# KINETIC STUDIES OF THE INTERACTION BETWEEN SPERGUALIN OR 15-DEOXYSPERGUALIN AND AMINE OXIDASE FROM BOVINE PLASMA

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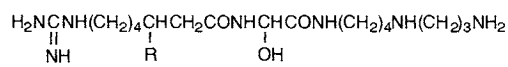
Spergualin (SG) and 15-deoxyspergualin (DSG) are derivatives of spermidine. Their substrate and inhibitor activities toward amine oxidase from bovine plasma were determined. SG was a good substrate of amine oxidase next to spermidine. The *Km* and $V_{max}$ for SG were 46.5 $\mu$M and 0.429 $\mu$M/minute, respectively, whereas the *Km* and $V_{max}$ for spermidine were 111 $\mu$M and 3.75 $\mu$M/minute, respectively. Thus SG has about two-fold stronger affinity to amine oxidase than spermidine, but its catalysis rate was one ninth of spermidine. In contrast, DSG was hardly oxidized and inhibited spermidine oxidation at low concentration. Affinity of both compounds for amine oxidase was determined by inhibition kinetics using benzylamine as substrate. SG and DSG competitively inhibited amine oxidase activity showing the *Ki* values of 175 $\mu$M and 7.46 $\mu$M, respectively.

Spergualin (SG) was discovered as an antitumor antibiotic from culture filtrate of *Bacillus laterosporus* BMG162-aF2[1]. The structure is (−)-(15*S*)-1-amino-19-guanidino-11,15-dihydroxy-4,9,12-triazanonadecane-10,13-dione[2] as shown in Fig. 1. 15-Deoxyspergualin (DSG), (±)-1-amino-19-guanidino-11-hydroxy-4,9,12-triazanonadecane-10,13-dione is a more active synthetic analog[3,4] of SG. Since their immunosuppressive activity has been known[5~7], DSG has been used as an immunosuppressant in organ transplant rejection in Japan and clinical trial for autoimmune diseases has been ongoing in Japan and Europe. From the unique structure a unique mechanism of action has been expected, but the molecular mechanism remains a mystery. SG and DSG enhance interleukin 2 production and natural killer activity in tumor-bearing mice[8]. They show strong immunosuppressive activity by different mechanism of cyclosporin A[9]. Cyclosporin A and FK506 bind to immunophilins in T cells and the formed complex inhibits calcineurin, $Ca^{++}$-calmodulin-dependent phosphatase which modifies NF-AT, a nuclear factor binding to interleukin 2 enhancer[10,11]. Thus production of interleukin 2 is inhibited by cyclosporin A and FK506. In the case of DSG interleukin 2 production is enhanced[9]. Recently, S. G. NADLER *et al.* reported that DSG bound to Hsc70, a member of the Hsp70 family of heat shock proteins[12] and they proposed putative mechanisms of action as an immunosuppressant. They discuss that Hsp70 may be a new class of immunophilins, or a carrier to translocate DSG to the nucleus or organelles where the drug may elicit its immunosuppressive effect. Molecular mechanisms of SG and DSG may become clear from further studies on Hsp70 function in immune response. However, there are proteins besides Hsc70 to which SG or DSG binds. We previously reported that SG and DSG interacted with amine oxidase in bovine serum[13,14]. H. HIBASAMI *et al.* reported that DSG was an inhibitor of spermine synthase, spermidine

Fig. 1. Structure of SG and DSG.



Spergualin R=OH (*S*)
15-Deoxyspergualin R=H

synthase and polyamine oxidase[15]. In this paper we describe studies of the effect of SG and DSG on amine oxidase. The both compounds showed stronger affinity to amine oxidase than spermidine, a natural substrate. DSG is a kind of amine oxidase inhibitor, and it is oxidized far slowly than SG. Interaction between SG or DSG and amine oxidase may be unable to be neglected in their biological activity, because their metabolites are supposed to be chemically active and unstable substances with aldehyde and amine groups.

## Materials and Methods

### Materials

Amine oxidase from beef plasma (60 units/g protein) was purchased from Sigma Chemical Company, U.S.A. Guaiacol (*o*-methoxyphenol) and spermidine phosphate were purchased from Wako Pure Chemical Industries, Ltd., Japan. Benzylamine HCl salt was purchased from Tokyo Kasei Kogyo Co., Ltd., Japan.

### Oxidation of Spermidine by Amine Oxidase

Oxidation of spermidine, SG or DSG as substrate by amine oxidase was assayed by determination of produced hydrogen peroxide[13,16]. Hydrogen peroxide was coupled with horseradish peroxidase and the generated hydrogen was trapped by the chromogenic donor, guaiacol. Incubation mixture (1.5 ml) contained 25 $\mu$l of 10 mM substrate, 25 $\mu$l of 20 mM guaiacol, 50 $\mu$l of 40 $\mu$g/ml of horseradish peroxidase, 1 ml of 0.1 M sodium phosphate buffer pH 7.2 and 0.1 ml of water or enzyme solution. In kinetic studies substrate and/or inhibitor concentrations were varied. The reaction was started by addition of enzyme solution (3.79 $\mu$unit/100 $\mu$l) and color formation was monitored continuously by a Beckman DU-8 spectrophotometer at 436 nm at 37°C. Hydrogen peroxide concentration was calculated, assuming that the molar extinction coefficient ($\varepsilon_{436}$) of the pigment is $2.55 \times 10^4\ M^{-1}\ cm^{-1}$ and one mol of pigment is produced by consumption of 4 mol of hydrogen peroxide[13].

### Oxidation of Benzylamine by Amine Oxidase

Oxidation of benzylamine was determined by an increase of $E_{250}$ by produced benzaldehyde[17] using a Beckman DU-8 spectrophotometer at 37°C. Incubation mixture (1.5 ml) contained 0.5 to 2 mM benzylamine in 0.05 M phosphate buffer, pH 7.2. Reaction was started by addition of enzyme solution (0.758 $\mu$unit/0.1 ml). Benzaldehyde concentration was calculated based on the $\varepsilon_{250}$ of benzaldehyde is $12.0 \times 10^3\ M^{-1}\ cm^{-1}$.

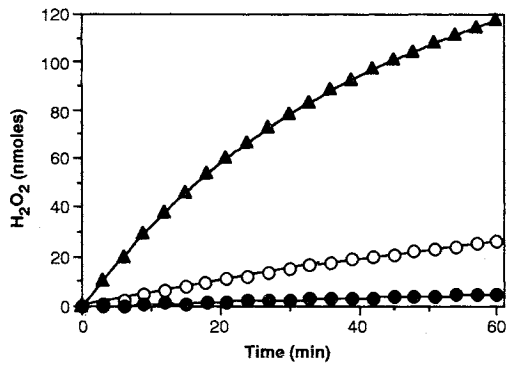
## Results

### Substrate and Inhibitor Activities of SG and DSG

Substrate activities of SG and DSG were compared with spermidine by determining $H_2O_2$ generation. As shown in Fig. 2, SG was more slowly oxidized by amine oxidase than spermidine, and oxidation of DSG was even slower, but still detectable. As shown in Fig. 3, SG hardly inhibited spermidine oxidation at 26.7 $\mu$M, whereas 1.33 and 2.67 $\mu$M of DSG inhibited it to 35 and 25%, respectively. This shows that DSG has higher affinity to amine oxidase but is oxidized by the enzyme in lower efficiency than spermidine.

Fig. 2. Amine oxidase substrate activities of SG and DSG.

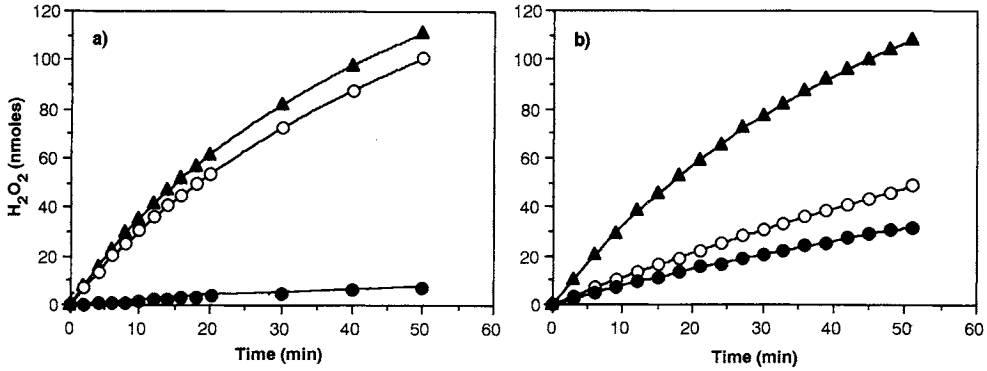
Incubation mixture (1.5 ml) contained 25 $\mu$l of 10 mM spermidine (▲), SG (○), or DSG (●) as substrate, 25 $\mu$l of 20 mM guaiacol, 50 $\mu$l of horseradish peroxidase (40 $\mu$g/ml), and amine oxidase (3.79 $\mu$ unit) in 0.1 M sodium phosphate buffer, pH 7.2.




Hydrogen peroxide was determined by monitoring pigment generation with a spectrophotometer.

Fig. 3. Effect of SG and DSG on oxidation of spermidine by amine oxidase.

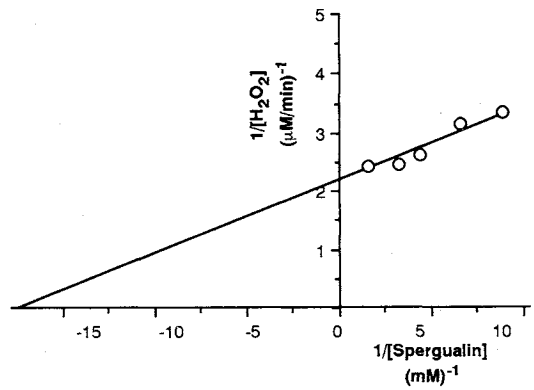
Incubation mixture was the same as Fig. 2 except for substrate concentrations. a) Spermidine (167 $\mu$M, ▲) and SG (26.7 $\mu$M, ●) were incubated with amine oxidase separately or at the same time (○). b) Spermidine (167 $\mu$M) was oxidized by amine oxidase with or without DSG (▲, no addition; ○, 1.33$\mu$M; and ●, 2.67 $\mu$M).




## The Michaelis Constant and the Maximum Velocity of SG

Steady state kinetic experiment was performed by determining initial velocities when SG concentrations were varied. From the Lineweaver-Burk plot as shown in Fig. 4, the Michaelis constant (*Km*) of SG was $46.5 \pm 9.5\,\mu$M (mean$\pm$SE) and the maximum velocity ($V_{max}$) was $0.4285 \pm 0.040\,\mu$M/minute (mean$\pm$SE). In the same condition *Km* and $V_{max}$ of spermidine were $111 \pm 10\,\mu$M and $3.75 \pm 0.32\,\mu$M/minute, respectively. SG has about twice higher affinity to amine oxidase than spermidine, but its catalytic efficiency was one ninth of spermidine. *Km* and $V_{max}$ of DSG could not be determined owing to the slow reaction rate.

Fig. 4. Lineweaver-Burk plot of oxidation of SG.




At different SG concentrations among 167 and 533 $\mu$M, initial velocities were determined by $H_2O_2$ generation with 3.79$\mu$ unit of amine oxidase. Reaction was monitored continuously for 2 hours by a spectrophotometer. Oxidation straightly progressed for 1 hour and initial velocities were obtained from the data to 30 minutes. The entire experiment was repeated with essentially the similar result. Curve fit and kinetic constants were obtained by non-linear regression analysis.

## The Inhibition of Amine Oxidase by Spermidine, SG and DSG using Benzylamine as a Substrate.

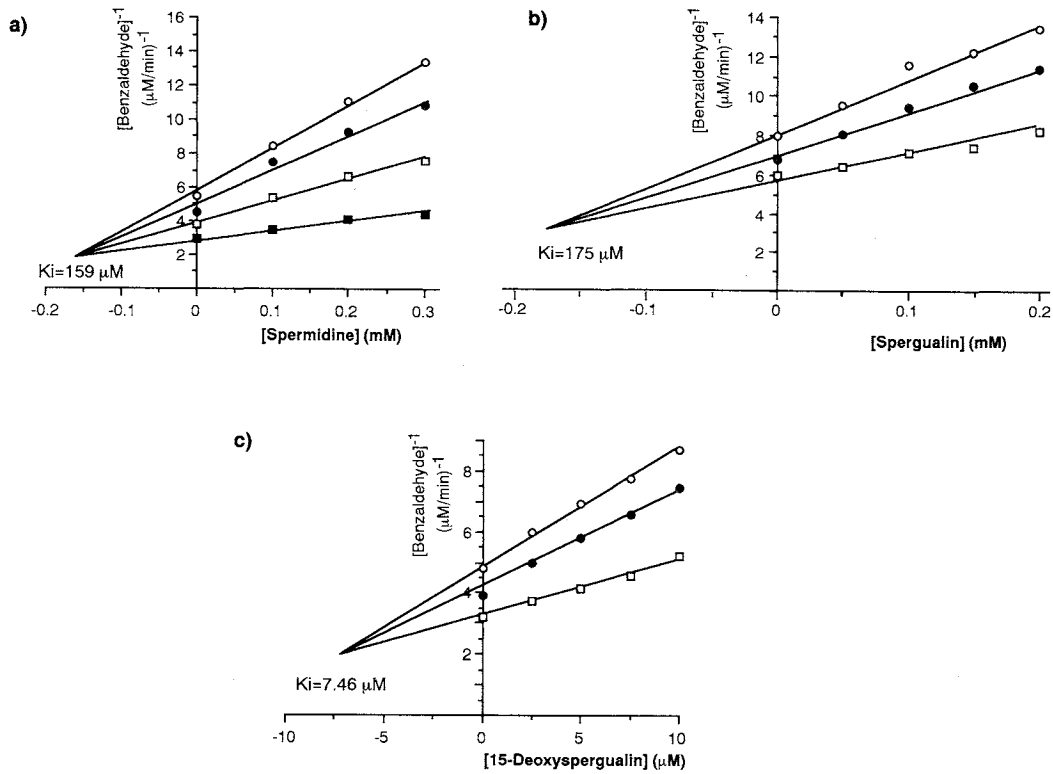
When benzylamine is used as substrate, the product, benzaldehyde can be determined without interference of spermidine, SG, DSG and their oxidized products. As shown in Fig. 5, spermidine, SG, DSG competitively inhibited benzylamine oxidation. The inhibition constant (*Ki*) of spermidine and SG were 159 $\mu$M and 175 $\mu$M, respectively. The *Ki* of DSG was 7.46 $\mu$M (Fig. 5c), indicating that DSG has about 20 times higher affinity to the amine oxidase than does SG.

## The Inhibition of Amine Oxidase by DSG using Spermidine as a Substrate

The *Ki* value of DSG was calculated from inhibition kinetic experiment using spermidine as substrate.

Fig. 5. Inhibition of benzylamne oxidation by spermidine, SG and DSG.

Inhibitory constants of spermidine, SG and DSG were determined by oxidation of benzylamine as substrate with 0.758 $\mu$ unit of amine oxidase. Benzylamine concentrations were 0.5 (○), 0.67 (●), 1 (□) and 2 mM (■).




Incubation time was 60 minutes and 100 minutes for a) and c), and for b), respectively. Curve fits and kinetic constants were obtained by non-linear regression analysis.

a) Dixon plot for spermidine inhibition.
b) Dixon plot for SG inhibition.
c) Dixon plot for DSG inhibition.

DSG oxidation could be neglected, because DSG concentrations were lower than those of spermidine, and DSG was poorly oxidized. DSG concentrations necessary for inhibition were between 2 and 8 $\mu$M, while spermidine concentrations were between 66.7 and 1,067 $\mu$M. As shown in Fig. 6, the inhibition by DSG was found to be noncompetitive type, with $K_{IS}$ and $K_{II}$ (the dissociation constants of the enzyme-inhibitor and enzyme-inhibitor-substrate complex, respectively) of 8.33 and 6.75 $\mu$M, respectively. The $K_{IS}$ and $K_{II}$ values were similar to that found from inhibition of benzylamine oxidation, but inhibition type was different.

## Discussion

In the immunosuppressive and antitumor activities DSG are stronger than SG, although their structural difference is only the absence or the presence of hydroxyl group in the C-15 position. In this paper we described the interaction of SG and DSG with amine oxidase as summarized in Table 1. This

Table 1. Summary of affinity to amine oxidase.

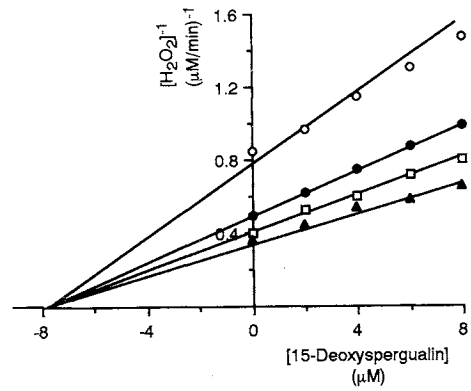
| | Substrate activity | | Inhibitor activity | |
|---|---|---|---|---|
| | *Km* ($\mu$M) | $V_{max}$ ($\mu$M/min) | *Ki* ($\mu$M) | Inhibition type |
| Spermidine | 111 | 3.75 | 159 | Competitive to benzylamine |
| Spergualin | 46.5 | 0.4285 | 175 | Competitive to benzylamine |
| 15-Deoxyspergualin | | | 7.46 | Competitive to benzylamine |
| | | | 8.33* | Noncompetitive to spermidine |
| | | | 6.75** | |

*Km* and $V_{max}$ (mean $\pm$ SE) of benzylamine were $1.048 \pm 0.25$ mM and $0.509 \pm 0.012$ $\mu$M/minute, respectively.
* The dissociation constant of the enzyme-inhibitor complex.
** The dissociation constant of the enzyme-inhibitor-substrate complex.

hydroxyl group is far from the spermidine moiety of SG and DSG, but the existence of which dramatically influenced to amine oxidase activity. SG with the hydroxyl group is a kind of substrate, but DSG without it is an inhibitor. The loss of the hydroxy group increased affinity to amine oxidase about 20 times, as the *Ki*'s are 175 $\mu$M for SG and 7.46 $\mu$M for DSG. Their affinity to amine oxidase is stronger than that to spermine synthase and spermidine synthase. *Ki*'s of DSG for spermine synthase and spermidine synthase are 215 and 340 $\mu$M, respectively[15]. SG and DSG may work as biological modifiers by giving some disturbance to the function of amine oxidase. Moreover the metabolites may have activity as biological modifiers, though products of the amine oxidase catalysis of SG and DSG or metabolites in animals, were too unstable to be isolated or examined for biological activities.

Fig. 6. Inhibition of spermidine oxidation by DSG.

Inhibitory constant of DSG was determined by Dixon plot with spermidine as substrate and 0.758 $\mu$ unit of enzyme. Substrate concentrations were 66.7 (○), 133 (●), 267 (□) and 533 (▲) $\mu$M. Incubation time was 10 minutes.




Curve fits and kinetic constants were obtained by non-linear regression analysis.

Amine oxidase from bovine plasma is dimer composed of identical subunits and contains Cu(II) ion and 6-hydroxydopa (topa)[18] as redox cofactors. It is thought that there are more than two enzyme forms in amine oxidase reaction from kinetic studies[19]. Inhibition patterns of azide and thiocyanate binding to the Cu(II) sites change from mixed to uncompetitive as the amine concentration increases. The switch of the inhibition pattern is supposed to be due to the binding of the anion inhibitors to both the resting enzyme and at least one other enzyme form generated during a reaction. Although the copper is not close to the substrate-binding site[20], the structure of SG and DSG may permit interaction with the copper by forming metal complex in addition to binding to substrate-binding site through their spermidine moiety. These complex properties may be related to why SG is substrate and DSG is an inhibitor, and why the inhibition type of DSG differs depending on substrates in amine oxidase reaction.

DSG was known to bind to Hsc70, a member of heat shock protein 70 (Hsp70) protein family which plays a role in immune responses[12]. It is unlikely that amine oxidase and Hsp70 have common structure as homology in peptide sequences and catalytic activities between Hsp70 and amine oxidase have not been found. SG and DSG binding is the first discovery as common properties between Hsp70 and amine oxidase.

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