Fusarium merismoides CORDA NR 6356, THE SOURCE OF THE PROTEIN KINASE C INHIBITOR, AZEPINOSTATIN

TAXONOMY, YIELD IMPROVEMENT, FERMENTATION AND BIOLOGICAL ACTIVITY

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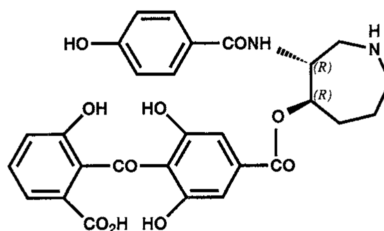
Fungal strain NR 6356, *Fusarium merismoides* Corda, was discovered as the source of the protein kinase C (PKC) inhibitor, azepinostatin. The strain was identified based on its growth on potato sucrose agar, slender conidial shape, characteristic polyphialide and production of abundant chlamydospores. *Fusarium aquaeductuum* Lagh. IMI 103658 and *Fusarium* sp. NR 7222 were also found to produce the same inhibitor. After single colony isolation and medium optimization trials, a more than 30-fold increase in the production of azepinostatin over the original culture was achieved.

Azepinostatin selectively and potently inhibited rat brain PKC with an IC_{50} value of 70 nM. Other enzymes utilizing ATP, including hexokinase, were not affected. The *Ki* of azepinostatin for PKC was 0.5 nM. The inhibition of PKC was competitive with ATP and uncompetitive with histone.

Protein kinase C (PKC) is involved in a wide range of cellular responses including oncogenesis and inflammation[1,2]. It can be activated by diacylglycerol[3] or tumor promoting phorbol ester[4,5], and it has the ability to phosphorylate lipocortin[6,7]. Although several inhibitors of PKC have been reported[8~10], they are either highly toxic or not very potent. During our microbial screening program aimed at finding new inhibitors of PKC, we discovered azepinostatin (Fig. 1), from the fermentation broth of *Fusarium merismoides* Corda. Although KULANTHAIVEL *et al.*[11] reported on the isolation of a compound identical to azepinostatin, balanol, from the culture broth of *Verticillium balanoides* (Drechs.) Dowsett, Reid and Hopkins, the producing fungi of azepinostatin belong to a genus different from the balanol producer. In addition, the biological data of balanol in detail, including its modes of action have not yet been described. Therefore, we report on the screening method, taxonomy and improvement of the yield of the producing organism, and the biological properties of azepinostatin.

Fig. 1. Structure of azepinostatin.



Materials and Methods

Materials

Materials used in this study were the following: calf thymus histone H1 (type III-S), phosphatidylserine, diolein and ATP (magnesium salt) from Sigma; staurosporine and K-252a from Kyowa Medecs; [γ-^{32}P]ATP from New England Nuclear.

Microorganisms

Fungal strain NR 6356 came from soil collected in Sigilia, Sri Lanka on September 16, 1984. It was isolated using a serial dilution method on M40Y medium. The other strain, NR 7222, was derived from soil in Barcelona, Spain collected on December 26, 1986. We isolated the latter strain by the Percoll density gradient method and cultured it on malt extract agar containing rose bengal. Several strains purchased from the Commonwealth Mycological Institute (CMI), Egham, Surrey, United Kingdom, and Centraalbureau voor Schimmelculture (CBS), Baarn, The Netherlands, were also examined and compared with NR 6356.

Taxonomy

All strains were taxonomically examined essentially as reported by BOOTH[12,13], ICHINOHE *et al.*[14] and BURGESS and LIDDELL[15]. In addition, Miura medium and dichloran peptone agar[16] were also used. A one-point inoculation was made in the center of the agar plates. The agar plates were alternately incubated in the dark and in artificial light every 12 hours at 24°C for 30 days. The color of the colonies was determined according to the Munsell system[17,18]. Fungal strains were fixed for scanning electron microscopy as reported by COLE and SAMSON[19]. The specimens were then dried in liquid CO_2 with a critical point drying apparatus (JCPD-5, JEOL, Japan). The dried specimens on brass were coated with about 100 Å of gold-palladium. The material was examined in a scanning electron microscope (JSM-35CF, JEOL, Japan) at 15 kV.

Screening Method

PKC was prepared from rat brain according to the method of KNOPF *et al.*[20]. PKC activity was assayed essentially by the method of KIKKAWA *et al.*[21]. In brief, the 200 μl reaction mixture contained 20 mM Tris-HCl (pH 7.4), 5 mM magnesium acetate, 0.5 mM $CaCl_2$, phosphatidylserine (100 μg/ml), diolein (10 μg/ml), 10 mM β-mercaptoethanol, histone H1 (1 mg/ml), 10 μM [γ-^{32}P]ATP (0.25 μCi), an appropriate amount of enzyme, and 20 μl of the sample solution (10% v/v). The mixture was incubated at room temperature for 10 minutes. The reaction was stopped by adding 60% TCA (100 μl) and the precipitate was then filtered out. The filters were washed three times with 10% TCA (200 μl for each) and then punched out into scintillation vials. The radioactivity on the filter was counted with a liquid scintillation counter.

Fermentation

The media used are listed in Table 1. For the flask cultures, we inoculated 2 ml of thawed stock culture (previously at −80°C) into 100 ml of seed medium (Medium A) in a 500-ml Erlenmeyer flask. The flask was shaken on a rotary shaker at 190 rpm at 27°C for 3 days and then 2 ml from the flask was inoculated separately into 100 ml of the production medium in 500-ml Erlenmeyer flasks. The flasks were shaken in the same way as described above. For the 50-liter and 200-liter fermentations, 2 ml of the seed culture described above was transferred into 500-ml Erlenmeyer flasks each containing 100 ml of Medium A. These second seed flasks were incubated in the same manner as the primary seed culture. Six of the second seed flasks were combined and transferred into a 50-liter jar fermentor (working volume: 30 liters). Thirty of the second seed flasks were used for the inoculation of a 200-liter jar fermentor (working volume: 150 liters). The 50-liter and 200-liter jar fermentors were operated at 27°C with agitation speeds of 300 and 150 rpms and aerations of 30 and 150 liters/minute, respectively. Dissolved oxygen (DO) and pH were automatically monitored during the fermentation.

Table 1. List of media.

Ingredient (%)[a]	Medium			
	A	SO1	SO2	SO3
Potato starch	2	0.5	2	3
Glucose	2	0.5	0.5	0.5
Toast soya	2	0.5	0.5	0.5
Yeast extract	0.5	0.125	0.125	0.125
NaCl	0.25	0.063	0.063	0.063
Trace element[b]	0.1	0.025	0.025	0.025
$CaCO_3$	0.32	0.08	0.08	0.08

[a] w/v except for metal mixture (in % v/v).

[b] Trace element is a solution containing 50 mg/ml $ZnSO_4 \cdot 7H_2O$, 5 mg/ml $CuSO_4 \cdot 5H_2O$ and 5 mg/ml $MnCl_2 \cdot 4H_2O$. The pH of each medium was adjusted to 7.0 before adding $CaCO_3$. Nissan Disfoam CA-115 was added at the concentration of 0.03% for the flask culture and 0.3% for the jar fermentor.

Single Colony Isolation

A conidial suspension made from a well-grown

slant culture was diluted 10^{-3} to 10^{-5} and spread onto malt extract agar plates for the isolation of single colonies. The plates were incubated at 27°C for several days. The colonies grown on the plates were isolated and cultured on slants. The activity of each isolate against PKC was tested by direct inoculation of each slant culture into Medium A in a flask.

Results and Discussion

Approximately 10,000 fungi were screened to discover a novel PKC inhibitor and only two fungal strains, NR 6356 and NR 7222, were found which showed potent inhibitory activity against PKC. Strain NR 6356 showed 80~98% inhibitory activity against PKC, whereas NR 7222 showed 62~75% inhibition when a 10% broth supernatant was added to the reaction mixture. The inhibitory activity of both strains was found to be attributed to the compound, azepinostatin, but since NR 6356 had the higher activity, azepinostatin was only isolated from the cultured broth filtrate of NR 6356 by HP-20, *n*-butanol extraction, QAE-sephadex A-25 and silica gel chromatography (data not shown). We mainly focused on the taxonomy and yield improvement of the strain showing the greater inhibitory activity, NR 6356.

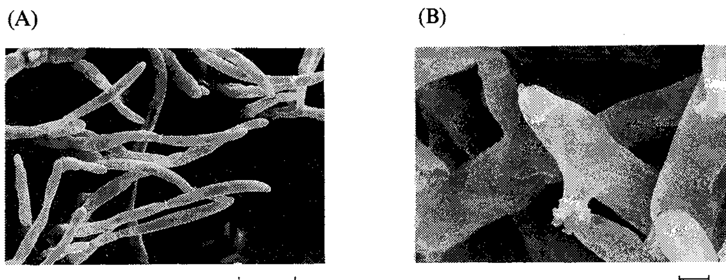
Taxonomy of the Producing Strains

Colonies of NR 6356 grew slowly on potato sucrose agar (PSA), reaching 9 mm in diameter after 4 days at 25°C. They bore no visible aerial hyphae, which contributed to a flat leathery appearance. Occasionally distinct erumpent hyphae (funiculose to fasciculate) were formed in the center of the colony. Colonies on PSA were cream-colored (Munsel: 2.5YR9/2). After 1 month, the culture turned a dull, dark brown. The conidiophores consisted of simple lateral phialides or a basal cell with a cluster of phialides. The phialides were $10\sim31\times2.3\sim4.0\,\mu$m, usually single and bore one small but distinct collarette at the tip (Fig. 2 (A)). Sometimes polyphialides had two or three conidial-forming lateral pores (Fig. 2 (B)). The macroconidia were $24\sim50\times2.1\sim5.0\,\mu$m, 0 to 5 septate, fusiform, slightly curved, with rounded apical and hardly differentiated basal cells; no micro-conidia were observed. The chlamydospores ($4.0\sim7.3\times3.3\sim4.6\,\mu$m) were abundantly produced intercalarily or terminally. The chlamydospores were globose to oblong especially in older cultures on Miura medium and occurred singly, in pairs, or sometimes formed a chain. Teleomorph was not observed.

The boat-shaped, hyaline, phragmospore with a marked foot-cell is characteristic of the anamorph genus *Fusarium* Link[22]. This anamorph genus compiled by BOOTH[12] into 12 sections includes 51 species and varieties. Among these species, fungal strain NR 6356 was closely related to *Fusarium aquaeductuum* Lagh. or *Fusarium merismoides* Corda based on the following observations: the colonies of strain NR 6356

Fig. 2. Scanning electron micrographs of *F. merismoides* NR 6356.

(A) Phialides. Bar represents 10 μm. (B) Polyphialide. Bar represents 1 μm.



grew extremely slow (less than 1 cm in diameter after 4 days), were cream-colored, and did not have micro-conidia. *Fusarium aquaeductuum* Lagh., an anamorph of *Nectria purtonii* (Grev.) Berk. forms single phialides with one terminal conidial forming collarette, has curved fusoid and somewhat slender macro-conidial with a single central septum measuring $15\sim45\times3\sim3.5\,\mu$m, and does not produce chlamydospores. *Fusarium merismoides* Corda usually known to be a soil saprophyte, forms polyphialides, has less slender macro-conidia with 3 to 4 transverse septa measuring $30\sim40\times3.5\sim5\,\mu$m, and produces chlamydospores. Therefore, several cultures of these two species purchased from CMI and CBS were compared with strain NR 6356. The color of *F. aquaeducutuum* IMI 103658 was a light yellowish orange (7.5YR9/2) which was slightly more reddish than the cream-colored (10YR9/2) *F. merismoides* IMI 105043 after 4 days of growth on PSA. Colonies of *F. merismoides* IMI 105043 grew much slower than *F. aquaeductuum* IMI 103658 and CBS 734.79 reaching 4 mm in diameter after 4 days and 16 mm after 10 days; whereas, the colony diameter of the latter two strains reached 31 and 25 mm after 10 days, respectively. The phialides and chlamydospores formed were distinct, which agreed with BOOTH[12]; *i.e.*, *F. aquaeductuum* IMI 103658 and CB 734.79 produced single phialides and no chlamydospores. Furthermore, *F. merismoides* IMI 105043 formed abundant intercalary chlamydospores on cornmeal agar. Conidial length within each above species is variable; whereas, conidial width is constant. *F. merismoides* formed less slender conidia than *F. aquaeductuum*. The distribution of the conidial size of NR 6356 was identical to that of IMI 105043. Therefore, strain NR 6356 was identified as *Fusarium merismoides* Corda. The other strain, NR 7222 grew slowly, showing ocher to dark red orange colonies. This strain also formed 4-septate macroconidia, $26\sim35.5\times3.0\sim4.0\,\mu$m. These characteristics indicated that NR 7222 was included in the genus *Fusarium*, but it may be a different species mainly due to the color of its colonies. However, further identification work was not conducted; therefore, NR 7222 was tentatively assigned to *Fusarium* sp. NR 7222.

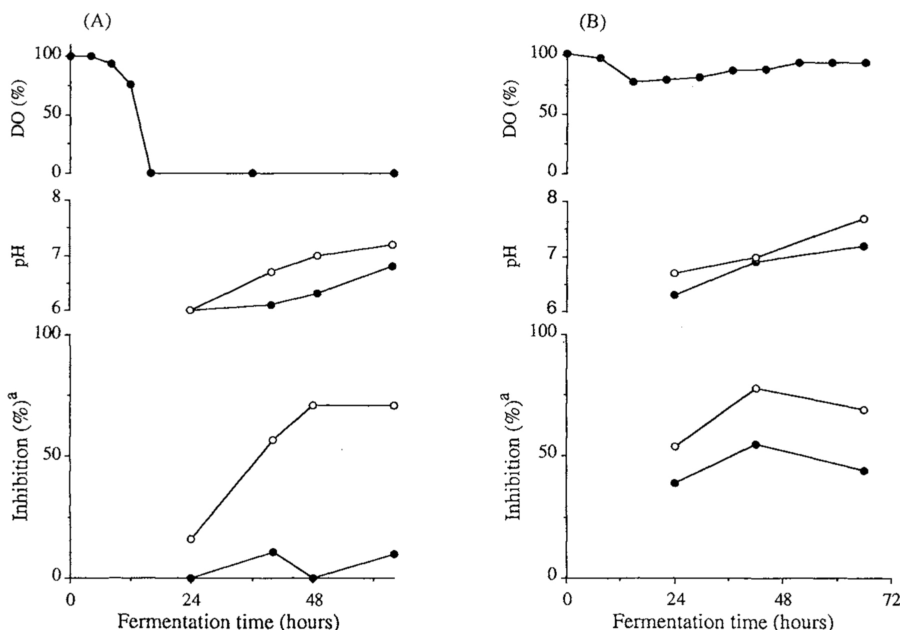
Production of secondary metabolites in fungi is sometimes correlated with conventional classification: related fungal species or genera often produce the same or structurally related metabolites. Thus, we cultured *F. aquaeductuum* IMI 103658 and CBS 734.79, and *F. merismoides* IMI 105043 in Medium A to see if they would also produce azepinostatin. We found that *F. aquaeductuum* IMI 103658 produced azepinostatin (0.3 μg/ml) and *Fusarium* sp. NR 7222 also produced azepinostatin (0.14 μg/ml), although the productivity of the latter was a little lower than that of NR 6356 (0.28 μg/ml). We concluded that azepinostatin may be a secondary metabolite of other species or strains within the genus *Fusarium*. In addition, a quite different genus, *Verticillium balanoides*, was reported to produce the same inhibitor[11]. Further study is needed to determine whether this PKC inhibitor is exclusively found in these two genera or whether it could be isolated from other genera if they were grown under different culture conditions.

Medium Optimization

Azepinostatin was initially produced in flasks using Medium A. In the scale-up fermentation from the flask culture to a 50-liter jar fermentor, however, Medium A was found to be unsuitable for production and only 10% inhibition was detected (Fig. 3(A)). We speculated that one of the critical reasons for this was the increase in the viscocity of the culture broth accompanied by vigorous growth which caused depletion of dissolved oxygen during the early stage of fermentation. In order to limit the growth, the concentration of Medium A in the flask cultures was diluted up to one sixth (Table 2). The productivity of the one half dilution of Medium A was the most favorable and, even in the one fourth dilution (Medium

Fig. 3. Time course of azepinostatin production in a 50-liter jar fermentor and flask culture.

(A) Medium A, (B) Medium SO1, ● jar fermentor, ○ flask culture.



[a] Broth supernatant was added at 10% volume of the reaction mixture as described in the Materials and Methods.

Table 2. Effect of dilution of Medium A in flask culture on azepinostatin production.

Medium	Growth (%)[a]	IC_{50} (%)[b]
A	31	0.75
1/2A	23	0.27
1/4A (=SO1)	19	0.80
1/6A	19	>3

[a] Packed cell volume (3,000 rpm for 10 minutes).
[b] IC_{50} was defined as the concentration of broth supernatant in the reaction mixture as described in the Materials and Methods at which PKC was inhibited by 50%.

Table 3. Effect of concentration of carbon and nitrogen sources in Medium SO1 on azepinostatin production.

Medium	IC_{50} (%)[b]
SO1 [Potato starch 2%][a] (=SO2)	0.025
SO1 [Glucose 2%][a]	0.40
SO1 [Yeast extract 0.5%][a]	>3
SO1 [Toast soya 2%][a]	>3
SO1	0.80
A	0.75

Fermentation including SO1 and A was done at the same time.
[a] The concentration of only one ingredient in Medium SO1 was changed as shown in parenthesis.
[b] See Table 2.

SO1), its productivity was the same as that of the undiluted Medium in the flask. Although the growth in Medium SO1 was somewhat limited as shown in Table 2, when Medium SO1 was used for 50-liter jar fermentation, azepinostatin production was almost recovered concomitantly with a higher level of dissolved oxygen (Fig. 3 (B)).

Next, we examined the concentration of carbon and nitrogen sources on the production of azepinostatin in the base of Medium SO1 (Table 3). When the concentration of potato starch was changed from 0.5 to 2.0% (Medium SO2), the productivity was much greater than that of Medium A or Medium SO1. There was a little stimulation of the productivity when glucose was increased from 0.5 to 2.0%. In contrast with carbon sources, when the concentration of nitrogen sources increased, the production was

repressed, suggesting that azepinostatin production was affected by the carbon-nitrogen ratio. When the concentration of potato starch in Medium SO1 was increased to 3%, the inhibitory activity was slightly improved from 73% to 79% inhibition. It was thus decided to use this concentration of potato starch, and the medium was named SO3.

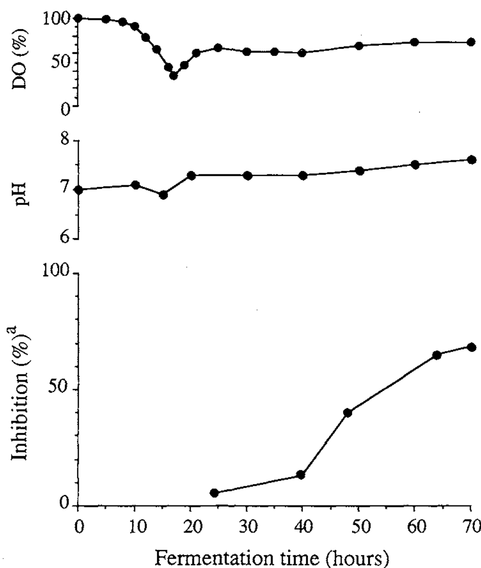
Single Colony Isolation

We conducted single colony isolation with culture NR 6356 to obtain better and uniform productivity. Among 200 single colonies, one isolate, SC16 was able to produce three times as much azepinostatin as the parent strain NR 6356; in Medium A and in Medium SO2.

Production

A typical time course of the production of azepinostatin with the selected strain SC16 and improved Medium SO3 in a 200-liter jar fermentor is shown in Fig. 4. The dissolved oxygen and pH decreased to their lowest levels after 17 hours. The production reached its maximum (14 μg/ml) after 70 hours. This productivity was much more than that of the original flask culture using Medium A (0.28 μg/ml).

Fig. 4. Time course of azepinostatin production in a 200-liter jar fermentor.



A broth supernatant was added at 0.3% volume of the reaction mixture as described in the Materials and Methods.

Physico-chemical Properties

The physico-chemical properties of azepinostatin (Fig. 1) are summarized in Table 4.

Biological Properties

Table 5 shows the biochemical properties of azepinostatin and known potent PKC inhibitors

Table 4. Physico-chemical properties of azepinostatin.

Appearance	Yellow amorphous powder
FAB-MS (m/z)	551 $(M+H)^+$
Molecular formula	$C_{28}H_{26}N_2O_{10}$
UV λ_{max}^{MeOH} nm (ε)	213 (18,600), 258 (9,600), 274 (9,020), 285 (sh), 360 (1,380)
$\lambda_{max}^{MeOH+NaOH}$ nm (ε)	217 (19,570), 292 (13,500)
IR ν_{max}^{KBr} cm^{-1}	3600 (br), 2500, 1720, 1705, 1640~1530
$[\alpha]_D^{25}$	$-62.2°$ (c 1.0, MeOH)
Color reaction Positive:	Ninhydrin, $FeCl_3$, 2,4-DNP, I_2
Solubility Soluble:	MeOH, DMSO, DMF
Slightly soluble:	H_2O
Insoluble:	Et_2O, Benzene, EtOAc

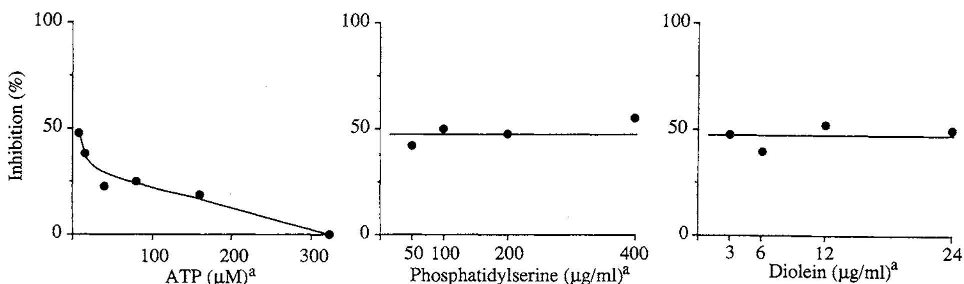
Table 5. Biochemical properties of PKC inhibitors.

Compound	IC_{50} (μM)		(A)/(B)	Ki (M)
	Cytotoxicity (A)	PKC inhibition (B)		
Azepinostatin	>45	0.07	>650	5.0×10^{-10}
Staurosporine	0.09	0.006	15	7.0×10^{-10}*
K-252a	0.96	0.033*	—	2.5×10^{-8}*

* Data from literature.

such as staurosporine and K-252a. Azepinostatin inhibited PKC in a dose dependent manner, and its 50% inhibitory concentration (IC_{50}) for rat brain PKC was 70 nM in the standard assay. The *Ki* value of azepinostatin was 5.0×10^{-10} M which was a remarkably low *Ki* value, indicating that azepinostatin

Fig. 5. Effects of ATP, phosphatidylserine and diolein on the inhibition of PKC by azepinostatin.



[a] Each concentration was varied in the reaction mixture as described in the Materials and Methods. Azepinostatin was added to become 70 nM (IC_{50} value) in the above reaction mixture.

Fig. 6. Inhibition of PKC activity by azepinostatin and staurosporine.

Each concentration in the below figures was varied in the reaction mixture as described in the Materials and Methods. (A) Azepinostatin, (B) Staurosporine.

(A)

(A-1)

(A-2)

(B)

(B-1)

(B-2)

had the highest affinity for PKC. We tested the inhibitory activity of azepinostatin, staurosporine and K-252a against hexokinase which was chosen as an example of "non-protein" kinases. All of them were found to be inactive against hexokinase (data not shown). This suggests that these inhibitors do not act as simple "ATP-mimetics" which can suppress the activity of any enzyme utilizing ATP as a substrate, but act as inhibitors which preferentially interact with protein kinases. So, we conclude that azepinostatin was a potent selective inhibitor of PKC.

The cytotoxicity of azepinostatin, which was determined by the inhibition of HeLa cell growth, was much lower than that of staurosporine or K-252a (Table 5). Azepinostatin at 70 μM showed no antibacterial activity against *Bacillus subtilis* and no antifungal activity against *Candida albicans*.

A greater concentration of ATP lessened the inhibitory effect of azepinostatin, whereas PKC activators (phosphatidylserine and diolein) did not affect the inhibitory potency of azepinostatin (Fig. 5). These results suggest that azepinostatin acts on the ATP-binding site of PKC. The mode of action of azepinostatin was compared with staurosporine by Lineweaver-Burk plots (Fig. 6). Both azepinostatin and staurosporine inhibited PKC activity competitively with ATP (Fig. 6(A-1) and 6(B-1)). However, a difference was observed in the effect of these inhibitors on the interaction between PKC and histone. The mode of PKC inhibition by azepinostatin was found to be uncompetitive with histone (Fig. 6(A-2)), whereas that by staurosporine was noncompetitive (Fig. 6(B-2)). These results suggest that the mode of action of azepinostatin is different from that of staurosporine in that azepinostatin interacts with the histone-enzyme complex.

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