STUDIES ON WF-3681, A NOVEL ALDOSE REDUCTASE INHIBITOR

I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

MOTOAKI NISHIKAWA, YASUHISA TSURUMI, TAKAYUKI NAMIKI, KEIZO YOSHIDA and MASAKUNI OKUHARA

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Toyosato-machi, Tsukuba-gun, Ibaraki 300-26, Japan

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WF-3681 was isolated from a cultured filtrate of *Chaetomella raphigera* as a novel inhibitor of aldose reductase. It was extracted with ethyl acetate and then purified with silica gel chromatography. Its molecular formula was determined to be $C_{13}H_{12}O_5$ by elemental analysis and high resolution electron impact mass spectrometry. IC_{50} of WF-3681 was 2.5×10^{-7} M for partially purified aldose reductase of rabbit lens.

Insulin therapy can greatly improve the life span of diabetic patients, yet they still suffer from the complications associated with diabetes, such as cataract[1], neuropathy[2], retinopathy[3,4] and nephropathy[5].

Aldose reductase (EC 1.1.1.21) (Fig. 1), which catalyzes the conversion of glucose to sorbitol, stimulates the accumulation of sorbitol in the tissues where these complications develop during hyperglycemia.

Aldose reductase inhibitors, such as sorbinil, tolrestat and epalrestat, have been shown to reduce tissue sorbitol content in diabetic animals and also recently reported to be clinically potent as the therapeutic agents for diabetic complications. These evidences strongly suggest that sorbitol accumulation plays a critical pathophysiological role in the development of diabetic complications.

In the process of searching for a novel aldose reductase inhibitor from microbial products, we discovered WF-3681 which inhibited the enzyme specifically.

In this paper, the taxonomy of the producing organism, fermentation, isolation procedures, physico-chemical and biological properties of WF-3681 are described.

Materials and Methods

Enzyme Preparation

Rabbit lenses were obtained from the eye of male Japanese white rabbit after sacrificing the animal by bleeding. Lenses were homogenized in 3 volumes of cold distilled water in a Teflon homogenizer and then centrifuged at $10,000\times g$ for 60 minutes to remove insoluble material. The supernatant was dialyzed overnight against 0.05 M sodium chloride. The dialyzed lens homogenate was used in the enzymatic reaction as a partially purified aldose reductase. All procedures for enzyme preparation were carried out at 4°C.

Fig. 1. The sorbitol pathway.

$$\text{D-Glucose}+\text{NADPH}+\text{H}^+ \xrightarrow[\text{Aldose reductase}]{} \text{Sorbitol}+\text{NADP}^+$$

$$\text{Sorbitol}+\text{NAD}^+ \xrightarrow[\text{Sorbitol dehydrogenase}]{} \text{D-Fructose}+\text{NADH}+\text{H}^+$$

Determination of Enzymatic Activity

Aldose reductase activity was measured according to the method of HAYMAN and KINOSHITA[6] with slight modification. Oxidation of NADPH, a cofactor for aldose reductase, to NADP was determined spectrophotometrically using an automatic reaction-rate analyzer LKB-8600 at 340 nm. In routine enzymatic assay, the reaction mixture contained 50 mM Na-phosphate buffer (pH 6.2), 0.125 mM NADPH, 400 mM lithium sulfate, enzyme solution and 3 mM *dl*-glyceraldehyde as a substrate in a total volume of 1 ml. The reaction was started by the addition of *dl*-glyceraldehyde and NADPH. The reaction-rate was measured for 2 minutes. In the kinetic study of Lineweaver-Burk plot, several substrate concentration was employed as described in results.

Fermentation

A loopful of *Chaetomella raphigera* No. 3681 on mature slant culture was transferred into 250-ml Erlenmeyer flasks containing 80 ml of the seed medium and inoculated at 28°C for 72 hours. A 200-liter jar fermentor containing 160 liters of production medium was inoculated with 1,600 ml of the seed broth and cultured at 28°C for 84 hours under aeration of 100 liters per minute and agitation of 300 rpm. The composition of the seed and production media are shown in Table 1.

Results

Identification of Strain No. 3681

The strain No. 3681 was originally isolated from a soil sample collected at Fukuoka-city, Fukuoka Prefecture, Japan. The strain was a sphaeropsidaceous fungus characterized by pycnidia with a raphe and some setae (Fig. 2). Its mycological characteristics were as follows.

Colonies on malt extract agar grew rapidly, attaining 7.0 cm in diameter after 2 weeks at 25°C. The colony surface was plane, cottony, yellowish brown to dark brown. Dark brown pycnidia and creamy sporodochia were produced on the surface. The reverse of the colony was dark yellowish brown to dark brown. Cultures on corn meal agar grew more rapidly than that on malt extract agar, attaining 7.5 cm in diameter under the same conditions. The surface of the colony was plane, thin and subhyaline to white. The reverse was the same. The pycnidia and sporodochia were abundantly formed as brownish black and creamy dots on agar surface.

The pycnidia of strain No. 3681 were superficial, ellipsoidal or reniform, with a raphe, 190 to 290 μm long and 100 to 190 μm thick. The raphe was composed of a narrow row of thin-walled cells bordered by thick-walled cells, and

Table 1. Media used for production of WF-3681.

Seed medium (%)		Production medium (%)	
Corn starch	2.0	Corn starch	3.0
Corn steep liquor	2.0	Corn steep liquor	3.0
Glucose	1.0	Glucose	2.0
Cotton seed flour	1.0	Peanut powder	1.0
Dry yeast	0.5	Dry yeast	0.5
$CaCo_3$	0.2	$CaCO_3$	0.2
pH adjusted to 6.0		pH adjusted to 6.0	

Fig. 2. Photograph of pycnidia and sporodochia of strain No. 3681 (scale: 200 μm).

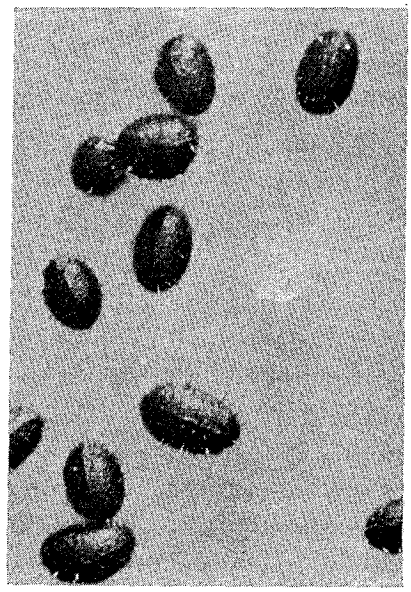


Fig. 3. Photograph of conidiophores and conidia of strain No. 3681 (scale: 20 μm).

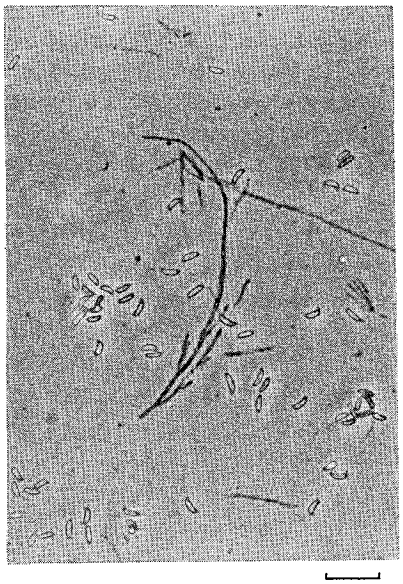


Fig. 4. Isolation procedure of WF-3681.

Fermentation broth
↓ filtered
Filtrate (120 liters)
↓ Diaion HP-20 column chromatography
eluted with MeOH
Concentrate
↓ washed with EtOAc (pH 9.0)
EtOAc extraction (pH 2.0)
Concentrate
↓ silica gel column chromatography
n-hexane - EtOAc (1 : 1)
Active fractions
↓ silica gel column chromatography
$CHCl_3$ - MeOH (50 : 1)
Active fractions
↓ crystallization from a mixture of ether - benzene
Crystal (1.6 g)

extending lengthwise over the upper part of the pycnidia. The setae were septate, brown, club-shaped, 30~60×3~4 μm in size, and beset with the upper part of pycnidia. The conidiophores were formed from inner pycnidial wall. They were hyaline, septate, filiform, irregularly branched, 20~40 μm long and 1.5~2 μm thick, and produced blastconidia from the apex or the side of cells (Fig. 3). The conidia were hyaline, one-celled, oblong to allantoid, sometimes having appendages at each end, and 6~8×2~2.5 μm. The cream-colored sporodochia were ampulliform to lageniform with setae at the upper point and produced conidial masses on their heads. The conidiophores, conidia and setae of sporodochia were the same morphs as that of pycnidia. The vegetative hyphae were septate, hyaline, smooth and branched. The hyphal cells were cylindrical and 2 to 6.5 μm thick. The chlamydospores were absent.

The strain WF-3681 grew at the temperature range from 9 to 39°C with the growth optimum at 27 to 31°C.

From above-mentioned characteristics and the description of STOLK (1963), we concluded that the strain No. 3681 belonged *Chaetomella raphigera* Swift, and named it *Chaetomella raphigera* No. 3681.

Isolation Procedure

The flow diagram of the isolation procedure described below is shown in Fig. 4.

The cultured broth was filtered with an aid of diatomaceous earth. The filtrate (120 liters) was then passed through a Diaion HP-20 column chromatography (20 liters) at pH 7.0 and the column was eluted with methanol (80 liters) after washing the column with water. The eluate was concentrated *in vacuo* to 8 liters of aqueous solution. The concentrate was washed with ethyl acetate (8 liters) at pH 9.0 and then extracted twice with 8-liter portions of ethyl acetate at pH 2.0. The extract was concentrated *in vacuo*. The resultant material was applied to a silica gel column chromatography (1 liter) and the column was eluted with a mixture of *n*-hexane - ethyl acetate (1 : 1). The fractions containing active material were combined and applied to a silica gel column chromatography

Fig. 5. IR spectrum of WF-3681 (Nujol).

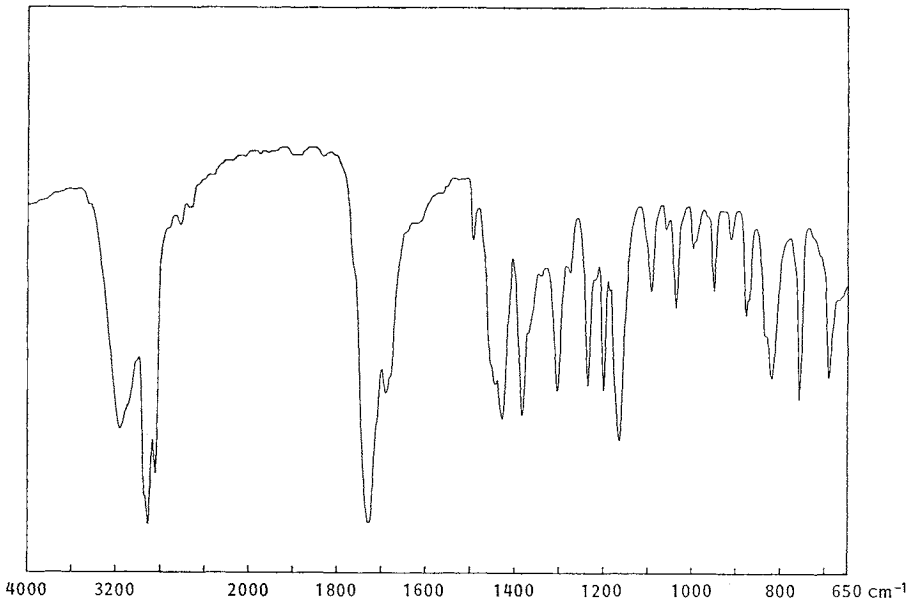
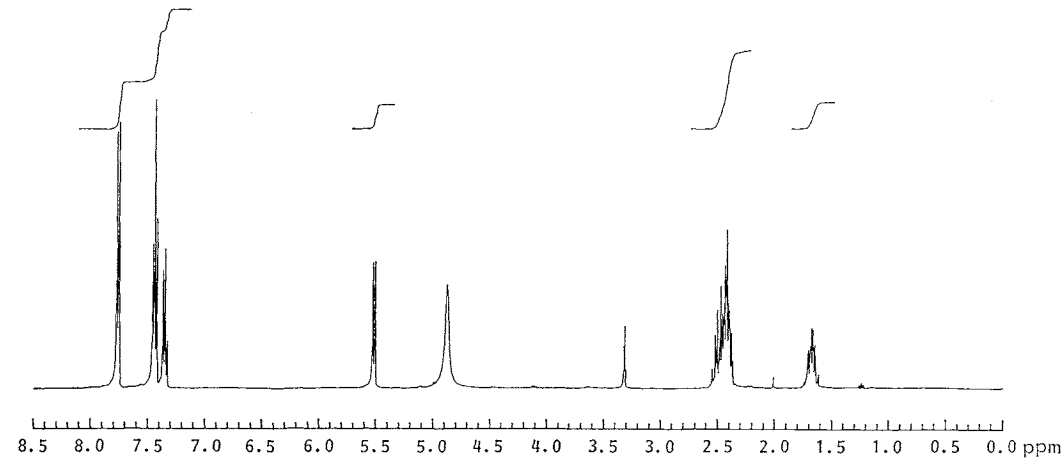


Fig. 6. ^{1}H NMR spectrum of WF-3681 (400 MHz, CD_3OD).



(0.3 liter) and eluted with a mixture of chloroform - methanol (50 : 1). The active fractions were dried *in vacuo* to give an oily material which was crystallized from a mixture of ether - benzene as colorless prisms. The yield of crystal was 1.6 g.

Physico-chemical Properties

IR and ^{1}H NMR spectra of WF-3681 are shown in Figs. 5 and 6. The other physico-chemical properties of WF-3681 are summarized in Table 2. WF-3681 is soluble in methanol, acetone, ethyl acetate, ether and insoluble in benzene, *n*-hexane and water. The color reactions of WF-3681 are positive in cerium sulfate, iodine vapor and ferric chloride and negative in ninhydrin, Molisch, Ehrlich and Dragendorff. Thin-layer chromatography was carried out on a silica gel sheet (Merck chromatogram sheet $60F_{254}$) using the solvent systems of chloroform - methanol (3 : 1) and *n*-hexane -

Table 2. Physico-chemical properties of WF-3681.

Appearance	Colorless prism
MP (°C)	177~179
$[\alpha]_D^{25}$ (*c* 1.0, EtOH)	0°
Molecular formula	$C_{13}H_{12}O_5$
Anal Found:	C 63.13, H 4.98.
Calcd:	C 62.90, H 4.87.
EI-MS obsd:	*m/z* 248.066
Calcd for $C_{13}H_{12}O_5$:	*m/z* 248.068
UV λ_{max}^{MeOH} nm (ε)	285 (17,900)
$\lambda_{max}^{MeOH-NaOH}$ nm (ε)	320 (14,900)

EI-MS: Electron impact mass spectrum.

Table 3. Inhibition of aldose reductase by WF-3681 and sorbinil.

Inhibitor	IC_{50} value (M)[a]
WF-3681	2.5×10^{-7}
Sorbinil[b]	4.2×10^{-7}

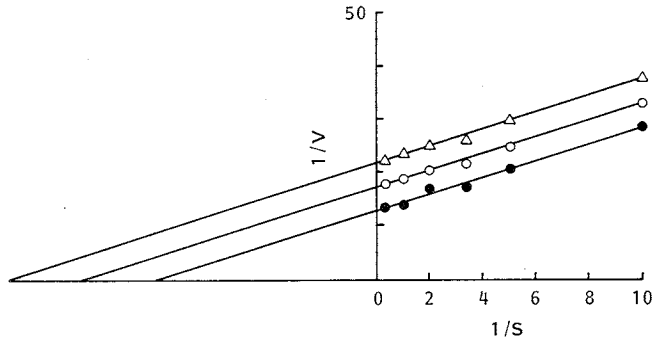
[a] Evaluated *in vitro* against rabbit lens aldose reductase.

[b] *S*-6-Fluorospirochroman-4,4′-imidazolidin-2′,5′-dione.

Fig. 7. Lineweaver-Burk plot of inhibition of rabbit lens aldose reductase by WF-3681.

The abscissa represents the reciprocal of glyceraldehyde concentration between 1×10^{-4} M and 3×10^{-3} M.

WF-3681 concentrations: ● Control, ○ 7.5×10^{-8} M, △ 2×10^{-7} M.



acetone - acetic acid (100 : 100 : 1). The Rf values of WF-3681 were 0.7 and 0.5, respectively. The chemical structure of WF-3681 will be described in succeeding papers[7].

Biological Properties

WF-3681 inhibited aldose reductase with an IC_{50} of 2.5×10^{-7} M as shown in Table 3. The kinetic study of WF-3681 was performed in Lineweaver-Burk plot for aldose reductase. The result is shown in Fig. 7. WF-3681 inhibited aldose reductase uncompetitively with *dl*-glyceraldehyde as substrate.

WF-3681 revealed no antimicrobial activity at the concentration of 5 mg/ml by pulp assay method against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Candida albicans*. LD_{50} of WF-3681 in *dd*Y mice was greater than 750 mg/kg ip.

Discussion

Aldose reductase is considered to play an important role in the development of diabetic cataract, neuropathy, retinopathy and possibly nephropathy.

Recently, several aldose reductase inhibitors[8], such as sorbinil[9,10], tolrestat and epalrestat[11], have been developed to the stage of clinical evaluation.

In the course of our screening program, we discovered WF-3681 as a potent aldose reductase inhibitor of microbial origin. WF-3681 inhibited partially purified rabbit lens aldose reductase with an IC_{50} of 2.5×10^{-7} M uncompetitively.

Chemical structure of WF-3681 was found to be a novel type as an aldose reductase inhibitor and its derivatives are now being assessed their therapeutic effect on the diabetic animal model. *In vivo* activities of WF-3681 and its analogues will be reported in due course.

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References

1) KINOSHITA, J. H.: Mechanism initiating cataract formation. Proctor Lecture Invest. Ophthalmol. 13: 713~724, 1974
2) GABBAY, K. H.: The sorbitol pathway and the complications of diabetes. N. Engl. J. Med. 288: 831~836, 1973
3) KUWABARA, T. & D. G. COGAN: Retinal vascular patterns VI. Mural cells of the retinal capillaries. Arch. Ophthalmol. 69: 492~502, 1963
4) AKAGI, Y.; P. F. KADOR, T. KUWABARA & J. H. KINOSHITA: Aldose reductase in human retinal mural cells. Invest. Ophthalmol. Visual Sci. 24: 1516~1519, 1983
5) CORDER, C. N.; J. H. BRAUGHLER & P. A. CULP: Quantitative histochemistry of the sorbitol pathway in glomeruli and small arteries of human diabetic kidney. Folia Histochem. Cytochem. 17: 137~146, 1979
6) HAYMAN, S. & J. H. KINOSHITA: Isolation and properties of lens aldose reductase. J. Biol. Chem. 240: 877~882, 1965
7) NAMIKI, T.; M. NISHIKAWA, Y. ITOH, I. UCHIDA & M. HASHIMOTO: Studies on WF-3681, a novel aldose reductase inhibitor. II. Structure determination and synthesis. J. Antibiotics 40: 1400~1407, 1987
8) KADOR, P. F.; W. G. ROBISON & J. H. KINOSHITA: The pharmacology of aldose reductase inhibitors. Annu. Rev. Pharmacol. Toxicol. 25: 691~714, 1985
9) JASPAN, J.; R. MASELLI, K. HEROLD & C. BARTKUS: Treatment of severely painful diabetic neuropathy with an aldose reductase inhibitor: Relief of pain and improved somatic and autonomic nerve function. Lancet 1983-II: 758~762, 1983
10) JUDZEWITSCH, R. G.; J. B. JASPAN, K. S. POLONSKY, C. R. WEINBERG, J. B. HALTER, E. HALAR, M. A. PFEIFER, C. VUKADINOVIC, L. BERNSTEIN, M. SCHNEIDER, K.-Y. LIANG, K. H. GABBAY, A. H. RUBENSTEIN & DANIEL PORTE, Jr.: Aldose reductase inhibition improves nerve conduction velocity in diabetic patients. N. Engl. J. Med. 308: 119~125, 1983
11) GOTO, Y.; N. OIKAWA, Y. AKANUMA & M. KIKUCHI: Clinical study on a new aldose reductase inhibitor (ONO-2235) in diabetic neuropathy. J. Jpn. Diabet. Soc. 28: 89~99, 1985