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Synthesis of new P_3CS derivatives and their mitogenic activity on in vitro mice splenocytes

Maria Pappalardo [a,*], Ennio Bousquet [a], Agata Annino [a], Gabriella Lombardo [b], Renato Bernardini [b], Giuseppe Ronsisvalle [a]

[a] Department of Pharmaceutical Science, School of Pharmacy, School of Medicine, University of Catania, Viale Andrea 6, 95125 Catania, Italy
[b] Department of Pharmacology, School of Medicine, University of Catania, Viale Andrea 6, 95125 Catania, Italy

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Abstract

Vaccination against tumors represents a relevant issue in current human cancer therapy. The N-terminal part of the lipoprotein from the outer membrane of *Escherichia coli*, tripalmitoyl-*S*-glyceryl-Cys-Ser (P_3CS) and analogs with longer aminoacidic sequence are polyclonal activators for B-lymphocytes. Previous study reported that their *N*-2,2,2-trichloroethoxycarbonyl (Troc) derivatives increase immunocyte mitogenic activity. Therefore, in order to obtain compounds of greater activity and to investigate relationships between molecular structure of *S*-glyceryl skeleton and biological activity, we synthesized new Troc derivatives of P_3CS. The mitogenicity of compounds was determined in vitro, by measuring in vitro [^{3}H]-thymidine incorporation into splenocytes from Balb/c mice. Concentrations of compounds ranged from 0 to 64 μg/ml. In particular, *S*-[2,3-bis(trichloroethoxycarbonyloxy)]-*N*-trichloroethoxycarbonyl dipeptide derivative exhibited significant mitogenic activity endowed with high pharmacological potency. These new series of compounds could be used as potent immunoadjuvants for the development of novel synthetic vaccines for tumor immunotherapy.
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Keywords: Tripalmitoyl-*S*-glyceryl-Cys-Ser derivative; Tumor immunotherapy; Lipopeptides; Immunoadjuvant; Synthetic vaccine; Mitogenicity

1. Introduction

The lipopeptides derived from the N-terminal portion of Braun's lipoprotein, the major component of the outer membrane of *Escherichia coli* and other Enterobacteriacee, are potent B-lymphocyte activators [1–3]. They constitute novel and potent immunoadjuvants in mice, rabbit and other species, markedly enhancing the immune response when covalently coupled to antigens or given in mixture with conventional antigens, inducing antigen-specific antibodies in vivo and in vitro.

The lipopeptides are non-toxic, non-pyrogenic immunoadjuvants, unable to induce tissue damage when injected; thus, they are ideal candidate molecules to replace Freund's adjuvant avoiding side effects of the latter. The Braun's lipoprotein is composed of 58 aminoacids [4], bearing three N-terminal fatty acids bound to glycerocysteine as part of a biologically active region [5]. The natural lipoprotein contains a mixture of different fatty acids, palmitic acid being the main component, whereas the synthetic analog *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine (P_3Cys-Ser-Ser-Asn-Ala) contains palmitoyl residues only.

The synthetic lipopentapeptide P_3Cys-Ser-Ser-Asn-Ala exhibits the same mitogenic properties as the native lipoprotein and being chemically synthesized, does not contain endotoxin contaminations as found in various other bacterial products [6,7]. Variations in the native lipopeptide structure related to chain length and amino acid sequence of the peptide moiety, as well as modifications of the lipoaminoacid P_3Cys-OH, lead to reduction or even complete loss of the adjuvant activity [8].

Moreover, the minimal structure for biological activity is showed by the lipodipeptide *N*-palmitoyl-*S*-[2,3-

* Corresponding author.
E-mail address: mrspld@mbox.unict.it (M. Pappalardo).

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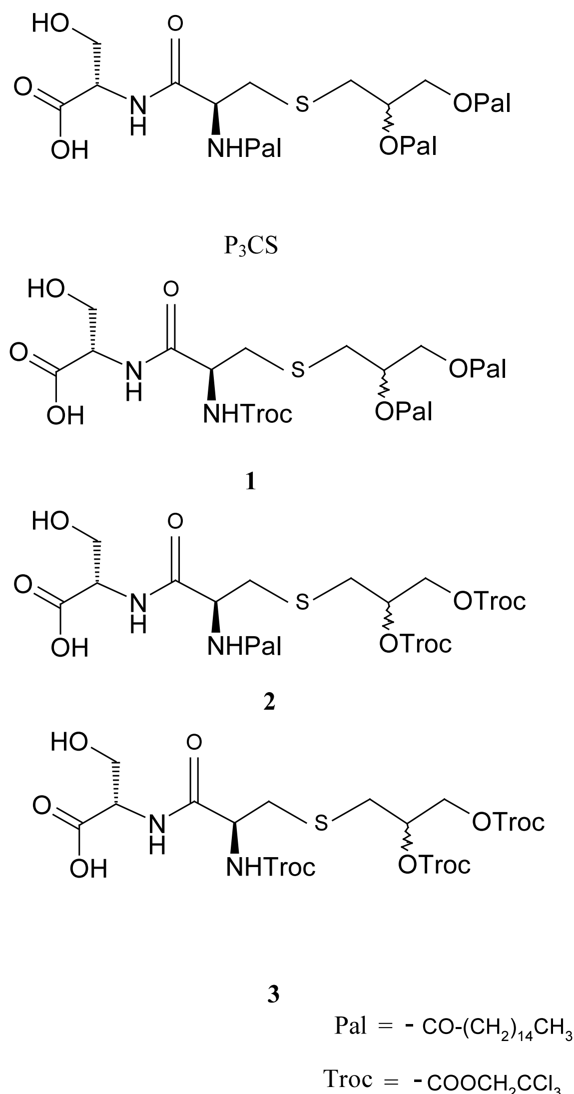

Chart 1. Chemical structure of P_3CS derivatives synthesized in this study.

bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-serine, P_3CS (Chart 1), whereas the lipoaminoacid P_3Cys-OH lacking a polar peptide moiety is almost inactive [9,10].

Covalent association of lipopeptide immunoadjuvant P_3CS to a glycopeptide has been proposed as a model of synthetic vaccine and elicited an immune response to tumor-associated carbohydrate antigens [11,12].

However, little is known on the different contribution offered to specificity of B-lymphocyte signaling activation by the plasma membrane interaction of lipid anchor group and polar headgroup of the mitogenic P_3Cys-Ser-lipopeptide [13,14].

Several studies have reported that activation process of B-lymphocytes elicited by lipopentapeptide and its analogs is strongly depending on chirality of asymmetric carbon atom C-2 of the (*S*)-glyceryl moiety [6,8,15]. Such evidence, along with the results reported by studies on lipopentapeptide derivatives with altered glycerol skeleton, suggests that glycerol moiety could play a more relevant role with respect to cysteinyl part in recognition process of synthetic lipopeptides [16,17]. It has also been reported that introduction of 2,2,2-trichloroethoxycarbonyl (Troc) group on cysteine residue of lipotetra- and pentapeptide greatly enhances the mitogenic activity [17–20], likely as a consequence of drastic alterations of physical properties, such as solubility and micellar state. Therefore, in order to acquire more information about the relationship between molecular requirements of *S*-glycerocysteine moiety and interactions with the lymphocyte plasma membrane, we have synthesized new derivatives of lipodipeptide P_3CS (**2**–**3** Chart 1) characterized by structural modifications obtained with insertion of Troc groups into the glycerol moiety.

We have also synthesized the compound with Troc group on cysteine residue (**1** Chart 1) for a comparison of the biological activity with the other lipodipeptides synthesized.

In addition, to determine whether the compounds synthesized could exert immunostimulating effects, we have tested their mitogenic activity on dispersed mouse splenocytes in culture in vitro.

2. Chemistry

Target compounds were synthesized according to the reaction sequence shown in Chart 2.

P_3CS was obtained as described by Prass et al. [13].

Compound **4** was obtained according to the method reported by Kurimura et al. [20].

Alkylation of thiol **4** with racemic 3-bromo-1,2-propandiol and triethylamine (TEA) gave **6** as mixture of diastereoisomers. The esterification of diol **6** with palmitic acid (Pal-OH), *N*,*N′*-dicyclohexylcarbodiimide (DCC) in presence of a catalytic amount of 4-dimethylaminopyridine (DMAP) gave **8** which was deprotected of the *tert*-butyl group with trifluoroacetic acid (TFA) affording **11**.

Compound **11** was condensed with O-*tert*-butyl-(*S*)-serine *tert*-butyl ester [H-Ser(Bu^t)-(OBu^t)] by the DCC/1-hydroxybenzotriazole (HOBT) method to give **14** which was deprotected to afford desired final compound **1**.

The synthesis of the thiol **5** and its alkylation to give the compound **7** were carried out according to the method reported by Wiesmüller et al. [6].

Esterification of compounds **6** and **7** with 2,2,2-trichloroethylchloroformate (Troc-Cl) in pyridine afforded **9** and **10**, respectively.

The deprotection of the *tert*-butyl groups with TFA yielded compounds **12** and **13**; subsequent coupling with

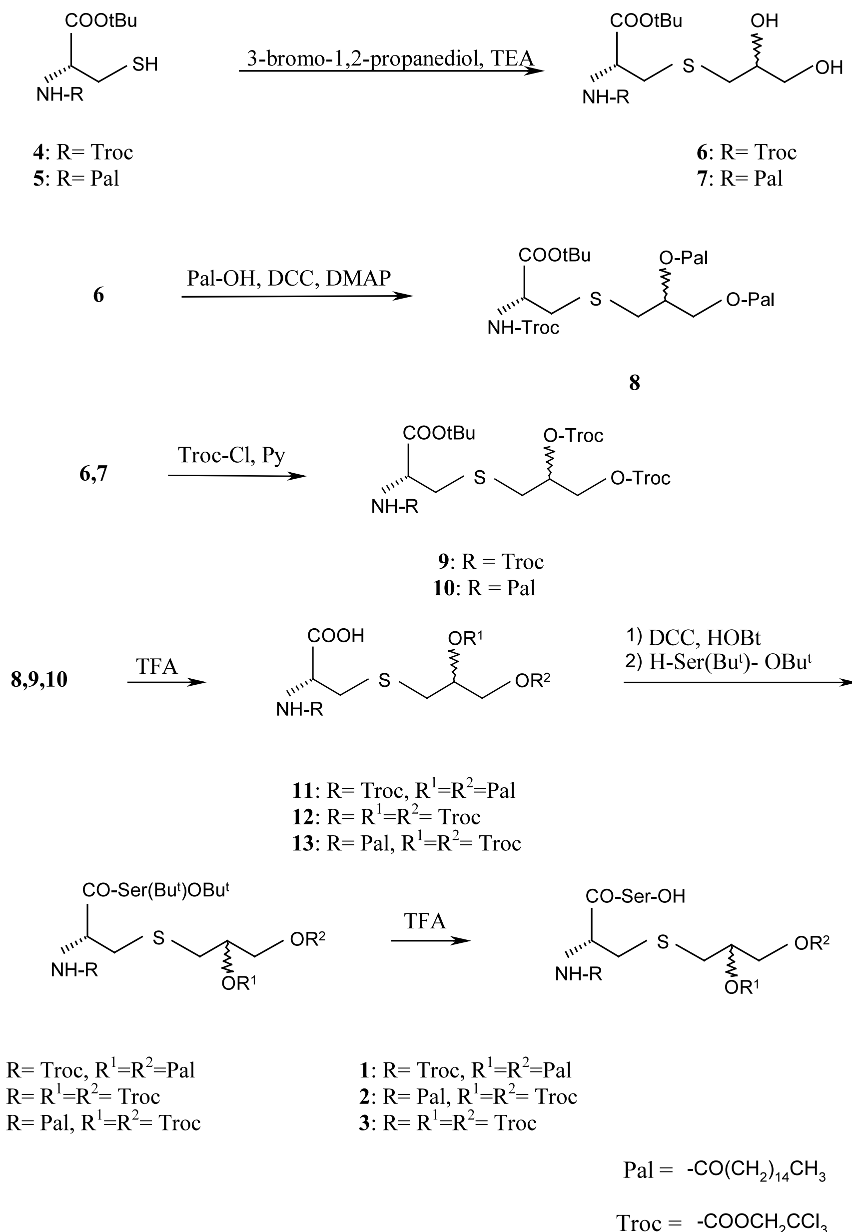

Chart 2. Preparation of lipopeptides **1**–**3**.

H-Ser(Bu^t)-(OBu^t) was carried out by the DCC/HOBT method to give **15** and **16**.

The final deprotection of the *tert*-butyl groups by treatment with TFA yielded desired compounds **2** and **3**. The structures of synthesized compounds were confirmed by analysis of the infrared (IR), ^{13}C NMR and FAB-MS spectra.

3. Experimental

3.1. Chemistry

^{13}C NMR spectra were recorded on a Varian Inova-200 spectrometer, using tetramethylsilane as an internal standard. FAB-MS was recorded on a Finnigan MHT 90 mass spectrometer. Analytical thin-layer chromatography was performed on Silica gel plates 60 F_{254} (Merck) with detection by quenching of UV fluorescence and by spraying with 50% NaClO and 1% KI in soluble starch. Column chromatography was carried out on Merck Silica gel 60 (mesh size 0.040–0.063 mm).

3.2. *S-[2,3-Dihydroxy-(2RS)-propyl]-N-2,2,2-trichloroethoxycarbonylcysteine tert-butyl ester (**6**)*

3-Bromo-1,2-propanediol (5.6 g, 36.1 mmol) and TEA (3.6 g, 36.1 mmol) were added to *N*-2,2,2-trichloroethoxycarbonylcysteine *tert*-butyl ester **(4)** (1.27 g, 3.6 mmol) in dry dimethylformamide (DMF)

(30 ml). After stirring for 15 min at 80 °C and 15 h at r.t., CH_2Cl_2 (300 ml) was added to the reaction mixture. The CH_2Cl_2 solution was washed with 5% citric acid (3 × 150 ml) and water (saturated with N_2). After drying over sodium sulfate, the solvent was removed in vacuo. The oily residue was purified by silica gel column chromatography using $CHCl_3$/MeOH/*n*-Hexane (15:1:1 v/v/v) as eluent to give **6** (1.15 g, 75%) as an yellow oil.

IR (neat)/cm: 3330, 1739. ^{13}C NMR ($CDCl_3$) δ: 27.9 (COOtBu-CH_3); 35.7 (*S*-glyceryl-CH_2); 36.6 (Cys-CH_2); 52.6 (Cys-CH); 64.9, 65.2 (*S*-glyceryl-OCH_2); 70.2 (S-glyceryl-CH); 74.6 (Troc-CH_2); 83.2 (tBu-C_q); 95.2 (Troc-CCl_3); 153.9 (Troc-CO); 168.9 (Cys-CO).

3.3. *S-[2,3-Bis-(palmitoyloxy)-(2RS)-propyl]-N-2,2,2-trichloroethoxycarbonyl-(R)-cysteine tert-butyl ester (**8**)*

Pal-OH (1.6 g, 6.24 mmol), DMAP (80 mg, 0.65 mmol) and DCC (1.3 g, 6.3 mmol) were added to a stirred solution of diol **6** (1.13 g, 2.65 mmol) in $CHCl_3$ (25 ml).

After being stirred for 4 h at r.t., the mixture was added of $CHCl_3$ (50 ml) and the precipitated dicyclohexylurea was filtered off. The organic solution was washed with 5% citric acid (3 × 80 ml), water (80 ml), 5% $NaHCO_3$ (3 × 80 ml) and water (3 × 80 ml). After drying over Na_2SO_4 and evaporating to dryness, the residue crystallized from $CHCl_3$/MeOH (1:3 v/v) to yield a colourless product (1.67 g, 70%). m.p. 42–43 °C. Rf: 0.9 ($CHCl_3$/MeOH/*n*-Hexane 7.5:0.5:0.5 v/v/v).

IR (KBr)/cm: 3300, 1739. ^{13}C NMR ($CDCl_3$) δ: 14 (Pal-CH_3); 22.6, 24.9 (Pal-CH_2); 27.9 (COOtBu-CH_3); 29.1, 29.4, 29.6, 31.9 (Pal-CH_2); 33.2 (*S*-glyceryl-CH_2); 34.2 (Pal-CH_2); 35 (Cys-CH_2); 54.5 (Cys-CH); 63.4 (S-glyceryl-OCH_2); 70.2 (*S*-glyceryl-CH); 74.6 (Troc-CH_2); 83.2 (tBu-C_q); 95.2 (Troc-CCl_3); 153.9 (Troc-CO); 168.9 (Cys-CO); 173.3 (Pal-CO).

3.4. *S-[2,3-Bis-(palmitoyloxy)-(2RS)-propyl]-N-2,2,2-trichloroethoxycarbonyl-(R)-cysteine (**11**)*

TFA (3 ml) was added to **8** (0.27 g, 0.3 mmol) at r.t. After being stirred for 2 h, the acid was removed in vacuo, and the residue dissolved in CH_2Cl_2 (50 ml). After washing with water (3 × 30 ml), the solution was dried over Na_2SO_4 and evaporated to dryness. The residue was chromatographed on silica gel with $CHCl_3$/MeOH/*n*-Hexane (7.5:0.5:0.5 v/v/v) as an eluent to give **11** as a colourless oil (0.22 g, 90%). Rf: 0.4 (ethyl acetate saturated with water).

^{13}C NMR ($CDCl_3$) δ: 14 (Pal-CH_3); 22.6, 24.9 (Pal-CH_2); 29.1, 29.4, 29.6, 31.9 (Pal-CH_2); 33 (*S*-glyceryl-CH_2); 34.2 (Pal-CH_2); 34.3 (Cys-CH_2); 54 (Cys-CH); 63.4 (*S*-glyceryl-OCH_2); 70.2 (*S*-glyceryl-CH); 74.6 (Troc-CH_2); 95.2 (Troc-CCl_3); 153.9 (Troc-CO); 173.3 (Pal-CO); 174.6 (Cys-COOH).

3.5. *S-[2,3-Bis-(palmitoyloxy)-(2RS)-propyl]-N-2,2,2-trichloroethoxycarbonyl-(R)-cysteinyl-O-tert-butyl-(S)-serine tert-butyl ester (**14**)*

11 (0.25 g, 0.3 mmol) was activated in CH_2Cl_2 (6 ml) with DCC (70 mg, 0.34 mmol) and HOBT (45 mg, 0.34 mmol) in DMF (3 ml) at 0 °C. After 30 min H-Ser(Bu^t)-OBu^t (75 mg, 0.34 mmol) was added. After being stirred for 15 h at r.t., the precipitated dicyclohexylurea was filtered off and the filtrate was concentrated in vacuo. The residue was dissolved in AcOEt (5 ml) and dicyclohexylurea was filtered off again. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2 (50 ml) and washed with 5% $NaHCO_3$ (3 × 25 ml) and water (3 × 25 ml). After drying over Na_2SO_4, the solvent was evaporated in vacuo. The residue was chromatographed on silica gel with CH_2Cl_2/MeOH/C_6H_6 (7:0.5:3 v/v/v) as an eluent to give **14** as a colourless oil (0.18 g, 60%). Rf: 0.6 (ethyl acetate saturated with water).

^{13}C NMR ($CDCl_3$) δ: 14 (Pal-CH_3); 22.6, 24.9 (Pal-CH_2); 27.3 (OtBu-CH_3); 27.9 (COOtBu-CH_3); 29.1, 29.4, 29.6, 31.9 (Pal-CH_2); 34 (*S*-glyceryl-CH_2); 34.2 (Pal-CH_2); 34.3 (Cys-CH_2); 53.1 (Cys-CH); 54.5 (Ser-CH); 61.2 (Ser-CH_2); 63.5 (*S*-glyceryl-OCH_2); 70.2 (*S*-glyceryl-CH); 74.3 (OtBu-C_q); 74.7 (Troc-CH_2); 83.2 (COOtBu-C_q); 95.3 (Troc-CCl_3); 154.4 (Troc-CO); 169.8 (Ser-CO); 170 (Cys-CO); 173.3 (Pal-CO).

3.6. *S-[2,3-Bis-(palmitoyloxy)-(2RS)-propyl]-N-2,2,2-trichloroethoxycarbonyl-(R)-cysteinyl-(S)-serine (**1**)*

TFA (3 ml) was added to **14** (0.3 g, 0.3 mmol). After being stirred for 2 h at r.t., the mixture was concentrated in vacuo and the residue was chromatographed on silica gel with CH_2Cl_2/MeOH/AcOH (9:1:0.01 v/v/v) as an eluent to give **1** (0.17 g, 60%) as a white powder. Rf: 0.25 ($CHCl_3$/MeOH/AcOH 9:1:0.01 v/v/v). $[\alpha]_D = +3.8°$ (c 1.04, $CHCl_3$).

^{13}C NMR ($CDCl_3$) δ: 14 (Pal-CH_3); 22.6, 24.9, 29.1, 29.4, 29.6, 31.9 (Pal-CH_2); 34 (*S*-glyceryl-CH_2); 34.2 (Pal-CH_2); 34.3 (Cys-CH_2); 53.1 (Cys-CH); 54.5 (Ser-CH); 62.4 (Ser-CH_2); 63.5 (*S*-glyceryl-OCH_2); 70.2 (S-glyceryl-CH); 74.7 (Troc-CH_2); 95.3 (Troc-CCl_3); 154.8 (Troc-CO); 170.1 (Cys-CO); 173.3 (Pal-CO); 173.7 (Ser-COOH). FAB-MS *m/z*: 933 $(M+H)^+$.

3.7. *S-[2,3-Bis-(trichloroethoxycarbonyloxy)-(2RS)-propyl]-N-2,2,2-trichloroethoxycarbonyl-(R)-cysteine tert-butyl ester (**9**)*

2,2,2-Trichloroethoxycarbonyl chloride (0.21 g, 1 mmol) was added dropwise under stirring to the

solution of diol **6** (0.43 g, 1 mmol) in pyridine (8 ml) at 0 °C. After being stirred for 45 min at 0 °C, H_2O (2 ml) was added. After being stirred for 15 min at r.t., the solution was washed with 5% citric acid, water and evaporated in vacuo. The oily residue was subjected to column chromatographed on silica gel with C_6H_6/Ethyl acetate (6:1 v/v) as an eluent to give **9** as a colourless oil (0.5 g, 65%). Rf: 0.4 (C_6H_6/Ethyl acetate 6:1 v/v).

^{13}C NMR ($CDCl_3$) δ: 27.9 (COOtBu-CH_3); 33.4, 33.6 (*S*-glyceryl-CH_2); 35.1, 35.3 (Cys-CH_2); 54.5 (Cys-CH); 67.3 (*S*-glyceryl-OCH_2); 74.6 (Troc-CH_2); 75.4 (*S*-glyceryl-CH); 83.5 (COOtBu-C_q); 95.2 (Troc-CCl_3); 153.3, 153.6, 153.9 (Troc-CO), 168.8 (Cys-CO).

*3.8. S-[2,3-Bis-(trichloroethoxycarbonyloxy)-(2RS)-propyl]-N-2,2,2-trichloroethoxycarbonyl-(R)-cysteine (**12**)*

TFA (3 ml) was added to **9** (0.23 g, 0.3 mmol) at r.t. After being stirred for 30 min, the acid was removed in vacuo. The residue was purified by precipitation from diethyl ether/light petroleum 30–50 °C (1:4 v/v) and dried in vacuo with diphosphorus pentoxide. Yield 0.15 g, 70%. Rf: 0.2 (CH_2Cl_2/MeOH/AcOH 9:1:0.01 v/v/v).

^{13}C NMR ($CDCl_3$) δ: 32.3, 32.4 (*S*-glyceryl-CH_2); 34.5, 34.7 (Cys-CH_2); 53.6, 53.8 (Cys-CH); 67.3 (*S*-glyceryl-OCH_2); 74.6 (Troc-CH_2); 75.4 (*S*-glyceryl-CH); 95.2 (Troc-CCl_3); 153.3, 153.6, 154.2 (Troc-CO), 173.7 (Cys-COOH).

*3.9. S-[2,3-Bis-(trichloroethoxycarbonyloxy)-(2RS)-propyl]-N-2,2,2-trichloroethoxycarbonyl-(R)-cysteinyl-O-tert-butyl-(S)-serine tert-butyl ester (**15**)*

12 (0.21 g, 0.3 mmol) was activated in CH_2Cl_2 (6 ml) with DCC (70 mg, 0.34 mmol) and HOBT (45 mg, 0.34 mmol) in DMF (3 ml) at 0 °C. After 30 min H-Ser(Bu^t)-OBu^t (75 mg, 0.34 mmol) was added. After being stirred for 15 h at r.t., the precipitated dicyclohexylurea was filtered off and the filtrate was concentrated in vacuo. The residue was dissolved in AcOEt (5 ml) and dicyclohexylurea was filtered off again. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2 (50 ml) and washed with 5% $NaHCO_3$ (3 × 25 ml) and water (3 × 25 ml).

After drying over Na_2SO_4, the solvent was evaporated in vacuo. The residue was chromatographed on silica gel with $CHCl_3$/MeOH (9:1 v/v) as an eluent to give **15** as a colourless oil (0.17 g, 60%). Rf: 0.5 ($CHCl_3$/MeOH 9:1 v/v).

^{13}C NMR ($CDCl_3$) δ: 27.3 (OtBu-CH_3); 27.9 (COOtBu-CH_3); 34.6 (*S*-glyceryl-CH_2); 35.5 (Cys-CH_2); 53.1 (Cys-CH); 53.8–54.5 (Ser-CH); 61.2 (Ser-CH_2); 67.3 (*S*-glyceryl-OCH_2); 74.3 (OtBu-C_q); 74.6 (Troc-CH_2); 75.4 (*S*-glyceryl-CH); 83.5 (COOtBu-C_q); 95.2 (Troc-CCl_3); 153.3, 153.6, 154.2 (Troc-CO), 169.8 (Ser-CO); 170.2 (Cys-CO).

*3.10. S-[2,3-Bis-(trichloroethoxycarbonyloxy)-(2RS)-propyl]-N-2,2,2-trichloroethoxycarbonyl-(R)-cysteinyl-(S)-serine (**3**)*

TFA (3 ml) was added to **15** (0.27 g, 0.3 mmol). After being stirred for 30 min at r.t., the mixture was concentrated in vacuo and the residue was chromatographed on silica gel with CH_2Cl_2/MeOH/AcOH (9:1:0.01 v/v/v) as an eluent to give **3** (0.14 g, 60%) as a white powder. Rf: 0.35 ($CHCl_3$/MeOH/AcOH 9:1:0.01 v/v/v). $[\alpha]_D = +9.2°$ (c 1.3, $CHCl_3$)

^{13}C NMR ($CDCl_3$) δ: 32.6 (*S*-glyceryl-CH_2); 34.6 (Cys-CH_2); 54.1 (Cys-CH); 54.5 (Ser-CH); 62.4 (Ser-CH_2); 67.5 (*S*-glyceryl-OCH_2); 74.6 (Troc-CH_2); 75.4 (*S*-glyceryl-CH); 95.2 (Troc-CCl_3); 153.3, 153.6, 154.2 (Troc-CO), 170.5 (Cys-CO); 174.8 (Ser-COOH). FAB-MS m/z: 808 $(M+H)^+$.

*3.11. S-[2,3-Bis-(trichloroethoxycarbonyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteine tert-butyl ester (**10**)*

2,2,2-Trichloroethoxycarbonyl chloride (0.21 g, 1 mmol) was added dropwise under stirring to the solution of diol **7** (0.5 g, 1 mmol) in pyridine (8 ml) at 0 °C.

After being stirred for 45 min at 0 °C, H_2O (2 ml) was added. After being stirred for 15 min at r.t., the solution was washed with 5% citric acid, water and evaporated in vacuo.

The oily residue was subjected to column chromatographed on silica gel with $CHCl_3$/MeOH/C_6H_6 (8.5:0.2:0.2 v/v/v) as an eluent to give **10** as a colourless oil (0.55 g, 65%). Rf: 0.85 ($CHCl_3$/MeOH/*n*-Hexane 15:1:1 v/v/v).

^{13}C NMR ($CDCl_3$) δ: 14 (Pal-CH_3); 22.6, 25.5 (Pal-CH_2); 27.9 (COOtBu-CH_3); 29.3, 29.4, 29.6, 31.9 (Pal-CH_2); 35.3 (*S*-glyceryl-CH_2); 35.4 (Cys-CH_2); 36.5 (Pal-CH_2); 52.5 (Cys-CH); 68.3 (*S*-glyceryl-OCH_2); 75.2 (*S*-glyceryl-CH); 76.3 (Troc-CH_2); 83.4 (COOtBu-C_q); 93.8 (Troc-CCl_3); 153.4 (Troc-CO); 169.5 (Cys-CO); 173.2 (Pal-CO).

*3.12. S-[2,3-Bis-(trichloroethoxycarbonyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteine (**13**)*

TFA (3 ml) was added to **10** (0.28 g, 0.3 mmol) at r.t.

After being stirred for 30 min, the acid was removed in vacuo and the residue dissolved in CH_2Cl_2 (50 ml). After washing with water (3 × 30 ml), the solution was dried over Na_2SO_4 and evaporated to dryness to give **13** as a colourless oil (0.22 g, 95%). Rf: 0.2 (CH_2Cl_2/MeOH/C_6H_6 8:1:2 v/v/v).

^{13}C NMR ($CDCl_3$) δ: 14 (Pal-CH_3); 22.6, 25.5 (Pal-CH_2); 29.3, 29.4, 29.6, 31.9 (Pal-CH_2); 32.3 (Cys-CH_2); 34.2 (*S*-glyceryl-CH_2); 36.5 (Pal-CH_2); 52.5 (Cys-CH); 68.3 (*S*-glyceryl-OCH_2); 74.6 (Troc-CH_2); 75.2 (*S*-glyceryl-CH); 93.8 (Troc-CCl_3); 153.4 (Troc-CO); 173.2 (Pal-CO); 173.5 (Cys-COOH).

*3.13. S-[2,3-Bis-(trichloroethoxycarbonyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteinyl-O-tert-butyl-(S)-serine tert-butyl ester (**16**)*

13 (0.23 g, 0.3 mmol) was activated in CH_2Cl_2 (6 ml) with DCC (70 mg, 0.34 mmol) and HOBT (45 mg, 0.34 mmol) in DMF (3 ml) at 0 °C. After 30 min H-Ser(Bu^t)-OBu^t (75 mg, 0.34 mmol) was added. After being stirred for 15 h at r.t., the precipitated dicyclohexylurea was filtered off and the filtrate was concentrated in vacuo. The residue was dissolved in AcOEt (5 ml) and dicyclohexylurea was filtered off again. After evaporation of the solvent, the residue was dissolved in CH_2Cl_2 (50 ml) and washed with 5% $NaHCO_3$ (3 × 25 ml) and water (3 × 25 ml). After drying over Na_2SO_4, the solvent was evaporated in vacuo. The residue was chromatographed on silica gel with CH_2Cl_2/MeOH/C_6H_6 (8:1:2 v/v/v) as an eluent to give **16** as a colourless oil (0.19 g, 65%). Rf: 0.25 (CH_2Cl_2/MeOH/C_6H_6 8:1:2 v/v/v).

^{13}C NMR ($CDCl_3$) δ: 14 (Pal-CH_3); 22.6, 25.5 (Pal-CH_2); 27.4 (OtBu-CH_3); 27.9 (COOtBu-CH_3); 29.3, 29.4, 29.6, 31.9 (Pal-CH_2); 34.8 (S-glyceryl-CH_2); 35.4 (Cys-CH_2); 36.5 (Pal-CH_2); 52.4 (Cys-CH); 55.1 (Ser-CH); 61.1 (Ser-CH_2); 67.5 (S-glyceryl-OCH_2); 73.8 (OtBu-C_q); 74.6 (Troc-CH_2); 75.2 (S-glyceryl-CH); 83.4 (COOtBu-C_q); 94.2 (Troc-CCl_3); 153.5 (Troc-CO); 169.5 (Ser-CO); 170 (Cys-CO); 173.2 (Pal-CO).

*3.14. S-[2,3-Bis-(trichloroethoxycarbonyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteinyl-(S)-serine (**2**)*

TFA (3 ml) was added to **16** (0.29 g, 0.3 mmol). After being stirred for 30 min at r.t., the acid was removed in vacuo and the residue was chromatographed on silica gel with CH_2Cl_2/MeOH/AcOH (9:1:0.01 v/v/v) as an eluent to give **2** as a colourless oil (0.15 g, 60%). Rf: 0.25 (CH_2Cl_2/MeOH/AcOH 9:1:0.01 v/v/v). $[\alpha]_D = +10°$ (c 0.4, $CHCl_3$).

^{13}C NMR ($CDCl_3$) δ: 14 (Pal-CH_3); 22.6, 25.5 (Pal-CH_2); 29.3, 29.4, 29.6, 31.9 (Pal-CH_2); 34.7 (*S*-glyceryl-CH_2); 35.2 (Cys-CH_2); 36.5 (Pal-CH_2); 52.4 (Cys-CH); 55.2 (Ser-CH); 62.7 (Ser-CH_2); 67.5 (*S*-glyceryl-OCH_2); 74.6 (Troc-CH_2); 75.2 (*S*-glyceryl-CH); 94.2 (Tro-CCl_3); 153.5 (Troc-CO); 170.4 (Cys-CO); 173.2 (Pal-CO); 174.6 (Ser-COOH). FAB-MS *m/z*: 871 $(M+H)^+$.

4. Biological activity

4.1. Preparation of immunoadjuvants in DMF/medium (DM)

The lipopeptide was dissolved in a minimum quantity of DMF. Then, the solution was diluted in medium, constituted by RPMI-1640 (GIBCO, Life Technologies, Paisley, Scotland) and 10% fetal calf serum (FCS, GIBCO), to obtain a solution 0.5% in DMF, not toxic for the cells.

4.2. Animals

Adult female Balb/c mice, 6–10 weeks old (Charles River Italia S.p.A, Calco, LC, Italy). Mice were housed, 6 per cage, in standard conditions (24 ± 1 °C, 12 h light/dark cycle, commercial rat chow and tap water available ad libitum) for at least 5 days prior to sacrifice.

4.3. Mouse splenocyte cultures and [methyl-^{3}H]-thymidine incorporation test

Mice were sacrificed by decapitation, according to current GLP guidelines. Spleen was sterilely minced using the tip of a syringe needle (21 gauge) and the resulting splenocyte suspension was then stratified onto Ficoll gradient (1:2 w/v) (Ficoll-Paque, Pharmacia-Biotech, Uppsala, Sweden) and centrifuged at 3000 rpm/15 min, to separate splenocytes from other cellular elements. The resulting pellet was washed twice in PBS and suspended in RPMI-1680 medium supplemented with 10% FCS. A volume of 100 μl (2×10^5 cells) of cell suspension and 100 μl of each solution containing graded concentrations (0–64 μg/ml) of the compounds were placed in a 96-well microplate (Tissue Culture Plate 96-well, U-bottom, Falcon, Becton Dickinson Labware, Oxnard, CA) and incubated at 37 °C for 72 h in a 5% CO_2 atmosphere. Then, cells were incubated for 6 h with 0.625 μCi/well (in 10 ml) of [methyl-^{3}H]-thymidine (Amersham Life Science, Little Chalfont, TRA-120, specific activity 5 Ci/mmol), harvested by means of an automatic apparatus (Skatron INC, Sterling, USA), collected onto fiber glass filters (Skatron) and mixed with 3.5 ml of scintillation liquid (Instagel, Beckman). Radioactivity was counted for 1 min in a β-counter. Results were expressed as the mean of count per minute. All experiments were run in triplicate and at least twice.

EC50 was calculated for all used compounds. Percent increase of mitogenic activity of splenocytes was calculated on the basis of the maximally active concentration of each compound and according to the following formula:

$$\left[\frac{\text{stimulated}^3\text{H-Thyincorporation}}{\text{basal}^3\text{H-Thyincorporation}} \times 100\right] - 100$$

4.4. *Statistical analysis of results*

All data were subjected to one-way analysis of variance, followed by a Duncan's test. Significance was accepted for $P < 0.05$.

5. Results and discussion

To test mitogenic activity of synthetic lipodipeptides **1–3,** compared to P_3CS activity, we measured [^{3}H]thymidine incorporation in Balb/C mice dispersed splenocytes incubated with graded concentrations of each compound (Fig. 1).

All the compounds tested exhibited significant, concentration-dependent mitogenic activity.

We used the mitogen Concanavalin A [21], known to stimulate splenocyte proliferation through a specific interaction with their membranes, as a positive control.

Among compounds tested, lipodipeptide **1** and **3** showed the highest potency.

In addition, lipopeptide 3 was about fourfold more potent than the reference compound P_3CS, whereas lipopeptide 1 was about equivalent to the latter (Table 1).

Maximal [^{3}H]-thymidine incorporation with lipopeptides **1–2** and P_3CS was achieved, respectively at the following concentrations: 32, 32 and 64 μg/ml, whereas

Table 1
EC_{50} values and max. increase of P_3CS and analogs

Materials tested	EC_{50} μg/ml	Max.% increase	μg/ml
P_3CS	8.6	134	64
Lipopeptide 1	9.9	584	32
Lipopeptide 2	18.4	57	32
Lipopeptide 3	1.9	167	4

lipodipeptide **3** reached its maximal effect at significantly lower concentrations (4 μg/ml).

All compounds showed a concentration-related cytotoxicity, reflected by the decline of their mitogenic activity, as indicated by the decline of [^{3}H]-Thy incorporation at high concentrations (higher than 64 μg/ml) (Fig. 1).

The lipodipeptide P_3CS and its analogs reported in this study, are characterized by an amphiphilic structure constituted of a polar headgroup (Ser residue) and a lipidic anchor (acylic chains).

Such structural elements could differently cooperate in mitogenicity activity of these compounds.

Thus, the molecular mechanism underlying lipopeptide mitogenicity probably involves either a specific interaction of the polar headgroup with membrane binding proteins, or an a specific interaction by insertion of the idrophobic region into the lipid membrane layer.

Based on mitogenic activity profile showed by our compounds, the trichloroethoxycarbonyl group on cysteine residue (lipodipeptides **1** and **3**) seems to contribute positively to specific membrane interactions. In fact the lipodipeptide **1**, bearing Troc group only on the

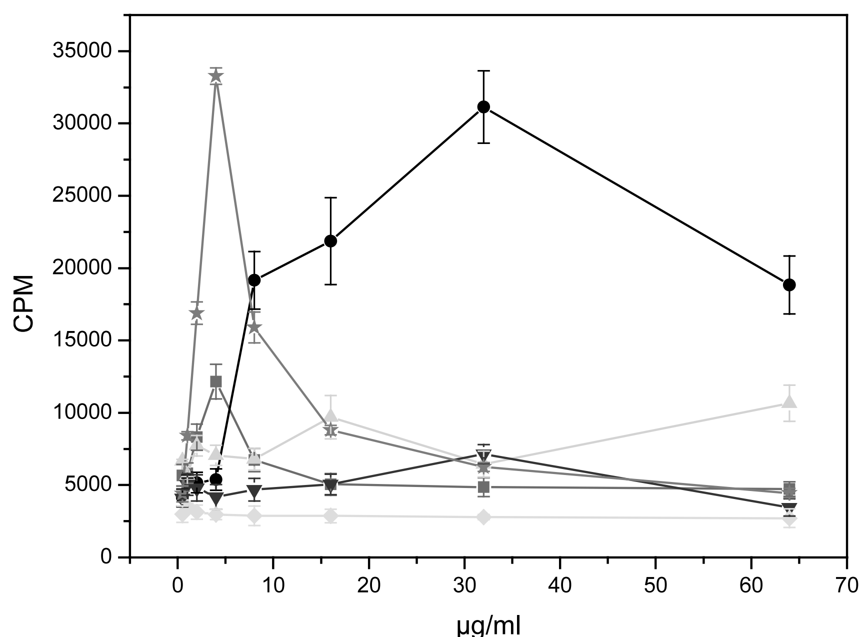

Fig. 1. Dose-response curves for [^{3}H]thymidine incorporation splenocytes of Balb/c mice after stimulation with P_3CS (▲), lipopeptides 1 (●), 2 (▼), 3 (■), untreat (◇) and Concanavalin A (☆).

cysteine residue, exhibits a mitogenic activity significantly higher than P_3CS; such proliferative effect on splenocyte responsiveness is further potentiated by the presence of Troc groups also on *S*-glyceryl skeleton (lipodipeptide **3**).

It is plausible to hypothesize that Troc groups cooperate in orienting polar headgroup into lipid membrane through their a specific additional interactions more effectively than palmitoyl chains [22].

On the other hand, if the cysteine residue supports palmitoyl chain, the Troc groups may not have relevant impact on the biological activity. Such experimental results seem to suggest that in this our class of compounds the NH-functionality of cysteine residue could play a critical role if compared to the *S*-glyceril part for the activation process specificity.

Possible use of P_3CS analogs, if admixed or covalently bound to antigens or haptens, could be envisioned as potent immunoadjuvants for development of novel synthetic antitumor vaccines.

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