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Synthesis and modulation of cytokine production by two new adamantane substituted acyclic desmuramyldipeptide analogs

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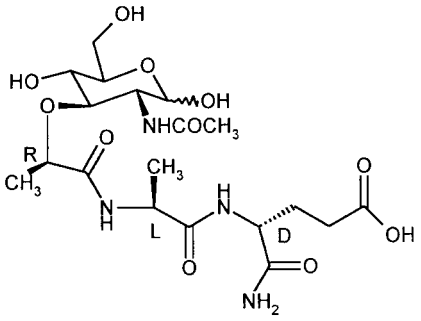
Two new adamantyl-desmuramyldipeptides LK 415 and LK 517 with 1-adamantylcarboxamido moiety as a replacement for muramyldipeptide's *N*-acetylglucosamine fragment were synthesized. Their efficacy to modulate the production of cytokines was measured *in vitro* in ionomycin and phorbol-12-myristate-13-acetate (PMA) activated cultures of human peripheral blood mononuclear cells (PBMC), co-incubated with the substances tested. The results were compared with the activity of muramyldipeptide (MDP). All three substances are strong up-regulators of IL-12 synthesis and hence of the IFNγ synthesis as well. While MDP and LK 415 are relatively ineffective in modulation of IL-2, IL-4 and IL-10 production *in vitro*, the synthesis of all three cytokines is cosiderably up-regulated when peripheral blood mononuclear cells are co-incubated with LK 517. It seems likely that the introduction of the diethyl phosphonate moiety into LK 517 is of great importance for the augmented T-cell cytokine production.

1. Introduction

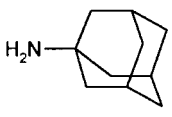
Polymeric peptidoglycans are the constituents of the bacterial cell wall possessing remarkable biological activities, in particular as potent immunomodulators. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (muramyldipeptide or MDP) represents the smallest immunologically active peptidoglycan monomer [1]. To improve its immunomodulatory activity and pharmacodynamic profile, as well as to reduce its side effects, such as pyrogenicity, transient leukopenia, induction of arthritis, and somnogenic activity [2], numerous MDP analogs have been synthesized and immunologically evaluated [3, 4]. Structure-activity relationship studies have shown that the intact *N*-acetyl-D-glucosamine fragment is not essential for the immunostimulant activity of MDP and analogs. Derivatisation of the sugar moiety as well as its replacement with various acyl groups thus represents an important step in the design and synthesis of new immunomodulators based on MDP.

The adamantane derivative amantadine has been used for the prophylaxis and chemotherapy of many viral diseases and Parkinson's disease [5]. It is known that introduction of the adamantyl moiety into substances with known biological activity could improve their pharmacological properties. Compounds containing the adamantyl residue bound to the essential part of MDP L-Ala-D-iGln might exhibit both antiviral and immunomodulatory activity, as demonstrated by adamantylamide dipeptide [6, 7] and (adamant-2-yl)glycine-L-Ala-D-iGln stereoisomers [8].

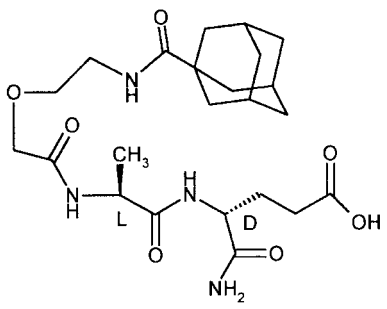
We wanted to apply the approach of combining two biological active components in a single synthetic compound by introducing 1-adamantylcarboxamido moiety as a re-



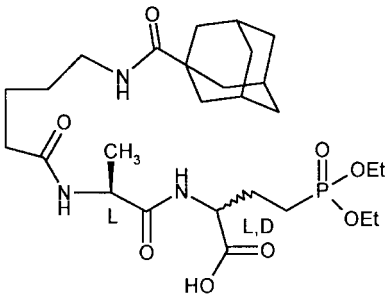
MDP



Amantadine



LK 415



LK 517

placement for MDP's *N*-acetylglucosamine fragment. A replacement of the highly hydrophilic sugar part by a hydrophobic adamantane ring is expected to improve the pharmacodynamic profile of new MDP analogs as well as their pharmacokinetic profile. Thus, the adamantane substituted desmuramyldipeptide LK 415 has been designed and synthesized. To obtain further SAR information, the isoglutamine moiety of LK 415 has been replaced by its phosphono analog 2-amino-4-(diethoxyphosphoryl)butanoic acid, and a spacer connecting the adamantane and dipeptide part has been replaced by methylene bioisostere, giving the compound LK 517.

Recently attention has focused on cytokine interfering drugs, that suppress or stimulate specific pathways in immune and inflammatory responses. The production of cytokines is a key event in the activation and effector phases of innate and specific immunity and serves to mediate and regulate these responses. So far, several experimental models have adressed the influence of MDP and some analogs on the production of cytokines [9–12]. In an effort to further define the effect of compounds of the MDP series, we present the synthesis and the efficacy of two new adamantyl desmuramyldipeptides, LK 415 and LK 517, in the modulation of the cytokine production. IL-12, TNFα, IFNγ, IL-2, IL-4, and IL-10 were measured in *in vitro* ionomycin and phorbol-12-myristate-13-acetate (PMA) polyclonally activated human peripheral blood mononuclear cell (PBMC) cultures. Taking into account its profound immunomodulatory potential, MDP was used as a comparative substance.

2. Investigations, results and discussion

2.1. Synthesis of the compounds

2-{2-[(1-Adamantylcarbonyl)amino]ethoxy}acetic acid (**1**) [13] was coupled with benzyl L-alanyl-D-isoglutaminate hydrochloride (**2**) [14] using diphenylphosphoryl azide (DPPA) as a coupling reagent to give the compound **3**. Hydrogenolysis over palladium on charcoal in methanol afforded the target compound LK 415 (Scheme 1). 1-Adamantanecarbonyl chloride **4** was reacted with 5-aminopentanoic acid to furnish 5-(1-adamantylcarbonyl)aminopentanoic acid **5**, which was coupled with benzyl (2 R,S)-4-diethylphosphoryl-2-[(L-alanyl)-amino]butanoate hydrochloride **6** [15] to give benzyl ester **7**. The compound **7** was finally hydrogenated and the product LK 517 was obtained (Scheme 2).

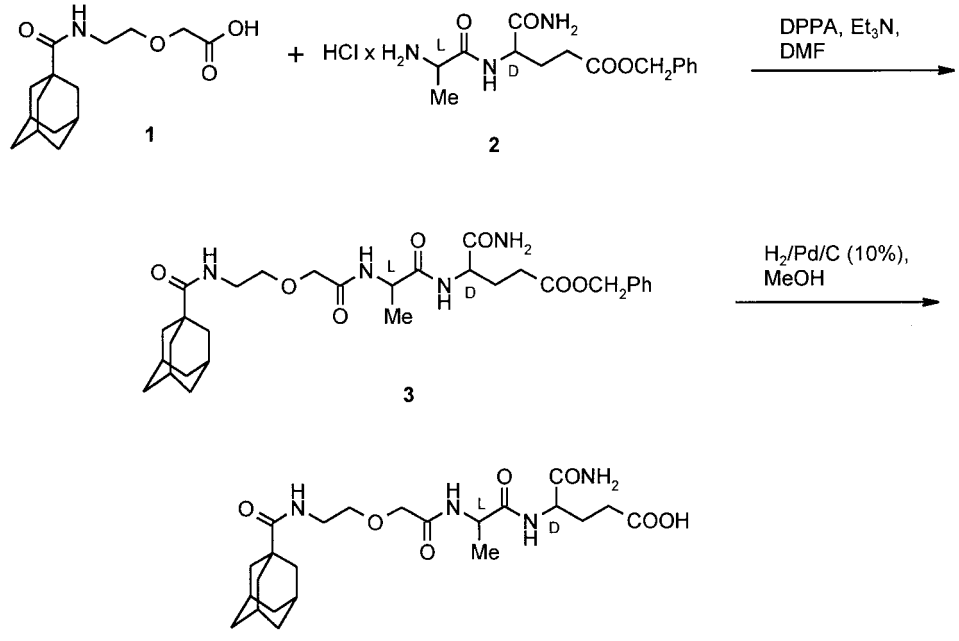
2.2. Modulation of cytokine production

The immunomodulating activity of LK 415 and LK 517 was evaluated as the efficacy to modify IL-12, TNFα, IFNγ, IL-2, IL-4, and IL-10 synthesis, induced in human PBMC cultures by ionomycin and PMA. PBMC were isolated from the buffy coat (donor 1). Following ionomycin and PMA stimulation with added LK 415 (ionomycin + PMA + LK 415) and LK 517 (ionomycin + PMA + LK 517) respectively, supernatants were screened for the content of 6 different cytokines. The level of a single cytokine obtained after co-incubation of PBMC with ionomycin and PMA and the substances tested was compared with the cytokine level obtained after solely ionomycin and PMA activation. Besides that, MDP as well as established immunomodulating substance was included in the study using the same model but with PBMC from another buffy coat (donor 2). In both experiments the baseline production of cytokines was measured in nonstimulated PBMC cultures, with added RPMI 1640 only. The cytokine concentrations are displayed in Tables 1 and 2.

The stimulation/suppression indexes were calculated (Table 3) to evaluate the immunomodulating activity of the substances as described elsewhere [12] and with the purpose of overcoming variable cytokine production in PBMC cultures of healthy blood donors [16].

All three substances tested are strong up-regulators of IL-12 synthesis. It seems that there is a difference in stimulation of macrophages to produce TNFα or to produce Il-12. As we reported previously, the induction of a

Scheme 1



Scheme 2

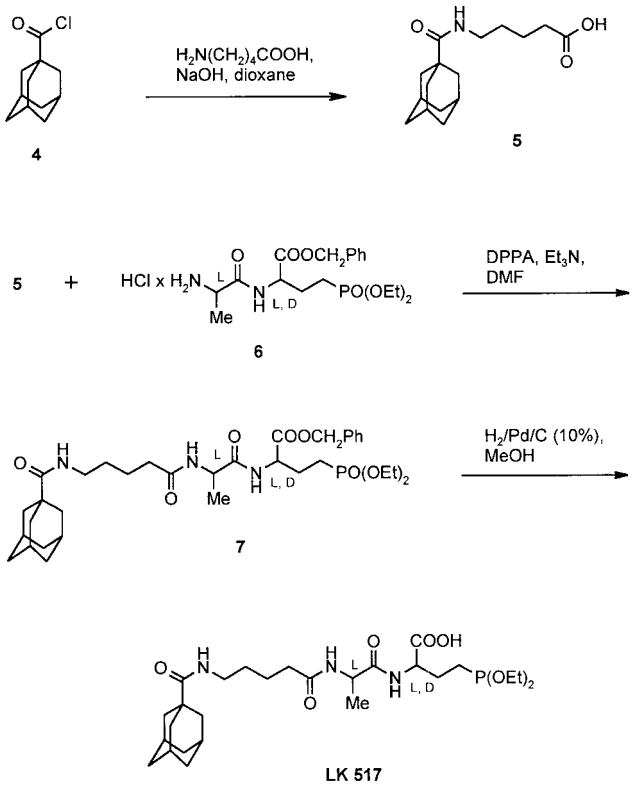


Table 1: Concentrations of cytokines (pg/ml) in culture supernatants of ionomycin and PMA activated human PBMC and in supernatants of cultures co-incubated with LK 415 and LK 517 respectively, together with the baseline production of cytokines in nonstimulated culture (donor 1)

Cytokine	Nonstimulated culture (RPMI 1640)	Ionomycin/PMA	Ionomycin/PMA + LK 415	Ionomycin/PMA + LK 517
IL-12	10.7	93.9	122	167
TNFα	<3.00[a]	3220[a]	4979[a]	2902[a]
IFNγ	<1.00	4486	6047	8190
IL-2	<1.00	1449	1570	1732
IL-4	<1.00	15.7	15.4	18.8
IL-10	3.34	39.6	44.6	52.0

[a] From reference [12].

Table 2: Concentrations of cytokines (pg/ml) in culture supernatants of ionomycin and PMA activated human PBMC and in supernatants of cultures co-incubated with MDP, together with the baseline production of cytokines in nonstimulated culture (donor 2)

Cytokine	Nonstimulated culture (RPMI 1640)	Ionomycin/PMA	Ionomycin/PMA + MDP
IL-12	1.16	117	207
TNFα	5.90	2116	2838
IFNγ	<1.00	10940	15255
IL-2	<1.00	10480	9965
IL-4	5.56	16.4	14.4
IL-10	7.00	62.0	59.0

Table 3: Immunomodulating effect of LK 415 and LK 517 respectively, compared to the effect of MDP on cytokine production in ionomycin and PMA activated human PBMC cultures. The results are expressed in stimulation/supression indexes[a]

Cytokine	Stimulation/suppression indexes[a]		
	Ionomycin/PMA + LK 415	Ionomycin/PMA + LK 517	Ionomycin/PMA + MDP
IL-12	1.30	1.78	1.77
TNFα	1.55	0.90	1.34
IFNγ	1.35	1.83	1.39
IL-2	1.08	1.20	0.95
IL-4	0.98	1.20	0.85
IL-10	1.13	1.31	0.97

[a] Stimulation/suppression index = [ionomycin/PMA cytokine concentration]/[ionomycin/PMA + immunomodulating substance]. Cytokine concentration indexes below 0.8 and above 1.2 are considered as suppression and stimulation index, respectively [12]

diethyl phosphate moiety to the molecule shows a tendency to downregulate the TNFα secretion [12], which obviously does not interfere with augmentated IL-12 production as we can see with LK 517. The up-regulation of IL-12 is followed by increased IFNγ synthesis, which is positively regulated by IL-12 [17]. On the other hand, IFNγ is a well-defined inducer of TNFα synthesis [17]. But even in the cultures coincubated with LK 517 the increased amount of IFNγ does not overcome suppressed TNFα synthesis, although the IFNγ production here is the most abundant (stimulation index 1.83). It seems that the diethyl phosphonate moiety introduced into the molecule of LK 517 plays an important role in TNFα suppression despite the augmented IL-12 and IFNγ production.

While MDP and LK 415 are relatively ineffective in T-cell stimulation for producing IL-2, IL-4 and also IL-10, LK 517 is highly effective in this compartment. The synthesis of all three cytokines is considerably up-regulated when PBMC are co-incubated with LK 517. In addition, the highest IFNγ production in LK 517 containing cultures could be partially due to the direct impact of LK 517 molecule on T-cells. In this case the diethyl phosphonate moiety introduced to the molecule of LK 517 seems to have stimulating effect on T-cells to produce cytokines.

3. Experimental

3.1. Chemistry

All reagents and solvents were of commercial grade and used as such. Melting points were determined on a Reichert hot stage microscope and are uncorrected. Optical rotations were measured on a Perkin-Elmer 1241 MC polarimeter. Elemental C, H, N analyses were performed at the Faculty of Chemistry and Chemical Engineering, University of Ljubljana, on a Perkin-Elmer elemental analyzer 240 C. All the results were in an acceptable range. IR spectra were measured by a Perkin-Elmer FTIR 1600 instrument from KBr peleted samples. MS were obtained by a Micromass AutospecQ mass spectrometer using FAB ionization. NMR spectra were obtained on a Bruker Avance DPX 300 instrument. ^{1}H NMR was done at 300.13 MHz with tetramethylsilane as an internal standard and ^{31}P NMR was done at 121 MHz using H_3PO_4 as an external standard.

3.1.1. N-(2-{2-[(1-Adamantylcarbonyl)amino]ethoxy}acetyl)-L-alanyl-D-isoglutamic acid benzyl ester (**3**)

To a solution of 2-{2-[(1-adamantylcarbonyl)amino]ethoxy}acetic acid (**1**) (0.802 g, 3 mmol) and benzyl L-alanyl-D-isoglutaminate hydrochloride (**2**) (1.030 g, 3 mmol) in dry DMF (10 ml) diphenylphosphoryl azide (DPPA) (0.78 ml, 3.6 mmol) in DMF (5 ml) was added at 0 °C while stirring. After the addition of DPPA, Et_3N (0.92 ml, 6.6 mmol) was added. The stirring was continued for 2 h at −5 °C–0 °C and 24 h at RT. AcOEt (150 ml) was added and the solution was extracted subsequently with 10% citric acid (48 ml), H_2O (33 ml), saturated $NaHCO_3$ solution (48 ml), H_2O (48 ml), and saturated NaCl solution (24 ml). The organic phase was dried (anh.

$MgSO_4$), and the solvent removed under reduced pressure. Column chromatography (silica gel, acetone) of the residue gave colorless crystals; yield: 0.902 g (53%); m.p.: 57–64 °C; IR (KBr, cm^{-1}) 3405.0, 2906.2, 1734.7, 1654.1, 1534.5, 1452.9, 1385.1, 1261.0, 1167.1, 1116.2, 978.8, 910.1, 752.3; ^{1}H NMR ($CDCL_3$) δ(ppm) = 1.39 (d, 3 H, J = 7.0 Hz, $\underline{CH_3}CH$), 1.65–2.02 (m, 15 H, adamantyl), 1.93–2.02 and 2.17–2.26 (2 m, 1 H each, CH_2-β-i-Gln), 2.48 (t, 2 H, J = 7.3 Hz, CH_2-γ-i-Gln), 3.48 (dt, 2 H, J = 5.0 Hz, J = 5.0 Hz, $NH\underline{CH_2}CH_2$), 3.57 (t, 2 H, J = 5.0 Hz, $CH_2\underline{CH_2}O$), 3.96 (s, 2 H, OCH_2CO), 4.39–4.50 (m, 1 H, CH-i-Gln), 4.30–4.58 (m, 1 H, CH-Ala), 5.11 (s, 2 H, $\underline{CH_2}Ph$), 6.23 and 6.32 (2s, 1 H each, $CONH_2$), 6.98 (broad s, 1 H, NH), 7.25–7.34 (m, 6 H, phenyl and NH), 7.73 (d, 1 H, J = 8.1 Hz, NH); $[\alpha]_D^{20} = -5.54°$ (c = 0.45, MeOH); FAB-MS m/z 570 $(M + H)^+$.
$C_{30}H_{42}N_4O_7$

3.1.2. N-(2-{2-[(1-Adamantylcarbonyl)amino]ethoxy}acetyl)-L-alanyl-D-isoglutamic acid (LK 415)

Compound **3** (500 mg, 0.87 mmol) was dissolved in MeOH (15 ml) and hydrogenated for 1 h over 10% Pd/C (72 mg) at RT and normal pressure. After filtration the solvent was removed in vacuo to give colorless crystals; yield: 0.338 g (97%); m.p.: 84–86 °C, IR (KBr, cm^{-1}) 3372.1, 2906.8, 1707.0, 1654.1, 1533.3, 1450.5, 1287.6, 1255.2, 1173.0, 1137.2, 977.4, 673.4; ^{1}H NMR ($CDCl_3$) δ(ppm) = 1.25 (d, 3 H, J = 6.9 Hz, $\underline{CH_3}CH$), 1.58–2.06 (m, 17 H, adamantyl and CH_2-β-i-Gln), 2.20 (t, 2 H, J = 7.6 Hz, CH_2-γ-i-Gln), 3.39 (dt, 2 H, J = 5.0 Hz, J = 5.0 Hz, $NH\underline{CH_2}CH_2$), 3.46 (t, 2 H, J = 5.0 Hz, $CH_2\underline{CH_2}O$), 3.88 (s, 2 H, OCH_2CO), 4.10–4.26 (m, 1 H, CH-i-Gln), 4.28–4.40 (m, 1 H, CH-Ala), 7.10 (broad s, 1 H, NH), 7.47 (t, 1 H, J = 5.4 Hz, NH), 7.78 (d, 1 H, J = 7.1 Hz, NH), 8.16 (d, 1 H, J = 8.4 Hz, NH); $[\alpha]_D^{20} = -3.52°$ (c = 0.41, MeOH); FAB-MS m/z 480 $(M + H)^+$.
$C_{23}H_{36}N_4O_7$

3.1.3. 5-[(1-Adamantylcarbonyl)amino]pentanoic acid (**5**)

To a solution of 5-aminopentanoic acid (14 mmol, 1.64 g) in a mixture of H_2O (15 ml) and dioxane (15 ml) 2 M NaOH (28 mmol, 14 ml) was added. The resulting mixture was cooled to 0 °C and a solution of 1-adamantanecarbonyl chloride (**4**, 14 mmol, 2.78 mmol) in dry dioxane (25 ml) was added dropwise while stirring. The ice bath was removed and the reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated in vacuo, the residue dissolved in 50 ml of H_2O and acidified to pH = 3 with 2 M HCL. The separated precipitate was filtered off and purified by CC (silicagel, $CHCl_3$/MeOH = 15/1); yield: 2.55 g (65%); m.p.: 127–130 °C; IR (KBr, cm^{-1}) 3390.6, 2900.2, 2850.1, 2510.8, 1710.1, 1605.2, 1540.5, 1450.4, 1290.4, 1245.7, 1090.0, 820.3, 750.6; ^{1}H NMR ($CDCl_3$) δ(ppm) = 1.50–2.10 (m, 19 H, adamantyl and $CH_2\underline{CH_2}\underline{CH_2}$), 2.39 (t, 2 H, J = 6.9 Hz, $\underline{CH_2}COOH$), 3.26 (dt, 2 H, J = 6.3 Hz, J = 6.3 Hz, $NH\underline{CH_2}$), 5.79 (broad s, 1 H, NH), 9.90 (broad s, 1 H, COOH); FAB-MS m/z 280 $(M + H^+)$.
$C_{16}H_{25}NO_3$

3.1.4. Benzyl (2S,R)-2-{[(2S)-2-({5-[(1-adamantylcarbonyl)amino]pentanoyl}amino) propanoyl]amino}-4-(diethoxyphosphoryl)butanoate (**7**)

The product was synthesized from **5** (3 mmol, 796 mg) and benzyl (2*S,R*)-4-diethylphosphonyl-2-[(L-alanyl)amino]butanoate hydrochloride (**6**, 3 mmol, 1.2 g) as described above for compound **3**. It was purified by CC (silicagel, $CHCl_3$/MeOH = 15/1); yield: 1.09 g (55.5%); as an oil; IR (KBr, cm^{-1}) 3306.3, 2906.1, 2851, 1741.7, 1642.8, 1532.3, 1451.7, 1370.5, 1238.4, 1027.2, 966.3, 752.2; ^{1}H NMR (DMSO-d_6) δ(ppm) = 1.12–1.26 (m, 9 H, $\underline{CH_3}CH$ and 2 $CH_2\underline{CH_3}$), 1.28–1.48 (m, 4 H, $CH_2\underline{CH_2}\underline{CH_2}$), 1.58–2.00 (m, 19 H, adamantyl, $\underline{CH_2}$-β, CH_2-γ), 2.05–2.15 (m, 2 H, $\underline{CH_2}CO$), 3.00 (dt, 2 H, J = 6.5 Hz, $NH\underline{CH_2}$), 3.88–4.02 (m, 4 H, 2 $\underline{CH_2}CH_3$), 4.25–4.40 (m, 2 H, 2CH), 5.05–5.20 (m, 2 H, $\underline{CH_2}Ph$), 7.25–7.40 (m, 6 H, aromatic and NH), 7.90 (t, 1 H, J = 6.5 Hz), 8.30 (8.25*) (d, 1 H, J = 7.5 Hz, NH), (*two sets of signals due to diastereoisomers); ^{31}P NMR (DMSO-d_6) δ(ppm) = 31.87, 31.96; $[\alpha]_D^{20} = -17.88$ (c = 0.43, MeOH); FAB-MS m/z 662 $(M + H)^+$.
$C_{34}H_{52}N_3O_8P$ (661.8) × H_2O

3.1.5. (2S,R)-2-{[(2S)-2-({5-[(1-Adamantylcarbonyl)amino]pentanoyl}amino)propanoyl]amino}-4-(diethoxyphosphoryl)butanoate (LK 517)

Compound **7** (661 mg, 1 mmol) was dissolved in glacial acetic acid (10 ml) and hydrogenated for 6 h over 10% Pd/C (100 mg) at RT and normal pressure. After filtration the solvent was removed in vacuo to give white crystals; yield: 0.553 g (97%); m.p.: 59–62 °C; IR (KBr, cm^{-1}) 3360.9, 2909.1, 2852.4, 1734, 1653.8, 1540.4, 1456.6, 1212.2, 1165.9, 1047.7, 1024.6, 968.8; ^{1}H NMR (DMSO-d_6) δ(ppm) = 1.15–1.25 (m, 9 H, $\underline{CH}\underline{CH_3}$ and 2 $CH_2\underline{CH_3}$), 1.31–1.55 (m, 4 H, $CH_2\underline{CH_2}\underline{CH_2}$), 1.60–2.00 (m, 19 H, adamantyl, $\underline{CH_2}$-β, CH_2-γ), 2.12 (t, 2 H, J = 6.8 Hz, CH_2CO), 2.95–3.05 (m, 2 H, $NH\underline{CH_2}$), 3.88–4.02 (m, 5 H, 2 $\underline{CH_2}CH_3$ and CH), 4.17–4.30 (m, 1 H, CH), (the position of the COOH and NH proton signals was not established); ^{31}P NMR (DMSO-d_6) δ(ppm) = 33.65, 33.69; $[\alpha]_D^{20} = -18.09$ (c = 0.42, MeOH); FAB-MS m/z 594 $(M + Na)^+$, 610 $(M + K)^+$.
$C_{27}H_{46}N_3O_8P$ (571.6) × 1.5 H_2O

3.2. Modulation of cytokine synthesis

The desmuramyldipeptide compounds, LK 415 and LK 517, and MDP (Sigma, Germany) (11 μmol) used for *in vitro* stimulation of human PBMC were dissolved in dimethyl sulfoxide (DMSO, Sigma) and further diluted in RPMI 1640 (Sigma) supplemented as described below so that the final concentration of DMSO did not exceed 0.1%.

3.2.1. Cell culture

Human PBMC from the buffy coat of two healthy blood donors were isolated by density gradient centrifugation with Ficoll-Paque (Pharmacia, Sweden). The cells were cultured in RPMI 1640 supplemented with 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 2 mmol L-glutamine (Sigma), 20 mmol Hepes (Sigma) and 10% heat-inactivated AB normal human serum (Sigma) for the TNFα production or 5% heat-inactivated foetal calf serum (Sigma) for the production of other cytokines. 1×10^6 cells (final culture volume 1.5 ml) were plated in 24-well culture plates (Nunc, Denmark) with either a combination of a test compound (11 μmol) and ionomycin (500 nmol) and PMA (3.33 ng/ml) or medium alone, at 37 °C, and free cell supernatants were collected at 40 h and stored at −70 °C before being evaluated for 6 cytokines.

3.2.2. Measurement of cytokines

The concentration of cytokines (pg/ml) was measured by commercially available ELISA kits, TNFα from Innogenetics (Belgium), IL-2, IL-4, IL-10, IL-12 and IFNγ from Endogen (USA). A modulatory capacity of a cytokine synthesis by a test substance was evaluated with an index according to the ionomycin and PMA induced cytokine production [12].

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