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Short Communication

Synthesis, Characterization, and Biological Activity of Amino Acid Derivatives of the Heteropolytungstophosphoric Acid

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Summary. Compounds of phosphotungstic acid (*WPA*) containing the amino acids alanine (*WPA–Ala*) or glycine (*WPA–Gly*) as counter cations were synthesized and characterized by elemental analysis, thermal analysis, and IR spectroscopy. Cellular toxicity was assessed by the trypan blue exclusion method, and the antiviral activity of *WPA* and the modified *WPA* compounds was tested against herpes simplex viruses (HSV) type 1 and type 2. Biological assays indicate that the newly synthesized compounds exhibit no evident cytotoxic effects on Vero cells and negligible antiviral activity against HSV-1 and HSV-2.

Keywords. Heteropoly compounds; Amino acids; IR spectroscopy; Cellular toxicity; Antiviral activity.

Introduction

The 12-tungstophosphoric acid, $H_3PW_{12}O_{40} \times nH_2O$ (*WPA*), a heteropoly acid of tungsten(VI) and phosphorus(V), belongs to a large class of nano-sized early

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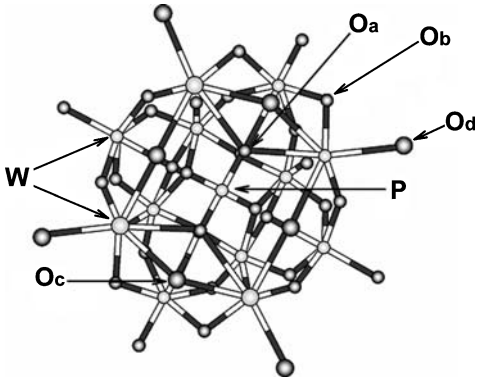

Fig. 1. The *Keggin* structure of $(PW_{12}O_{40})^{3-}$; Oa – oxygen atoms bound to heteroatoms, Ob – corner-sharing oxygen-bridges, Oc – edge-sharing oxygen-bridges, and Od – terminal oxygen atoms

transition metal oxygen ion complexes called polyoxometalates (POMs) that are of utmost practical importance in a great variety of applications (as catalysts, analytical reagents, superionic proton conductors, sensors, *etc*.) [1, 2]. The principal structural unit of *WPA* is the *Keggin* anion $(PW_{12}O_{40})^{3-}$ consisting of a central PO_4^{3-} tetrahedron surrounded by 12 WO_6 octahedra [3] (Fig. 1). The *Keggin* anions are interconnected by hydrogen-bonded water molecules forming in a solid state a unique ionic structure that consists of heteropolyanions and countercations, which underlies the distinctive physicochemical properties of POMs, such as the high proton and electron mobility [4, 5].

In recent years the interest in POMs applications in clinical medicine, primarily as anticancer and antiviral agents, has increased. The antiviral activity of POMs against viruses causing vesicular stomatitis, poliomyelitis, german measels, *Rausher* leukaemia, rabies, and HIV-AIDS is especially emphasized [6–8]. The principal obstacle to a wider application of POMs in bio-medicine is their inorganic derivation that is often coupled to marked toxicity *in vivo* (hepatotoxity, renal toxicity, and thrombocytopenia) [6]. For that reason, synthesis of novel POMs modified in such a way to cause less cellular toxicity while retaining antiviral activity is required. Thus, since the first POM modified with an organic polyanion bound covalently to an organometallic group $[(Cp\text{Ti})PW_{11}O_{39}]^{4-}$ ($Cp = \eta^5\text{–}C_5H_5$) was synthesized by *Klemperer* [9] in 1978, a large number of organometallic-substituted heteropolyanions have been synthesized, both by metal substitution and organic derivatization, and characterized so far [10–14].

WPA as well as other POMs are the subject of our longtime research during which we resolved its structure, conductive properties, dielectric relaxation phenomena and defined a variety of protonic species in detail [4, 5, 15–20]. The aim of this study is to synthesize novel *WPA* compounds that are modified by amino acids alanine and glycine as countercations (*WPA–Ala* and *WPA–Gly*), characterize them by elemental analysis, thermal analysis, and IR spectroscopy, and investigate their value for bio-medical applications by examining their toxicity and antiviral potential. According to published data antiviral properties of unmodified *WPA* and *WPA* modified with *N*-methyl-2-pyrrolidone ($(NMP)_2(H^+)_3$) or $(Na^+)_3$ as counter ions, were tested against the HIV-1 virus, showing an effective concentration that sup-

presses the virus by 50%, EC_{50}[1] $= 14\,\mu M$, for *WPA* [21] and different efficiency for the modified compounds [6]. However, we found no data on the antiviral activity of *WPA* itself against the herpes simplex virus (HSV), an enveloped *DNA* virus belonging to the Herpesviridae family that has a complex structure and a comparably large genome coding for numerous protein products. Given the globally widespread prevalence of HSV [22] and the lack of data on the effect of *WPA* and *WPA*-derived compounds on HSV, we examined the antiviral properties of *WPA*, *WPA–Ala*, and *WPA–Gly* against the HSV type 1 and type 2 and their toxicity on the Vero cells used for viral isolation.

Results and Discussions

Elemental Analysis

The elemental analysis revealed a yield of 67% for *WPA–Ala* based on $H_3PW_{12}O_{40}$. Anal. for $C_{12}H_{43}N_4O_{54}PW_{12}$: calcd C 4.31, N 1.67, H_2O 3.23, P 0.93, W 65.97; found C 2.98, N 1.83, H_2O 3.33, P 0.92, W 67.0. For *WPA–Gly* the yield was 71% based on $H_3PW_{12}O_{40}$. Anal. for $C_8H_{35}N_4O_{54}PW_{12}$: calcd C 2.92, N 1.70, H_2O 3.28, P 0.94, W 67.09; found C 2.86, N 1.67, H_2O 3.22, P 0.93; W 66.9. According to the elemental analysis four molecules of amino acids make a molecular complex with one *Keggin* anion, which is in agreement with the T_d symmetry of the *Keggin* anion. Contents of tungsten and phosphorus in the modified compounds were also verified by inductively coupled plasma optical emission spectroscopy (ICP-AES). The obtained values were in good agreement with values expected from theoretical inference, confirming that the *Keggin* anion is preserved in all investigated compounds.

Thermal Analysis

Results on thermal analysis of *WPA* can be found in the literature [4]. The modified compounds, *WPA–Ala* and *WPA–Gly*, show endothermic transitions in the region from room temperature to 400°C. *WPA–Gly* exhibits two phase transitions; one at 125°C corresponding to dehydration of crystalline water and the other at 320°C corresponding to melting of the organic component. In the case of *WPA–Ala*, three endothermic processes are evident in the same temperature region; the transition at 75°C corresponds to dehydration of crystalline water, that at 157°C corresponds to sublimation of the organic component and the melting was found at 310°C. In the case of both modified compounds melting is followed by partial decomposition of the organic components, which was evident from the TGA curves.

Infrared Spectroscopy

The infrared spectra were recorded in the range from 4000 to 350 cm^{-1}. In the IR spectra characteristic frequencies of the carbonyl group are found at *ca.* 1500 cm^{-1}

[1] Effective concentration EC_{50} is defined as the concentration of the test substance that produces an antiviral effect in 50% of test organisms

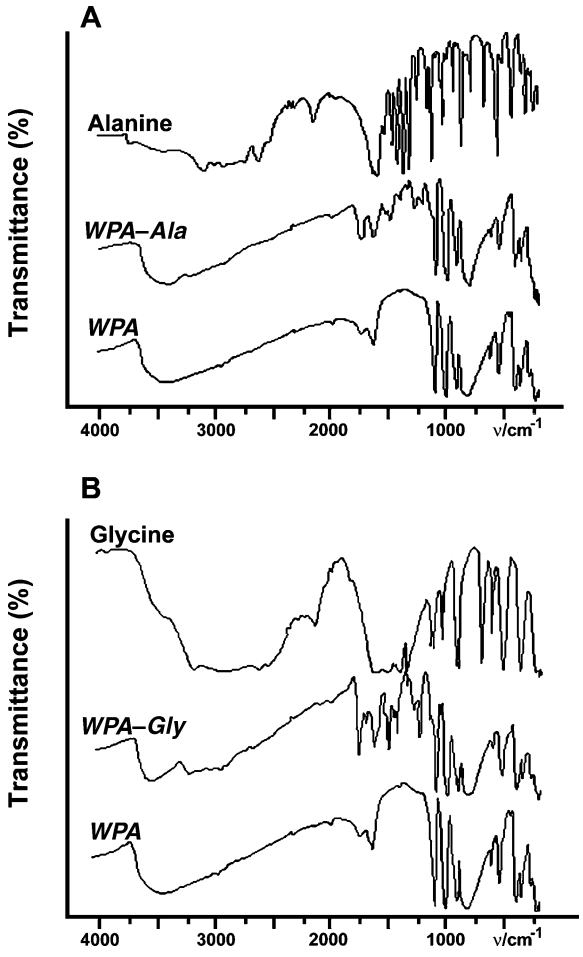

Fig. 2. IR spectra of the heteropolytungstophosphoric acid (*WPA*), its amino acid derivatives *WPA–Ala* and *WPA–Gly*, and free alanine and glycine

and those of the *Keggin* anion in the range below 1100 cm^{-1} (1079s, 983s, 891s, 808s,b, 592m, 522s, 382s and 1075s, 1042w, 989s, 975s, 890s, 806vs, 595w, 512s, 388s for *WPA–Ala* and *WPA–Gly*, respectively).

Comparing the IR spectra of the newly synthesized compounds with the IR spectrum of the parent *WPA* (Fig. 2), differences that could be attributed to the formation of hydrogen bonds between the amino acids and *WPA* were observed. The most notable distinctions observed in the IR spectrum are the splitting and shifting of bands that are characteristic for the host's network of *Keggin* anions and of the stretching and bending vibrations that correspond to hydrogen bonds. For example, the vibration band at 982 cm^{-1} for pure *WPA*, which corresponds to the ν_1 (PO_4) vibration, splits into two 989 and 975 cm^{-1} in the case of *WPA–Gly*, and the band characteristic for the ν (W–PO_4) vibration, which appears at 377 cm^{-1} for pure *WPA*, is shifted to 388 cm^{-1} for *Gly* and 382 cm^{-1} for the *Ala* modified *WPA*. This confirmed that the W–Oc (Oc, edge sharing oxygen) bonds were strengthened while the P–O and W–Ob (Ob, corner bridging oxygen) bonds were weakened in the modified *WPA* compounds. Comparing the IR spectra of *WPA–Ala* and

Table 1. Cellular toxicity assay; effect of *WPA*, *WPA–Ala*, and *WPA–Gly* on the viability of Vero cells

WPA		*WPA–Ala*		*WPA–Gly*	
Concentration	Viability	Concentration	Viability	Concentration	Viability
μM	%	μM	%	μM	%
0	97.7	0	97.4	0	97.2
100	93.8	100	96.9	100	98.3
50	94.9	50	97.7	50	98.0
25	94.5	25	98.0	25	98.2
12.5	95.2	12.5	98.0	12.5	99.0

WPA–Gly with those of the corresponding amino acid precursor, it can be noted the bands arising from *Ala* and *Gly* remain almost unchanged in position, but change in their intensities and overlap with bands characteristic for protonic species [4].

Cellular Toxicity and Antiviral Activity

As a first step of analysis, toxicity of *WPA* and the novel compounds *WPA–Ala* and *WPA–Gly* (*WPA*s) was examined on Vero cells grown in culture. Cell viability was assessed using the trypan blue vital dye exclusion method [23, 24]. All concentrations of *WPA*s used in the study were compatible with viability of Vero cells in culture for up to 72 hours. As measured by the trypan blue exclusion method, the polyoxometal compounds tested in this study showed no cytotoxic effects on Vero cells grown in culture independently of the used concentrations (Table 1).

It was not possible to discriminate unambiguously between the toxicity of the parent and modified *WPA*s in this particular assay, although some trend in lower toxicity for modified *WPA*s can be noticed (Table 1). Low cellular toxicity that was observed, especially for modified *WPA*s, can be viewed as a promising trait for prospective bio-medical applications. Therefore, we plan to expand our future investigation and study the toxicity of *WPA*s on other cell types.

Antiviral effect of *WPA*s was tested by quantifying reduction in infectious viral titers, as described in the experimental part. The yield reduction assay showed virtually no antiviral activity of *WPA*s on the reference strains of HSV-1 and HSV-2 inoculated into the Vero cells, as the viral titer in the treated cells remained unchanged and equal to the tissue culture infectious dose 50%, $TCID_{50}$[2] $= 10^{3.3}$, in the control plates.

It was shown in previous studies (reviewed in Ref. [21]) that heteropolytungstates readily incorporate in the plasma membrane and are efficiently taken up by Vero, as well as other cell types. The primary mechanism of antiviral action is assumed to be due to the inhibition of surface viral proteins and/or enzymes, most probably the viral reverse transcriptase. The absence of notable antiviral activity of the *WPA*s tested in the present study could be due to the complexity of the herpes

[2] Tissue culture infectious dose equals to virus titer that successfully infects 50% of inoculated cultures.

simplex virus structure, its *DNA* genome and related particular features of its replication, unlike the *RNA* viruses on which the activity of several POMs was previously tested [25].

To sum up, tungstophosphoric acid derivatives modified with amino acids alanine and glycine were successfully synthesized, structurally characterized, and their potential as antiviral agents against the Herpes simplex virus type 1 and type 2 was evaluated in the present study. The newly synthesized compounds showed virtually no toxicity on Vero cells grown in culture and exhibited little antiviral properties against HSV-1 and HSV-2. Since the potentials of the newly synthesized compounds for bio-medical applications cannot be evaluated using one cell and virus type only, the results obtained so far should be considered as preliminary results in a comprehensive study including other cell types and viruses that is to follow.

Experimental

WPA was synthesized according to the *Drachels* method [26], starting from $Na_2WO_4 \cdot 2H_2O$ (Aldrich Chemical Company, Milwaukee, USA) and recrystallized twice from bidistilled water. The amino acids were purchased from Loba Chemie, India. All other chemicals and solvents (H_3PO_4, HCl, diethyl ether), were of commercial *p.a.* reagent-grade and were used without further purification.

Preparations of WPA–Ala and WPA–Gly

A water solution of *WPA* $\cdot 6H_2O$ (5 g *WPA* in 20 cm^3 of water) was added very slowly to a solution of alanine (0.5 g) in 1.0 *M* HCl (10 cm^3), with continuous stirring. The stirring was continued for 1 h after mixing the reagents. Within 30 min, a white microcrystalline compound began to separate out. The stirring was turned off and the reaction mixture was allowed to stay at room temperature for three days. The precipitated solid (*WPA–Ala*) was collected by filtration, washed thoroughly with water, and dried in vacuum over P_4O_{10}. *WPA–Gly* was synthesized following the above procedure using glycine instead of alanine.

Physical Measurements

Microanalyses (C, H, N) were performed using a Perkin-Elmer 240C elemental analyzer. TG-DSC was performed in nitrogen atmosphere on a SDT 2960 TA instrument, calibrated with CaC_2O_4 and Al_2O_3. IR spectra were obtained on a Perkin-Elmer 983G spectrometer with samples prepared as KBr pellets and mulls in Nujole and Fluorolube oils.

Toxicity and Antiviral Assays

Vero cells (African green monkey kidney cells) were grown in minimal essential medium (MEM) (Torlak Intitute, Belgrade, Serbia and Montenegro) with 2% *L*-glutamine, supplemented with 5% fetal calf serum (FCS) (PAA laboratories, Pashing, Austria), and maintained in MEM supplemented with 2% FCS.

Twenty-four well mini-plates were seeded with Vero cells in growth medium at 1×10^5 cells/well and incubated over night at 37°C in 5% CO_2 and humidified air. By the next day a confluent cell layer was present in each well. The growth medium was replaced by the maintenance medium containing the investigated compounds at serial-doubling dilutions ranging from 1.0×10^{-4} to 1.25×10^{-5} *M*, in quadruplicate. Control wells received the nonmodified maintenance medium. The plates were then incubated for 72 h under the same conditions as indicated above. Thereafter the maintenance medium was removed, the cells were detached with 0.25% trypsin solution, collected and re-suspended. 0.1% solution of trypan blue in PBS was added to equal volumes of cell suspension. Viability counts were

then carried out for each well individually and expressed as percentage of viable cells per well. Average values were presented in Table 1.

Antiviral activity of *WPA*s was tested by yield reduction assay. The yield reduction assay in microtiter plates was used to compare the titer of HSV type-1 and type-2 in the control medium to that in the presence of different *WPA*s concentrations. Tests were conducted in quadruplicates. HSV-1 and HSV-2 reference strains were used at $10^{3.3}$ $TCID_{50}$. Growth medium (GM) was removed from confluent monolayers and wells were infected with 50 mm^3 of 10^{-2} to 10^{-7} dilutions of the starting viral suspensions in drug-free maintenance medium and adsorption was carried out at 37°C in 5% CO_2 in humidified air for one hour. After adsorption, unadsorbed virus was removed and maintenance medium, containing serial-doubling dilutions of *WPA*s ranging from 1.00×10^{-4} to 1.25×10^{-5} M in the test wells, or drug-free maintenance medium in the control wells, was added. The plates were incubated at 37°C in 5% CO_2 in humidified air for 72 h and cytopathic effect (CPE) appearance read. Virus yield was measured as $TCID_{50}$ in test and control wells.

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