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Synthesis and Immunomodulatory Activity of Some Novel Amino Acid Germinates

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Amino acid germanate compounds are synthesized by the chemical reaction of germanium dioxide with a selected amino acid in the presence of an alkali medium at a suitable elevated temperature. Amino acids included in the novel synthesis include histidine, methionine and glutathione. The study evaluated their effects on induction of interleukin-12 and interferon-γ in Swiss albino Webster rats. The new germanium compounds, especially bis-methionino germanate, are considered as good interferon inducer that significantly enhance the immunologic function.

Keywords: Amino acid germanate-immunomodulatory, Interferon-γ, Interleukin-12

INTRODUCTION

Organic germanium has found to be an impressive immunostimulant. Several studies have demonstrated its marked anti-tumor effects [1], interferon-inducing activity [2] and have restored immune function in immune depressed animals. Organic germanium compounds demonstrate their anticancer activity in laboratory animals infected with a wide range of different cancers as immunostimulant, which has been shown to be mediated by activation, and stimulation of T-cells, natural killer cells, lymphokine and macrophage activity [3]. Organic germanium's immune enhancing properties were also noted in many studies particularly in case of cancer and arthritis [4-6].

Badawi reported the inhibitory effect of germanium(IV) sodium ascorbate on the *in vitro* reverse transcriptase of human immunodeficiency virus [7]. The role of germanium citrate lactate was proved to be of a great protective value against schistosomal complications [8].

Histidine is used as a chelating agent in some cases of arthritis, methionine is one of the sulpher-containing amino acids, which act as a good free radical scavenger, and glutathione prevents and treats a wide range of degenerative diseases [9]. In this study, we investigated the effect of some novel synthesized compounds titled, bis-histidino germanate(I), bis-methionino germanate(II), and bis-glutathiono germanate(III) on production of serum cytokines such as interferon-γ (IFN-γ) and interlukin-12 (IL-12) in untreated controls rats in order to assess these compounds as immunomodulators.

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EXPERIMENTAL

Chemicals

Reagent grade germanium dioxide, histidine, methionine, glutathione, and sodium carbonate *were obtained from Aldrich chemical company.*

General Procedure for the Synthesis of Amino Acid Germinates

Germanium dioxide (0.01 mol, 1.05 g), sodium carbonate (0.01 mol, 1.06 g) and 50 ml of distilled water were mixed,

Table 1. Micro Elemental Analysis of Amino Acid Geminates

Amino acid	C (%)		H (%)		N (%)		S (%)		Ge (%)	
germinates	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found
(I)	36.31	35.17	4.03	4.83	21.18	20.39	-	-	18.30	18.13
(II)	31.20	31.46	5.20	4.76	7.28	7.71	16.64	16.15	18.88	18.91
(III)	34.26	34.74	4.57	4.97	11.99	11.41	9.14	9.93	10.36	10.11

Table 2. FTIR Characteristic Band of Amino Acid Germinates

Amino acid germinates	Ge=O	Ge-N	N-H indole	-CONH-	$> {}^{+}NH_2$	$-CO_2^-$
(I)	1625	1330	3400	-	3105	1456
(II)	1611	1351	-	-	3155	1464
(III)	1661	1408	-	3463-3442	3160	1446

then refluxed to 100-150 °C with agitation until complete solubility of germanium dioxide. To the reaction mixture was added 0.02 mol histidine, methionine, or glutathione with continuous refluxing and stirring for 2 h [10]. Cooling and evaporation of water were then carried out to give crude product, which was crystallized from ethyl alcohol to yield: bis-histidino germinate(I), bis-methionino germinate(II) or bis-glutathiono germanate(III), respectively. The resulting compounds were characterized by Micro elemental analysis (VARIO EL ELEMENTAR) (Table 1), fourier Transform infrared (FT-IR) (ATI Mattson Genesis FT-IR^{TM}) (Table 2), and Mass spectroscopic analysis (MS) (mass spectrometer HP model Ms 5988, using chemical ionization technique).

Determination of the Partition Coefficient of Amino Acid Germinates

Water and a suitable non-miscible solvent such as octanol (1:1) were added to the amino acid germanate (0.004 mol), shaked vigorously at the ordinary temperature in a stopperd bottle, and then immersed in thermostatic water at 25 °C. After attainment of equilibrium, the bottle was allowed to stand in the thermostat for 30 min to ensure complete separation of the two liquid layers. The concentration of amino acid germinates in the aqueous layer was determined sepectrophotometrically [11]. From the concentration of amino acid germinate in water, its concentration in octanol layer was then calculated. The partition coefficients were obtained from equation K = concentration in octanol/concentration in water. Here, increase in K value means increase in lipophilicity.

Determination of Surface Tension

Surface tension measurements [12] for the amino acid germinates were carried out using a DuNouy Ring Tensiometer Type 8451 Krus (Hamburg, Germany) at a concentration of 0.05% in water. The resulting data are recorded in Table 4.

Animals

Female Swiss albino rats weighing 130-150 g (obtained from the breeding unit of the National Research Center, Cairo, Egypt) were used in these experiments. The rats were housed under optimal ventilation and illumination conditions at a temperature of 20-25 °C with relative humidity 60-70% and maintained on stock diet formulated to meet rat's nutrient requirements and free supply of water.

Gamma Irradiation Procedure

Irradiation process was carried out using a Gamma Cell-40

Table 3. Partition Coefficient (K) of Amino Acid Germinates

Amino acid germanate	K
(I)	0.869
(II)	0.631
(III)	0.539

Table 4. Surface Tension of Amino Acid Germinates

Amino acid germanate	Surface tension (dyne cm^{-1})
(I)	54
(II)	44
(III)	55

(cesium-137 irradiation unit) at National Center for Radiation Research and Technology (NCRRT), Cairo. The dose rate was 0.667 Gy min^{-1} as calibrated at the time of investigation.

Following animal groups were tested: (Group 1) animals treated with saline (control); (Group 2) animals irradiated rats, subjected to γ-irradiation (6.5 Gy); (Group 3) animals treated with single dose of glutathione-germanate (150 mg kg^{-1} body weight); (Group 4) animals treated with single dose of histidine-germanate (150 mg kg^{-1} body weight); (Group 5) animals treated with single dose of methionine-germanate (150 mg kg^{-1} body weight); (Group 6) animals treated with glutathione-germanate (150 mg kg^{-1}) 30 min before exposure to γ-irradiation (6.5 Gy); (Group 7) animals treated with single dose of histidine-germanate (150 mg kg^{-1}) 30 min before γ-irradiation exposure; (Group 8) animals treated with single dose of methionine-germanate (150 mg kg^{-1}) 30 min before γ-irradiation exposure.

Six rats were sacrificed from each group at one and three days post-irradiation. The blood was collected by heart puncture in sterile tubes and serum was separated by centrifugation at 3000 rpm for 15 min.

Assay of IL-12

ELISA technique was used for *in vitro* quantitative determination of rat interleukin-12 (rIL-12) in rat serum [13]. The assay recognizes both natural and recombinant rIL-12 (p70) as well as the free p40 subunits. The minimum detectable dose of rIL-12 was < 5 pg ml^{-1}.

Assay of INF-γ

Enzyme Linked Immuno Sorbent Assay technique (ELISA) was used for *in vitro* quantitative determination of rat interferon-gamma (rIFN-γ) in rat serum [14].

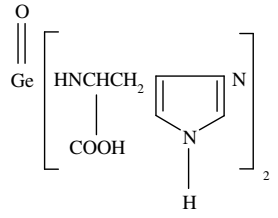
Statistical Analysis

Results were expressed as meam ± standard error (S.E.). Differences between the groups was determined by Student's t-test [15]. Scatter plots and linear regression coefficient between the biochemical parameters were done.

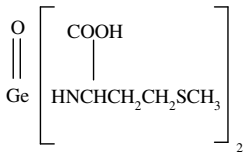
RESULTS AND DISCUSSION

Synthesis of Amino Acid Germinates

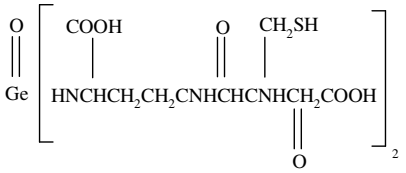
The chemical reaction between germanium dioxide and histidine, methionine and glutathione takes place in a basic medium as a catalyst. The structures of the products can be denoted as follows:



Bis-histidino germanate(I)



Bis-methionino germanate(II)



Bis-glutathiono germanate(III)

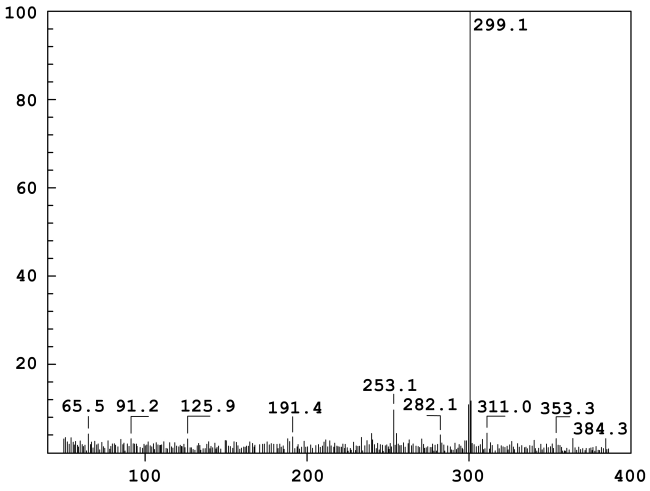
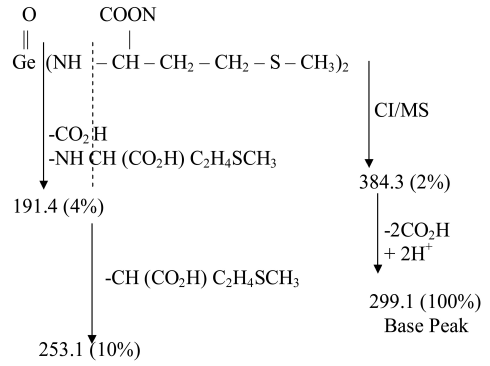


Fig. 1. Mass spectrum of compound II.



Scheme 1. Fragmentation pattern of compound II.

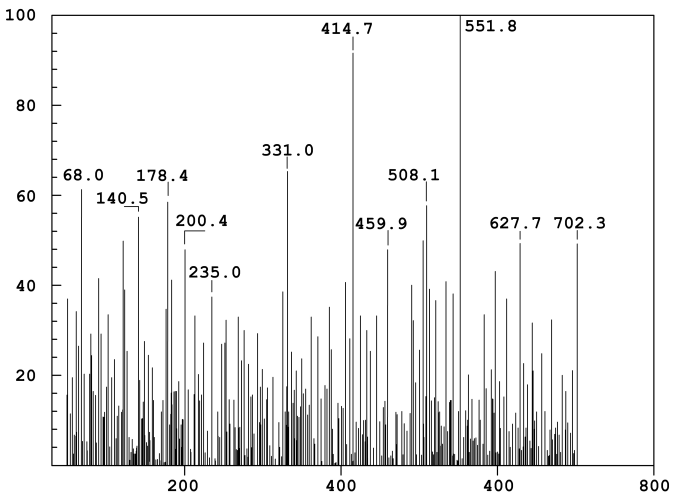
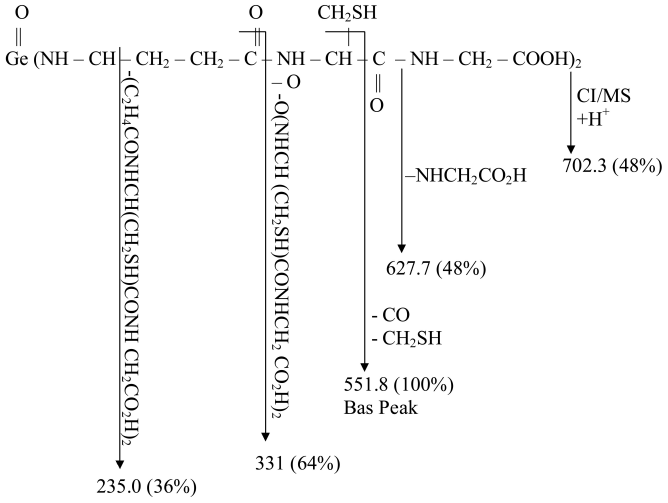


Fig. 2. Mass spectrum of compound III.



Scheme 2. Fragmentation pattern of compound III.

The structure of compounds I, II, and III were established by correct micro elemental analysis (Table 1), IR spectra showing the characteristic band of amino acid germinates due to bonding to germanium (Table 2), mass spectra of compounds II and III (shown in Figs. 1 and 2). The fragmentation patterns (Schemes 1 and 2) show the mass peaks (m/e) and their relative abundances (%) for the fragments.

Assay of Interleukin-12 and Interferon-γ

As indicated in Table 5, exposure of animals to single dose (6.5 Gy) whole body γ-irradiation exhibited significant decrease in the level of interleukin-12 at one and three days post-radiation exposure. Administration of germinate compounds increased the level of interleukin-12; especially, GSH-germanate and methionine-germanate recorded 55.52% and 63.95%, respectively, at one day post-treatment. The

Table 5. Levels of Serum ILK-12 (Pg ml^{-1}) in Rats Treated with Amino Acid Germinates after one and Three Days

Amino acid germinates	ILK-12 (Pg ml^{-1})	
	One day	Three days
Control	32.62 ± 1.28	32.99 ± 1.64
(I)	40.08 ± 2.08	33.50 ± 1.77
%Change from control	122.87%	101.55%
(II)	53.48 ± 1.35	90.70 ± 4.53
%Change from control	163.95%	274.93%
(III)	50.73 ± 1.29	38.71 ± 1.95
%Change from control	155.52%	117.34%

Table 6. Levels of Serum IFN-γ (Pg ml^{-1}) in Rats Treated With Amino Acid Germinates after one and Three Days

Amino acid germinates	ILK-12 (Pg ml^{-1})	
	One day	Three days
Control	44.50 ± 1.10	43.99 ± 2.19
(I)	50.72 ± 1.24	56.82 ± 2.84
%Change from control	113.98 %	129.17 %
(II)	68.50 ± 1.10	57.04 ± 2.85
%Change from control	153.93 %	129.67 %
(III)	62.53 ± 1.23	47.80 ± 2.39
%Change from control	140.52 %	108.66 %

highly significant elevation of IL-12 was continued and became more pronounced after three days of methionine-germanate injection (174.93), as compared to the control group.

On the other hand, injection of glutathione-germanate compound 30 min before radiation exposure induced a highly significant increase ($P < 0.001$) of the interleukin-12 level at one day post-radiation (149.20%), while treatment of animals with either histidine-germanate or methionine-germanate pre-radiation exposure recorded slight significant increases ($P < 0.05$) after one day (114.50% and 114.19%, respectively) and highly significant increases after three days post exposure (193.60% and 134.74%, respectively).

Results in Table 6 show that radiation exposure resulted in a $P < 0.001$ in the level of γ-interferon only after one day of exposure. However, after three days, no significant changes were observed.

Administration of germinate compounds to animals enhanced the production of interferon-γ in the following order: methionine germinate > glutathione germinate > histidine germinate, which recorded significant increases of 153.93%, 140.52%, and 113.82%, respectively. Treatment with histidine germinate or methionine germinate maintained the high level of interferon-γ up to the third day. Treatment of animals with glutathione germinate pre-radiation exposure elevated the levels of interferon-γ which recorded highly significant increases after one and three days post-irradiation (145.39% and 160.29%) while histidine and methionine germinate compounds increased significantly the levels of IFN-γ amounting 133.51% and 127.57%, respectively, at 3 days post-irradiation.

It is well known that immune response is one of the most important defense mechanisms of the body against cancer and infection. The induction of cytokines including IL-12 and IFN-γ play a protective role in the defense mechanism against many diseases [16].

Cytokines, hormone like proteins, produced by stimulated cells and tissues, are found to protect animals from immunodeficiency and infection by intracellular or extra cellular pathogens [17]. IFN-γ and IL-12 are primary cytokines, which are important for the induction of T-cell-mediated immunity. IL-12 is a cytokine that exerts immunoregulatory effects on cells [18]. It is observed that the obtained amino acid germinated enhance IL-12 and IFN-γ induction and, hence, it is expected that they increase the natural killer T-cells activity, the macrophage activity and the lymphocytes activity.

Data presented in Tables 5 and 6 show the increasing IF-γ and IL-12 induction after treating with the amino acid germinates I-III, where bis-methionino germinate II recorded the highest rate of IFN-γ induction. It is known that methionine reacts with adenosine triphosphate (ATP), which is transformed to bioactive form S-adenosyl methionine (SAMe). IL-12 turns to SAMe gene in T-cells [19]. There is a previous report which ascertains the effect of SAMe on T-lymphocytes and cytokines in an experimental model of surgical sepsis

[20].

A chronic administration of L-methionine (500 mg kg^{-1} i.p. twice daily) to nude mice is reported to increase the number of dopaminergic receptor. These mice have no thymus and have the advantage of presenting pure B-lymphocytes to observation [21,22].

Glutathione (GSH) shows an important role in the immune and enhancing effect of dietary whey protein, a rate limiting substrate for the synthesis glutathione, which is necessary for lymphocyte proliferation [23].

Increasing GSH concentration in relevant tissues is recorded to show anti-tumor effect on low volume of tumor *via* stimulation of immunity through the GSH pathway [24].

Thus, it seems that both bis-methionino and bis-glutathiono germanate significantly increase the levels of serum IL-12 and IFN-γ by mechanism which act as neurochemical messengers to the immune system.

Structure-Activity Relationship

In addition to the previous discussion supporting the reason of the high immunoenhancing activity of compounds II and III, the partition coefficient may affect this activity. The partition coefficient (K) predicts the passage of a drug between lipid membrane (nonaqueous) and the aqueous environment, where the lipid-soluble substance will cross such a membrane by simple dissolving in, and diffusing across, the lipid layer. Thus, the data given in Table 3 show that K value of compound II is higher than that of compound III. In addition, compound II has higher percentage value of Ge and S, as compared with compound III (Table 1).

Although bis-histidino germinate I contains the highest percentage of Ge and has higher K value, it shows less interferon induction in the first day, while higher interferon induction is observed in the third day this might be due to the higher liphophilicity of compound I, which facilitates its retention inside the cell exerting accumulated activity.

In addition, the variation in the biological activity of the three germinates I-III can be attributed to the difference in the surface activity, which refers to fast crossing of cell membrane. Data recorded in Table 4 reveals that bis-methionino germanate has the most pronounced surface activity, reducing water surface tension from 72 to 44 dyne cm^{-1}, which might be one of the factors explaining its higher immunomdulatory activity.

CONCLUSIONS

This study reports the synthesis of novel amino acid germinates and their evaluating as immunomodulators. The selected amino acids used were histidine, methionine and glutathione. The induction of IL-12 and IFN-γ were measured. It was found that both bis-methionino germanate and bis-glutathiono germanate significantly increase the levels of serum IL-12 and IFN-γ *via* mechanisms which act as neuro chemical messengers to the immune system. This allows the communication, orchestration and execution of the immune system strategies that might provide sufficient immune responses to cancer diseases in future research.

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