Synthesis of a B-Lymphocyte Activating α-Methylserine Containing Lipopentapeptide

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Dedicated to Professor Dr. Ernst Bayer on the occasion of his 60th birthday

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The lipopentapeptide S-[2,3-bis(palmitoyloxy)-2-R.S-propyl]-N-palmitoyl-[R]-cysteinyl-[R]-α-methylseryl-[R]-α-methylseryl-[S]-asparaginyl-[S]-alanine (Pam,Cys-a-MeSer-a-MeSer—Asn—Ala) has been synthesized as an analogue of the immunoadjuvant Pam,Cys—Asn—Ala (TPP) via fragment condensations. The two [R]-α-methylserine residues were introduced instead of [L]-serine in order to obtain a mitogenic lipopeptide resistant against proteases. As the parent lipopeptide TPP the analytically pure analogue with MeSer was found to induce enhanced cell proliferation of spleen lymphocytes.

S-[2,3-bis(palmitoyloxy)propyl]-N-palmitoyl-L-cysteinyl-L-seryl-L-seryl-L-asparaginyl-L-alanine (TPP), which represents the N-terminal part of the lipoprotein from the outer membrane of E. coli, has been synthesized by condensation of S-[2,3-bis(palmitoyloxy)-2-R.S-propyl]-N-palmitoyl-R-cysteine (Pam,Cys—OH) (1) with the tetrapeptide ester H-Ser(Bu')—Ser(Bu')—Asn—Ala—OBu' [1]. TPP possesses a mitogenic activity comparable to that of the native lipoprotein. TPP and other analogues of lipoprotein cause a polyclonal activation of the B-lymphocyte pool and a stimulation of the reticuloendothelial system. They are able to enhance weakly pronounced immune responses upon simultaneous administration with the antigen (adjuvant effect). Moreover, antigen-specific immune response in vivo can be enhanced by covalently coupling the lipopeptide adjuvants to weakly immunogenic peptides or other haptens [2—4].

Since the immunogenic lipopeptide conjugates may be attacked by enzymes after administration, we systematically develop enzyme resistant analogues. Unspecific esterases may slowly degrade the lipid part of the nitrogen and proteases can hydrolyse the peptide moiety. This degradation of the lipopeptide adjuvants counteracts to a longer lasting immunoadjuvant activity. Lipopeptides, which are stable against proteases and esterases, should therefore be more potent adjuvants.

Since it was not advisable to change too many structural parameters simultaneously and because of the tedious multistep synthesis of the lipopentapeptide, we decided to improve the protease resistance at first. Therefore the lipopeptide 12 (Fig. 1) was synthesized, which has the amino acid sequence of TPP with the only difference that both serine residues are substituted by two α-methylserine residues.

Peptide syntheses using α-methylserine were reported recently [5]. Similar to α-methylalanine (Aib) peptides [6, 7] α-MeSer-peptides should adopt conformationally strongly restricted structures. α-Methylserine containing peptides are not degradable by proteases [8], therefore carboxypeptidases can degrade this lipopeptide only to the lipotripeptide or lipotetrapeptide. Since Pam,Cys-Ser was found to exhibit about 50% of the biological activity of the complete lipoprotein [9] one would expect mitogenic activity also for Pam,Cys—α-MeSer—α-MeSer. However, the mitogenic activity of 12 could be considerably altered due to configurational

Abbreviations: α-MeSer = α-methylserine; Pam,Cys = S-[2,3-bis(palmitoyloxy)-2-R.S-propyl]-N-palmitoyl-[R]-cysteinyl; TPP = S-[2,3-bis(palmitoyloxy)-2-R.S-propyl]-N-palmitoyl-cysteinyl-[R]-asparaginyl-[S]-alanine; DCC = dicyclohexylcarbodiimide; DMF = N,N-dimethylformamide; HOBT = 1-hydroxybenzotriazol; NMM = N-methylmorpholine; TDM = 4,4'-bis(dimethylamino)-diphenylmethane; Tos—OH = p-Toluolsulfonic acid; FD—MS = field desorption mass spectrometry; TLC = thin-layer chromatography.

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or conformational effects of the sterically hindered [R]-α-MeSer residues respectively.

**Experimental Part**

*Thin-layer chromatography:* Rf values were determined in equilibrated glass chambers using pre-formed plates Kieselgel 60 F254 (Merck No. 5714) and the following solvent systems (v/v):

1. Ethyl acetate saturated with water; II chloroform/methanol 8:2; III chloroform/methanol/water 65:25:4; IV chloroform/methanol/acetic acid 90:10:1; V chloroform/methanol 9:1; VI chloroform/acetic acid 14:1; VII chloroform/methanol 95:5; VIII chloroform/methanol/acetic acid 90:10:5; IX chloroform/2-propanol/acetic acid 90:10:1; X 1-butanol/acetic acid/water 4:1:1.

For detection ninhydrin and chloride/4,4'-bis-(dimethylamino)diphenylmethane were used.

**Amino acid analysis:** As α-methylalanine (Aib) and isovaline (Iva) [10] the α-methylated serine gives also considerably reduced colour yields with ninhydrin in the amino acid analyzer. Pam-Cys—OH gives at least three products appearing in the chromatogram as discussed elsewhere [1]. Therefore standards containing equimolar amounts of α-MeSer, Pam-Cys—OH, Asn and Ala were hydrolysed (6 N HCl, 110 °C, 24 h) and analyzed under the same conditions as the probes.

**Spectroscopy:** 1H NMR spectra were recorded on Tesla BS-487C (80 MHz) with TMS as internal standard. 13C NMR spectra were measured on Bruker WP-80 (20.115 MHz) and Bruker WM 400 (100.62 MHz). Optical rotations were measured on the Perkin-Elmer 141 photopolarimeter. FD-MS spectra were measured on a MAT 711A (Varian); ion source temperature 50 °C.

**Benzzyloxycarbonyl-[R]-O-tert-butyl-α-methylerine methyl ester (Z-[R]-α-MeSer(Bu')-OMe) (3)**

Z-[R]-α-MeSer—OH (2) (2.53 g, 10 mmol, [α]D = −5.8 (c = 1, MeOH), m.p. 91–92 °C) was esterified at 0 °C with ethereal solution of diazomethane, and the solution was evaporated in vacuo. The oily residue was dissolved in dichloromethane (25 ml), a catalytic amount of sulfuric acid was added, and the solution was placed in a pressure flask, cooled to −20 °C, saturated with isobutene in excess (20 ml) and shaken for 3 d at room temp. An additional amount of isobutene was added, and the shaking was continued for 2 d. The oily product 3 was sufficiently pure for further work was isolated after usual washing and evaporation procedure as a neutral fraction. Yield 3.20 g (96%); 1H NMR (CDCl3), δ [ppm]: 1.06 (s, 9H); 1.49 (s, 3H); 3.45, 3.70 (AB system, 2H, J = 8.5 Hz); 3.50 (s, 3H); 5.00 (s, 2H); 5.35 (s broad, 1H); 7.31 (s, 5H).

H2N\text{O}5 (323.38)

Calcd C 63.14 H 7.79 N 4.33,
Found C 62.90 H 7.60 N 4.90.

**Benzzyloxycarbonyl-[R]-O-tert-butyl-α-methylerine methyl ester p-toluene-sulfonate**

(Tos-OMe-H-[R]-α-MeSer(Bu')-OMe) (5)

Z-[R]-α-MeSer(Bu')-OMe (3) (1 g, 3.1 mmol) was dissolved in methanol (30 ml) and Tos-OMe (0.54 g, 3.1 mmol) was added. Hydrogenation was carried out in the presence of 10% Pd/C (0.2 g) for 3 d at atmospheric pressure (TLC control). The catalyst was removed, and the solution was evaporated in vacuo. The oily product 5 (1.11 g, 96%) was used for subsequent coupling without purification.

1H NMR of the free amino acid ester H-[R]-α-MeSer(Bu')-OMe (5) in the presence of shifting reagent Eu(tfc) showed no splitting of Me and Bu' group signals. It means that enantiomeric purity of both H-[R]-α-MeSer(Bu')-OMe (5) and its educt Z-[R]-α-MeSer(Bu')-OMe (3) was sufficient. Signal splitting is observed in the case of racemic H-[α-MeSer(Bu')-OMe].

**Benzzyloxycarbonyl-[R]-O-tert-butyl-α-methylerine methyl ester p-toluene-sulfonate**

(Z-[R]-α-MeSer(Bu')-H-[R]-α-MeSer(Bu')-OMe) (6)

Z-[R]-α-MeSer(Bu')-OH (4) (0.93 g, 3 mmol) was activated in DMF (10 ml) at 0 °C with HOBt [11]
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(0.41 g, 3 mmol) and DCC (0.62 g, 3 mmol, dissolved in CH₂Cl₂) for 20 min.

Tos-ΟΗ-Η-[R]-α-MeSer(Bu′)-ΟMe (5) (1.11 g, 3 mmol) in DMF and triethylamine (0.42 ml, 3 mmol) was added. Stirring was continued for 1 h at 0 °C for 12 h at room temp. The solvent was removed in vacuo, and ethyl acetate was added to the residue. After removal of the precipitated N,N′-dicyclohexylurea the solution was washed with 1 N NaHSO₄, 1 N NaHCO₃, brine, water and dried over Na₂SO₄. Evaporation in vacuo yielded crude product which was purified by silica gel flash chromatography using CHCl₃/CH₃OH (98:2) as eluent. The yield of oily dipeptide derivative 6 was 0.63 g (43.8%). \( \alpha \) = 10.52 (c = 0.8, CH₃OH); \( R_f(V) = 0.88; R_f(VI) = 0.84; \) \( R_f(VII) = 0.81. \)

C₂₅H₄₀N₂O₈ (480.59)

Caled C 62.48 H 8.39 N 5.83

Found C 62.95 H 8.50 N 6.10.

Benzyloxy carbonyl-[R]-O-tert-butyl-a-methylseryl-\[R\]-O-tert-butyl-a-methylserine (Z-[R]-α-MeSer(Bu′)-[R]-α-MeSer(Bu′)-OH) (7)

Z-[R]-α-MeSer(Bu′)-[R]-α-MeSer(Bu′)-OMe (6) (0.63 g, 1.3 mmol) was saponified in methanol (5 ml) and 1 N NaOH (2 ml) at room temp, for 48 h. After evaporation of methanol, the residue was dissolved in water (10 ml), washed with ether and acidified with KHSO₄. The product was extracted with three portions of ethyl acetate, washed with water and dried over MgSO₄. Evaporation of the solvent yielded 0.58 g of crude product 7 which was purified by column chromatography (silica gel 60–280 mesh) using CHCl₃/CH₃OH/CH₃COOH (90:10:5) as eluent. The yield of oily dipeptide acid 7 was 0.43 g (71%). \( \alpha \) = +10.14 (c = 0.8, CH₃OH); \( R_f(V) = 0.61; R_f(X) = 0.67; R_f(X) = 0.81. \)

The \( ^1 \)H NMR spectrum of the homodipeptide derivative 7 (Fig. 2) shows no signals splitting indicating a high purity.

C₂₅H₃₈N₂O₇ (466.56)

Caled C 61.78 H 8.21 N 6.00

Found C 62.13 H 8.30 N 5.95.

Benzyloxy carbonyl-[R]-O-tert-butyl-a-methylseryl-\[R\]-O-tert-butyl-a-methylseryl-[S]-asparaginyl-[S]-alanine-tert-butylester-(H-[R]-α-MeSer(Bu′)-[R]-α-MeSer(Bu′)-[S]-Asn-[S]-Ala-OBu′) (9)

Z-[R]-α-MeSer(Bu′)-[R]-α-MeSer-[S]-Asn-[S]-Ala-OBu′ (8) (120 mg, 0.26 mmol) was activated in a small volume of DMF at 0 °C with HOBt [1] (36 mg, 0.26 mmol) and DCC (54 mg, 0.26 mmol) for 30 min. H-[S]-Asn-[S]-Ala-OBu′ (8) [1] (81 mg, 0.31 mmol) in DMF and NMM (30 μl, 0.26 mmol) were added. After 4.5 h the solvent was removed in vacuo, and the residue was digerated in ethyl acetate. The precipitated N,N′-dicyclohexylurea was removed by centrifugation. The solution was washed several times with 5% KHSO₄, 5% NaHCO₃ and water and dried over Na₂SO₄. The solvent was evaporated, and the residue was chromatographed on Sephadex LH-20 (column 80 × 2.5 cm) in chloroform/methanol (1:1). Chlorine/TDM-positive fractions containing pure tetrapeptide 9 were combined, the solvent was removed in vacuo, and the residue was lyophilized from tert-butanol. Yield 95 mg (52%); m.p. 76 °C. Amino acid analysis: Ala 1.00(1); Asx 1.05(1).

C₂₅H₄₀N₂O₈ (707.86)

Caled C 62.95 H 8.50 N 6.10

Found C 61.94 H 8.78 N 9.64.

[R]-O-tet-butyl-a-methylseryl-[R]-O-tet-butyl-a-methylseryl-[S]-asparaginyl-[S]-alanine-tert-butylester-(H-[R]-α-MeSer(Bu′)-[R]-α-MeSer(Bu′)-[S]-Asn-[S]-Ala-OBu′) (10)

(Z-[R]-α-MeSer(Bu′)-[R]-α-MeSer-[S]-Asn-[S]-Ala-OBu′) (9) (80 mg, 0.11 mmol) was hydrogenated in methanol in the presence of Pd/C. After about 90 min (TLC-control) the catalyst was removed by centrifugation, and the solution was evaporated in vacuo. Like the educt 9 the product 10 is ninhydrin-negative, and it could be detected only with Cl₃/TDM. The oily tetrapeptide ester 10 was dried in vacuo for several hours before the next coupling step. Yield: 60 mg (95%); TLC: \( R_f(1) = 0.05; R_f(II) = 0.58; R_f(III) = 0.82; R_f(IV) = 0.13. \)

C₂₇H₅₇N₅O₈ (573.73)

S-[2,3-Bis(palmitoyloxy)-2-R,S]-propyld-N-palmitoyl-[R]-cysteiny-[R]-O-tet-butyl-a-methylseryl-[R]-O-tet-butyl-a-methylseryl-[S]-asparaginyl-[S]-alanine-tert-butylester (Pam₃Cys-[R]-α-MeSer(Bu′)-[R]-α-MeSer(Bu′)-[S]-Asn-[S]-Ala-OBu′) (11)

Pam₃Cys-ΟH (1) [1] (95 mg, 0.11 mmol) in DMF (2 ml) and DCC (21 mg, 0.1 mmol) were stirred at 10 °C, N,N′-Dicyclohexylurea precipitated, and after 30 min H-[R]-α-MeSer(Bu′)-[R]-α-MeSer(Bu′)-[S]-Asn-[S]-Ala-OBu′ (10) (60 mg, 0.1 mmol) in DMF (1 ml) and NMM (20 μl, 0.18 mmol) were added. After 30 h the solvent was removed in vacuo, the residue was taken up in chloroform/methanol (1:1) and chromatographed on Sephadex LH-20 (column 80 × 2.5 cm) in this solvent. The fractions containing
the lipopentapeptide 11 were combined, and the solvent was removed. The residue was dissolved in tert-butanol and lyophilized. Yield: 105 mg (69%); m.p.: 39°C; amino acid analysis: Ala 1.00(1); α-MeSer 1.76(2); TLC: Rf(I) = 0.72; Rf(II) = 0.85; Rf(III) = 0.97; Rf(IV) = 0.77. FD-MS (see Fig. 3): (M+H)⁺ = 1467.

C₈₁H₁₅₂N₆O₁₄S (1466.19)
Calcd C 66.35 H 10.45 N 5.73 S 2.19.
Found C 67.53 H 10.98 N 4.60 S 1.77.

Results and Discussion

The synthesis of the lipopentapeptide 12 (Fig. 1) includes a 2+2 fragment condensation of Z—a-MeSer(Bu')—MeSer(Bu')—OH (7) with H—Asn—Ala—OBu' (8) [1] using the DCC/HOBt procedure [11]. The Z-protected dipeptide 7 was built up with MeSer derivatives of the R configuration, which are somewhat easier accessible than the S enantiomers [5].

After catalytic hydrogenolysis of the Z-protecting group of tetrapeptide 9 the resulting ninhydrin-negative tetrapeptide ester 10 was obtained, which was coupled with HOBr/DCC to tripalmitoyl-S-glyceryl-L-cysteine (Pam₃Cys—OH (1)). As expected from experiences with α-methylalanine (Aib) peptides couplings with the N-terminal sterically hindered α-alkyl-amino acids [7, 10, 12], α-methylserine [5] should give poor yields despite long reaction times. However, a surprisingly good yield of lipopentapeptide 11 of about 70% after a coupling period of 30 h was found. The deprotected lipopentapeptide 12 was obtained by treatment with anhydrous trifluoroacetic acid for one hour.

The products of the lipopeptide synthesis were characterized by amino acid analysis, field-desorption mass spectrometry (FD-MS), ²H and ¹³C NMR spectroscopy and elemental analysis. As an example the ¹³C NMR spectrum of the dipeptide Z-α-

![Fig. 1. Scheme of the synthesis and structural formula of the mitogenic lipopentapeptide Pam₃Cys—a-MeSer—MeSer—Asn—Ala—OH (12).](image-url)
MeSer(OBu')-α-MeSer(OBu')-OH (7) (Fig. 2) shows no signals splitting, which proves that the sample contains only one diastereomer.

The key step of the successful synthesis is also proved by the FD-MS of the fully protected lipopentapeptide 11 (Fig. 3). The acidolysis of Pam,Cys—OH (1) (6 N HCl, 110 °C, 18 h) does not give a single product, but a mixture of cystine and S-glyceryl-cysteine, which has a retention time comparable to that of Asp, and in addition a not yet characterized product [1]. α-Methylserine appears with low intensity of its peak between Asp and Ala (Fig. 4). Careful systematic elaborations for the quantitative [13] and configurational [14] determination of α-alkyl-α-amino acids including α-MeSer were published recently.

The biological activity of the new analog of the lipoprotein was tested in vitro. Increasing amounts of lipopentapeptide were added to spleen lymphocytes

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**Fig. 2.** $^{13}$C NMR spectrum of the dipeptide $\text{Z-[R]-α-MeSer(Bu')-}[R]-α-\text{MeSer(Bu')-OH}$ (7) ($c = 139 \text{ mg/ml}$; $^2$H$_2$-DMF; 26,000 scans; 20,115 MHz).

**Fig. 3.** FD-MS of the fully protected lipopentapeptide Pam,Cys-α-MeSer(Bu')-α-MeSer(Bu')-Asn-Ala-OBu' (11) ($M^+$ calc. 1466).
of the mouse, and the B-cell proliferation induced was determined by incorporation of $[^3\text{H}]$-thymidine into the DNA of the lymphocytes as described elsewhere [1, 9]. The proliferation increased already at low doses of 0.01–0.1 μg/ml with increasing amounts of the mitogen (Fig. 5). Up to concentrations of more than 100 μg/ml no lysis of the cell was observed. This is an encouraging result for the design of more lipopeptide analogs of this type.

In long-term in vivo experiments the immunoadjuvant activity of lipopeptide 12 is tested at present in comparison to TPP (W. G. Bessler and S. Schlecht, Freiburg). The mitogenicity test will include also a comparison of the (R)-α-MeSer containing lipopeptide 12 described here with the corresponding analog containing (S)-α-MeSer residues and the L-Ser containing TPP.

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Fig. 4. Chromatogram of the amino acid analysis of the total hydrolyzate (6 N HCl, 110 °C, 18 h) the fully protected lipopentapeptide 11.

Fig. 5. Dose response curve for $[^3\text{H}]$-thymidine incorporation into DNA of splenocytes of C3H/HeJ mice induced by Pam3Cys-[R]-α-MeSer-[R]-α-MeSer-Asn-Ala-OH (for experimental details see refs. 1 and 9).