YUA001, a Novel Aldose Reductase Inhibitor Isolated from Alkalophilic *Corynebacterium* sp. YUA25

I. Taxonomy, Fermentation, Isolation and Characterization

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YUA001 is a novel aldose reductase inhibitor produced from alkalophilic *Corynebacterium* sp. YUA25 isolated from soil. YUA001 was purified from the supernatant of culture broth by successive silica gel column chromatography, Sephadex LH20-100 gel column chromatography, and HPLC. From instrumental analysis, molecular formula of YUA001 is $C_{13}H_{19}NO_2$ and its molecular weight is 221. It exhibits potent aldose reductase inhibition activity and has no antimicrobial activity against some Gram-positive or Gram-negative bacteria, fungi and yeast.

Aldose reductase (EC 1, 1, 1, 21) is an NADPH-dependent oxidoreductase that catalyzes the conversion of glucose to sorbitol in the polyol pathway. These biochemical changes elevated intracellular sorbitol level, which lead to the development of diabetic complication[1] such as cataract[2], neuropathy[3], retinopathy[4,5] and nephropathy[6]. Inhibitors of aldose reductase have been shown to reverse these biochemical changes and have been proven effective in delaying and even preventing several diabetic pathologies. Thus, aldose reductase has become an attractive pharmacological target for the treatment of diabetic complications[7].

Yu's had been discovered new enzyme and gene in alkalophilic bacteria from soil[8,9].

During screening process for aldose reductase inhibitors, a novel aldose reductase inhibitor has been isolated from the supernatant of fermentation broth of alkalophilic *Corynebacterium* sp. YUA25.

The present paper describes the taxonomy, production, isolation, physico-chemical properties and biological activities of YUA001.

Materials and Methods

Fermentation

A loopful of mature slant culture of alkalophilic *Corynebacterium* sp. YUA25 was inoculated into a seed medium (5 ml) containing glucose 2%, yeast extract 0.5%, polypeptone 0.5%, K_2HPO_4 0.1%, $Mg_2SO_4 \cdot 7H_2O$ 0.02%, Na_2CO_3 1% in 50 ml glass tube and cultured at 30°C for 24 hours on a rotary shaker.

Fermentation studies were carried out in jar fermenters. A seed culture was shaken in the above mentioned glass tube and then transferred at the rate of 5% to 200 ml of the same medium in 1-liter flask, which was cultured at 30°C for 24 hours on a rotary shaker with 150 rpm. 200 ml of seed culture was inoculated into 4 liters of same medium as above mentioned in a 5-liter jar fermenter which was cultured at 30°C for 48 hours under aeration of 6.25 liters/minute and agitation of 450 rpm.

Assay of Aldose Reductase Activity

Aldose reductase was prepared from pig kidney. The tissue was dissected from the connective tissue, weighed and homogenized in 2 volumes of 10 mM sodium phos-

phate buffer (pH 7.0). The homogenate was centrifuged at 14,000×*g* for 30 minutes and the sediment was discared. The supernatent was precipitated by ammonium sulfate. Ammonium sulfate fraction was carried out by adding solid ammonium sulfate to obtain 35% and 70% precipitates. The final precipitate was resuspended in 10 mM sodium phosphate buffer (pH 7.0) and dialyzed with the same buffer. The dialyzed fraction was used in the enzymatic reacton. Aldose reductase activity[10] was measured on a Shimadzu UV1201 spectrophotometer by monitoring nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm. In enzymatic assays, the reaction mixture contained 200 mM sodium phosphate buffer (pH 7.0), 0.125 mM NADPH, 400 mM lithium sulfate, enzyme solution and 10 mM DL-glyceraldehyde in a total volume of 1.0 ml. The reaction was started by the addtion of NADPH and was measured for 5 minutes. The effect of aldose reductase inhibitor was determined by comparing the O.D. at 340 nm of enzyme reaction mixture with aldose reductase inhibitor.

Assay of Antimicrobial Activities

Antimicrobial activity of YUA001 was determined by paper-disk assay method in nutrient media for Gram-positive and Gram-negative bacteria and in YM media for fungi and yeasts.

Results

Identification of Alkalophilic *Corynebacterium* sp. YUA25

Morphological characteristics of alkalophilic *Corynebacterium* sp. YUA25 isolated from soil were shown in Fig. 1. This strain was rod-type and had no flagella and cilia. Colonies were yellow when exposed to light.

Fig. 1. Scanning electron microscopic photograph of alkalophilic *Corynebacterium* sp. YUA25.

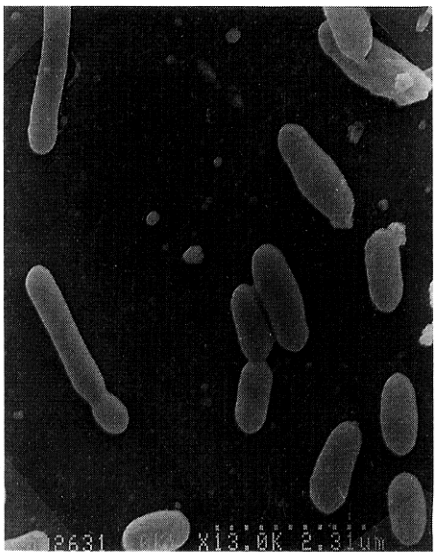


Fig. 2. Time course of the production of YUA001 from alkalophilic *Corynebacterium* sp. YUA25.

Incubation temperature; 30°C, Impeller speed; 450 rpm, Aeration rate; 1.25 vv/minute.
■; O.D., ●; YUA001, ◆; pH.

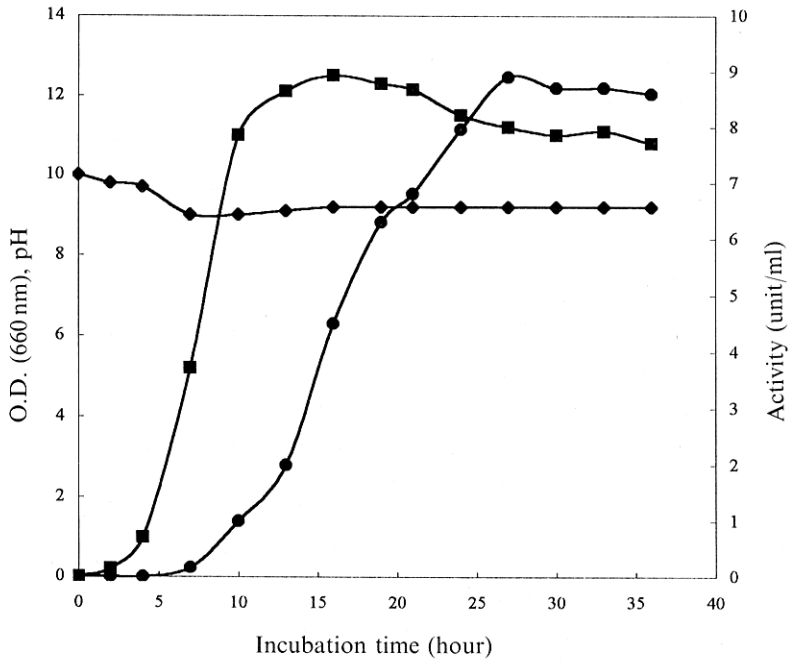
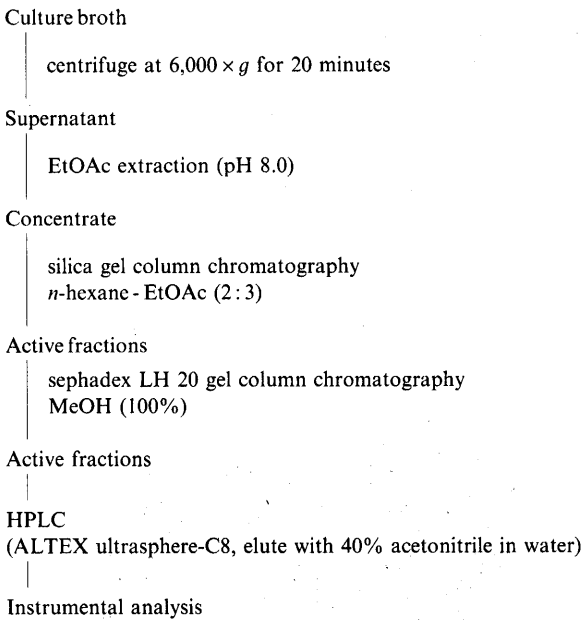


Fig. 3. Purification procedure of YUA001.



This strain was Gram's positive by Gram's staining and Ryu Nonstaining KOH technique[11] and non-spore forming strain by spore staining. Also this one was strict aerobe by oxygen requirement test and had both oxydase and catalase activity. This strain showed positive reaction to acid-fast test and no motility by motility test. Furthermore, this strain revealed resistance to lysozyme and penicillin.

Production of YUA001 by Alkalophilic *Corynebacterium* sp. YUA25

The growth of alkalophilic *Corynebacterium* sp. YUA25 was monitored by optical density measured by Shimadzu UV 1201 spectrophotometer of 660 nm. The production of YUA001 was monitored by the inhibition of aldose reductase. A time course of YU001 is shown in Fig. 2.

Isolation and Purification

The cultured broth was centrifuged in 6000 × *g* for 20 minutes. After pH adjusted to 8.0, the culture supernatant, it was extracted by ethyl acetate in 1 : 1 volume. This extraction procedure was carried out three times. The extract was concentrated in a rotary vacuum evaporator and charged to a column of normal phase Silica Gel (column size, 2.7 × 30 cm: flow rate, 257 ml/hour). This column has been equilibrated with a mixture of ethyl acetate and *n*-hexane (6 : 4) and eluted with same solvent. The active elute was concentrated with a rotary vacuum evaporator again and the concentrate was dissolved in dehydrated methanol. The methanol solution was charged to a column of Sephadex LH 20 Gel which has been equilibrated with methanol. The active fractions were pooled and concentrated *in vacuo*. The pooled active material was subjected to high performance liquid chromatography (HPLC) purification. HPLC was carried out on Altex ultrasphere-C8 column, 0.46 cm (i.d.) × 25 cm and monitored at 224 nm. Mobile phase was a mixture of acetonitrile and distilled water (4 : 6). The active fractions had a retention time of 6.1 minutes at a flow rate of 0.8 ml/minute. The overall purification procedure is presented in Fig. 3.

Table 1. Physico-chemical properties of YUA001.

Appearance	White powder
MP (°C)	103
Molecular formula	$C_{13}H_{19}NO_2$
Mass spectrum (*m/z*)	222 (M + H, ESI-MS)
UV spectrum	
λ_{max} nm (MeOH)	224, 276
TLC (normal phase silica gel plate)	
Rf[a]	0.4

ESI-MS: Electrospray Ionization Mass Spectrum.
[a] Solvent system: Ethyl acetate - *n*-hexane, 3 : 2.

Physico-chemical Properties

The physico-chemical properties of YUA001 are summarized in Table 1. The ^1H NMR, ^{13}C NMR and ESI-MS spectra are represented in Figs. 4, 5, and 6, respectively. The ^{13}C NMR showed 5 signals in the sp^2-carbon region, one (179.4 ppm) of which is attributable to a ketone. The remaining four signals at 116.2, 130.7, 131.3, 156.9 ppm indicated the presence of 1,4-disubstituted phenyl ring in 1. In the sp^3-carbon region, six signals were assignable to two methyl carbon (12.2, 16.1 ppm), four methylene or tertiary methine carbon (28.3, 35.8, 42.1, 43.9 ppm). The 500 MHz ^1H NMR spectra (DMSO-d_6) confirmed the deduced structure of 1. In sp^3 hydrogen areas, two methyl-group hydrogen (H_{3h} (t), H_{3g} (d)) resonated at 0.76 and 0.94 ppm respectively. $H_{f1,2}$, H_e, $H_{d1,2}$, and $H_{c1,2}$ hydrogen was indicated at 1.25 (m), 1.45 (m), 2.09 (q), and 2.57 (t) ppm, respectively. In sp^2 hydrogen areas, 6.65 (d) and 6. 98 (d) ppm signals were attributable to $H_{a1,2}$ and $H_{b1,2}$ of 1,4-disubstituted phenyl ring. As expected, H_i of phenol OH resonated at around 9.1 ppm and H_j of amide NH at 7.73 ppm.

Fig. 4. ^{1}H NMR spectrum of YUA001.

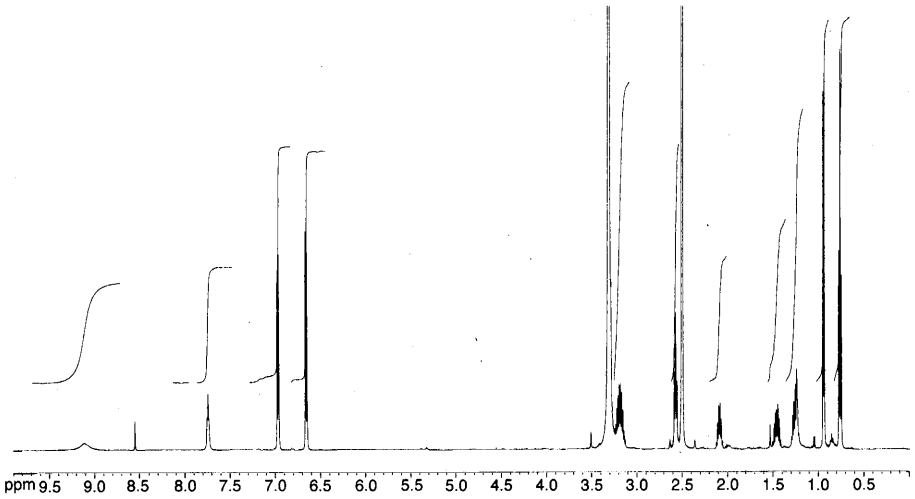
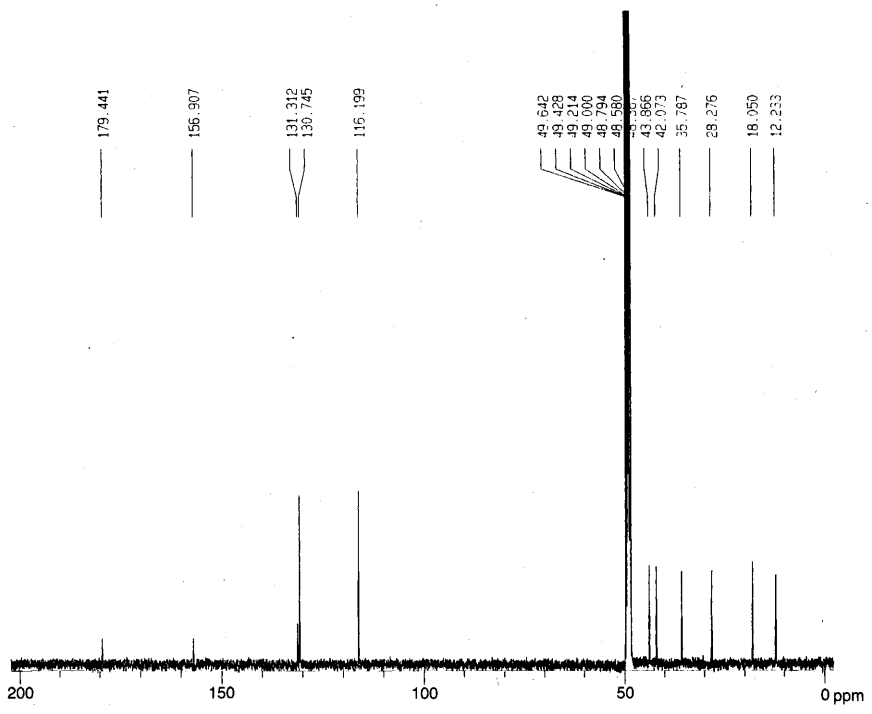


Fig. 5. ^{13}C NMR spectrum of YUA001.



The molecular formula of YUA001 was determined to be $C_{13}H_{19}NO_2$ and molecular weight (m/z) to be 221 by these data and positive ion mode m/z 222 (M+H), 263 (M+MeCN+H), 443 (2M+H) in the electrospray ionization (ESI) MS. The chemical structure of YUA001 was shown in Fig.7. For the confirmation of molecular weight of YUA001, we made YUA001 derivatives by methylation and acetylation. By ESI-MS (positive), molecular weights of methyl and acetyl YUA001 derivatives were determined to be 235 and 263 (data not shown), respectively. Color reaction are as follows: YUA001 gave positive reactions to sulfuric acid and *p*-anisaldehyde, though negative to bromocresol green, vanillin-sulfuric acid, hydrazine sulfate, ninhydrin, phenol sulfuric acid, anthrone, orcinol, bromothymol blue, silver nitrate-pyrogallol, iodine-potassium iodide, perchloric acid and aluminum chloride.

Biological Activities

YUA001 revealed no antimicrobial activity at the

Fig. 6. ESI-MS spectrum of YUA001.

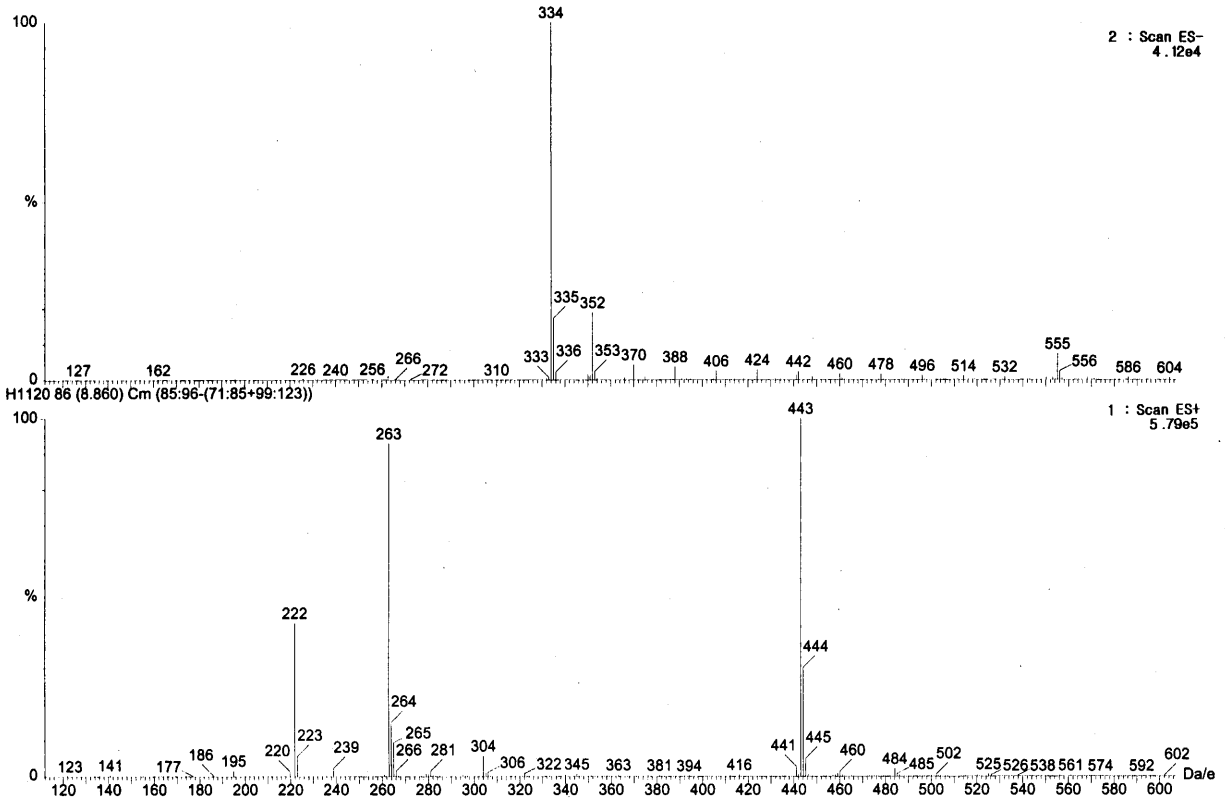
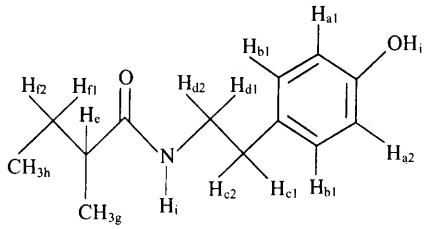


Fig. 7. The chemical structure for YUA001.



concentration of 1 mg/ml for *Staphylococcus aureus*, *Staphylococcus fecalis*, *Bacillus subtilis*, *Bacillus megatrium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus oryzae* and *Penicillium reoquefortii*.

YUA001 inhibited aldose reductase and it's IC_{50} value was 1.8×10^{-3} M (Table 2). The IC_{50} value for tolreatat was 1.6×10^{-5} M in the same experiment. The kinetic study of YUA001 was performed in Lineweaver-Buck plot for aldose reductase. As shown in Fig. 8. YUA001 inhibited aldose reductase noncompetitively with *dl*-glyceraldehyde as substrate.

Table 2. Inhibition of aldose reductase by YUA001 and tolrestat.

Inhibition	IC_{50} value (M)[a]
YUA001	1.8×10^{-3}
Tolrestat[b]	1.6×10^{-5}

[a] Evaluted *in vitro* against pig kidney aldose reductase.

[b] *N*-[[6-Methoxy-5-(trifluoromethyl)-1-naphthalenyl]thioxomethyl]-*N*-methylglycine.

Discussion

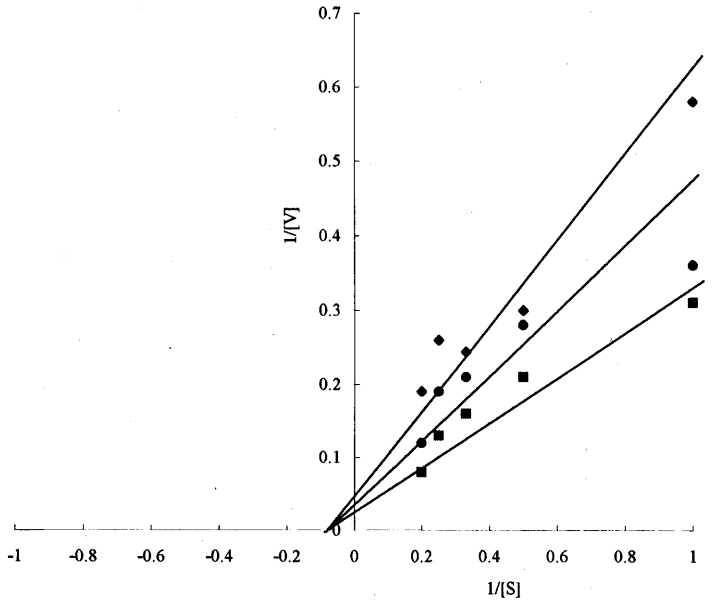
In the course of our screening for aldose reductase inhibitors, we isolated alkalophilic *Corynebacterium* sp. YUA25 from Korea soil which produced YUA001.

From instrumental analysis, YUA001 had 223 *m/z* in DI-MS and FAB-MS (data not shown) but 221 *m/z* in ESI-MS. Although the reason is not clear, it is assumed that carbonyl group of YUA001 could be changed to hydroxyl group, taking two hydrogens.

In vitro aldose reductase assay using partially purified pig kidney enzyme, YUA001 had weak inhibition activity compared to previously reported inhibitor, but chemical

Fig. 8. Lineweaver-Burk plot of inhibition of pig kidney aldose reductase by YUA001.

YUA001 concentration: ■; No inhibitor, ●; 1.0×10^{-3} M, ◆; 2.0×10^{-3} M.



structure of YUA001 was found to be a novel compound. We are trying to increase the inhibition activity of YUA001 by chemical derivation and its derivatives will be reported in due course.

Acknowledgment

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