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In Vitro Study of the Relationship between the Structure of Ginsenoside and Its Antioxidative or Prooxidative Activity in Free Radical Induced Hemolysis of Human Erythrocytes

ZAI-QUN LIU,*,† XU-YANG LUO,† GUO-ZONG LIU,† YAN-PING CHEN,§ ZHI-CAI WANG,§ AND YUN-XIU SUN†

Department of Organic Chemistry and Center for Teaching, College of Chemistry, Jilin University, Changchun 130023, China

Ginsenoside, the major active component in *Panax ginseng*, which has been used in traditional Chinese medicine, contains a series of derivatives of the triterpene dammarane being attached by some sugar moieties. To clarify the relationship between the structure of ginsenoside and its properties, 11 individual ginsenosides, along with the central structures of ginsenoside, protopanaxadiol and protopanaxatriol, are used in 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) induced hemolysis of human erythrocytes, a good experimental model to research free radical induced membrane damage and to evaluate the antioxidative or prooxidative activities of various antioxidants conveniently. It is found that the central structures of ginsenosides, either protopanaxadiol or protopanaxatriol, play a prooxidative role in AAPH-induced hemolysis of erythrocytes. As to the individual ginsenoside, if there are no sugar moieties attached to the 20-position of the triterpene dammarane, the ginsenoside acts as a prooxidant, that is, Rg3, Rh2, and Rg2. A glucose attached to the 6-position instead of the 20-position sugar moieties can make the ginsenoside an antioxidant, that is, Rh1. The antioxidants among ginsenosides follow two different mechanisms that can be expressed mathematically by the Boltzmann equation, that is, Rc and Rb1, and a polynomial equation, that is, Re, Rd, R1, Rg1, Rb3, and Rh1. The orders of antioxidative ability are Rc > Rb1 and Re > Rd > R1 > Rg1 > Rb3 > Rh1, respectively.

KEYWORDS: Structure−activity relationship; antioxidant; prooxidant; ginsenoside; free radical; erythrocytes; hemolysis

INTRODUCTION

Free radical induced peroxidation of membrane lipids, which is associated with a variety of pathological events, has attracted much interest (*1*, *2*). Both natural and synthetic antioxidants have been used to suppress radicals to protect the membrane lipids against free radical peroxidation. Therefore, antioxidant therapy has become an attractive strategy (*3*, *4*). The erythrocyte membrane contains abundant polyunsaturated fatty acids that are very susceptible to free radical induced peroxidation. Because the generation rate of free radicals from the decomposition of 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) at physiological temperature can be easily controlled, this water-soluble azo compound can be used as a free radical initiator to peroxidize the erythrocyte membrane to induce hemolysis eventually. Hence, the AAPH-induced hemolysis of erythrocytes provides a good experimental model to research the free radical induced membrane damage and to evaluate the antioxidative or prooxidative activities of various antioxidants conveniently (*5*, *6*).

Panax ginseng has been used in traditional Chinese medicine since the Han dynasty some 2000 years ago. The major active components in *P. ginseng* are called ginsenosides, containing a series of derivatives of the triterpene dammarane being attached by some sugar moieties. Although there are many works that deal with the effect of ginsenosides on lipid metabolism, immune function, cardiovascular system, etc. (*7*−*9*), reports are often contradictory due to the fact that mixtures of ginsenosides are used and the experimental systems are not the same (*10*). We have reported the antioxidative/prooxidative and synergistic effects with α-tocopherol on AAPH-induced hemolysis (*11*). However, the relationship between the structure of ginsenoside and its antioxidative or prooxidative activities is not clear. Presented here is the structure−activity relationship research of 11 individual ginsenosides, along with their central structures, protopanaxadiol (PD) and protopanaxatriol (PT), on AAPH-induced hemolysis of human erythrocytes. The relationship between positions of sugar moieties in the molecule of ginse-

* Address correspondence to this author at the Department of Organic Chemistry, College of Chemistry, Jilin University, No. 119 Jiefang Road, Changchun 130023, China (fax +86 431 8949334; e-mail zaiqunliu@mail.jlu.edu.cn).
† Department of Organic Chemistry.
§ Center for Teaching.

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noside and its activity is revealed, and the antioxidative activities of various individual ginsenosides are also expressed mathematically by the Boltzmann equation and a polynomial equation, demonstrating that ginsenosides follow two different mechanisms to protect erythrocytes against AAPH-induced hemolysis. The orders of antioxidative activity of various ginsenosides are also given.

MATERIALS AND METHODS

AAPH was purchased from Aldrich and used as received. Various individual ginsenosides were extracted from *P. ginseng* root, and the structures were also determined following the methods of the literature (*12*−*14*).

The experimental process of erythrocyte hemolysis is as described as in the literature (*5*, *6*, *11*). Human erythrocytes were collected from a healthy adult donor by venipuncture into heparin. Before experiment, erythrocytes were washed three times with phosphate-buffered saline (PBS: 150 mM NaCl, 8.1 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4, pH 7.4) to remove the plasma (*15*). During the last washing the erythrocytes were centrifuged at exactly 3000 rpm (the diameter of the rotor was 24 cm) for 10 min to obtain a constantly packed cell volume, and then erythrocytes were added to PBS to obtain a 3.0% (v/v) suspension (*16*). Ginsenosides were dissolved in PBS; if ginsenoside cannot be dissolved completely in PBS, dimethyl sulfoxide (DMSO) was added to generate a homogeneous solution (*11*). Then the ginsenoside solution was added to a 3.0% suspension of erythrocytes in PBS (pH 7.4) and incubated at 37 °C (±0.1 °C) for 3 h. An AAPH/PBS solution was then injected into the above mixture to initiate hemolysis. In every experiment the concentration of AAPH was kept at 40 mM. After incubation for 3 h with the addition of AAPH, the percentage of hemolysis was determined by measuring the absorbance of the supernatant of the erythrocytes at 540 nm of the visible spectrum and compared with that of complete hemolysis. The percentage of hemolysis in the controls was 100% in the presence of AAPH and was <3.0% in the absence of AAPH. Every experiment was repeated three times, and the results were reproducible within 10% experimental error. Experimental points shown in figures were the average value of three parallel data.

Mathematical statistics were carried out by Origin Server software, version 5.0, and the statistically significant difference of experimental data was >0.99.

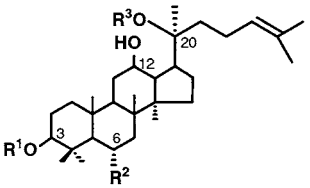
RESULTS AND DISCUSSION

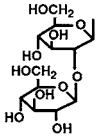
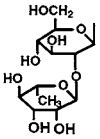
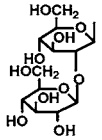
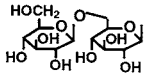
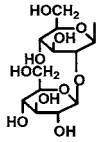
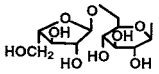
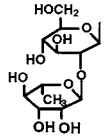
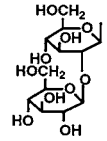
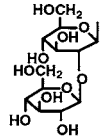
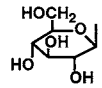
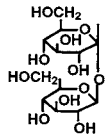
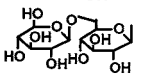
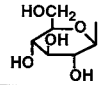
Effect of Mixtures of Protopanaxadiols and Protopanaxatriols on AAPH-Induced Hemolysis. Ginsenosides are various derivatives of the triterpene dammarane, and, in general, can be divided into two groups according to the positions at which sugar moieties are attached. The characteristic structure of the group of protopanaxadiols is due to sugar moieties attaching to the ring of the triterpene dammarane at the 3-position, that is, Rg3, Rd, Rc, Rh2, Rb1, and Rb3. As to the group of protopanaxatriols, the sugar moieties attach to the ring of the triterpene dammarane at the 6-position, that is, Rg1, Rg2, Rh1, Re, and R1, as listed in **Table 1**.

To determine the effect of the mixture of ginsenosides on AAPH-induced hemolysis of human erythrocytes, the mixture of protopanaxadiol (PDm) and the mixture of protopanaxatriol (PTm) with various amounts were added to the human erythrocytes/PBS (pH 7.4) system, to which AAPH at the final concentration of 40 mM was added as initiator, respectively.

Figure 1 shows that PDm decreases the hemolysis percentage with the increase of the amount. PTm, however, increases the hemolysis percentage amount-dependently. These facts reveal that ginsenosides play two roles in AAPH-induced hemolysis of erythrocytes: PTm acts as prooxidant to increase hemolysis percentage, and PDm acts as an antioxidant to protect erythrocytes against AAPH-induced hemolysis.

Table 1. Structures of Various Ginsenosides



Ginsenosides	R^1	R^2	R^3
PD	H	H	H
PT	H	OH	H
Rh2		H	H
Rg3		H	H
Rg2	H		H
Rb1		H	
Rc		H	
Re	H		
Rd		H	
R1	H		
Rg1	H		
Rb3		H	
Rh1	H		H

Effect of the Central Structures of Ginsenosides, Protopanaxadiol and Protopanaxatriol, on AAPH-Induced Hemolysis. To clarify the function of every part of the molecule of ginsenoside on AAPH-induced hemolysis, the protopanaxadiol and protopanaxatriol groups were hydrolyzed to remove all of

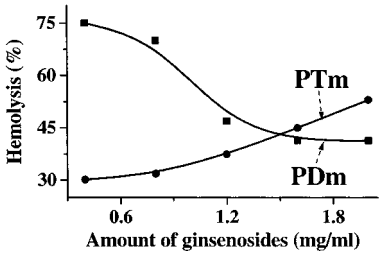


Figure 1. Effects of the mixture of ginsenosides on AAPH-induced hemolysis of erythrocytes.

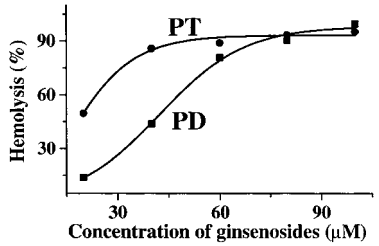


Figure 2. Prooxidative effects of protopanaxadiols (PD) (in the absence of AAPH) and protopanaxatriols (PT) (in the presence of AAPH).

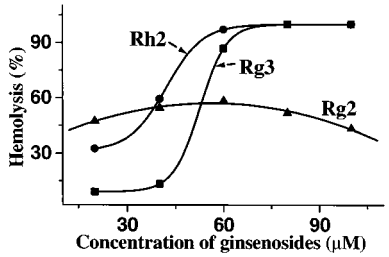


Figure 3. Prooxidative effects of Rg3 (in the absence of AAPH), Rh2, and Rg2 on AAPH-induced hemolysis of erythrocytes.

the sugar moieties and to obtain two kinds of central structure of ginsenoside, protopanaxadiol (PD) and protopanaxatriol (PT), respectively. Then, PD and PT were added to the erythrocytes/PBS system.

Figure 2 shows that the hemolysis percentages were increased along with the increase of addition of PT and PD, respectively. In particular, PD can lead to hemolysis even in the absence of AAPH as initiator. This demonstrates that the central structure of ginsenoside, either protopanaxadiol or protopanaxatriol, cannot suppress the AAPH-induced hemolysis of erythrocytes. Contrarily, these structures play a prooxidative role in this case.

Relationship between the Structure of Ginsenoside and Its Antioxidative or Prooxidative Activity. To clarify the structure−activity relationship of various individual ginsenosides, 11 individual ginsenosides were isolated from the mixtures of the protopanaxadiol and protopanaxatriol groups and added to the AAPH-induced hemolysis system, respectively.

It can be seen in **Figure 3** that the hemolysis percentages are increased with the increase of the concentration of Rg3 (in the absence of AAPH as initiator) and Rh2. For Rg2, only a high concentration can suppress the hemolysis. Therefore, Rg3, Rh2, and Rg2 act as prooxidants. On the contrary, **Figures 4** and **5** show that the other ginsenosides, Rb1, Rc, R1, Rd, Re, Rb3, Rg1, and and Rh1, function as antioxidants because the hemolysis percentages are decreased remarkably with the increase of ginsenoside concentration added to the AAPH-induced hemolysis system. By comparing the structures of prooxidants with those of antioxidants, one can find that the absence of sugar moieties attaching to the 20-position of the triterpene dammarane makes Rh2, Rg3, and Rg2 prooxidant.

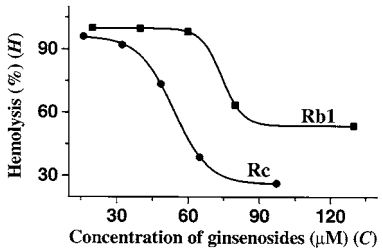


Figure 4. Antioxidative effects of Rb1 and Rc on AAPH-induced hemolysis of erythrocytes.

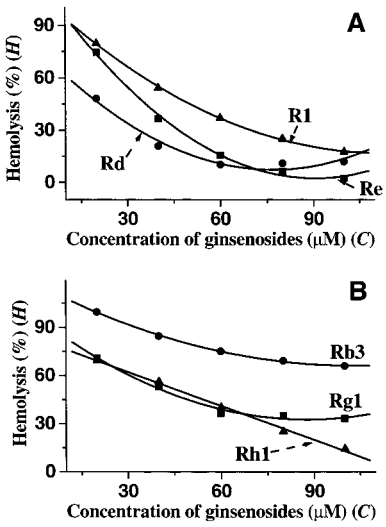


Figure 5. Antioxidative effects of R1, Rd, and Re (A) and Rb3, Rg1, and Rh1 (B) on AAPH-induced hemolysis of erythrocytes.

However, for Rh1, although without the above characteristic structure, one glucose moiety attaching to the 6-positon of the triterpene dammarane can also lead Rh1 to be an antioxidant. Therefore, sugar moieties at the 20-position or a glucose moiety at the 6-position makes ginsenoside an antioxidant. However, we have found that Rg2 was an antioxidant and Rd was a prooxidant in a previous work (*11*), and the obtained result in this work is contradictory. This is because the PBS used in the previous work contained 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, and 5 mM glucose (*17*), and there is no glucose contained in PBS used in this work (150 mM NaCl, 8.1 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4) (*15*). This reveals that the additional glucose in the experimental system also affects the antioxidant or prooxidant effects of ginsenosides on AAPH-induced hemolysis. Although the interaction between the additional glucose in the experimental system and the ginsenosides, Rg2 and Rd, respectively, should be researched in detail, this result also demonstrates that the glucose either in the experimental system or attaching to the ring of the triterpene dammarane affects the antioxidant or prooxidant activity of ginsenosides in AAPH-induced hemolysis.

Mathematical Expression of Antioxidative Activities of Ginsenosides. To compare the antioxidative activities of Rb1, Rc, R1, Rd, Re, Rb3, Rg1, and Rh1, the experimental curves in **Figures 4** and **5** are expressed mathematically by the Boltzmann equation (eq 1) and a polynomial equation (eq 2).

$$H = (A_1 - A_2)/(1 + e^{(C-C_0)/dC}) + A_2 \quad (1)$$

$$H = A_1C + A_0C^2 + A_2 \quad (2)$$

H stands for hemolysis percentage, and C refers to the

Table 2. Constants and Coefficients in the Boltzmann Equation of Rb1 and Rc[a]

ginsenoside	C_0	dC	A_1	A_2
Rc	54.1	7.30	95.9	26.0
Rb1	74.3	4.31	99.9	53.7

[a] The antioxidative activity of the above ginsenosides follows the Boltzmann equation, $H = (A_1 - A_2)/(1 + e^{(C-C_0)/dC}) + A_2$.

Table 3. Constants and Coefficients in the Polynomial Equation of Re, Rd, R1, Rg1, Rb3, and Rh1[a]

ginsenoside	A_1	A_0	A_2
Re	−2.59	0.0143	119
Rd	−1.86	0.0121	78.8
R1	−1.65	0.00736	109
Rg1	−1.47	0.00839	97.1
Rb3	−0.995	0.00484	117
Rh1	−0.703	0.0	83.1

[a] The antioxidative activity of the above ginsenosides follows the polynomial equation, $H = A_1C + A_0C^2 + A_2$.

concentration of ginsenoside. In eq 1, A_1, A_2, C_0 and dC are the constants of the equation. The physical meanings of C_0 and dC can be designated *balance concentration* and *concentration sensitivity*, respectively. In eq 2, A_0 and A_1 are the coefficients, which can also be designated *concentration sensitivity*, and A_2 is the constant. The constants and coefficients of the Boltzmann equation are listed in **Table 2** and those of the polynomial equation in **Table 3**.

The experimental results for Rb1 and Rc follow the Boltzmann equation, and those for R1, Rd, Re, Rb3, Rg1, and Rh1 follow the polynomial equation, demonstrating that the antioxidative mechanism of Rb1 and Rc is quite different from that of other antioxidants.

As listed as in **Table 2**, the *balance concentration*, C_0, of Rc, 54.1 μM, is lower than that of Rb1, 74.3 μM, indicating that the valid antioxidative concentration of Rc is lower than that of Rb1. Furthermore, the *concentration sensitivity*, dC, of Rc, 7.30, is larger than that of Rb1, 4.31, revealing that the antioxidative activity of Rc increases more remarkably with the increase of the concentration of Rc than Rb1 does. This reveals that the antioxidative activity of Rc is better than that of Rb1. As listed in **Table 3**, becausethe coefficient of C^2, A_0, is much smaller than that of C, A_1, the influence of C^2 on the hemolysis percentage, H, is so small that it can be neglected. According to the value of A_1, these ginsenosides can be divided into three groups. The first group contains Re, which has an A_1 of −2.59, the smallest value among these six ginsenosides, indicating that the hemolysis percentage, H, is decreased efficiently with the increase of the concentration of Re. By structural comparison, this is because rhamnose and glucose moieties attached to the 6-position benefit the antioxidative activity of Re (*11*). As to the second group, although the A_1 values of Rd, R1, and Rg1, −1.86, −1.65, and −1.47, are similar, two glucose moieties attaching to the 3-position make Rd a better antioxidant than the same sugar moieties at the 6-position as in R1 and much better than only one glucose attaching to the 6-position as in Rg1. The last group contains Rb3 and Rh1. The similarity of the A_1 values of Rb3 and Rh1, −0.995 and −0.703, indicates their antioxidative activities are similar, although their structures are quite different.

Conclusion. Briefly, the central structures of ginsenosides, either protopanaxadiol (PD) or protopanaxatriol (PT), play a prooxidative role in AAPH-induced hemolysis of erythrocytes; in particular, PD generates hemolysis of erythrocytes even in the absence of initiator. To the individual ginsenoside, if there are no sugar moieties attached to the 20-position of the triterpene dammarane, the ginsenoside acts as a prooxidant in AAPH-induced hemolysis, that is, Rh2 and Rg2, even in the absence of AAPH, that is, Rg3. A glucose attached to the 6-position instead of the 20-position sugar moieties can also make the ginsenoside an antioxidant, that is, Rh1. The antioxidants among ginsenosides follow two different mechanisms that can be expressed mathematically by the Boltzmann equation, that is, Rc and Rb1, and the polynomial equation, that is, Re, Rd, R1, Rg1, Rb3, and Rh1. The orders of antioxidative ability are Rc > Rb1 and Re > Rd > R1 > Rg1 > Rb3 > Rh1, respectively. Sugar moieties attaching to different positions of the triterpene dammarane generate various ginsenosides, which play different roles in AAPH-induced hemolysis, demonstrating there are complicated interactions between sugar moieties and the central structure, the triterpene dammarane. This kind of interaction within one molecule of ginsenoside should be researched in detail.

LITERATURE CITED

(1) Rice-Evans, C. A.; Diplock, A. *Free Radical Biol. Med*. **1993**, *15*, 77−96, and references cited therein.

(2) Bland, J. S. *J. Nutr. Environ. Med.* **1995**, *5*, 255−280, and references cited therein.

(3) Spooren, A. G.; Evelo, C. A. *Blood Cells Mol. Dis*. **1997**, *23*, 323−336.

(4) Nakamura, T.; Ogasawara, M.; Koyama, I.; Nemoto, M.; Yoshida, T. *Biol. Pharm. Bull*. **1993**, *16*, 970−972.

(5) Miki, M.; Tamai, H.; Mino, M.; Yamamoto, Y.; Niki, E. *Arch. Biochem. Biophys*. **1987**, *258*, 373−380.

(6) Niki, E.; Komuro, E.; Takahashi, M.; Urano, S.; Ito, E.; Texao, K. *J. Biol. Chem*. **1988**, *263*, 19809−19814.

(7) Chen, X. In *Proceedings of the International Symposium on Natural Antioxidant*; Packer, L., Traber, M. G., Xin, W., Eds.; AOCS: Champaign, IL, 1996; pp 179−187.

(8) Kim, H.; Chen, X.; Gillis, C. N. *Biochem. Biophys. Res. Commun*. **1992**, *189*, 670−676.

(9) Rimar, S.; Lee-Mengel, M.; Gillis, C. N. *Pulm. Pharmacol*. **1996**, *9*, 205−209.

(10) Gillis, C. N. *Biochem. Pharmacol*. **1997**, *54*, 1−8, and references cited therein.

(11) Liu, Z.-Q.; Luo, X.-Y.; Sun, Y.-X.; Chen, Y.-P.; Wang, Z.-C. *Biochim. Biophys. Acta* **2002**, *1572*, 58−66.

(12) Soldati, F.; Sticher, O. *Planta Med*. **1980**, *39*, 348−357.

(13) Shibata, S.; Tanaka, O.; Shoji, J.; Saito, H. Chemistry and pharmacology of *Panax*. In *Economic and Medicinal Plant Research*; Wagner, H., Hikino, H., Farnsworth, N. R., Eds.; Academic Press: New York, 1985; Vol. 1, pp 217−284.

(14) van Breeman, R. B.; Huang, C.-R.; Lu, Z.-Z.; Agnes, R.; Fong, H. S.; Fitzloff, J. F. *Anal. Chem.* **1995**, *67*, 3985−3989.

(15) Zavodnik, I. B.; Lapshina, E. A.; Rekawiecka, K.; Zavodnik, I. B.; Bartosz, G.; Bryszewska, M. *Biochim. Biophys. Acta* **1999**, *1421*, 306−316.

(16) Ma, L.-P.; Liu, Z.-Q.; Zhou, B.; Yang, L.; Liu, Z.-L. *Chin. Sci. Bull*. **2000**, *45*, 2052−2056.

(17) David, J. M.; Scott, G.; Richard, H. S.; Richard, M. *Biochim. Biophys. Acta* **1999**, *1415*, 342−348.

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