117. Synthesis and Antiviral Activity of Novel Adamantylpeptides

by **Branka Vranešić***, **Jelka Tomašić**, and **Stanislav Smerdel**

Institute of Immunology, Rockefellerova 2, 41000 Zagreb, Croatia

and **Darko Kantoci**

Institute 'Ruđer Bošković', Bijenička c. 54, 41000 Zagreb, Croatia

and **Fabio Benedetti**

Università di Trieste, Via Valerio 22, I-34127 Trieste

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A novel type of compound that contains a peptide specific for bacterial peptidoglycans (immunomodulatory activity) and an adamantyl residue (antiviral activity) was synthesized. Thus, L-Ala-D-γGln was linked to DL-Gly(Ada) yielding two diastereoisomers **3a** and **3b**, *i.e. ambo*-Gly(Ada)-L-Ala-D-γGln (γGln = isoglutamine-4-amino-4-carbamoylbutanoic acid; Ada = adamantyl). The diastereoisomers were easily separated by column chromatography (silica gel) and characterized, but only the use of a stereospecific enzyme led to the assignment of the configuration of the Gly(Ada) moiety in the two diastereoisomers. The cytotoxic effect and antiviral activity of the novel adamantyltripeptides **3a** and **3b**, observed as the cell survival and the infectivity reduction of the influenza A virus H1N1 and H3N2 strains, respectively, were studied on MDCK cells *in vitro*. Both **3a** and **3b** exhibited low cytotoxicity for MDCK cells and reduced the infectivity of the tested influenza A virus strains, but to a different extent on different strain.

Introduction. – Polymeric peptidoglycans are the ubiquitous constituents of bacterial cell walls, built of large polysaccharide chains and short peptide units. Peptidoglycans have received increasing attention in the last 15 years due to their remarkable biological activities, particularly as potent immunomodulators. Versatile biological activities of natural and synthetic peptidoglycans were widely investigated and well documented [1].

Muramyl-peptides represent structural copies of the monomers of the peptidoglycans of bacterial cell walls, and *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP, for muramyl-dipeptide) has been known since 1974 as the smallest synthetic adjuvant-active molecule capable of replacing whole *Mycobacteria* in *Freund*'s adjuvant [2]. Several hundred chemically defined MDP analogs and derivatives were synthesized since, in order to modulate, preferably improve, the properties of the parent molecule [3].

On the other hand, some compounds containing an adamantyl residue exhibit antiviral activity; amantadine (= *Symmetrel*®), *e.g.*, is currently used in therapy of viral diseases [4] [5]. In general, introducing an adamantyl moiety to substances with known biological activity improves their pharmacological properties and enhances their activity [6]. Compounds containing the adamantyl structure were described as sedatives, antitumor agents, antibiotics, hypoglycemics, antidepressants, drugs for *Parkinson*'s disease, *etc.*

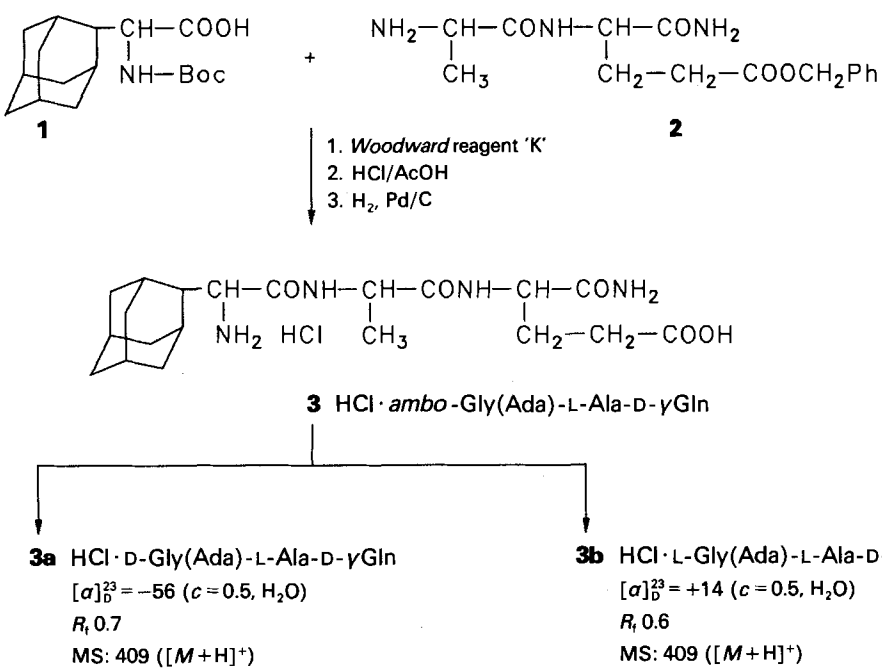
In view of a novel approach of stimulating host immune response using immunomodulators to enhance resistance against viruses, relatively few efforts were made, so far, to

prepare novel compounds containing both the muramyl-dipeptide structure (peptidoglycan structures, in general) and an adamantyl residue. Only a few reports [7–10] concerning the synthesis of a novel adamantyl-amide dipeptide that consisted of an adamantyl moiety and a dipeptide characteristic for MDP were reported. Preliminary tests showed that this compound exhibits both immunomodulating and antiviral properties in several experimental models.

Our aim was to synthesize novel compounds containing the adamantyl residue coupled to peptides characteristic for peptidoglycan structures. It could be expected that some of these compounds might exhibit antiviral properties in parallel with immunomodulating activity and antitumor activity. The synthesis of new adamantyltripeptides in which the peptide portion is coupled to the adamantane through a C–C bond at position 2 of the adamantane molecule is described, starting from DL-(adamant-2-yl)glycine (DL-Gly(Ada)) and L-alanyl-D-isoglutamine (L-Ala-D-γGln; γGln = isoglutamine = 4-amino-4-carbamoylbutanoic acid) and using appropriately protected starting materials. The formed diastereoisomers **3a**/**3b** were separated and investigated for antiviral activity separately. The effect on the influenza A virus strains *in vitro* was tested and compared to that of amantadine and rimantadine [11]. Antitumor and immunomodulating properties are published separately [12].

Results and Discussion. – *Chemistry.* For the successful synthesis of *ambo*-Gly(Ada)-L-Ala-D-γGln (**3**), the amino group of DL-Gly(Ada) was masked with the Boc protective group (→ **1**) [13], and dipeptide L-Ala-D-γGln was applied in the form of benzyl ester **2** (*Scheme*). The peptide-bond formation was achieved using *Woodward*'s reagent 'K' [14]

Scheme



[15]. The condensation product was easily isolated after addition of H_2O, the Boc protection was then cleaved with HCl/AcOH and the benzyl ester removed by catalytic hydrogenation (H_2, Pd/C). On TLC ($CHCl_3$/i-PrOH/MeOH/H_2O/AcOH 20:15:6:4:2) the product **3** was nonhomogeneous, showing two peptide-reagent-positive spots (R_f 0.6 and 0.7). The mixture was successfully separated by column chromatography (silica gel) into two components which were supposed to be D-Gly(Ada)-L-Ala-D-γGln (**3a**) and L-Gly(Ada)-L-Ala-D-γGln (**3b**), respectively. To confirm this hypothesis, **3a** and **3b** were subjected to MS, optical rotation, and hydrolysis. The MS of **3a** and of **3b** revealed the presence of an identical amino-acid sequence and of an adamantyl-containing glycine moiety as well as the ion $[M + H]^+$ at m/z 409 for both. The optical rotation of **3a** and **3b** was different ($[\alpha]_D = -56$ and $+14$, resp.). As expected, total acid hydrolysis of **3a** and **3b** yielded hydrolysates of the same required composition (Gly(Ada), Ala, and Glu; by TLC (PrOH/NH_3 7:3 or BuOH/AcOH/H_2O 60:15:25)).

To establish the absolute configuration of the diastereoisomers **3a** and **3b**, their hydrolysates were submitted to enzymatic oxidation using L-amino-acid oxidase at pH 7 [16a] and the resulting incubation mixtures compared by TLC with the starting hydrolysates. Thus, no L-Ala was detected in the incubation mixture from **3a** (L-Ala oxidized), and only D-Glu (from D-γGln) was present in the incubation mixture from **3b** (L-Gly(Ada) and L-Ala oxidized). The same procedure (hydrolysis and subsequent enzymatic oxidation) was performed with each **3a** and **3b** and D-amino-acid oxidase at pH 8.5 and 37° for 24 h [16b]. TLC revealed that D-Glu reacted very poorly with D-amino-acid oxidase [17] and D-Gly(Ada) seemed to be a resistant D-amino acid. The results obtained with D-amino acid oxidase showed that the enzymatic approach to the configuration determination of amino acids is limited to the substrates oxidizable by the assay enzyme. The described amino-acid oxidase experiments not only established the absolute configuration of isomers **3a** and **3b**, but also documented the preservation of the L-Ala configuration during the peptide synthesis.

The peptides **3a** and **3b** most probably could have been synthesized easier by use of enantiomerically pure (adamant-2-yl)glycine. The reason that justifies the use of the enantiomeric mixture DL-Gly(Ada) is a very convenient way of its preparation [18] and an extremely poor separation of the DL-Gly(Ala) enantiomers by classical methods.

It has to be stressed that both diastereoisomers **3a** and **3b** are apyrogenic, noncytotoxic (in high concentrations), and H_2O-soluble. All these remarkable properties of synthesized adamantyltripeptides rendered them very suitable for biological testing.

Cytotoxic and Antiviral Effect of Diastereoisomers **3a** *and* **3b** in vitro. The cytotoxic effect and antiviral activity of the novel adamantylpeptides, observed as the cell survival and the infectivity reduction of the tested influenza A virus strains, were studied *in vitro*. Both **3a** and **3b** as well as DL-Gly(Ada) and L-Ala-D-γGln were better tolerated by *Madin-Darby* canine kidney (MDCK) cells, grown *in vitro*, than *Symmetrel®*. The final concentrations of up to 200 μg in 50 μl volume/well did not affect the morphology of the cells and the capability of cell growth after one subsequent passage. Thus, the *in vitro* cytotoxicity of **3a** and **3b** was four times lower than that of *Symmetrel*.

The minimum *IC*'s (inhibitory concentration) of *Symmetrel* amounted to 12.5 μg in the final test volume of 200 μl of both the H1N1 and H3N2 strains of influenza A virus and caused the reduction of the infectivity for more than 1 $\log_{10}$ when compared to the virus control. The same concentration (12.5 μg) of **3a** was active for the H1N1 strain, but

Table. *Infectivity Reduction of Influenza A Virus Subtypes for ⩾ 1.0 log_{10} Produced by the Tested Compounds in MDCK Cells*

Viral subtype	*IC*'s [μg/200 μl]				
	Symmetrel®	**3a**	**3b**	DL-Gly(Ada)	L-Ala-D-γGln
H1N1	12.5	12.5	100	50	200
H3N2	12.5	200	50	50	50

more than 200 μg were required to obtain the reduction of the same potency level for the H3N2 strain of influenza A virus. On the other hand, a minimum of 100 μg of **3b** affected the replication of the H1N1 strain, and 50 μg were required to reduce the infectivity of the H3N2 strain of influenza A virus. A similar effect to that obtained with **3b** was achieved with DL-Gly(Ada) and L-Ala-D-γGln, the essential building blocks of the synthesized isomers.

Both isomers **3a** and **3b** reduced the infectivity of the tested influenza A virus H1N1 and H3N2 strains, but at different minimum *IC*'s. Thus, **3a** gave a result equal to that obtained with *Symmetrel*® on the H1N1 influenza A virus strain. On the contrary, the effect on the H3N2 strain was achieved with a 16 times higher concentration of **3a** than it was necessary with *Symmetrel*®. In comparison to the latter, almost 4 to 8 times higher concentrations of **3b** were required to produce the reduction of H3N2 and H1N1 influenza A virus strains potency. The essential building blocks of **3a** and **3b** had a very similar effect on both strains of influenza A virus to that of **3b**, particularly on the H3N2 strain.

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Experimental Part

General. Adamantanone, *N,N'*-dicyclohexylcarbodiimide, and di(*tert*-butyl) dicarbonate were obtained from *Fluka Chemie AG* (Switzerland). *Woodward*'s reagent 'K' (3-(2-ethylisoxazol-5-io)benzenesulfonat), Et_3N, D-glutamic acid, and *N*-hydroxysuccinimide were purchased from *Merck* (Darmstadt, FRG). L-Amino acid oxidase (*EC 1.4.3.2*) type IV, purified from *Crotalus adamanteus* venom, was purchased from *Sigma Chemical Co.* (St. Louis, MO, USA). Column chromatography (CC): silica gel (*Merck*, 0.040–0.063 mm). TLC: 'Kieselgel' *60* HF_{254} plates (*Merck*); detection with ninhydrin and chlorine-iodine reagent for peptides. M.p.: uncorrected. Optical rotations: automatic polarimeter *AA-10* (*Optoelectronic Design Engineers*). Absorbance at 230 nm: *Perkin-Elmer Lambda 3* UV-VIS spectrophotometer. ^{1}H- and ^{13}C-NMR Spectra (300 MHz, internal Me_4Si): *Varian-Gemini-300* spectrometer; δ in ppm.

Prior to biological testing, **3a** and **3b** were applied to a column of *Bio-Gel P-2* (minus 400 mesh, wet; *Bio-Rad Laboratories*, Richmond, USA) and fractions pooled, concentrated, lyophilized, and tested for pyrogenicity. Total hydrolyses of **3a** and **3b** were carried out in 6M HCl, at 100° for 16 h. Synthesized **3a** and **3b** (see below) were used throughout the testing of antiviral activity. They were dissolved in MEMH (Minimum Essential Medium with *Hank*'s salts) and sterilized by 0.2 μm pore size membrane filtration; amantadine (= *Symmetrel*®), DL-Gly(Ada) and L-Ala-D-γGln, used for comparisons, were treated in the same way.

Biological Materials. MDCK cells were grown in the tissue culture 96 flat bottom well plates [8]. The suspension of cells in MEMH supplemented with 10% foetal calf serum was adjusted to contain 10^4 of cells/100

µl/well. The cell-seeded plates were incubated for 24 h at 36° in a CO_2 incubator before use for cytotoxicity and antiviral effect studies. One strain with H1N1 and the other strain with H3N2 set of influenza A virus antigens, grown in the allantoic cavity of 11-day-old embryonated hen's eggs, were used.

DL-*(Adamant-2-yl)glycine*. From adamantanone according to the previously described procedure [18].

N*[(*tert*-Butyloxy)carbonyl]*-L-*alanyl*-D-*isoglutamine Benzyl Ester*. From benzyl D-isoglutaminate hydrochloride and pentachlorophenyl *N*-[(*tert*-butyloxy)carbonyl]-L-alaninate according to [19].

L-*Alanyl*-D-*isoglutamine Benzyl Ester Hydrochloride* (**2**·HCl). *N*-[(*tert*-Butyloxy)carbonyl]-L-alanyl-D-isoglutamine benzyl ester (1 g, 2.5 mmol) was dissolved in AcOH (50 ml) saturated with HCl and the soln. left at r.t. for 2 h (monitoring by TLC (BuOH/AcOH/H_2O 12:3:5)). AcOH was evaporated and the residual AcOH co-evaporated several times with benzene: 844 mg (100%) of **2**·HCl. Amorphous solid. R_f 0.5 (solvent, see above).

N-*[(*tert*-Butyloxy)carbonyl]*-DL-*(adamant-2-yl)glycine* (**1**). To a soln. of DL-(adamant-2-yl)glycine (314 mg, 1.5 mmol) in dioxane/H_2O 2:1 (9 ml) and 1N NaOH (5 ml), di(*tert*-butyl) dicarbonate (361 mg, 1.66 mmol) was added slowly and dropwise at r.t. After stirring for 2 h, an additional amount of di(*tert*-butyl) dicarbonate (100 mg, 0.46 mmol) was added dropwise within 30 min. After stirring for 24 h at r.t., the mixture was concentrated, acidified to pH 3 with sat. $KHSO_4$ soln., and extracted with AcOEt (3 15-ml portions), the combined extract washed with H_2O (2 × 10 ml), dried (Na_2SO_4), and evaporated, and the crude product recrystallized from AcOEt/hexane: 450 mg (97%) of colorless crystals. M.p. 168–170°. $[\alpha]_D^{23} = -27$ ($c = 1$, $CHCl_3$). R_f 0.92 (BuOH/AcOH/H_2O 12:3:5). ^{1}H-NMR ($CDCl_3$): 10.71 (*s*, COOH); 4.6–4.5 (*m*, CH(α)); 2.0–1.6 (*m*, 15 H, Ada); 1.44 (*s*, Me_3C). ^{13}C-NMR ($CDCl_3$): 178.05 (COOH); 155.76 (Me_3COCO); 80.14 (Me_3C); 55.8 (C(α), Gly); 47.29, 38.66, 37.98, 31.66, 29.63, 27.82, 27.59 (Ada); 28.33 (Me_3C). Anal. calc. for $C_{17}H_{27}NO_4$ (309.41): C 65.99, H 8.80, N 4.52; found: C 66.22, H 9.08, N 4.27.

N-*[(*tert*-Butyloxy)carbonyl]*-ambo-*(adamant-2-yl)glycyl*-L-*alanyl*-D-*isoglutamine Benzyl Ester. Woodward*'s reagent 'K' (97 mg, 0.38 mmol) was added to MeCN/dimethylformamide (DMF) 2:1 (2 ml) and cooled to 0°. The soln. of **1** (120 mg, 0.38 mmol) in MeCN/DMF 2:1 (3 ml) and Et_3N (50 µl, 36 mg, 0.38 mmol) were added. The mixture was stirred for 1.5 h at 0–5°. Subsequently, **2**, obtained from **2**·HCl (130 mg, 0.38 mmol) and Et_3N (50 µl, 0.38 mmol) in MeCN/DMF 2:1 (1 ml), was added, the mixture stirred overnight at r.t., the solvent evaporated, and H_2O (20 ml) added to the residue. Upon keeping the mixture at 0–5° for 2 h, a colorless solid separated (220 mg, 95%). The crude material was submitted to CC (silica gel, AcOEt). After concentration to a smaller volume, a colorless solid (210 mg, 91%) separated. Recrystallization from AcOEt/hexane yielded an anal. sample of the completely protected tripeptide. M.p. 114–116°. R_f 0.49 (AcOEt). ^{1}H-NMR ($CDCl_3$): 7.33 (*s*, Ph); 5.11 (*s*, $PhCH_2$); 2.6–2.3 (*m*, $CH_2(\gamma)$, Gln); 2.3–2.0 (*m*, $CH_2(\beta)$, Gln); 2.0–1.5 (*m*, 15 H, Ada); 1.39 (*s*, Me_3C); 1.35 (*d*, Me(β), Ala). ^{13}C-NMR ($CDCl_3$): 173.87, 172.80, 172.52 (4C, CO); 156.09 (Me_3COCO); 135.78, 128.56, 128.27 (Ph); 79.91 (Me_3C); 66.54 ($PhCH_2$); 55.70 (C(α), Gly); 52.43 (C(α), Gln); 51.98 (C(α), Ala); 49.71, 48.87, 46.45, 38.60, 37.98, 30.53, 29.40, 27.88, 27.71 (Ada); 31.89 (C(γ), Gln); 28.39 (C(β), Gln, Me_3C); 18.23 (Me(β), Ala). Anal. calc. for $C_{32}H_{46}N_4O_7$ (598.74): C 64.19, H 7.74, N 9.36; found: C 64.37, H 7.69, N 9.48.

ambo-*(Adamant-2-yl)glycyl*-L-*alanyl*-D-*isoglutamine Hydrochloride* (**3**). *N*-[(*tert*-Butyloxy)carbonyl]-DL-(adamant-2-yl)glycyl-L-alanyl-D-isoglutamine benzyl ester (350 mg, 0.6 mmol) was dissolved in AcOH (25 ml) saturated with HCl and the soln. left at r.t. for 4 h (monitoring by TLC (BuOH/AcOH/H_2O 12:3:5)). AcOH was evaporated and the product precipitated with abs. Et_2O. The obtained Boc-deprotected benzyl ester hydrochloride was dissolved in 90% aq. EtOH (20 ml) and hydrogenated over 10% Pd/C (80 mg) at r.t./4 atm for 4 h. The catalyst was filtered off and the filtrate evaporated. CC (silica gel, $CHCl_3$/i-PrOH/MeOH/H_2O/AcOH 20:15:6:4:2) of the crude product yielded diastereoisomers **3a** and **3b** as colorless glossy foams.

D,L,D-*Isomer* **3a**: 100 mg (22.4%). R_f 0.7 ($CHCl_3$/i-PrOH/MeOH/H_2O/AcOH 20:15:6:4:2). $[\alpha]_D^{23} = -56$ ($c = 0.5$, H_2O). ^{1}H-NMR ((D$_6$)DMSO): 9.3 (br. *s*, COOH); 8.96 (1 H, NH(α), Gln); 7.17, 6.96 (2 *s*, $CONH_2$); 4.25 ('*quint.*', CH(α), Ala); 3.01–3.84 (*m*, 2 H, CH(α) of Gln, CH(α) of Ada); 2.56–1.48 (*m*, 19 H, CH(γ) of Gln, $CH_2(\beta)$ of Gln, Ada); 1.19 (*d*, Me(β), Ala). ^{13}C-NMR (D_2O): 182.52 (COOH); 177.44, 175.86, 170.94 (C=O); 55.98 (C(α), Gln); 55.03 (C(α), Gly); 50.35 (C(α), Ala); 48.03, 38.95, 38.80, 38.07, 34.54, 31.59, 30.19, 28.22, 28.06, 27.97 (Ada); 32.12 (C(γ), Gln); 28.12 (C(β), Gln); 17.39 (Me(β), Ala). FAB-MS (glycerol matrix): 409 ($[M + H]^+$).

L,L,D-*Isomer* **3b**: 180 mg (40.4%). R_f 0.6 (solvent as for **3a**). $[\alpha]_D^{23} = +14$ ($c = 0.5$, H_2O). ^{1}H-NMR (D_2O): 4.35 (*q*, CH(α), Ala); 4.22–4.18 (*m*, CH(α), Gln); 4.07 (*d*, CH(α), Gly); 2.21 (*t*, $CH_2(\gamma)$, Gln); 2.07–1.99 (*m*, $CH_2(\beta)$, Gln); 1.93–1.57 (*m*, 15 H, Ada); 1.36 (*d*, Me(β), Ala). ^{13}C-NMR (D_2O): 182.17 (COOH); 177.11, 174.77, 170.80 (C=O); 55.12 (C(α), Gln); 54.31 (C(α), Gly); 50.79 (C(α), Ala); 46.61, 38.56, 38.32, 37.72, 34.30, 31.43, 29.36, 28.41, 27.88, 27.83 (Ada); 31.82 (C(γ), Gln); 28.14 (C(β), Gln); 17.06 (Me(β), Ala). FAB-MS (glycerol matrix): 409 ($[M + H]^+$).

Enzymatic Oxidation of L-*(Adamant-2-yl)glycine* (obtained from **3b**) *with* L-*Amino-Acid Oxidase*. Upon total hydrolysis of **3b** (6M HCl, 100°, 16 h), HCl was evaporated and the residue dissolved in phosphate buffer (pH 6.5). By addition of 1N NaOH (20 µl), the pH of the soln. was adjusted to 7. L-Amino-acid oxidase (80 µl of aq. soln.: 7.0 mg protein/ml, 8.9 units/mg protein) was added and the mixture incubated at 37° for 24 h. TLC ($PrOH/NH_3$ 7:3 and $BuOH/AcOH/H_2O$ 60:15:25; comparison with hydrolysate): no L-Ala and L-Gly(Ada); only spot for D-Glu.

Enzymatic Oxidation of the Hydrolysate Obtained from **3a**. The same procedure (hydrolysis and enzymatic oxidation with L-amino-acid oxidase) was repeated for **3a**. TLC (see above): no L-Ala, spots for D-Gly(Ada) and D-Glu.

Enzymatic Oxidations with D-*Amino-Acid Oxidase*. Hydrolysates of **3a** and **3b** were incubated in *Tris* buffer pH 8.5 at 37° for 24 h with D-amino-acid oxidase (80 µl of aq. soln.: 5 mg protein/ml, 21.7 units/mg protein). TLC (see above): D-Glu reacted very poorly with D-amino-acid oxidase and D-Gly(Ada) not at all.

Testing of the Toxicity for MDCK Cells. The solns. of **3a**, **3b**, *Symmetrel®*, DL-Gly(Ada), and L-Ala-D-γ-Gln were adjusted to contain in 50 µl volumes, when added to 150 µl culture medium/well, the final concentration from 6.25 to 800 µg in two-fold steps. After 72 h of incubation, the cells were observed for morphological changes and the ability for further multiplication by one passage using trypsin release and subsequent growth in MEMH with added serum at 36°.

Testing of Antiviral Effect in MDCK Cells. The potency of two influenza A virus strains was tested by inoculation of serial 10-fold dilutions in MEMH/trypsin-containing medium but without serum to the MDCK cells. The dilutions of viruses in 50-µl volumes per well were added to the 3 times washed cell monolayers. After 48 h of incubation, viral replication was detected by appearance of CPE and by quantitating the specific haemagglutinin released in the culture medium. Chick RBC haemagglutination assay (HA) was used for this purpose. $CCID_{50}$ for each viral strain was determined by the method of *Kärber* [20].

To study the inhibitory effect of **3a**, **3b**, *Symmetrel®*, DL-Gly(Ada), and L-Ala-D-γGln, the following procedure was adopted. MDCK monolayer was washed 3 times with MEMH and each well received 100 µl of MEMH/trypsin-containing medium. Two-fold dilutions of the compounds prepared in MEMH in 50 µl volumes, starting from the highest concentration of 200 µg to the lowest of 6.25 µg were added in the appropriate wells. A 50 µl of MEMH with 1000, 100, 10, and 1 $CCID_{50}$ of the tested viral strains were added to the wells with cells. The corresponding controls of MDCK cells alone, MDCK cells with, either tested compounds (in various dilutions), or influenza A virus received 50 and 100 µl of MEMH, resp.

After 72 h of incubation, the infectivity titre was determined by HA as it is described for the determination of the potency of influenza A virus strains.

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