J. Enzyme Inhibition, 1993, Vol. 7, pp. 27–32
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DEOXYRIBONUCLEASE INHIBITORS PRODUCED BY STREPTOMYCETES

KEITAROU SUZUKI, KAZUHIKO NAGAO, MASAFUMI GOTO and MASARU UYEDA*

Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862, Japan

(Received 9 November 1992)

Streptomyces sp. strain No. A-5838 produces three types of inhibitors of DNase II. Two of them, DNI-2 and DNI-3, were distinguished from the previously reported DNase II inhibitors, 5838-DNI and 5923-DNI, by their inhibitory profiles towards phosphodiesterases. DNI-2 has M_r 654, and is considered to be a coproporphyrin. DNI-3 is an acidic substance with M_r about 60,000 as estimated by gel filtration. The inhibitory activities of both inhibitors were shown to be temperature-dependent whereas only that of DNI-2 was pH-dependent.

KEY WORDS: Deoxyribonuclease inhibitors, DNase inhibitor, DNase II, *Streptomyces*.

INTRODUCTION

In the search for inhibitors of deoxyribonucleases (DNases), we have screened the products isolated from various streptomycetes cultured in our laboratory. In previous papers,[1-2] we have reported the DNase II inhibitors, 5923-DNI and 5838-DNI, produced from culture filtrates of *Streptomyces* sp. strain No. A-5923 and strain No. A-5838 respectively, and described the purification and some properties of both inhibitors.

In the present paper, we report the isolation of two new inhibitors, DNI-2 and DNI-3, from *Streptomyces* sp. strain No. A-5838 and some of their inhibition and structural characteristics.

MATERIALS AND METHODS

Enzymes

DNase II (EC 3.1.4.6) from porcine spleen, RNase A (EC 3.1.27.5) from bovine pancreas, phosphodiesterase I (EC 3.1.4.1) from *Crotalus atrox* venom, phosphodiesterase II (EC 3.1.16.1) from bovine spleen, 5′-nucleotidase (EC 3.1.3.5) from *Crotalus adamantus* venom and alkaline phosphatase (EC 3.1.3.1) from bovine intestine

* Correspondence.

were obtained from Sigma Chemicals. DNase I (EC 3.1.4.5) from bovine pancreas and nuclease S_1 (EC 3.1.30.1) from *Aspergillus oryzae* were obtained from Boehringer Mannheim Yamanouchi. Nuclease P_1 from *Penicillium citrinum* was obtained from Yamasa Shoyu Co. Ltd.

Substrates

DNA from salmon and thymidine-5′-monophosphate-*p*-nitrophenylester (Sigma Chemicals), RNA from yeast and adenosine-5′-monophosphate (Kohjin Co. Ltd.), thymidine-3′-monophosphate-*p*-nitrophenylester (Merck Co.), and *p*-nitrophenyl phosphoric acid disodium salt (Nacalai Tesque Inc.) were used.

Enzyme Reactions

Activities of DNase I, DNase II,[3] RNase A,[4] nuclease P_1[5] and nuclease S_1[6] were determined by measuring absorbance at 260 nm of the acid-soluble hydrolysate liberated from the substrate by each enzyme reaction. Inorganic phosphate released by 5′-nucleotidase was measured by the method of Fiske-SubbaRow.[7] Activities of phosphodiesterase I,[8] phosphodiesterase II[9] and alkaline phosphatase[10] were determined from absorbance at 400 nm of *p*-nitrophenol liberated from each substrate.

Inhibitory activities of DNI-2 and DNI-3 during purification procedures were determined by using DNase II. One inhibitory unit (IC_{50}) is defined as that amount which reduces the activity of DNase II by 50%. The assay conditions for inhibitory activity have been described previously.[2]

Production of DNI-2 and DNI-3

The seed culture medium containing 2% glucose, 3% starch, 1% soybean flour, 1% corn steep liquor, 0.5% peptone, 0.3% NaCl and 0.5% $CaCO_3$ (pH 7) was inoculated with spores from a slant culture of *Streptomyces* strain No. A-5838. The strain was grown at 28°C for 24 h on a rotary shaker (180 rpm, 5 cm radius). The resultant culture (200 ml) was transferred to 5 litres of the same medium in a 10 litre-jar fermentor, and cultivated under aerobic conditions (aeration; 3 litres/min, agitation; 200 rpm) at 28°C for 1 day.

Isolation of DNI-2 and DNI-3

After cultivation, the mycelium and other residues were removed by filtration. DNI-2 was obtained from the culture filtrate by adjusting to pH 3 and extracting with ethyl acetate; DNI-3 and 5838-DNI remained in the aqueous layer. The organic layer was concentrated *in vacuo* and applied to a Diaion HP-10 (Mitsubishi Chemical Industries, Ltd.) column. After washing with 60% methanol, DNI-2 was eluted with 100% methanol. The eluate was subjected to Sephadex LH-20 column chromatography with methanol. The fractions containing DNI-2 as shown by assay using DNase II were combined and chromatographed on a silica gel column using chloroform-methanol-water (5:5:1). The active fractions obtained were combined and evaporated to give DNI-2.

DNI-3 was obtained from the culture filtrate by adding solid ammonium sulfate to 80% saturation. The precipitate containing DNI-3 was dissolved in 0.01 M Tris-HCl

buffer (pH 8) and dialysed against the same buffer for 2 days. The dialysate was then applied to a DEAE-cellulose column which had been equilibrated with 0.01 M Tris-HCl buffer (pH 8) and DNI-3 eluted with the same buffer containing linear concentrations of NaCl (0.5–1.5 M). The active fractions were combined and solid ammonium sulfate was added to 80% saturation. The resulting precipitate was dialysed against 0.02 M Tris-HCl buffer containing 0.1 M KCl for 2 days. The dialysate was then applied to a Sephadex G-75 column previously equilibrated with the above dialysing buffer, and eluted with the same buffer. Then the gel filtration of the eluate containing DNI-3 was repeated twice on Sephadex G-75. The active fractions were combined and dialysed against distilled water and then lyophilized.

RESULTS AND DISCUSSION

Fermentation and Isolation

Streptomyces sp. strain No. A-5838 produced three types of DNase II inhibitors, designated as 5838-DNI, DNI-2 and DNI-3, in the culture filtrate. Changes in inhibitory activity during cultivation and the isolation procedure for 5838-DNI have been described previously.[2] Table 1 gives the summaries of the purification steps for DNI-2 and DNI-3 from the culture filtrate. The yields of DNI-2 and DNI-3 obtained from 1000 ml of the culture filtrate were 0.25 mg and 4.5 mg respectively.

Physicochemical Properties

DNI-2 was obtained as reddish powder, which was soluble in methanol, ethanol and ethylacetate but insoluble in water. The ultra violet and visible absorption maxima of DNI-2 in the methanol were at 393 (Soret band), 496, 531, 564, and 619 nm. As shown in Figure 1, the spectrum of DNI-2 was analogous to the typical absorption spectra of the etio-type of porphyrins as shown by λ_{max} at 393 nm and 496 nm.[11] The molecular weight of DNI-2 was estimated to be 654 from the result of fast atom bombardment mass spectrometry (FAB-MS) using a JMS-DX303HF mass spectrometer. From these results, the structure of DNI-2 was considered to be a coproporphyrin as shown in Figure 2.

DNI-3 was soluble in water but insoluble in aqueous acid and organic solvent. The inhibitor showed end absorption in the UV spectrum, and gave a negative reaction with ninhydrin,[12] phenol-H_2SO_4,[13] Fiske-SubbaRow,[7] and sodium

Table 1 Summaries of purification steps of DNI-2 and DNI-3

DNI-2	Total activity (unit)	Yield (%)	DNI-3	Total activity (unit)	Yield (%)
Culture filtrate	61800	100.0	Culture filtrate	61800	100.0
AcOEt layer	4760	7.7	Precipitate[c]	52107	84.3
Crude solution[a]	1285	2.1	Crude solution[d]	26547	43.0
Active fraction[b]	941	1.5	Active fraction[e]	2909	4.7
Purified DNI-2	50	0.1	Purified DNI-3	1573	2.5

[a]After column chromatography on Diaion HP-10. [b]After gel filtration on Sephadex LH-20. [c]By solid ammonium sulfate. [d]After column chromatography on DEAE-cellulose. [e]After gel filtration on Sephadex G-75.

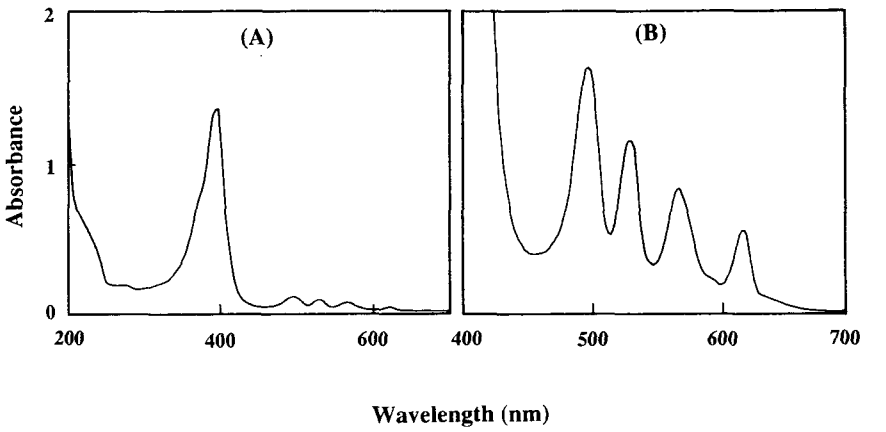

Figure 1 UV and visible absorption spectra of DNI-2 in methanol at concentrations of (A) 9 μg/ml and (B) 122 μg/ml.

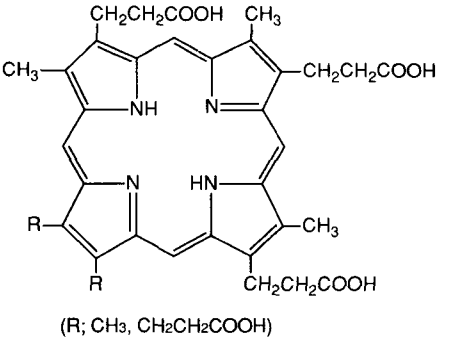

Figure 2 Proposed structure of DNI-2.

rhodizonate.[14] DNI-3 absorbed on an anion exchange resin such as DEAE-cellulose, and its molecular weight was estimated to be about 60,000 by gel filtration on Sephadex G-75 column. From these results, DNI-3 was considered to be an acidic substance with high molecular weight. Further investigation will be necessary to elucidate the structure of the inhibitor.

Effects of pH and Temperature on Inhibitory Activity

The inhibitory activities of DNI-2 and DNI-3 against DNase II were measured over the pH range pH 3–7. As shown in Figure 3(A), the activity of DNI-2 was pH-dependent with a maximum at pH 6. The activity of DNI-3 was independent of pH.

The inhibitory activities determined at various temperatures from 20°C to 70°C at pH 5 are shown in Figure 3(B). The inhibitory activities of both inhibitors were shown to be temperature-dependent and decreased with increasing temperature.

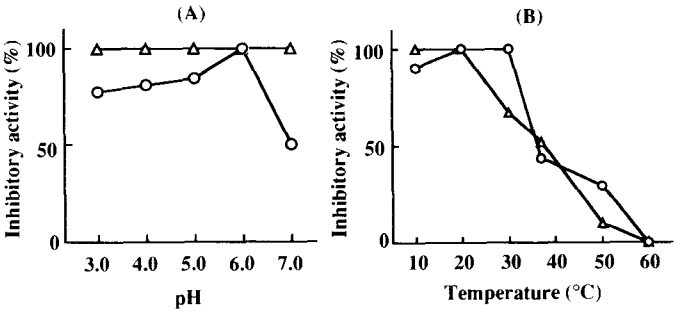

Figure 3 Effects of pH and temperature on the inhibitory activities of (○) DNI-2 and (△) DNI-3. DNI-2 and DNI-3 were used at concentrations of 60 μg (9.2×10^{-5} M) and 76 μg (1.3×10^{-6} M) per ml respectively in the incubation mixture.

Table 2 Inhibitory spectra of DNase II inhibitors produced by streptomycetes

	Amount required to cause 50% inhibition (μg/ml)			
Enzyme	DNI-2	DNI-3	5838-DNI	5923-DNI
DNase II	60	76	610	31
DNase I	>1000	>1000	>1000	41
RNase A	>1000	>1000	>1000	>1000
Nuclease P_1	>1000	>1000	>1000	>1000
Nuclease S_1	>1000	>1000	550	101
Phosphodiesterase I	0.4	0.2	167	>1000
Phosphodiesterase II	17	12	>1000	183
5′-Nucleotidase	111	>1000	>1000	ND
3′-Nucleotidase	>1000	>1000	>1000	ND
Alkaline phosphatase	>1000	>1000	>1000	ND

ND; not determined.

Inhibitory Spectra

The effects of DNI-2 and DNI-3 on various enzymes were examined, and the results are compared with those of 5838-DNI and 5923-DNI (see Table 2). DNI-2 and DNI-3 inhibited DNase II, phosphodiesterase I or II, but did not inhibit DNase I, RNase A, nuclease P_1, nuclease S_1, 3′-nucleotidase or alkaline phosphatase. DNI-2 was distinguished from DNI-3 in that DNI-2 inhibited 5′-nucleotidase. DNI-2 and DNI-3 were also distinguished from 5838-DNI and 5923-DNI in that DNI-2 and DNI-3 inhibited both phosphodiesterases (I and II) and did not inhibit nuclease S_1.

K_i Values of DNI-2 and DNI-3 against Phosphodiesterase I

The type of reversible inhibition of DNI-2 or DNI-3 against phosphodiesterase I was determined by a Lineweaver-Burk plot. As shown in Figure 4, both inhibitors inhibited in a competitive manner, and the K_i values were 2.22×10^{-11} M and 9.32×10^{-13} M

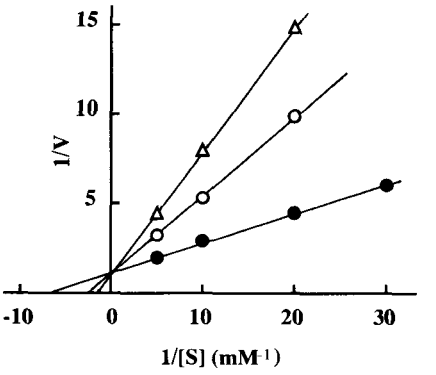

Figure 4 A Lineweaver-Burk reciprocal plot of substrate (thymidine-5′-monophosphate-*p*-nitrophenylester) concentration against rate of hydrolysis by phosphodiesterase I with (○) DNI-2 or (△) DNI-3, and (●) without these inhibitors. DNI-2 and DNI-3 were used at concentrations of 0.4 μg (6.1×10^{-7} M) and 0.2 μg (3.3×10^{-9} M) per ml respectively in the incubation mixture.

respectively. The K_m value for the enzyme with thymidine-5′-monophosphate-*p*-nitrophenylester as substrate was 1.00×10^{-8} M.

DNase inhibitors of high molecular weight have been isolated from animal tissues such as liver,[15] spleen[16] and thymus.[17] However, these inhibitors only inhibit DNase I and do not inhibit DNase II. On the other hand, among four inhibitors isolated from streptomycetes in our studies, 5838-DNI,[2] DNI-2 and DNI-3 inhibited DNase II but not DNase I, and 5923-DNI inhibited both enzymes.[1] These inhibitors with distinct inhibitory properties may be useful as tools in studies on the biological functions of DNases in the cell.

References

1. Uyeda, M., Uehara, K., Deguchi, M., Tsuchiyama, S., Suzuki, K. and Shibata, M. (1991) *J. Enz. Inhib.*, **4**, 359.
2. Uyeda, M., Nagao, K., Kido, Y., Suzuki, K., Hara, Y. and Yamamura, R. (1992) *J. Enz. Inhib.*, **6**, 157.
3. Kunits, M. (1950) *J. Gen. Physiol.*, **33**, 365.
4. Richards, F.M. and Wyckoff, H.W. (1971) In *The Enzymes*, (Boyer, P.D. ed.) Vol. 4, p. 647. New York: Academic Press.
5. Fujimoto, M., Fujiyama, K., Kuninaka, A. and Yoshino, H. (1974) *Agric. Biol. Chem.*, **38**, 2141.
6. Shishido, K. and Ando, T. (1972) *Biochim. Biophys. Acta*, **287**, 477.
7. Fiske, C.H. and SubbaRow, Y. (1925) *J. Biol. Chem.*, **66**, 375.
8. Razzell, W.E. (1963) In *Methods in Enzymology*, (Kaplan, N.O. ed.) Vol. 6, p. 236. New York: Academic Press.
9. Razzell, W.E. and Khorana, H. (1961) *J. Biol. Chem.*, **236**, 1144.
10. Engstrom, L. (1961) *Biochim. Biophys. Acta*, **52**, 36.
11. Smith, K.M. (1975) *Porphyrins and Methalloporphyrins*, p. 19. Amsterdam: Elsevier Scientific Publishing Co..
12. Lee, Y.P. and Takahashi, Y. (1966) *Anal. Biochem.*, **14**, 71.
13. Dubois, M., Giles, K.A., Hamilton, J.K., Roberts, P.A. and Smith, F. (1956) *Anal. Chem.*, **28**, 350.
14. Terho, T.T. and Hartiala, K. (1971) *Anal. Biochem.*, **41**, 471.
15. Lesca, P. (1976) *J. Biol. Chem.*, **251**, 116.
16. Lindberg, M.U. (1967) *Biochemistry*, **6**, 323.
17. Lindberg, M.U. and Skoog, L. (1970) *Eur. J. Biochem.*, **13**, 326.