Novel biological function of sialic acid (*N*-acetylneuraminic acid) as a hydrogen peroxide scavenger

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Abstract We have found that *N*-acetylneuraminic acid (NANA) consumes toxic hydrogen peroxide (H_2O_2) under physiological conditions. Close investigation of this finding revealed that NANA was oxidized by an equimolar amount of H_2O_2 to provide its decarboxylated product, 4-(acetylamino)-2,4-dideoxy-D-glycero-D-galacto-octonic acid (ADOA). To date, there have been little data on this reaction, and its physiological significance has not been discussed. Examining the detoxification of H_2O_2 in cultured cells with NANA, we were able to confirm that the cell death caused by H_2O_2 was suppressed by NANA in a dose-dependent manner. These results revealed a novel role for NANA as a reactive oxygen scavenger. It is known that terminal NANA residues are removed by neuraminidase and that free NANA molecules are recycled or degraded by enzymes. We propose that released monomeric NANA is the potent defense molecule against oxidative damage.

Key words: Sialic acid; Hydrogen peroxide; Decarboxylation; Detoxification; Reactive oxygen scavenger

1. Introduction

Sialic acids, a family of monosaccharides comprising more than 30 different variations of neuraminic acid, are known to exist in animals and some microorganisms [1]. Since Blix isolated sialic acid from bovine submaxillary gland mucin (BSM) in 1936 [2], a great deal of effort has been directed toward elucidating the biological functions of these sugars, especially *N*-acetylneuraminic acid (NANA). NANA occupies the terminal position of many naturally occurring glycoconjugates including glycoproteins and glycolipids. Most studies on NANA have focused primarily on cell protection, fertilization, cell differentiation, cell adhesion, immunology, inflammation and tumors [1,3,4]. The importance of NANA was confirmed by the lethal phenotype in the development of mice defective in NANA synthesis [5]. However, the unique α-ketocarboxylic acid structure of NANA itself has been curiously overlooked in these investigations. We propose here a new hypothesis with regard to these considerations, namely that NANA functions as a reactive oxygen scavenger in the host.

In the course of our study on innate immune factors, we had analyzed the cytotoxic mechanism of sea hare antineoplastic proteins. These proteins confirm an L-amino acid oxidase (LAAO), commonly known as a cytotoxic enzyme in the snake venom [6,7]. LAAO has been known to catalyze the conversion of L-amino acids into α-keto acids. When LAAO was added to RPMI-1640 cell culture medium containing approximately 6.8 mM of free L-amino acids, it was observed that a cytotoxic concentration of H_2O_2 was produced as a byproduct of the reaction. It was also confirmed that co-addition of catalase into the medium suppressed the cell death caused by sea hare LAAOs. Therefore we expected the cytotoxicity of sea hare LAAOs to be reduced by the addition of an eliminator of H_2O_2. In previous studies on inhibitory substances for the cytotoxicity of sea hare antineoplastic proteins, we have found that NANA acted like a catalase in the system [8,9]. These results prompted us to concentrate our attention on the reaction of NANA with H_2O_2.

2. Materials and methods

2.1. Materials

NANA (enzymatically synthesized, Code 00648-24, Nacalai Tesuque, Japan) was dissolved in water and the pH of the solution was adjusted to 7.0–7.5 with sodium hydroxide. The H_2O_2 solution used for atomic absorption spectrophotometry, which did not contain any stabilizer agents, was obtained from Wako Pure Chemical Co., Japan.

2.2. Analytical of the reaction product

The analysis of the compound was based on the familiar methods: Mass spectra were measured using fast atom bombardment-mass spectrometry (FAB-MS) and high-resolution (HR)-FAB-MS. ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra were recorded at 600 MHz (^{1}H-NMR) with a pulse Fourier transform NMR spectrometer using tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported in ppm downfield from TMS.

4-(Acetylamino)-2,4-dideoxy-D-glycero-D-galacto-octonic acid (ADOA): HR-MS (FAB^+) calculated for $C_{10}H_{20}O_9N$ 282.1189, found 282.1180; ^{1}H-NMR (600 MHz, CD_3OD): d 4.47 (1H, ddd, J = 9.6, 4.2, 1.8 Hz), 3.91 (1H, dd, J = 10.2, 1.2 Hz), 3.81 (1H, dd, J = 10.2, 1.8 Hz), 3.79 (1H, dd, J = 3.6, 11.4 Hz), 3.71 (1H, ddd, J = 9.0, 6.0, 3.6 Hz), 3.60 (1H, dd, J = 11.4, 6.0 Hz), 3.39 (1H, dd, J = 9.0, 1.2 Hz), 2.33 (1H, dd, J = 15.6, 9.6 Hz), 2.23 (1H, dd, J = 15.6, 4.2 Hz); ^{13}C-NMR (150 MHz, CD_3OD): d 180.5, 174.6, 72.4, 71.6, 69.7, 67.5, 65.2, 55.6, 48.6, 42.2, 22.5.

2.3. Measurement of H_2O_2 concentration

The concentration of H_2O_2 in the reaction mixture was measured with a PeroxiDetect™KIT (Cat. No. PD-1, Sigma, St. Louis, MO,

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Abbreviations: NANA, *N*-acetylneuraminic acid; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; LAAO, L-amino acid oxidase; BSA, bovine serum albumin; NMR, nuclear magnetic resonance; MS, mass spectrometry

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USA). One volume of reaction mixture or a standard H_2O_2 solution in a 96-well microtiter plate was incubated with nine volumes of aqueous peroxide color reagent for 30 min at room temperature, and the optical density at 550 nm was measured.

2.4. Cytotoxicity assay

For the cytotoxicity assay, EL-4 murine lymphoma cells were seeded at 2×10^4 cells/well in a 96-well microplate and cultured in 5% CO_2 at 37°C for 16 h. Before the assay, H_2O_2 and NANA in the RPMI-1640 cell culture medium (Sigma, cat. no. R0883, containing 10% fetal bovine serum, Iwaki Co. Japan) were preincubated for 3–6 h, and then the cells were added. Cellular viability was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. After the above incubation period, an MTT solution was added to the culture (500 μg/ml of MTT, final concentration) and incubation continued for 4 h. The absorbance of MTT formazan was measured at an optical density of 550–650 nm. Note that 100% indicates the absorbance of the untreated control.

3. Results and discussion

First, we tried to confirm the reactivity of H_2O_2 with NANA in solution. A reaction solution containing 150 mM of H_2O_2 and 100 mM of neutralized NANA was incubated at 37°C, and the concentration of H_2O_2 was measured at each time point. Even in such a minimal system, the reduction of H_2O_2 was reproducible. The reaction progressed in an almost rectilinear fashion for 0–90 min at a rate of 1.02 mM/min, and was saturated after 180 min at which point the 100 mM of NANA seemed to be consumed (Fig. 1A). This result suggested that the reaction requires just NANA and H_2O_2, at a ratio of 1:1. No such phenomenon was observed using related sugars such as *N*-acetyl mannosamine or *N*-acetyl muramic acid (data not shown). Therefore, this reaction is considered

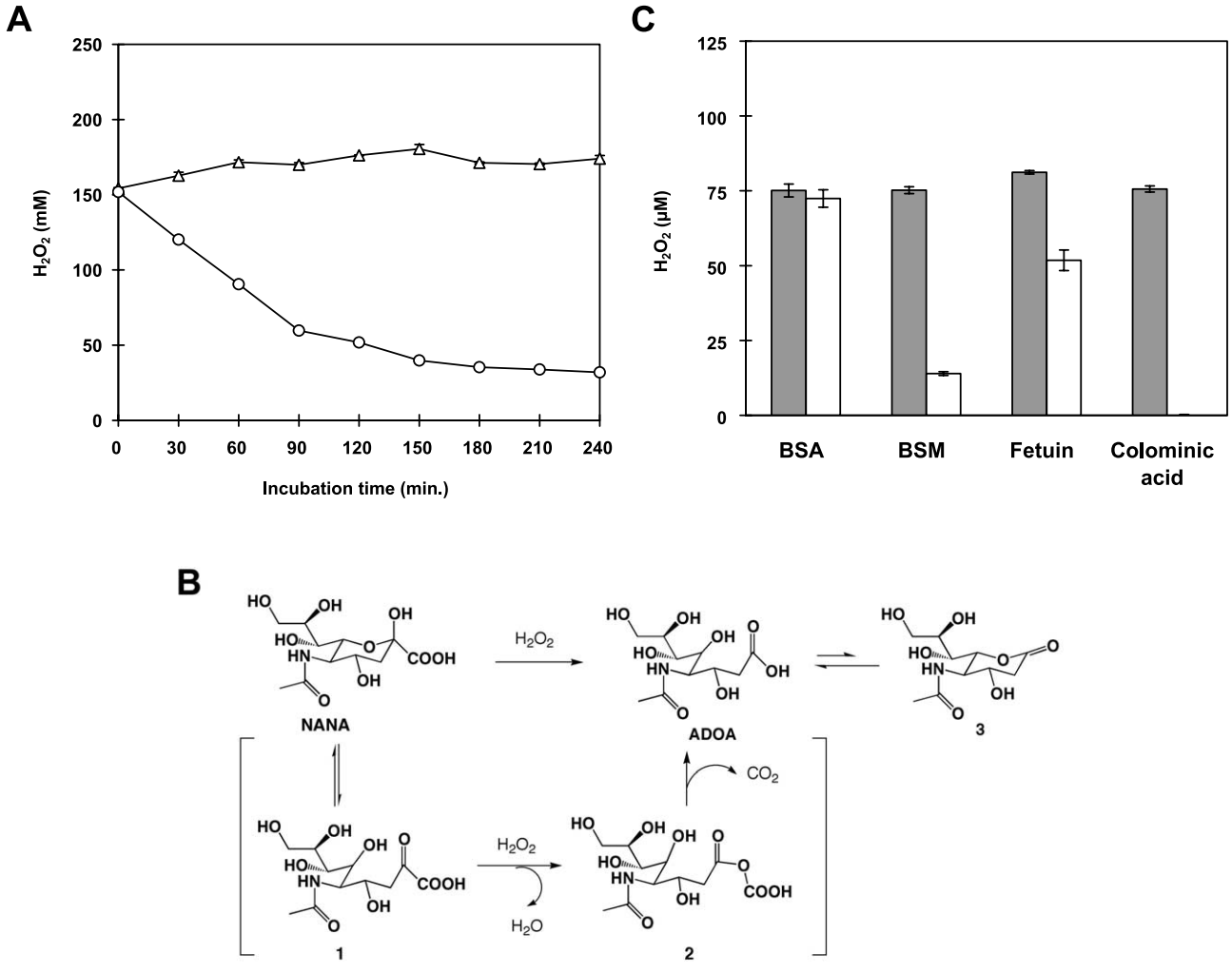

Fig. 1. Mechanism of decarboxylation of NANA by H_2O_2. A: Time course of H_2O_2 clearance by NANA. The reaction solution containing 150 mM H_2O_2 and 100 mM of neutralized NANA (○) or control (△) was incubated in the shaded box at 37°C. At the time points indicated on the horizontal axis, concentrations of H_2O_2 were measured. B: Reaction scheme for the decarboxylation of NANA by H_2O_2. NANA and its acyclic state **1** are in equilibrium in solution. **1** is reacted with H_2O_2 and converted to ADOA through the oxidized state **2**. ADOA is very stable and not cycled to **3**. C: H_2O_2-scavenging effect of neuraminidase digestion products. 0.2% of BSA, BSM, fetuin and colominic acid (poly-2,8-NANA) dissolved in a 100 μM H_2O_2/50 mM phosphate buffer solution (pH 7.5) was treated with (empty bar) or without (gray bar) 0.3 units/ml of neuraminidase (*Clostridium perfringens*) in the shaded box at 37°C. After three nights incubation, the H_2O_2 concentration was measured. Note that the spontaneous loss of H_2O_2 in the incubation period was approximately 25 μM. The NANA content of each substance described in the specification control is colominic acid sodium salt, 95%; BSM, 11.2%; fetuin, 7.3%; and BSA, 0%. The results shown in A and C are representative of three and four experiments; error bars indicate the standard deviation of triplicates.

dependent on the unique properties of NANA which has an α-ketocarboxylic acid group. To clarify the mechanism by which H_2O_2 is eliminated by NANA, the product in the reaction solution was analyzed.

A close analysis of the product by means of ^1H-, ^{13}C-NMR spectroscopy, and HR-MS proved that NANA was converted into the decarboxylated compound, ADOA, through the reaction (Fig. 1B). In short, NANA was oxidized by an equimolar amount of H_2O_2, which was the core of this matter. Although NANA usually exists as a cyclic structure in a solid state, it is assumed that its acyclic state, 2-keto-3-deoxy-nononic acid **1**, and cyclic NANA coexist at equilibrium in the solution. H_2O_2 oxidizes the keto position of **1** so that carbon dioxide was cleaved through the intermediate **2** to give acyclic ADOA just as like Baeyer–Villiger rearrangement. Further examination on the property of ADOA showed that structural stability of it is higher than its cyclic **3** under general conditions. This reaction seems to be ordinary from a chemical point of view [10]. However, little attention has been given to this in the sense that it is a hidden function of NANA. Considering the above results, it is obvious that NANA reacts with an equimolar amount of toxic H_2O_2 and provides ADOA with non-toxic water and carbon dioxide under physiological conditions.

A very small amount of free monomeric NANA is known to exist in tissues and organs. However, NANA is abundant as the residual sugar of sialoglycoproteins and sialoglycolipids. As shown in the reaction scheme in Fig. 1B, a free α-ketocarboxylic acid group of NANA is necessary for the reaction. Thus, NANA located at the non-reducing end and intra-sugar chain does not react with H_2O_2, because the α-keto carboxylic group is covered with a glycoside linkage in the α-ketoside bounds. Free NANA can exist temporarily, when it is cleaved from the sugar chain by neuraminidase. It was examined whether the products of digestion of sialo compounds by neuraminidase have the same effect as free monomeric NANA. Colominic acid (poly-2,8-NANA), homopolymeric sialic acids, and BSM and fetuin, sialoglycoproteins, are good substrates for neuraminidase. After three nights digestion, the products of these compounds eliminated H_2O_2 as expected (Fig. 1C). This effect was correlated with the NANA content of the substrate, thus the highest level of activity was observed in the colominic acid digest. On the other hand, bovine serum albumin (BSA), a non-sialoprotein, showed no activity to eliminate H_2O_2 even after neuraminidase treatment.

As NANA was observed to have an inhibitory effect on the cytotoxicity of sea hare LAAO [8,9], this reaction was considered a mechanism of detoxification. Thus, it had to be shown that the reaction product ADOA was non-toxic. H_2O_2 is a powerful cytotoxic agent which can destroy DNA and func-

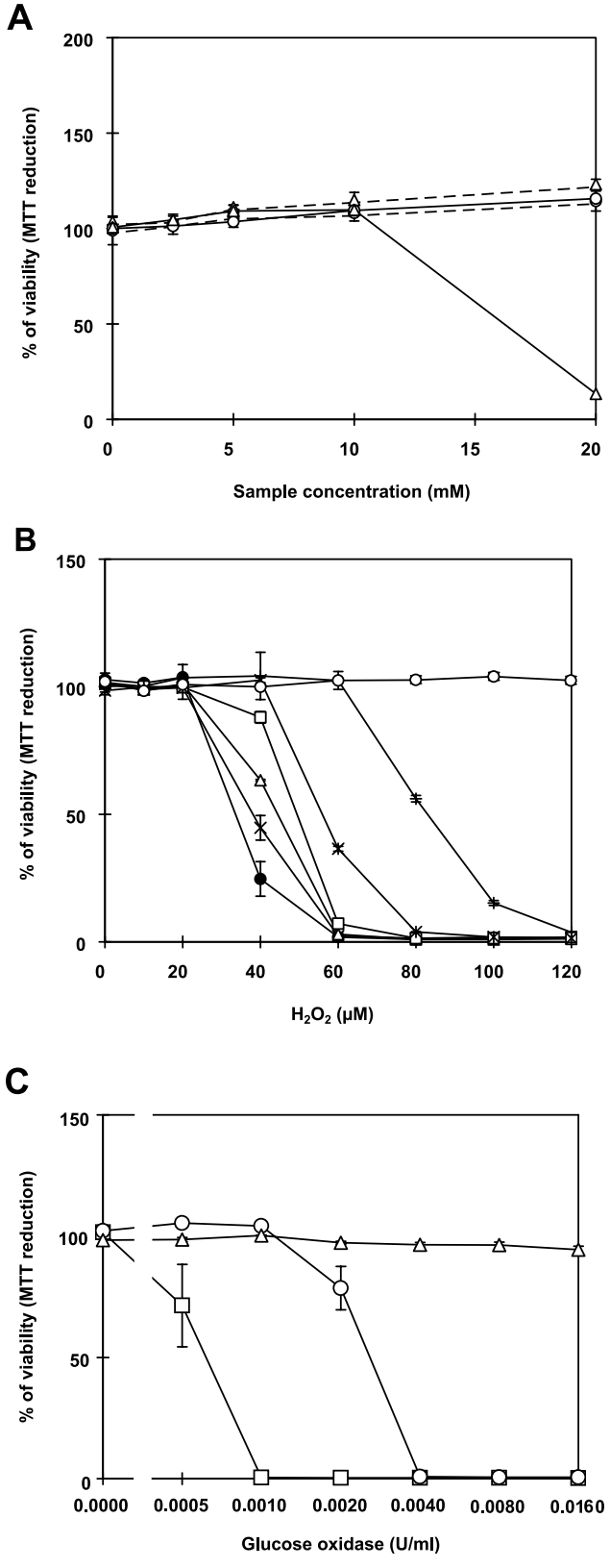

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Fig. 2. H_2O_2 detoxification effect of NANA. A: The non-toxic nature of ADOA was confirmed. EL-4 murine lymphoma cells were treated with 0–20 mM of NANA (○) or a reaction mixture of NANA and H_2O_2 (△) for 16 h. To eliminate the H_2O_2 retained in the reaction mixture, it was cotreated with (dotted line) or without (plain line) 100 units/ml of catalase. Cellular viability was determined by the MTT assay. B: Before cell culture, 0–120 μM of H_2O_2 in the RPMI-1640 medium was incubated for 4 h at 37°C, with 0 mM (●), 0.31 mM (×), 0.63 mM (△), 1.25 mM (□), 2.5 mM (∗), 5 mM (+) or 10 mM (○) of NANA. Then the EL-4 cells were cultured for 16 h in these media. C: RPMI-1640 medium was treated with 0–0.016 units/ml of glucose oxidase (*Aspergillus niger*) for 6 h at 37°C, together with 25 mM NANA (○), 10 units/ml catalase (△), or neither (□). Then the EL-4 cells were cultured for 16 h in each medium. Cellular viability in all experiments was determined by MTT assay. The error bars shown in this figure indicate the standard deviation of duplicates.

tion of cellular membranes by its oxidizability. EL-4 murine lymphoma cells were injured by H_2O_2 at 40 μM in vitro (Fig. 2B). In the reaction mixture obtained in the experiment shown in Fig. 1, an excess of H_2O_2 remained. So, the cytotoxicity of the reaction mixture is convincing. ADOA seemed to have no cytotoxicity in itself, because the cytotoxic effect completely suppressed by catalase (Fig. 2A). We propose that the reaction can be considering a novel H_2O_2 detoxification mechanism. The cytotoxicity of H_2O_2 abated on pretreatment with NANA in a dose-dependent manner. After 4 h pretreatment with 10 mM of NANA, the cytotoxicity of 120 μM H_2O_2 had completely disappeared (Fig. 2B). The cause of this was the removal of H_2O_2 from the culture medium by NANA (data not shown). Next, we tested the detoxification effect of NANA on cytotoxic proteins producing H_2O_2. Glucose oxidase (EC 1.1.3.4), previously known as the toxic antimicrobial protein notatin [11], produces D-gluconolactone and H_2O_2 from D-glucose and an oxygen molecule in the culture medium (RPMI-1640 medium containing approximately 11.1 mM of D-glucose), and injures cells by releasing H_2O_2. Again, pretreatment with 25 mM of NANA inhibited the cytotoxicity (Fig. 2C). Though preincubation with NANA was necessary to prevent the cytotoxicity in these experiments, NANA could inhibit the cytotoxicity of LAAO without preincubation as described previously [8,9]. It seems that this difference is due to the relatively slow reaction of NANA with H_2O_2. In the case of the H_2O_2 and glucose oxidase treatment, the cell was injured by H_2O_2 before an adequate reaction of NANA with H_2O_2. Meanwhile, NANA could inhibit LAAO cytotoxicity, because it seems to take longer for H_2O_2 to reach a cytotoxic concentration in the presence of LAAO (data not shown). These results show the potential of NANA which can detoxify H_2O_2.

Since the ubiquitous existence of sialic acids, biological functions according to amount and unique positioning in the sugar chain are studied. However, why sialic acids have a unique α-ketocarboxylic acid structure has not been considered at all. The data in this report show a novel potential of NANA, namely as an intrinsic antioxidant. H_2O_2 commonly occurs in aerobic metabolism, and in phagocytic immunity, where it is produced as a weapon against microorganisms. However, H_2O_2 is also highly toxic to the host, and a cause of cellular senescence and inflammatory disease. Therefore, it must be eliminated when not needed. H_2O_2 erasing enzymes such as catalase, glutathione peroxidase, and peroxiredoxins are known. However, H_2O_2 specific chemical scavenger is not known yet. It is expected that NANA should be the first member of this group. The reaction by NANA is a very safe system, because no dangerous oxygen radicals are generated. In the reaction, H_2O_2 and NANA are converted into H_2O and non-toxic carboxylic acid.

Until now, this novel but hidden role of NANA as the 2-keto-3-deoxy-nononic acid has been curiously overlooked. Considering the above results, we think that NANA might be a reactive oxygen scavenger. It is known that terminal sialic acid residues are removed by neuraminidase and that free sialic acid molecules are recycled or enzymatically degraded. What other fate awaits these residues after their cleavage from glycoconjugates? We propose that NANA is presented on the surface of cells and the mucosa to function as a defense against oxidative damage. In addition, the importance of the mechanisms controlling the concentration of H_2O_2 not only for detoxification but also in signal transduction is recognized, because H_2O_2 acts as a second messenger [12]. We expected that the reaction reported in this paper is a new control mechanism, and NANA acts as an intrinsic antioxidant in vivo.

References

[1] Angata, T. and Varki, A. (2002) Chem. Rev. 102, 439–469.
[2] Blix, G. (1936) Hoppe-Seiler's, Z. Phys. Chem. 240, 43–54.
[3] Crocker, P.R. (2002) Curr. Opin. Struct. Biol. 12, 609–615.
[4] Takamiya, K., Yamamoto, A., Furukawa, K., Yamashiro, S., Shin, M., Okada, M., Fukumoto, S., Haraguchi, M., Takeda, N., Fujimura, K., Sakae, M., Kishikawa, M., Shiku, H., Furukawa, K. and Aizawa, S. (1996) Proc. Natl. Acad. Sci. USA 93, 10662–10667.
[5] Schwarzkopf, M., Knobeloch, K.P., Rohde, E., Hinderlich, S., Wiechens, N., Lucka, L., Horak, I., Reutter, W. and Horstkorte, R. (2002) Proc. Natl. Acad. Sci. USA 99, 5267–5270.
[6] Iijima, R., Kisugi, J. and Yamazaki, M. (2003) Dev. Comp. Immunol. 27, 505–512.
[7] Du, X.Y. and Clemetson, K.J. (2002) Toxicon 40, 659–665.
[8] Kisugi, J., Kamiya, H. and Yamazaki, M. (1987) Cancer Res. 47, 5649–5653.
[9] Yamazaki, M., Kisugi, J. and Kamiya, H. (1989) Chem. Pharm. Bull. 37, 3343–3346.
[10] Jefford, C.W., Boschung, A.F., Bolsman, T.A., Moriatry, R.M. and Melnick, B.J. (1976) Am. Chem. Soc. 98, 1017–1018.
[11] Hiraishi, H., Terano, A., Razandi, M., Pedram, A., Sugimoto, T., Harada, T. and Ivey, K.J. (1994) J. Cell Physiol. 160, 132–134.
[12] Wood, Z.A., Poole, L.B. and Karplus, P.A. (2003) Science 300, 650–653.