (4N methanesulphonic acid, 18 h, 110°C; values in brackets are the expected values)

	Ala	Dhc[a]	Glx	Gly	Leu	Phe	Pro	Ser	Thr	Val
1	1.00(1)	–	0.96(1)	–	–	0.92(1)	2.91(3)	–	3.12(3)	–
2	1.00(1)	0.92(1)	0.94(1)	–	–	1.01(1)	2.90(3)	–	3.10(3)	–
3	1.00(1)	0.97(1)	0.90(1)	–	–	0.94(1)	2.85(3)	–	3.16(3)	–
4	1.00(1)	0.94(1)	0.92(1)	–	–	0.89(1)	2.84(3)	–	3.15(3)	–
5	2.00(2)	0.94(1)	0.97(1)	0.90(1)	1.01(1)	1.08(1)	3.01(3)	0.80(1)	3.22(3)	0.98(1)
6	1.00(1)	0.98(1)	0.91(1)	–	–	0.95(1)	2.91(3)	–	3.05(3)	–

[a]The recovery after hydrolysis and the colour yields with ninhydrin of Ser and Dhc are almost identical. Therefore, the Ser peak of the standard amino acid mixture was used for quantification of Dhc.

Electrospray Mass Spectrometry

Peptides **1** and **2** (*ca.* 100 μg) were dissolved in methanol/1% aqueous formic acid (1:1; 1 ml). The synthetic lipopeptides **3–6** (*ca.* 100 μg) were dissolved in a mixture of methanol/chloroform/10% aqueous formic acid (3:2:1; 1 ml) [17]. ES mass spectra were recorded on a triple-quadrupol mass spectrometer API III (*Sciex*, Thornhill, Ontario, Canada) equipped with an nebulizer-assisted electrospray ('ion spray') source. The solutions were introduced with a medical infusion pump (model 22, *Harvard Apparatus*, South Natick, USA) at a flow rate of 5 μl/min. For collision-induced dissociation (CID) experiments argon was used as collision gas.

Proliferation Assay

For the preparation of murine splenocytes, 6–8 week old BALB/c mice (Iovanovas, Kisslegg, Germany) were killed by cervical dislocation. Spleens were removed immediately, and splenic lymphocytes were prepared as described [18]. Proliferation assays to determine polyclonal lymphocyte activation *in vitro* were done in flat bottom microtitre plates (Falcon 3040, Becton Dickinson, Heidelberg, Germany). Cultures were performed for 48 h in 0.175 ml aliquots (3×10^5 cells/well) in RPMI 1640 medium (Gibco, Karlsruhe, Germany) supplemented with 3.3% heat-inactivated FCS fresh glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (5×10^{-5} M) in an atmosphere of 5% CO_2 in humidified air at 37°C. Equimolar concentrations of the substances were tested in 1:4 dilutions. Before harvesting, cultures were pulsed for 24 h by the addition of 23.1 kBq [^{3}H]-thymidine (Amersham, Braunschweig, Germany; specific activity 185 GBq/mol) to each well. Cells were broken by freezing overnight at – 20°C and thawing; cell debris were collected on glass-fibre filters with an automatic cell harvester (BetaPlate, Pharmacia, Freiburg, Germany). Incorporated radioactivity was determined by liquid scintillation counting. Results were expressed in c.p.m. as means of triplicate determinations.

RESULTS

Compounds **1–6** (Table I) were prepared by solid-phase peptide synthesis. In the bacterial cytochrome subunit the N-terminal Dhc-residue is *O,O'*-bisacylated with a 1:1 mixture of two isomeric 18:OH and three isomeric 18:1 fatty acids [1]. In contrast, our synthetic lipopeptides contained solely palmitoyl residues in order to simplify the synthesis, and because earlier studies on lipopeptides containing different kinds of fatty acids did not reveal pronounced differences in their immunological activities. **1**, which has no Dhc, constitutes the N-terminal decapeptide (2–10) of the cytochrome subunit; **2–6** contain Dhc, but differ in the number (0,2,3) and location (ester or amide bonded) of palmitoyl residues; **5** constitutes an analogue, which was obtained by N-terminal elongation of **4** with the sequence Ser-Leu-Val-Ala-Gly. This pentapeptide precedes the sequence of the mature cytochrome subunit and is part of the signal peptide, 20 amino acids long, of the lipoprotein [1]. A preliminary report on the synthesis of **4** and **5** was published previously [19].

Purification and Characterization

1 and **2** were purified by precipitation from ether, **3–6** by repeated precipitations from acetone. All analogues were characterized by electrospray mass spectrometry (ES-MS) [17], tandem mass

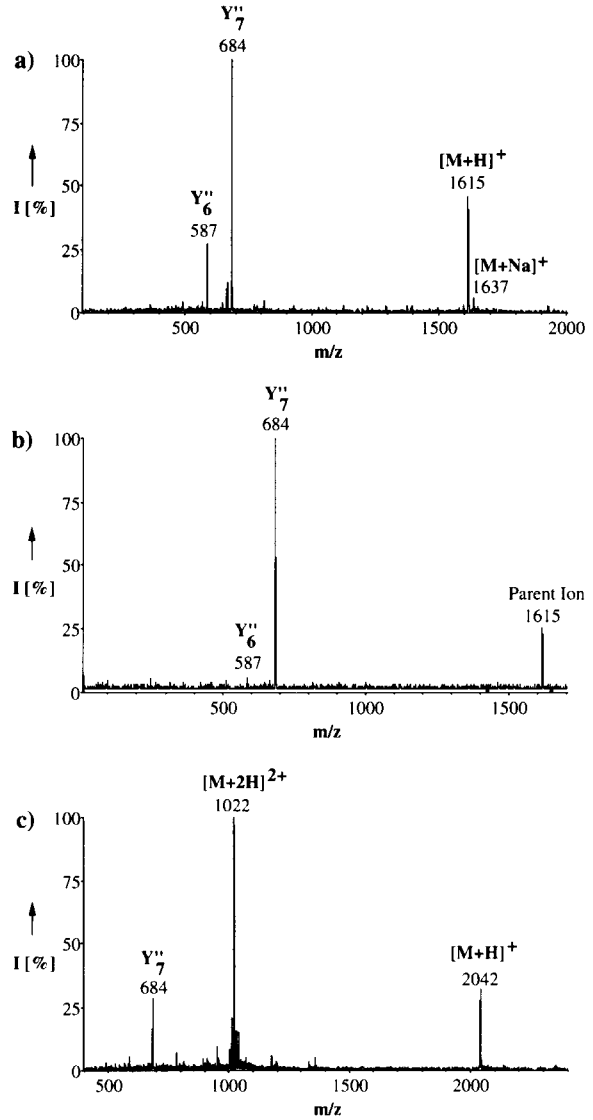

Figure 1 (a) ES mass spectrum of **4** showing the protonated molecular ion $[M+H]^+$ (*m/z* 1615) and the fragment ions Y''_6 and Y''_7 at *m/z* 587 and 684, respectively. (b) These fragment ions are also found as daughter ions after CID of $[M+H]^+$ with argon. (c) ES mass spectrum of **2** showing the singly and doubly protonated molecular ions $[M+H]^+$ (*m/z* 2042) and $[M+2H]^{2+}$ (*m/z* 1022), and the Y''_7 fragment ion at *m/z* 684.

spectrometry (ES-MS/MS), amino acid analysis and thin-layer chromatography. Analytical results are summarized in Table I and II. For **1**, **2**, **4** and **5** the correct sequence was confirmed by automated Edman-degradation (the Dhc-derivative gives a 'gap'). The ES mass spectra showed the expected protonated molecular ions of **1** to **6**. Figures 1(a) and (c) show the ES mass spectra of **4** and **5**, respectively. In these spectra fragment ions Y''_7 at *m/z* 684 and Y''_6 at *m/z* 587 indicate the cleavage of the labile ^{3}Glu – ^{4}Pro and ^{4}Pro – ^{5}Pro bonds, which occurs in the transport region of the ES ion source (nomenclature of fragment ions see [20]). These fragment ions were also obtained by CID of $[M+H]^+$ of **4** with argon in the MS/MS experiment (Figure 1(b)).

Triacyl lipopeptides (Pam_3Cys–peptides) interact very strongly with membranes [21] and are usually irreversibly adsorbed by the reversed-phase (RP) packing during HPLC. By contrast, it was possible to analyse the less lipophilic lipopeptides **4** and **5** by RP-8 HPLC with a standard solvent system (A = 1% trifluoroacetic acid in water, B = acetonitrile, linear gradient from 20 to 60% B in 30 min). Single peaks with retention times of 23.3 min (50% acetonitrile) and 26.7 min (55% acetonitrile) were obtained, which indicated the purity of these products.

Polyclonal Activation of Spleen Cells

Previous investigations showed that synthetic three-chain lipopeptides derived from *E. coli* lipoprotein can induce proliferation of murine spleen cells widely independent of the peptide part attached to the lipid moiety [5]. This finding made it possible to design immunoadjuvants with improved water solubility, such as the amphiphilic lipohexapeptide Pam_3Cys-Ser-$(Lys)_4$ [4, 5, 22] or lipopeptide–polyoxyethylene conjugates [23]. For the same reason, the incorporation of such lipopeptides into synthetic vaccines is of advantage [24]. In general, the removal of ester-or amide-bonded fatty acids from the Pam_3Cys-moiety of these lipopeptides results in a significant loss of biological activity (data not shown).

To our surprise compound **4**, although containing only two palmitoyl residues, proved to be a very potent splenocyte activator (Figure 2). Already below a concentration of 1 nM lipodecapeptide **4** enhanced the incorporation of [^{3}H]-thymidine into the DNA of BALB/c splenocytes by a factor of three in comparison to unstimulated cells. Thus, **4** had an even higher activity than the very potent three-chain immunoadjuvant Pam_3Cys-Ser-$(Lys)_4$ [5] (data not shown). Interestingly, the three-chain Pam_3Cys-peptide **6**, although being a potent activator at low concentrations, was not as active as the two-chain lipopeptide **4**. The nonapeptide **1** and the Dhc-peptide **2**, neither containing any fatty acid, were only marginally active. **3** containing one ester and one amide-bonded palmitoyl residue was a much less potent splenocyte stimulator than **4**, in which the two fatty acids were attached via ester-bonds. The

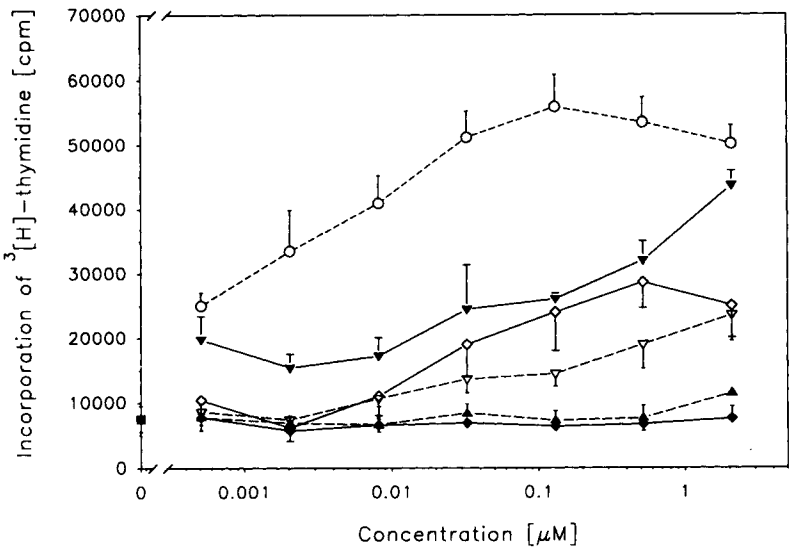

Figure 2 Dose–response curve for the incorporation of [^{3}H]thymidine into the DNA of BALB/c splenocytes after stimulation with **1** (◆), **2** (▲), **3** (▽), **4** (○), **5** (◇) and **6** (▼); control (■). 3×10^5 cells were cultured for 48 h in the presence of varying peptide and lipopeptide concentrations. 24 h before harvesting, the cells were pulsed with [^{3}H]thymidine. The values for the incorporated radioactivity (counts per minute, c.p.m.) are means of triplicate determinations (± standard deviation).

biological activity of **5**, which was obtained by elongation of **4** with the pentapeptide SLVAG, was also reduced in comparison to **4**.

DISCUSSION

Previous experiments have shown that potent B-cell mitogens in the mouse system are usually also good immunoadjuvants in the mouse and other systems. Therefore, it seems to be promising to use **4** or C-terminally shortened analogues thereof (the determination of the minimal structure required for biological activity of such two-chain lipopeptides is in progress) as adjuvant admixed to vaccines or to incorporate it into synthetic vaccines. Since the resultant lipopeptides are less lipophilic than Pam_3Cys-peptides, improved solubility of these conjugates in aqueous solutions is obtained facilitating purification and handling in biological assays (*ca.* 1 mg of **4** can be emulsified in 1 ml of water by ultrasonication forming opalescent micellar solutions). Although N-terminal elongation of the peptide chain of **4** may reduce the splenocyte stimulating properties, interesting conjugates, e.g. lipopeptides containing B-cell and CTL-epitopes separated by the lipoamino acid moiety, can be designed [25]. Up to now the synthesis of lipopeptides with lipids (e.g. palmitoylated residues) attached to residues within the sequence has been difficult. The Fmoc-protected lipoamino acids used in this study allow an easy access to synthetic analogues of such proteins. In addition, **5** and similar lipopeptides may serve as synthetic substrates for *N*-acyltransferases and signal peptidase II involved in the biosynthesis of bacterial lipoproteins [2].

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REFERENCES

1. K. A. Weyer, W. Schäfer, F. Lottspeich and H. Michel (1987). The cytochrome subunit of the photosynthetic reaction center from *Rhodopseudomonas viridis* is a lipoprotein. *Biochemistry 26*, 2909–2914.
2. H. C. Wu and M. Tokunaga, M. (1986). Biogenesis of lipoproteins in bacteria. *Curr. Top. Microbiol. Immunol. 125*, 128–157.
3. K.-H. Wiesmüller, W. G. Bessler and G. Jung (1983).

Synthesis of the mitogenic *S*-[2,3-bis(palmitoyloxy)propyl]-*N*-palmitoylpentapeptide from *Escherichia coli* lipoprotein. *Hoppe Seyler's Z. Physiol. Chem. 364*, 593–606.

4. P. Hoffmann, K.–H. Wiesmüller, J. Metzger, G. Jung and W. G. Bessler (1989). Induction of tumor cytotoxicity in murine bone marrow-derived macrophages by two synthetic lipopeptide analogues. *Biol. Chem. Hoppe-Seyler 370*, 575–582.
5. J. Metzger, K.–H. Wiesmüller, R. Schaude, W. G. Bessler and G. Jung (1991). Synthesis of novel immunologically active tripalmitoyl-*S*-glyceryl-cysteinyl lipopeptides as useful intermediates for immunogen preparations. *Int. J. Peptide Prot. Res. 37*, 46–57.
6. K.–H. Wiesmüller, G. Jung and G. Hess (1989). Novel low-molecular weight synthetic vaccine against foot-and-mouth disease containing a potent B-cell and macrophage activator. *Vaccine 7*, 29–33.
7. W. G. Bessler and G. Jung in: *Methods of Immunological Analysis* Vol. 2, *Samples and Reagents*, R. F. Masseyeff, W. H. Albert and N. A. Staines, Eds., p. 137–145, VCH Verlaggesellschaft, Weinheim 1993.
8. K.–H. Wiesmüller, W. Bessler and G. Jung (1992). Solid phase peptide synthesis of lipopeptide vaccines eliciting epitope specific B-, T-helper and T-killer cell response. *Int. J. Peptide Protein Res. 40*, 255–260.
9. K. Deres, H.–J. Schild, K.–H. Wiesmüller, G. Jung and H.–G. Rammensee (1989). *In vivo* priming of virus specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine. *Nature 342*, 561–564.
10. H. Schild, K. Deres, K.–H Wiesmüller, G. Jung and H.–G. Rammensee (1991). Efficiency of peptides and lipopeptides for *in vivo* priming of virus-specific cytotoxic T cells. *Eur. J. Immunol. 21*, 2649–2654.
11. J. W. Metzger, K.–H. Wiesmüller and G. Jung (1991). Synthesis of N_α-Fmoc protected derivatives of *S*-(2,3-dihydroxypropyl)-cysteine and their application in peptide synthesis. *Int. J. Peptide Prot. Res. 38*, 545–554.
12. E. B. Kester, C. J. Gaiser and M. E. Lazar (1943). Glycidyl esters of aliphatic acids. *J. Org. Chem. 8*, 550–556.
13. J. W. Metzger, K.–H Wiesmüller and G. Jung in: *Innovation and Perspectives in Solid Phase Synthesis, Peptides, Polypeptides and Oligonucleotides*. R. Epton, Ed., p. 433–436, Andover, Intercept Ltd 1992.
14. E. Atherton, C. J. Logan and R. C. Sheppard (1981). Peptide synthesis. Part 2. Procedures for solid-phase synthesis using N^α-fluorenylmethoxycarbonyl-amino-acids on polyamide supports. Synthesis of substance P and of acyl carrier protein 65–74 decapeptide. *J. Chem. Soc. Perkin I*, 538–546.
15. J. Metzger, A. Olma, M. T. Leplawy and G. Jung (1987). Synthesis of a splenocyte activating a-methylserine containing lipopentapeptide. *Z. Naturforsch. 42b*, 1195–1201.
16. J. Metzger, K.–H Wiesmüller and G. Jung in: *Peptides 1992*, C. H. Schneider and A. N. Eberle, Eds., p. 451–452, Escom, Leiden 1992.
17. J. W. Metzger, W. Beck and G. Jung (1992). Ion spray mass spectrometry of lipopeptide vaccines. *Angew. Chem. Int. Ed. Engl. 31*, 226–228.
18. W. Bessler and B. P. Ottenbreit (1977). Studies on the mitogenic principle of the lipoprotein from the outer membrane of *E. coli*. *Biochem. Biophys. Res. Commun. 76*, 239–246.
19. A. Beck-Sickinger and J. W. Metzger in: Peptides: Chemistry and Biology, J. A. Smith and J. E. Rivier, Eds., p. 472–473, Escom, Leiden 1992.
20. P. Roepstorff and K. Fohlmann (1984). Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom. 11*, 601.
21. J. W. Metzger, W. H. Sawyer, B. Wille, L. Biesert, W. G. Bessler and G. Jung (1993). Interaction of immunologically-active lipopeptides with membranes. *Biochim. Biophys. Acta 1149*, 29–39.
22. A. Reitermann, J. Metzger, K.–H. Wiesmüller, G. Jung and W. G. Bessler (1989). Lipopeptide derivatives of bacterial lipoprotein constitute potent immune adjuvants combined with or covalently coupled to antigen or hapten. *Biol. Chem. Hoppe-Seyler 370*, 343–352.
23. B. Kleine, W. Rapp, K.–H. Wiesmüller, M. Edinger, W. Beck, J. Metzger, R. Ataullakhanov, G. Jung and W. G. Bessler (1994). Lipopeptide-polyoxyethylene conjugates as mitogens and adjuvants. *Immunobiol. 190*, 53–66.
24. W. Beck, J. W. Metzger, K.–H Wiesmüller, A. Surovoy, B. Haas and G. Jung in: *Innovation and Perspectives in Solid Phase Synthesis, Peptides, Polypeptides and Oligonucleotides*, R. Epton, Ed., p. 343–347, Intercept Ltd, Andover 1992.
25. E. Borges, K.–H. Wiesmüller, G. Jung and P. Walden (1994). Efficacy of synthetic vaccines in the induction of cytotoxic T lymphocytes. Comparison of the costimulating support provided by helper T cells and lipoamino acids. *J. Immunol. Methods, 73*, 253–263.