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Search for New Synthetic Immunosuppressants. Antamanide Analogues Containing the Tetrazole Ring as a *cis*-Peptide Bond Mimetic

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Linear and cyclic antamanide (ANT) analogues with dipeptide segments Pro^2-Pro^3, Pro^7-Pro^8 and both of them replaced by their tetrazole analogue Pro-ψ[CN_4]-Ala, respectively, have been synthesized by SPPS method and cyclization with TBTU reagent. The peptides were examined for their immunosuppressive activity in a lymphocyte proliferation test (LPT).

Key words: antamanide, *cis*-peptide bond, 1,5-disubstituted tetrazole ring, immunosuppression

The serious problem of transplantology, a dynamically expanded branch of contemporary medicine, is the transplanted organ survival. Two effective immunosuppressants, cyclosporin A (CsA, Sandimmun®) [1] and FK-506 [2], are used in medical praxis as the potent drugs for the prevention of graft rejection after transplantation. A wider use of these therapeutics is limited by the side effects of CsA and FK-506. The search of new immunosuppressants, exhibiting the similar mechanism of action but devoid of toxicity, especially in the group of naturally existing immunomodulatory peptides and their analogues, is an important challenge for medicinal chemistry.

Antamanide (ANT) is a highly hydrophobic cyclic decapeptide of the sequence cyclo(Val^1-Pro^2-Pro^3-Ala^4-Phe^5-Phe^6-Pro^7-Pro^8-Phe^9-Phe^{10}), isolated by Wieland *et al*. [3] from the mushroom *Amanita phalloides.* A peculiarity of antamanide structure is the presence of two *cis*-configurated peptide bonds between each Pro-Pro dipeptide units, whereas the amide bonds between Val^1-Pro^2 and Phe^6-Pro^7 are locked in *trans*-geometry. Such a structure of ANT was postulated in 1969 [4,5], and has been confirmed by X-ray diffraction [6–8] and NMR [9,10].

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Antamanide demonstrates a quite strong immunosupressive activity in respect to both: humoral and cellular immune response [11], however, this activity is lower than that of cyclolinopeptide A (CLA). Linear antamanide fragments are also active, but higher concentration of these peptides exhibit some toxic effects.

To evaluate the significance of *cis*-geometry of Pro^2-Pro^3 and Pro^7-Pro^8 peptide bonds for ANT biological activity, we have synthesized three linear and three cyclic antamanide analogues, with dipeptide segments, Pro^2-Pro^3, Pro^7-Pro^8 and both of them replaced respectively, by their tetrazole analogue Pro-ψ[CN_4]-Ala. The 1,5-disubstituted tetrazole ring, ψ[CN_4], has been proposed as a surrogate for *cis*-amide bonds, making it a valuable tool in the design of conformationally constrained peptidic receptor probe [12–17]. The effects exerted by cyclic peptides on the immune response were compared with those produced by their linear precursors and with the effects exerted by CsA as well.

RESULTS AND DISCUSSION

Synthesis. The synthesis of desired peptides c(Pro^2-ψ[CN_4]-Ala^3)ANT **6,** c(Pro^7-ψ[CN_4]-Ala^8)ANT **8** and c(Pro^2-ψ[CN_4]-Ala^3)-(Pro^7-ψ[CN_4]-Ala^8)ANT **10** has been achieved, as is shown on example for peptide **10** (Fig. 2), by fragment condensation on the polymeric support (Merrifield type resin) using TBTU {O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate} or HATU {O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate} as a coupling reagent. The tetrazole building block has been synthesized separately in solution (Fig. 1).

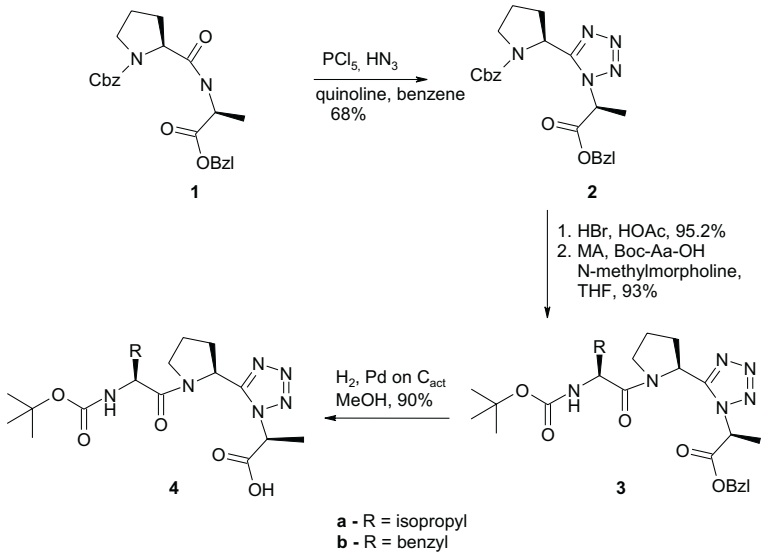

Figure 1. Synthetic scheme for tetrazole tripeptides.

Following the previously reported strategy [14,15], the fully protected dipeptide Z-Pro-Ala-OBzl **1** was converted into tetrazole analogue, Z-Pro-ψ[CN_4]-Ala-OBzl **2** using PCl_5 and hydrazoic acid in the presence of quinoline with full retention of configuration at both chiral centers. Careful treatment of dipeptides **2** with 30% HBr in acetic acid for 20 min gave the hydrobromide salt of H-Pro-ψ[CN_4]-Ala-OBzl. The hydrobromide was extended, from the N-terminus, by coupling with Boc-Val-OH and Boc-Phe-OH respectively to yield the protected tripeptides Boc-Val-Pro-ψ-[CN_4]-Ala-OBzl **3a** and Boc-Phe-Pro-ψ[CN_4]-Ala-OBzl **3b**. A mixed anhydride (MA) procedure with isobutyl chloroformate has been used as a method of choice to avoid diketopiperazine formation. Removal of the benzyl ester groups from **3a** and **3b** by hydrogenolysis in methanol over 10% Pd on charcoal lead to free acids Boc-Val-Pro-ψ[CN_4]-Ala-OH **4a** and Boc-Phe-Pro-ψ[CN_4]-Ala-OH **4b**. Both peptides **4a** and **4b** were used to the solid phase peptide synthesis procedure. The known proclivity of the C-terminal α-carbon of tetrazole dipeptides to epimerize [13–15] on prolonged base exposure led us to omit the neutralization step in the solid phase synthesis of peptides containing the tetrazole unit.

After being cleaved from the resin, the crude linear precursors **5**, **7** and **9** were cyclized by means of TBTU in the presence of HOAt (1-hydroxy-7-azabenzotriazole) and DIPEA (N,N-diisopropylethylamine) in dichloromethane (DCM) at a much lower concentration, than described for peptide cyclization reactions.

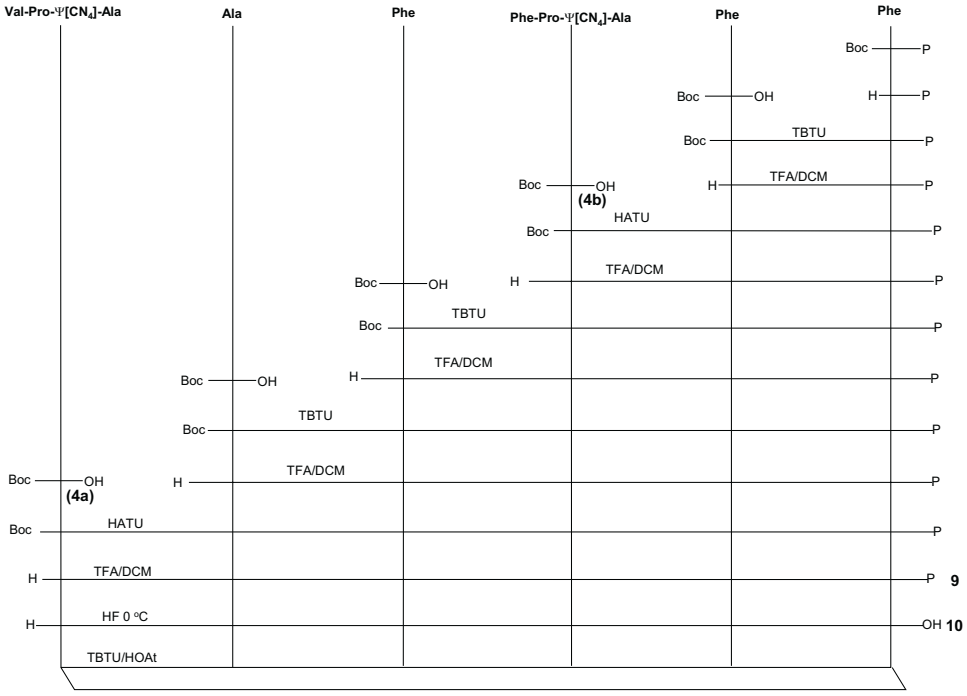

Figure 2. Synthetic scheme for c(Pro^2-ψ[CN_4]-Ala^3)-(Pro^7-ψ[CN_4]-Ala^8)-antamanide.

Crude cyclic peptides **6**, **9** and **10,** as well as their precursors **5**, **7** and **9** were purified by HPLC and characterized by FAB-MS and chromatography techniques (Table 1).

Table 1. Analytical data for antamanide analogues.

	Peptide	Yield[a] (%)	R_t[b] (min.)	MW[c] calcd/found
5	Linear (Pro^2-ψ[CN_4]-Ala^3)ANT	88.1	5.71	1163.32/1164.6
6	c(Pro^2-ψ[CN_4]-Ala^3)ANT	25.8	10.86	1146.31/1146.7
7	Linear (Pro^7-ψ[CN_4]-Ala^8)ANT	92.4	5.15	1163.32/1165
8	c(Pro^7-ψ[CN_4]-Ala^8)ANT	27.4	9.03	1146.3/1146.6
9	Linear (Pro^2-ψ[CN_4]-Ala^3)-(Pro^7-ψ[CN_4]-Ala^8)ANT	84.2	7.02	1163.28/1163.8
10	c(Pro^2-ψ[CN_4]-Ala^3)-(Pro^7-ψ[CN_4]-Ala^8)ANT	24.3	10.14	1146.27/1146.2

[a]Peptide content in a crude product as shown by analytical HPLC with linear gradient 50–95% B/25 min. (A) 0.05% trifluoroacetic acid in water and (B) 0.038% trifluoroacetic acid in acetonitrile/H_2O 90:10.
[b]Gradient as above.
[c]Molecular weight calculated/found by FAB-MS measurements.

It is worth to note that compound **10** is the first molecule containing two peptide bonds modified by the 1,5-disubstituted tetrazole ring, derived from natural peptide.

Biological studies. To evaluate the immunosuppressive activity of synthesized compounds a lymphocyte proliferation test (LPT) has been employed, which is a standard method of assessment of the effect of drug on immunological processes [18]. As opposed to earlier studies using animal models, human cell system (lymphocytes from healthy human volunteers) has been used for the assessment of the immunosuppressive activity. To exclude the toxic effect of synthesized compounds, as a prerequisite for LPT, an effect of synthesized compounds on viability of lymphocytes has been assessed. Only the concentrations of compounds that secured at least 90% cell viability, found by the dye exclusion method were considered as potentially useful for immunosuppressive activity testing (Table 2).

The linear compounds (**5, 7** and **9**) did not affect cells viability over the range of concentrations from 0.2–25 μg/ml. On the other hand, cyclic compounds (**6, 8** and **10**) demonstrated toxic activity at concentrations above 2 μg/ml.

A reference drug cyclosporine A demonstrated a dose dependent inhibition of PHA (phytohemaglutynine) stimulated proliferation of lymphocytes: 54%, 74% and 98% inhibition when tested in concentrations of 2.5, 10 and 25 μg/ml respectively (Table 3). In contrast neither the native ANT nor any of the synthesized compounds demonstrated any significant immunosuppressive activity over the range of concentrations, which were not toxic.

Assuming that any new compound with potential application as immunosuppressant in medicine should demonstrate at least comparable to cyclosporine A immunosuppressive activity, we can conclude that synthesized ANT analogues are not likely to be suitable as immunosuppressant. Our study does not exclude, that the examined compounds could have a immunomodulatory effect on the other immunological functions. In fact it has been previously noted [11] that the native compound demonstrated

Table 2. Viability of human peripheral blood lymphocytes after incubation.

	% of lymphocyte viability				% of lymphocyte viability								
	Linear (Pro^7-Ψ[CN$_4$]-Ala^8)ANT				c(Pro^2-Ψ[CN$_4$]-Ala^3)ANT			c(Pro^7-Ψ[CN$_4$]-Ala^8)ANT			c(Pro^2-Ψ[CN$_4$]-Ala^3)-(Pro^7-Ψ[CN$_4$]-Ala^8)ANT		
Concentration	After 24 h	After 48 h	After 72 h	Concentration	After 24 h	After 48 h	After 72 h	After 24 h	After 48 h	After 72 h	After 24 h	After 48 h	After 72 h
0.2 μg/ml	100	98.3	98.3	1 μg/ml	100	93	91	100	97.5	61	99	97	90
1 μg/ml	100	97.7	95.3	2 μg/ml	98	89.5	83	98	97	53	97	94.5	81
2.5 μg/ml	99.7	97.7	92	10 μg/ml	75	64.5	53.5	86.5	86.5	32	76.5	61.5	47
5 μg/ml	100	96	88	20 μg/ml	50.5	44	37.5	72	72	25	57.5	42.5	27.5
10 μg/ml	98.7	95	87										
25 μg/ml	95.3	92	80										

Table 3. Effect of compounds studied on a lymphocyte proliferation.

	% inhibition of lymphocyte proliferation									
Concentration [μg/ml]	Cyclosporin A	Native linear ANT	Linear (Pro^7-Ψ[CN$_4$]-Ala^8)ANT		Concentration [μg/ml]	Cyclosporin A	Native cANT	c(Pro^2-Ψ[CN$_4$]-Ala^3)ANT	c(Pro^7-Ψ[CN$_4$]-Ala^8)ANT	c(Pro^2-Ψ[CN$_4$]-Ala^3)-(Pro^7-Ψ[CN$_4$]-Ala^8)ANT
2.5	54	0	4.1		1	42	0	4	4	7
5	74	0	7.3		2	50	0	11	11	12
25	98	0	12		10	87	0	27	18	31
				Toxic concentrations	20	98	0	52	89	62

some immunosuppressive activity in animal models (mice), which was not confirmed in our model of LPT employing human lymphocytes.

EXPERIMENTAL

All solvents were purified by conventional methods. Evaporations were carried out under reduced pressure. Melting point were determined on a capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 250 MHz on a Bruker Avance model DPX 250. Splitting patterns are abbreviated as follows: (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet. The optical rotations were measured in a 1 dcm cell (1 ml) on a Horiba high speed automatic polarimeter at 589 nm (NaD line). For thin layer chromatography, 250 nm silica gel plates (Analtech, Merck) were used. The chromatograms were visualized with chlorine followed by starch/KI or ninhydrin. HPLC for all tetrazole fragments was performed on a LDC/Milton-Roy Analytical instrument using a Vydac C_{18} (0.46×25 cm) column, flow rate 1.0 ml/min., detection at 220 nm, and solvents (A) 0.05% trifluoroacetic acid in water and (B) 0.038% trifluoroacetic acid in acetonitrile/H_2O 90:10 in a gradient application. N^{α}-tert-butoxycarbonyl (Boc) protected amino acids were obtained from ChemImpex International. Coupling reagents (TBTU, HATU and HOAt) were obtained from Richelieu Biotechnologies Inc., PerSeptive Biosystems GmbH and Milipore respectively. Cyclosporin A (CsA) has been purchased in Sigma-Aldrich Co. and used as a DMSO (dimethylsulphoxide) solution. The preparation of Z-Pro-ψ[CN_4]-Ala-OBzl **2** as well as its precursor (Z-Pro-Ala-OBzl **1**) and Boc-Val-Pro-ψ[CN_4]-Ala-OH **4a** has been described elsewhere [15,19].

Boc-Phe-Pro-Ψ[CN_4]-Ala-OBzl (3b). *a) Removal of Z protecting group from dipeptide **2**.* A solution of 2.38 g (5.45 mM) of **2** in 5 ml acetic acid was treated with 16 ml of 30% HBr in acetic acid while stirring. After 20 min. at room temperature, the solution was poured into 100 ml of diethyl ether with vigorous stirring. The crystalline hydrobromide precipitated and was filtered off. The crystals were washed with diethyl ether (2×20 ml) and dried in vacuum over solid KOH to give 1.98 g (95.2%) of the dipeptide HBr salt as a very hygroscopic crystals. *b) Coupling with Boc-Phe-OH.* A solution of 1.38 g (5.19 mM) Boc-Phe-OH in 10 ml of THF was stirred at room temperature for 5 min. when N-methyl-morpholine 0.58 ml (5.19 mM) was added. The reaction mixture was cooled to –15°C and then isobutyl chloroformate 0.7 ml (5.19 mM) has been added followed by the addition of crystalline hydrobromide 1.98 g (5.19 mM) and N-methylmorpholine 0.58 ml (5.19 mM).The reaction mixture was stirred in –15°C for an hour, then was warmed to room temperature and stirred overnight. The solvent was removed in vacuo. The residue was taken up in ethyl acetate (50 ml) and washed with 1 N $NaHCO_3$ (3×20 ml), 1 N $NaHSO_4$ (3×20 ml), water (3×20 ml) and saturated NaCl solution (3×20 ml). The ethyl acetate solution, dried over anhydrous Na_2SO_4, was evaporated and desired tripeptide derivative isolated as a glassy powder. Yield 2.64 g (93%): $[\alpha]_D = -12.4$ (c = 1, MeOH); $R_f = 0.57$ (DCM:acetone, 30:1); HPLC purity 100%, $t_R = 14.25$ min. (gradient 50–70% B in 25 min.); FAB-MS [MH^+] = 548.5 calc. for $C_{30}H_{37}N_5O_5$ 547.6 ^{1}H NMR ($CDCl_3$) d: 1.37 (s, 9H, $(CH_3)_3C$); 1.93 (d, 3H, J = 7.5, Ala$\underline{CH_3}$); 1.94–1.97 (m, 2H, Pro$\beta\underline{CH_2}$); 2.29–2.35 (m, 2H, Pro$\gamma\underline{CH_2}$); 2.95 (d, 2H, J = 7.5, Phe$\beta\underline{CH_2}$); 3.46–3.50 (m, 2H, Pro$\delta\underline{CH_2}$); 4.16–4.28 (m, 1H, Phe$\alpha\underline{CH}$); 5.12–.15 (m, 1H, Pro$\alpha\underline{CH}$); 5.20 (m, 2H, Ph-$\underline{CH_2}$-); 5.52 (q, 1H, J = 7.5, Ala$\alpha\underline{CH}$); 7.00–7.40 (m, 11H, Phe$\underline{Ph}$, Bzl$\underline{Ph}$). 8.45 (d, 1H, J = 9.8, Phe$\underline{NH}$).

Boc-Phe-Pro-Ψ[CN_4]-Ala-OH (4b). Boc-Phe-Pro-Ψ[CN_4]-Ala-OBzl **3b** (2.64 g, 4.82 mM) in methanol (20 ml) was hydrogenated in the presence of 10% Pd/C at a pressure of 4–5 Kg/cm^2 on a Parr apparatus for 4 hours (monitored by TLC). After evaporation of filtered solution, the residual glassy powder was crystallized from ethyl acetate/hexane. Yield 1.98 g (90%); HPLC purity 98.6% $t_R = 9.74$ min (gradient 30–60% B in 25 min.). Without any further purification the free acid was used to SPPS procedure.

Solid phase peptide synthesis: Boc-Phe attached to chloromethylated Merrifield resin was prepared through esterification reaction performed in DMF in the presence of dry KF (6 eg.) at 50°C for 72 hours. The Boc-Phe-polymer after washing (5 × DMF, 5 × water, 5 × MeOH, 5 × DCM, 5 × MeOH) was dried under reduced pressure over KOH and P_2O_5 and substitution level of 0.479 mM/g was determined by weight gain. The linear peptides were synthesized by the standard SPPS methodology starting from 0.417 g (0.2 mM) Boc-Phe-resin. Standard single TBTU protocol was used for all single amino acid deriv-

atives and was repeated if Kaiser or Isatin (for proline residue) test was found positive. In all cases, where after second coupling Kaiser or Isatin test was slightly positive, the remaining free amino groups were acetylated with the aid of acetic anhydride in DCM. Standard single TBTU or HATU coupling protocol was used for tetrazole peptide units Boc-Val-Pro-Ψ[CN_4]-Ala-OH **4a** and Boc-Phe-Pro-Ψ[CN_4]-Ala-OH **4b**. The peptide resin were cleaved with anhydrous HF in the presence of anisole (~ 10%) at 0°C for 60 min. After HF removal under reduced pressure, the resin was washed several times with diethyl ether and then extracted with aqueous acetonitrile. Lyophilization of the extracts yielded crude linear peptides: **5** 205 mg (0.176 mM, 88.1% yield, 65.53% purity HPLC); **7** 215 mg (0.185 mM, 92.4% yield, 90.62% purity HPLC); **9** 196 mg (0.168 mM, 84.2% yield, 21.87% purity HPLC). Purification of crude linear precursors has been achieved by preparative HPLC (LCD Analytical) on reversed-phase column, [Vydac C_{18} 250×25 mm 10 mm column, l = 214 nm, flow 16 ml/min. with linear gradient 45–80% B in A (B: 0.038% TFA in 82% acetonitrile/water, A: 0.05% TFA in water) in 40 min.]. FAB-MS spectra confirmed expected structure of **5**, **7** and **9**. Linear precursors were cyclized by means of TBTU in the presence of HOAt and DIPEA in DCM at much lower concentrations (2–5 times dilution; 40 mg of peptide in 1000 ml of DCM), than usually described for "head to tail" peptide cyclization reactions. Crude cyclic peptides were purified in the same way as their linear precursors. The homogeneity of purified peptides was checked by analytical HPLC [Vydac C_{18} 250×4.6 mm 5 μm column, λ = 214 nm, flow 1 ml/min. with linear gradient 50–95% B in A (B: 0.038% TFA in 82% acetonitrile/water, A: 0.05% TFA in water) in 25 min.], for cyclic and linear peptides, respectively on Thermo Separation HPLC system equipped with AS3000 autosampler, P4000 pumps and scanning SpectraFocus detector and LDC/Milton-Roy Analytical instrument. The structures of the pure peptides were confirmed by FAB-MS spectra, which were recorded with Finningan MAT 95 Mass Spectrometer.

Biological tests: Immunosuppressive activity of linear and cyclic tetrazole analogues of antamanide was investigated by Lymphocyte Prolipheration Test (LPT) and compared to immunosuppressive effect cyclosporin A (CsA) as a reference immunosuppressant.

Assessement of cells viability: PBMC (Peripheral Blood Mononuclear Cells) were cultured in presence of serial dilutions of drugs for 24, 48 and 72 hours. After incubation the cells were mixed with 0.2% trypan blue and examined in microscopic slides. Blue cells were considered to be dead and % of viability was referred to 200 examined cells.

Assessement of immunosuppressive activity: LPT was performed according to the following methodology [20]: Lymphocytes (PBMC) were isolated from heparynized blood of healthy volunteers by gradient centrifugation on Ficoll–Hypaque (Pharmacia) [21]. After three washes in culture medium (RPMI–1640), PBMC were resuspended to a final concentration 2×10^6 cells/ml in the RPMI–1640 supplement with 10% Fetal Calf Serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all reagents from GIBCO – Germany). PBMC were cultured in four replicates at 200 μl/well in 96-well microtiter plates using different concentration of the examined analogues of antamanide and cyclosporin A. Cells were stimulated with phytohemaglutynine (PHA) at final concentration 10 μg/ml. Plates were incubated at 37°C in 5% CO_2 humidified atmosphere. After 48 hours of incubation, 1 μCi/well of radiolabelled thymidine (/methyl-3H/thymidine – Lacomed s.r.o. Czech Republic) was added. 24 hours later, cultures were harvested onto glass filters, and thymidine incorporation into DNA was measured in liquid scintillation counter (LKB) and expressed as CPM (count per minute). The suppressive effect of the study compounds was expressed as percentage of PHA – stimulated CPM values (without drugs).

Acknowledgments

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