BE-40644, a New Human Thioredoxin System Inhibitor Isolated from *Actinoplanes* sp. A40644

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In the course of our screening program for inhibitors of thioredoxin (TRX) system, a strain of *Actinoplanes*, A40644, isolated from a soil sample collected at Ohita Prefecture, Japan, was found to produce the first potent inhibitor of TRX system from a natural source. The structure of this novel compound, BE-40644, was elucidated as shown in Fig. 1. BE-40644 showed strong inhibitory activity against TRX system, while it showed weak inhibitory activity against glutathione reductase. This paper describes the strain, production, isolation, physico-chemical properties, structure elucidation and activity of this new inhibitor of TRX system.

Assay of TRX system essentially followed the modified method of KRAUSE *et al.*[1] and was carried out in the reaction mixture of 470 μl of 0.1 M Tris-HCl (pH 7.5) containing 2 mM EDTA, 15 μl of 0.25 mg/ml *E. coli* TRX (IMCO Co.), 20 μl of 0.023 mg/ml *E. coli* TRX reductase (IMCO Co.), 10 μl of 10 mg/ml bovine insulin (Sigma Chem. Co.) as substrate of TRX and 5 μl of dimethyl sulfoxide with or without test sample. The reaction was started by adding 15 μl of 0.2 mM NADPH solution at room temperature and after 30 minutes optical density decrease of NADPH consumption was read at 340 nm. Assay of human TRX system was carried out by the same method described above but the reaction mixture consisted of 80 μl of 0.1 M Tris-HCl (pH 7.5) containing 2 mM EDTA, 10 μl of 75 mg/ml human TRX produced by expressing as a fusion protein with glutathione S-transferase in *E. coli*, 10 μl of 0.214 mg/ml human TRX reductase (IMCO Co.), 2 μl of 10 mg/ml bovine insulin (Sigma Chem. Co.) as substrate of TRX and 1 μl of dimethyl sulfoxide with or without test sample and 3 μl of NADPH solution. To investigate the selectivity of test samples, inhibition against glutathione/glutathione reductase system was carried out. This counter assay was performed by the modified method of BREHE *et al.*[2] in a reaction mixture comprised of 750 μl of 0.1 M Tris-HCl (pH 7.5), 30 μl of oxidative dimer of glutathione (GSSG, 30.6 mg/ml), 30 μl of water, 10 μl of yeast GSSG reductase (Sigma Chem. Co., 5 IU/ml) and 20 μl of dimethyl sulfoxide with or without test sample. Two minutes after addition of 80 μl of 0.8 mg/ml NADPH solution, inhibitory activity was measured in the same way described above in TRX assay system.

Characterization of the strain followed the method adopted by the International Streptomyces Project (ISP)[3]. Strain A40644 had a brownish vegetative mycelium and bottle shaped sporangia on the surface of the agar media which had hair-like structures on their surfaces (Fig. 2). The spores were rod shaped or ellipsoidal and motile by a polar tuft of flagella. Chemotaxonomic analysis of strain A40644 revealed *meso*-diaminopimelic acid and glycine as distinguishing components of the cell wall. Xylose and arabinose were the major sugars in the whole-cell hydrolysate. These results indicated that the strain A40644 has a type IID cell wall of LECHEVALIER and LECHEVALIER[4]. The physiological properties and carbon source utilization of strain A40644 are shown in Table 1. The taxonomic features of strain A40644 resembled those of *Ampullariella pekinensis*[5] except for its characteristics of coagulation and peptonization of milk and liquefaction of gelatin (Table 1). However, a scientific name of *A. pekinensis* has not been approved by Bacteriological Code 1990 Revision and, in addition, the genus *Ampullariella* was transfered to the genus *Actinoplanes* Couch 1950[6,7]. Therefore strain A40644 was designated as *Actinoplanes* sp. A40644.

Fig. 1. Structure of BE-40644.

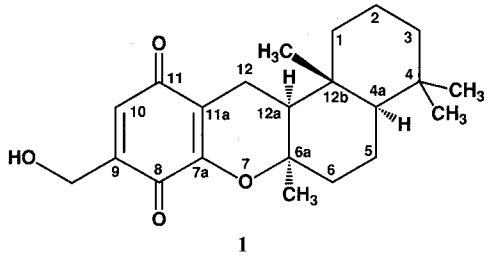

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Fig. 2. Scanning electron micrograph of sporangium of *Actinoplanes* sp. A40644.

Bar represents 4.29 μm.

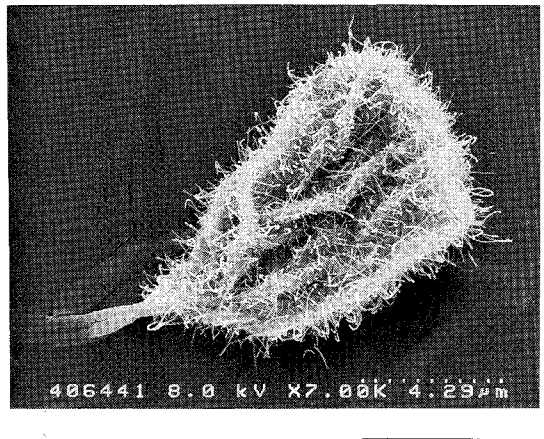


Table 1. Comparison of taxonomic characteristics of strain A40644 with "*Ampullariella pekinensis*" JMC3174.

	A40644	"*A. pekinensis*" JCM3174
Sporangial surface	Hair-like structures	Hair-like structures
Coagulation of milk	-	+
Peptonization of milk	-	+
Liquefection of gelatin	-	+
Melanoid production	-	-
Hydrolysis of starch	+	+
NaCl tolerance	≦2%	≦2%
Temperature range for growth	12 °C~37 °C	N.D.
Carbon utilization		
D-Glucose	+	+
D-Xylose	+	+
L-Arabinose	+	+
L-Rhamnose	+	+
D-Fructose	+	+
D-Galactose	+	+
Raffinose	-	±
D-Mannitol	+	+
i-Inositol	-	-
D-Galactose	+	+
Sucrose	+	+
Salicin	+	+

N.D.; Not determined

Table 2. Physico-chemical properties of BE-40644.

Appearance	yellow powder
Molecular weight	358.48
Molecular formula	$C_{22}H_{30}O_4$
HRFAB-MS (*m/z*)	
Calcd:	360.2301 as $C_{22}H_{32}O_4$
Found:	360.2314 $(M+2H)^+$
$[\alpha]_D^{20}$	-48.5° (c 0.887, MeOH)
UV λmax MeOH nm (ε)	204(15,200), 265(11,100), 400(900)
IR ν max (KBr) cm^{-1}	2945, 2926, 1676, 1651, 1633, 1610, 1369, 960
Color reaction	
positive	20% H_2SO_4

Table 3. ^1H and ^{13}C NMR signal assignments of BE-40644.

Number	^{13}C shift(δ ppm)[a] (multiplicity)	^1H shift(δ ppm)[b] (multiplicity, *J* in Hz)
1	39.8(t)	0.86(1H,m)
		1.79(1H,m)
2	18.2(t)	1.42(1H,m)
		1.55(1H,m)
3	41.5(t)	1.13(1H,dt,13.7,4.6)
		1.40(1H,m)
4	33.1(s)	
4a	55.0(d)	0.92(1H,m)
5	18.0(t)	1.57(2H,m)
6	39.6(t)	1.56(1H,m)
		2.35(1H,m)
6a	79.6(s)	
7a	153.5(s)	
8	181.9(s)	
9	144.1(s)	
10	131.5(d)	6.64(1H,t,1.8)
11	186.6(s)	
11a	119.9(s)	
12	16.6(t)	2.36(1H,dd,19.8,8.2)
		2.59(1H,d,19.8)
12a	48.4(d)	1.38(1H,d,8.2)
12b	38.3(s)	
4-Me(ax)	33.5(q)	0.88(3H,s)
4-Me(eq)	21.8(q)	0.80(3H,s)
6a-Me	27.1(q)	1.19(3H,s)
9-CH_2O-	59.4(t)	4.51(2H,br s)
9-$CH_2O\underline{H}$		2.21(1H,br s)
12b-Me	13.7(q)	0.72(3H,s)

All NMR spectra were measured in $CDCl_3$ by JEOL Alpha-500 spectrometer.

[a] Chemical shifts are shown with reference to $CDCl_3$ at 76.9 ppm.

[b] Chemical shifts are shown with reference to $CDCl_3$ at 7.26 ppm.

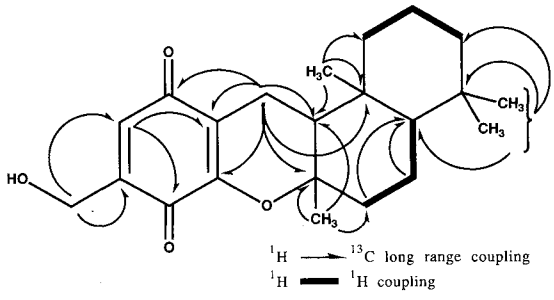
The strain A40644 has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan (No. FERM P-14196).

The strain A40644 on a slant agar medium was inoculated into two 500-ml volume Erlenmeyer flasks for cultivation which contained 100 ml of a culture medium comprised of glucose 0.2%, dextrin 2.0%, meat meal 0.5%, defatted rice bran 0.5%, defatted meat bone meal 0.1%, dried yeast 0.05%, $MgSO_4$ 0.025%, NaBr 0.025%, NaCl 0.25%, K_2HPO_4 0.05%, Fe_2SO_4 0.0002%, $CuCl_2$ 0.00004%, $MnCl_2$ 0.00004%, $CoCl_2$ 0.00004%, $ZnSO_4$ 0.0008%, $Ba_2B_4O_7$ 0.00008%, $(NH_4)_6Mo_7O_{24}$ 0.00024% (pH 7.2) and cultured on a rotary shaker (180 rpm) at 28°C for 72 hours. Two ml each of the culture broth was inoculated into 100 of 500-ml volume Erlenmeyer flasks containing 100 ml of the foregoing culture medium and incubated on a rotary shaker (180 rpm) at 28°C for 264 hours. A mycelial cake was obtained by filtration and was extracted twice with 4 liters and 3 liters of methanol. The methanol extracts were combined and concentrated under reduced pressure to about 300 ml and extracted twice with ethyl acetate (300 ml × 2). The evaporated residue of the ethyl acetate layer was dissolved with 200 ml of 90% aqueous methanol and evaporated after washing twice with *n*-hexane (200 ml × 2) to obtain 730 mg of crude active material. The crude material was successively purified by column chromatographies of silica gel (Kieselgel 60, Merck, 2 × 35 cm, chloroform - methanol (20 : 1)), Sephadex LH-20 (Pharmacia, 1.5 × 45 cm, methanol) and finally purified by HPLC (Capcellpak C18, Shiseido, 2 × 25 cm, acetonitrile - water (55 : 45), 5 ml/minute. BE-40644 was detected by the UV absorption at 254 nm). Pure 41.3 mg of BE-40644 was obtained from 10 liters of culture broth.

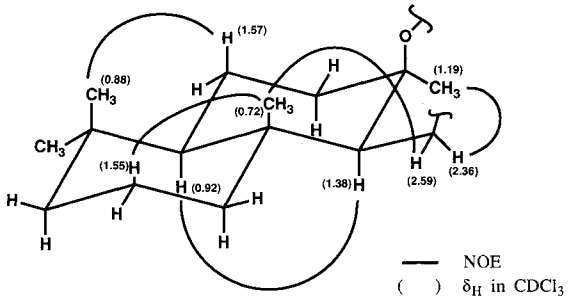
The physico-chemical properties of BE-40644 are

Fig. 3.

(a) HMBC and COSY correlations for BE-40644.



(b) NOEs observed in NOESY spectrum of BE-40644.



shown in Table 2. The BE-40644 (**1**) was obtained as yellow powder, soluble in chloroform and methanol and hardly soluble in water. High resolution positive FAB-MS analysis showed the molecular formula to be $C_{22}H_{30}O_4$ (calcd for $C_{22}H_{32}O_4$: *m/z* 360.2301 $(M+2H)^+$; found: *m/z* 360.2314). The UV spectrum of this compound in methanol showed absorption maxima at 204 (ε 15,200), 265 (ε 11,100), 400 (ε 900), which suggested the presence of a quinone moiety.

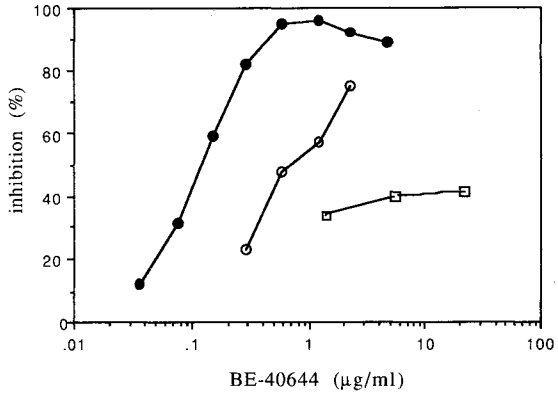
As shown in Table 3, the ^1H NMR spectrum (500 MHz, $CDCl_3$) of **1** showed four singlet methyl groups, seven methylene groups, one olefinic proton and two methine protons. The ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of **1** revealed the presence of 22 carbon atoms, supporting the above elemental composition. The structure of **1** shown in Fig. 1 was determined by modern NMR techniques including heteronuclear multiple bond correlation (HMBC) analysis (Fig. 3a), NOEs from NOESY (Fig. 3b), coupling constants between protons were also examined but the absolute chirality is still undetermined.

Fig. 4 shows the effect of BE-40644 on TRX or GSSG reductase enzymatic activity. BE-40644 selectively and potently inhibited both *E. coli* and human TRX systems. Its IC_{50} value was 0.12 μg/ml for *E. coli* TRX system and 0.80 μg/ml for human TRX system, while that for yeast GSSG reductase was greater than 22 μg/ml. When 5,5′-dithio-bis(2-nitrobenzoic acid) was used as substrate for *E. coli* TRX system in place of insulin, the IC_{50} was 0.35 μg/ml (data not shown).

Human TRX is a newly discovered 12Kd protein which

Fig. 4. Inhibitory effect of BE-40644 on TRX system and GSSG reductase.

E. coli TRX system (●), human TRX system (○), GSSG reductase (□).



has been detected and its cDNA has been cloned from culture supernatant of human T cell leukemia virus type I infected[8] and Epstein-Barr virus transformed[9] human cell lines. TRX is a disulfide reducing enzyme, and in *E. coli*, it has been initially reported as an essential component for DNA synthesis activating ribonucleotide reductase[10]. In humans, TRX has been demonstrated to be highly produced in cancer cell line especially virally transformed malignant cells and act as an autocrine growth factor and synergize with interleukin-1 and -2[9]. As a mechanism of this growth promoting effect, activation by TRX of phospholipase C-protein kinase C pathway of intracellular signal transduction has recently been reported[11]. In fact, our preliminary results show that BE-40644 can inhibit the growth of several cancer cell lines and exogenous addition of TRX in such condition can reverse this inhibition.

Consequently BE-40644, inhibitor of TRX, could be considered not only as a tool to investigate biological significance of TRX system but also as a candidate to be developed as anticancer agent.

Acknowledgments

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