J. Enzyme Inhibition, 1992, Vol. 6, pp. 157–164
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5838-DNI, A DEOXYRIBONUCLEASE INHIBITOR PRODUCED BY *STREPTOMYCES* SP. STRAIN NO. A-5838

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(*Received February 4 1992*)

5838-DNI, an inhibitor of deoxyribonuclease (DNase) II from porcine spleen was produced by *Streptomyces* sp. strain No. A-5838. The structure of 5838-DNI was shown to be 1,4,4a,5,12,12a-hexahydro-4,4a,11,12a-tetrahydroxy-3,8-dimethoxy-9-methoxycarbonyl-10-methyl-1,5,12-trioxo naphthacene. Although similar in structure to tetracenomycin C, which is an antibiotic against Gram-positive bacteria, 5838-DNI has different antibacterial activity.

5838-DNI was distinguished from 5923-DNI, a previously reported DNase II inhibitor, in inhibitory activity against each enzyme. 5838-DNI showed dependency of inhibition on pH and temperature, and inhibited phosphodiesterase I in a competitive manner. These data suggest that 5838-DNI is the first reported example of an inhibitor of microbial origin which is able to inhibit DNase II and phosphodiesterase I.

KEY WORDS: DNase inhibitor, inhibitor, *Streptomyces*, 5838-DNI.

INTRODUCTION

The biological functions of DNases are not well known, although they may be involved in important steps such as DNA replication, recombination, integration, excision after irradiation, or viral induction. The existence of a specific DNase inhibitor will facilitate studies concerning the role of DNases in the cells and the regulation of their activities. In this context, DNase inhibitors have been isolated from animal tissues such as liver,[1] thymus[2] and spleen,[3] and the involvement of DNases in some biological events have been investigated by the regulation of their activities using these inhibitors. However, little has been reported concerning inhibitors of DNases produced by microorganisms. In the search for inhibitors of DNases, we have screened various microorganisms isolated in our laboratory. In a previous paper,[4] we reported that an inhibitor of DNase II, 5923-DNI was produced by *Streptomyces* sp. strain No. A-5923, and the purification and some properties of the inhibitor were described.

Recently, we have also found that *Streptomyces* sp. strain No. A-5838 produced three types of inhibitors in the culture filtrate. One of them, designated 5838-DNI, was an inhibitor of both DNase II (EC 3.1.4.6) and phosphodiesterase I (EC 3.1.4.1). In this paper, we describe the purification procedure, the structure and some properties of 5838-DNI.

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MATERIALS AND METHODS

Materials

DNase II from porcine spleen, RNase A from bovine pancreas, phosphodiesterase I from *Crotalus atrox venom*, phosphodiesterase II from bovine spleen, 5′-nucleotidase from *Crotalus adamantus venom*, alkaline phosphatase from bovine intestine (type I), salmon DNA, thymidine-5′-monophosphate-*p*-nitrophenyl ester were obtained from Sigma Chemicals. DNase I from bovine pancreas, nuclease S_1 from *Aspergillus oryzae* were obtained from Boehringer Mannheim Yamanouchi. Nuclease P_1 from *Penicillium citrium* was obtained from Yamasa Shoyu Co. Ltd. RNA from yeast and adenosine-5′-monophosphate were obtained from Kohjin Co. Ltd. *p*-Nitrophenyl phosphoric acid sodium salt was obtained from Nacalai Tesque Inc. Thymidine-3′-monophosphate-*p*-nitrophenylester was obtained from Merck Co. *Eco* RI from *Escherichia coli* was obtained from Takara Shuzo Co. Ltd. *Dpn* I from *Diploccocus pneumoniae* was obtained from BRL. Plasmid pUC19 was obtained from BRL, and maintained in our laboratory.

Measurement of DNase Activity

DNase II activity was determined by measuring absorbance at 260 nm of acid-soluble hydrolysate liberated from DNA by the enzyme reaction.[5] The reaction mixture (0.5 ml) consisting of sodium acetate buffer (50 mM), EDTA · 2Na (10 mM), KCl (100 mM), DNase II (100 μg) and salmon DNA (250 μg) was incubated at 30°C for 30 min. After incubation, 0.1 ml of perchloric acid solution containing 0.75% uranyl acetate was added to terminate the reaction. After standing at 0°C for 10 min, the precipitate was removed by centrifugation, and then 0.2 ml of the supernatant was diluted to 3 ml with distilled water, and absorbance at 260 nm of the solution was measured by spectrophotometer (Hitachi U-2000). One inhibitor unit (IC_{50}) was defined as the amount which reduced the activity of DNase II by 50%.

Other Enzyme Reactions

Activity of DNase I, RNase A,[6] nuclease P_1[7] or nuclease S_1[8] was determined by measuring absorbance at 260 nm of acid-soluble hydrolysate liberated from substrate by each enzyme reaction. Inorganic phosphate released by 5′-nucleotidase was measured by the method of Fiske-SubbaRow.[9] Activity of phosphodiesterase I,[10] phosphodiesterase II[11] or alkaline phosphatase[12] was determined from absorbance at 400 nm of *p*-nitrophenol liberated from each substrate. Activities of *Eco* RI and *Dpn* I were measured by agarose gel electrophoresis after enzyme reaction.[13] Activity of DNA polymerase I was determined by measuring ^{32}P-CTP induced into acid-insoluble product by liquid scintillation counter.[14] The substrate concentrations used are given in Table IV.

Cultural Conditions for 5838-DNI Production by Strain No. A-5838

Streptomyces sp. strain No. A-5838 was cultivated under aerobic conditions (aeration; 3 l/min, agitation; 200 rpm) at 28°C in a 10 l jar fermentor containing 5 l of S-medium consisting of 2% glucose, 3% starch, 1% corn steep liquor, 1% soybean flour, 0.5% peptone, 0.3% NaCl and 0.5% $CaCO_3$ (pH 7.0).

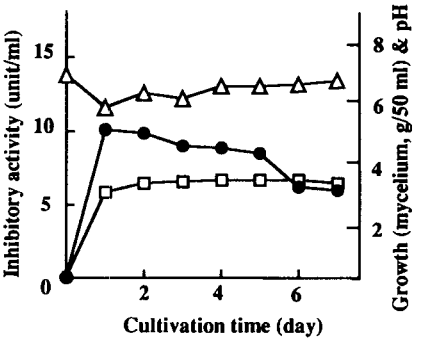


FIGURE 1 Time course of *Streptomyces* sp. strain No. A-5838 culture. (●) Inhibitory activity; (△) pH; (□) growth.

Instrumental Analyses

Optical rotation and melting point were determined by a Jasco DIP-360 digital polarimeter and a Yanagimoto micro melting point apparatus, respectively. IR spectrum was taken on a Hitachi 270-30 spectrophotometer. Molecular weight and molecular formula were estimated by using a JMS-DX303HF mass spectrometer (EI-MS). ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY and heteronuclear multiple bond correlation spectroscopy (HMBC) were measured by a JMN-GX400 spectrometer. ^{1}H and ^{13}C NMR spectra with TMS as internal standard were taken in DMSO-d_6 at 400 MHz and 100 MHz, respectively.

RESULTS AND DISCUSSION

Time Course of 5838-DNI Production by Strain No. A-5838

Cultivation of strain No. A-5838 was carried out under the cultural conditions previously described. Changes in inhibitory activity, growth of mycelium and pH of the broth are shown in Figure 1. The inhibitory activity increased with the growth of the mycelium and reached a maximum after 1 day of cultivation, and remained almost stable for 6 days thereafter.

Isolation of 5838-DNI

After cultivation, the mycelium and other residues were removed by filtration. The

TABLE I
Summary of purification steps of 5838-DNI from the culture filtrate

Step	Total inhibitory activity (units)	Yield (%)
Culture filtrate	28 080	100.0
AcOEt layer	2680	9.5
Active fraction I	820	2.9
Active fraction II	168	0.6
Purified 5838-DNI	100	0.4

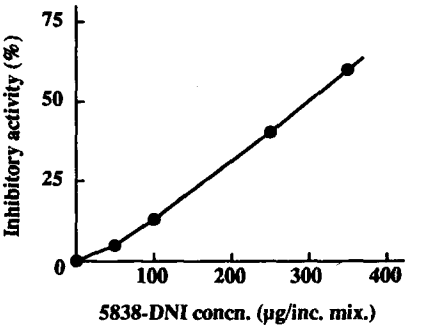


FIGURE 2 Inhibitory activity of 5838-DNI against DNase II. The concentration of 5838-DNI is expressed as μg in the reaction mixture (0.5 ml).

strain produced three types of DNase inhibitors in the culture filtrate. The culture filtrate was adjusted to pH 7 and extracted with ethyl acetate to give 5838-DNI. The other DNase inhibitors remained in the aqueous layer. The organic layer (AcOEt layer) containing 5838-DNI was concentrated *in vacuo* and applied to a Diaion HP-10 column (2.5 × 30 cm). After washing with 60% methanol, 5838-DNI was eluted with 100% methanol. The eluate (Active fraction I) was concentrated and subjected to Sephadex LH-20 column (1.8 × 40 cm) chromatography with methanol as a solvent. The active fractions (Active fraction II) were combined, concentrated and then chromatographed on a silica gel column (1.2 × 20 cm) with benzene and ethanol (9 : 1). The active fractions were combined and dried *in vacuo* (Purified 5838-DNI).

Table I gives a summary of the purification of 5838-DNI from the culture filtrate. Overall, 9.8 mg of 5838-DNI was obtained from 1000 ml of the culture filtrate with a yield of 0.4%. The low yield of 5838-DNI was due to the fact that the strain produces two inhibitors other than 5838-DNI, as described previously. As shown in Figure 2, about 300 μg of the inhibitor gave 1 unit in the assay system (0.5 ml) as described in Materials and Methods.

Physicochemical Properties and Structure of 5838-DNI

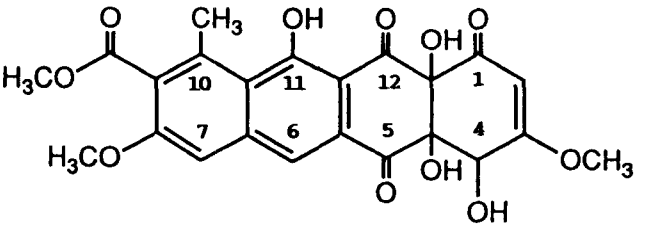
The physicochemical properties of 5838-DNI are summarized in Table II. 5838-DNI was obtained as yellow powder, m.p. 160°C, soluble in acetone and dimethyl sulfoxide but insoluble in water. The UV absorption maxima of 5838-DNI in methanol were

TABLE II
Physicochemical properties of 5838-DNI

Appearance	: yellow powder
Melting point	: 160°C
$[\alpha]_D^{20}$ (dioxane)	: + 29.6
Molecular formula	: $C_{23}H_{20}O_{11}$
Molecular weight	: 472 (EI-MS)
UV λmax (methanol) nm (ε)	: 287 (46 940)
	389 (11 776)
	406 (12 248)
IR νmax (KBr) cm^{-1}	: 3495, 1715
	1610, 1235

TABLE III

Chemical sifts and assignments for 5838-DNI



Assignment	^{13}C NMR (ppm)	Assignment	^{13}C NMR (ppm)	^{1}H NMR (ppm)
12	197.3	6	119.6	8.01(1H, s)
5	193.6	7	108.0	7.74(1H, s)
1	190.8	2	99.4	5.56(1H, s)
ester	174.0	4	69.0	4.79(1H, d)
3	167.1	OCH_3	56.6	3.73(3H, s)
11	165.7		56.3	3.94(3H, s)
8	156.9		52.5	3.89(3H, s)
5a	140.0	10–CH_3	20.4	2.76(1H, s)
10	136.5	11–OH		13.40(1H, s)
6a	128.2	12a–OH		7.08(1H, s)
10a	128.2	4–OH		5.92(1H, s)
9	119.7	4a–OH		5.92(1H, s)
11a	108.8			
4a	84.7			
12a	83.2			

at 287, 389 and 406 nm. The molecular weight of 5838-DNI was 472, and the molecular formula was calculated as $C_{23}H_{20}O_{11}$ from the results of EI-MS, ^{1}H NMR and ^{13}C NMR.

The structure of 5838-DNI was elucidated from ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY and HMBC spectra. Table III shows chemical shifts in the ^{1}H

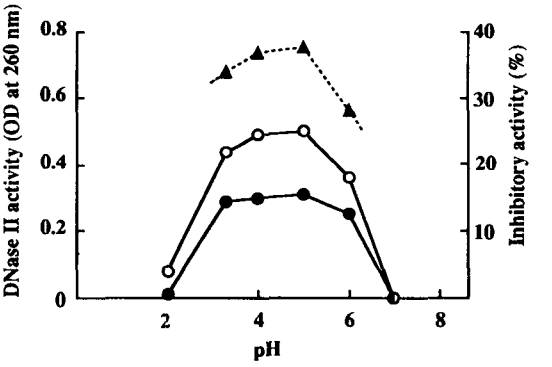


FIGURE 3 Effect of pH on the activity of DNase II with or without 5838-DNI. (○) Activity without 5838-DNI; (●) activity with 5838-DNI (0.98 μM); (▲) percentage inhibition.

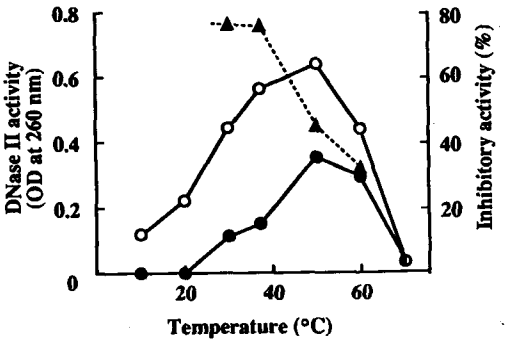


FIGURE 4 Effect of temperature on the activity of DNase II with or without 5838-DNI. (○) Activity without 5838-DNI; (●) activity with 5838-DNI (1.89 μM); (▲) percentage inhibition.

and ^{13}C NMR spectra of 5838-DNI, together with their assignments. The structure of 5838-DNI was determined to be 1,4,4a,5,12,12a-hexahydro-4,4a,11,12a-tetrahydroxy-3,8-dimethoxy-9-methoxycarbonyl-10-methyl-1,5,12-trioxo naphthacene. The chemical structure of 5838-DNI is similar to that of tetracenomycin C,[15] which is an antibiotic produced by *Streptomyces glaucescens*. 5838-DNI showed antibacterial activity against Gram-positive bacteria, but did not show activity against

TABLE IV

Inhibitory spectrum of 5838-DNI

Enzyme (EC)	Substrate (concn)[a]	IC_{50} (μM)
DNase II (3.1.4.6) from porcine spleen	DNA (500)	1.29
DNase I (3.1.4.5) from bovine pancreas	DNA (2000)	> 2.12
RNase A (3.1.27.5) from bovine pancreas	RNA (500)	> 2.12
Nuclease P_1 from *P. citrinum*	Denatured DNA (500)	> 2.12
Nuclease S_1 (3.1.30.1) from *A. oryzae*	Denatured DNA (500)	1.17
5′-Nucleotidase (3.1.3.5) from snake venom	Adenosine-5′-monophosphate (723)	> 2.12
DNA polymerase I (2.7.7.7) from *E. coli*	^{32}P-CTP (617×10^4 Bq)	0.53
Phosphodiesterase I (3.1.4.1) from *C. atrox venom*	Thymidine-5′-monophosphate -*p*-nitrophenylester (90)	0.35
Phosphodiesterase II (3.1.16.1) from bovine spleen	Thymidine-3′-monophosphate -*p*-nitrophenylester (230)	> 2.12
Alkaline phosphatase (3.1.3.1) from bovine intestine	*p*-Nitrophenyl phosphate (2217)	> 2.12
Eco RI from *E. coli*	Plasmid pUC19 (10)	0.27
Dpn I from *D. pneumoniae*	Plasmid pUC19 (10)	> 2.12

[a]Concentration was expressed as μg per 1 ml of reaction mixture.

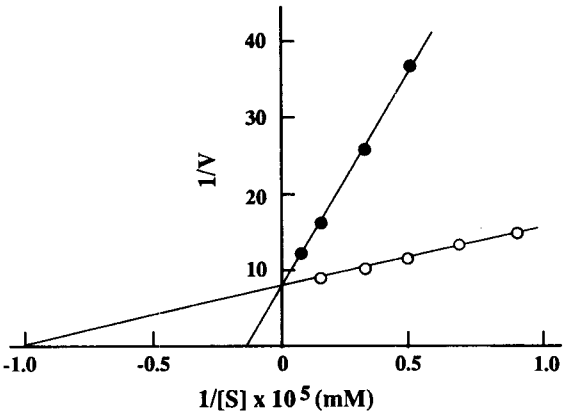


FIGURE 5 A Lineweaver–Burk reciprocal plot of substrate concentration against rate of hydrolysis by phosphodiesterase I with 5838-DNI (●), and without 5838-DNI (○). The inhibitor concentration used was 0.35 μM.

Gram-negative bacteria (data not shown). Since the antibacterial spectrum of 5838-DNI was different from that of tetracenomycin C, it is considered that 5838-DNI may be a stereoisomer of tetracenomycin C.

pH- and Temperature-Dependency of the Inhibitory Activity

DNase II reactions were carried out in buffers over a wide pH range and the effect of pH on the inhibitory activity of 5838-DNI was examined. As shown in Figure 3 DNase II activity, both in the presence and absence of 5838-DNI, was maximum at around pH 5. The inhibitory activity of 5838-DNI, calculated from these results, was shown to be pH-dependent.

DNase II activities with or without 5838-DNI were examined at incubation temperature from 10°C to 70°C at pH 5. Figure 4 shows that DNase II activity was maximum at 50°C and inhibitory activity of 5838-DNI was found to be temperature-dependent.

Inhibitory Spectrum of 5838-DNI

The effects of 5838-DNI on various enzymes were examined. As shown in Table IV, 5838-DNI inhibited DNase II, nuclease S_1, DNA polymerase I, phosphodiesterase I and *Eco* RI, but did not inhibit DNase I, RNase A, nuclease P_1, 5′-nucleotidase, phosphodiesterase II and *Dpn* I. With respect to DNase I and II, and/or phosphodiesterase I and II, the difference in inhibitory properties of 5838-DNI against these enzymes is of interest but the reason is unknown and remains to be examined. In a previous paper,[4] we reported a DNase inhibitor designated as 5923-DNI, which was produced by *Streptomyces* sp. strain No. A-5923. 5838-DNI was distinguished from 5923-DNI in that 5923-DNI inhibited phosphodiesterase II and did not inhibit phosphodiesterase I.

K_i *Value of 5838-DNI as an Inhibitor of Phosphodiesterase I*

The type of inhibition was determined by a Lineweaver–Burk plot of substrate (thymidine-5′-monophosphate-*p*-nitrophenylester) concentration against rate of hydrolysis of phosphodiesterase I in the presence and absence of 5838-DNI and the inhibition was seen to be competitive (Figure 5). The K_i value for 5838-DNI was 6.05×10^{-8}M (2.00×10^{-4}g/litre) and the K_m value for the enzyme was 1.00×10^{-8}M (4.38×10^{-6}g/litre).

DNase I inhibitor from calf spleen (M_r; 59,400)[3] and acid DNase inhibitor from beef liver (M_r; 21,500)[1] are known as specific inhibitors of DNase. Both inhibitors are high molecular weight proteins and do not inhibit DNase II. On the other hand, 5838-DNI (M_r; 472) as well as 5923-DNI (M_r; 2500) inhibited DNase II and did not inhibit DNase I. However, 5838-DNI was distinguished from 5923-DNI by their inhibitory activities against phosphodiesterase I and II. In conclusion, 5838-DNI is the first reported example of an inhibitor of microbial origin which is able to inhibit both DNase II and phosphodiesterase I.

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