WF-2421†, A NEW ALDOSE REDUCTASE INHIBITOR PRODUCED FROM A FUNGUS, *Humicola grisea*

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WF-2421 is a novel aldose reductase inhibitor produced by *Humicola grisea*. WF-2421 was purified from the culture filtrate by successive ion exchange chromatography and the chemical structure was assigned to be α-formamido-5'-(2-formamido-1-hydroxyethyl)-β,2',6-trihydroxy-3-biphenylpropanoic acid (**1**) on the basis of spectroscopic evidence.

The IC_{50} value of WF-2421 was 3×10^{-8} M against partially purified aldose reductase of rabbit lens.

Aldose reductase (EC 1.1.1.21) converts glucose to sorbitol in various tissues under conditions of hyperglycemia such as diabetes mellitus. Elevated intracellular sorbitol levels lead to the development of diabetic complications *e.g.* cataract[1], neuropathy[2], retinopathy[3] and nephropathy[4]. It has been expected, therefore that inhibition of aldose reductase activity may provide a pharmacological approach to a treatment of these diabetic complications.

In the process of searching for a novel aldose reductase inhibitor from microbial products, we recently discovered WF-2421 which inhibited lens aldose reductase activity.

In this paper, we describe the taxonomic studies of the producing strain, fermentation, isolation and physico-chemical properties of WF-2421.

The aldose reductase inhibitory activity of WF-2421 is also described.

Materials and Methods

Assay of Aldose Reductase Activity

Aldose reductase activity was measured according to the method of HAYMAN and KINOSHITA[5]. Rabbit lenses obtained from male Japanese white rabbits were homogenized in 3 volumes of cold distilled water and then centrifuged at $10,000 \times g$ for 60 minutes to remove insoluble material. The supernatant was dialyzed against 0.05 M sodium chloride. The dialyzed lens homogenate was used in the enzymatic reaction. Oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to NADP was determined spectrophotometrically at 340 nm. In routine enzymatic assays, the reaction mixture contained 50 mM sodium phosphate buffer (pH 6.2), 0.125 mM NADPH, 400 mM lithium sulfate, enzyme solution and 3 mM DL-glyceraldehyde as the substrate in a total volume of 1.0 ml. The reaction was started by the addition of DL-glyceraldehyde and NADPH and the reaction rate was measured for 2 minutes. The effect of an enzyme inhibitor was determined by including a solution (10 μl) of the inhibitor in the reaction mixture.

† WF-2421 is identical with FR900280 (MURAI *et al.*: Jpn. Kokai 72,144 ('90), Mar. 12, 1990).

Table 1. Media used for production of WF-2421.

Seed medium (%)		Production medium (%)	
Glucose	0.5	Glucose	2.0
Corn starch	2.0	Soluble starch	1.5
Corn steep liquor	1.0	Cotton seed flour	0.25
Dry yeast	1.0	Gluten meal	0.25
Soybean meal	1.0	Dry yeast	0.25
pH adjusted to 7.0		Wheat germ	0.25
		$CaCO_3$	0.2
		$FeSO_4 \cdot 7H_2O$	0.025

Fermentation

A loopful of the slant culture of *Humicola grisea* No. 2421 was inoculated into 500-ml Erlenmeyer flasks containing 160 ml of the seed medium and incubated on a rotary shaker at 28°C for 72 hours. A 200-liter jar fermenter containing 160 liters of production medium was inoculated with 800 ml of the seed broth and cultured at 28°C for 72 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.

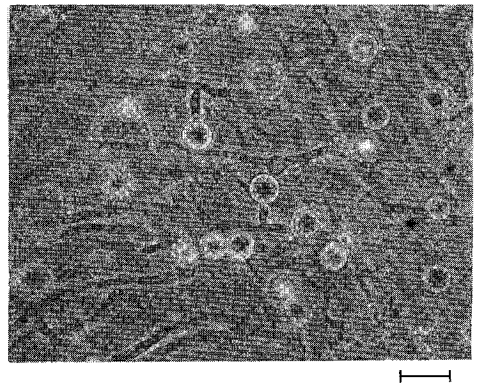
Results and Discussion

Characteristics of the Producing Strain

The strain No. 2421 was isolated from a solid sample collected at Tsuyama city, Okayama Prefecture, Japan.

On potato-glucose agar, this strain grew rapidly and formed white to gray colonies. Conidial structures were abundantly borne on the colony surface. Its conidiogenesis was holoblastic. Conidia were solitary, and produced directly on the vegetative hyphae or on short lateral branches (Fig. 1). They were smooth, dark brown, unicellular, thick-walled, globose or obovoid to pyriform, and $12 \sim 15\,\mu m$ i.d.

Fig. 1. Micrograph of conidial structures of *Humicola grisea* No. 2421. (scale: $10\,\mu m$).



This strain sometimes formed other condidial structures, consisting of phialides and phialoconidia. Phialides were mononematous, elongate, tapering at the top, and $8 \sim 16 \times 2 \sim 3\,\mu m$. Phialoconidia were hyalin, obovoid, and $2 \sim 3\,\mu m$ i.d. According to the taxonomic criteria,[6,7] we identified the strain as *H. grisea* Traaen 1914, on the basis of the above characteristics, and named it *H. grisea* No. 2421. The strain was deposited in the Fermentation Reserach Institute, Agency of Industrial Science and Technology, as FERM P-10135.

Isolation

The isolation procedure of WF-2421 is shown in Fig. 2. The cultured broth was filtered using diatomaceous earth as filter aid. The filtrate (150 liters) was passed through a column of activated carbon (Wako Pure Chemical Industries Ltd.) (20 liters) and the active principle adsorbed to the column was eluted with 80% aq acetone (60 liters). After concentration of the solution to 6 liters, the concentrate was

Fig. 2. Isolation procedure of WF-2421.

Fermentation broth
↓ filtered
Filtrate (150 liters)
↓ activated carbon-80% aq acetone
Concentrated
↓ DEAE-Sephadex A-25 (pH 6.5)-0.5 M NaCl
activated carbon-80% aq acetone
Concentrated
↓ silica gel-90% aq isopropyl alcohol
Active fractions
↓ CM-Sephadex C-25 (H^+)-water
pH was adjusted to 5.5 with NaOH
freeze-dry
White powder (24 g)

applied to a column of DEAE-Sephadex A-25 (1.2 liters) buffered with pH 6.5 phosphate buffer (1.2 liters). The column was washed with 0.1 M NaCl and then the active principle was eluted with 0.5 M NaCl (2 liters). The eluate was adsorbed on activated carbon and the active principle was eluted with 80% aq acetone. The desalted eluate was concentrated *in vacuo* to give an oily residue. The residue was mixed with silica gel (70~230 mesh) and applied to a silica gel column (0.5 liter) packed with 90% aq isopropyl alcohol and developed with the same solvent. The fractions containing the desired compound were combined and concentrated *in vacuo* to a volume of 80 ml.

This solution was chromatographed on a column of CM-Sephadex C-25 (H^+) (1.2 liters) and developed

Fig. 3. IR spectrum of WF-2421 (KBr).

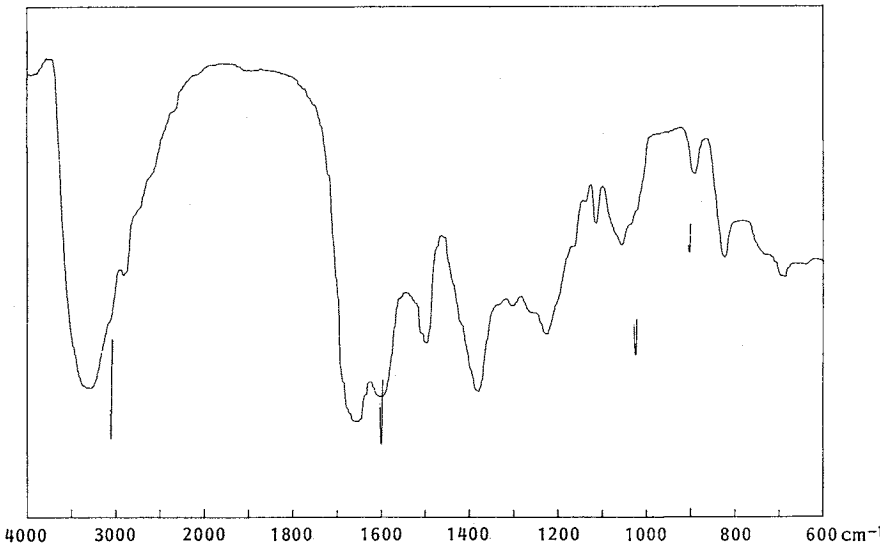
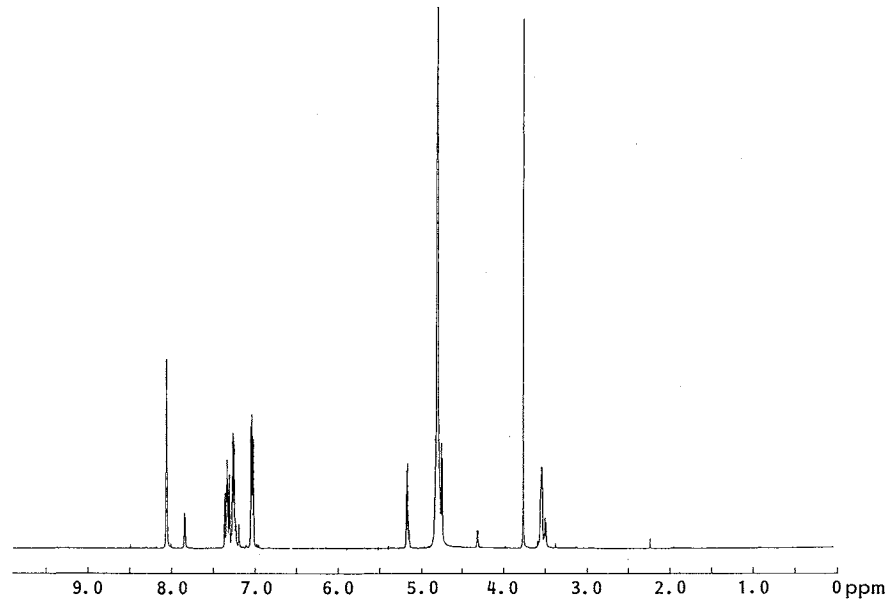


Fig. 4. ^1H NMR spectrum of WF-2421 (400 MHz, D_2O).



with water. The active fractions were adjusted to pH 5.5 with 1 N NaOH and lyophilized the sodium salt (24 g) in the form of a white powder.

Physico-chemical Properties

WF-2421 sodium salt is soluble in water and insoluble in acetone, chloroform and ethyl acetate.

Color reactions are as follows: Positive in cerium sulfate, Ehrlich and iodine vapor tests, negative in ninhydrin and Dragendorff tests. IR and ^{1}H NMR spectra of WF-2421 sodium salt are shown in Figs. 3 and 4, respectively. The other physico-chemical properties of WF-2421 sodium salt are summarized in Table 2. TLC was carried out on a silica gel sheet (Merck chromatogram sheet 60 F_{254}) using the solvent system of 90% aq isopropyl alcohol and isopropyl alcohol - acetic acid - water (18 : 1 : 1). The Rf values of WF-2421 sodium salt were 0.39 and 0.29, respectively.

Structure Elucidation

The molecular formula $C_{19}H_{19}N_2O_8Na$ for the sodium salt of WF-2421 was established by elemental analysis and mass spectrometry as shown in Table 2. The IR spectrum (Fig. 3) (1660 cm^{-1}) showed the presence of an amide group in the molecule. The ^{13}C NMR spectrum (Table 3) indicated that WF-2421 exists as an equilibrium mixture of two isomers in solution (*ca.* 5 : 1 in D_2O) and revealed the presence of 19 carbon signals which are assignable to one carboxylate (δ 178.34 (for the major isomer and so forth)), two formamides (δ 166.70 and 167.15), 12 aromatic carbons (δ 118.94 ~ 155.94), three methines (δ 76.26, 61.86 and 74.22), and one methylene (δ 47.32). The presence of the formamide groups was corroborated by the ^{1}H NMR spectrum (Fig. 4), which showed two formyl protons at δ 8.06 (s) and 8.04 (s) for the major isomer (δ 7.84 and 7.82 for the minor isomer)†. The ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY experiments revealed the presence of partial structures A, B, C, and D as shown in Fig. 5. The

Table 2. Physico-chemical properties of WF-2421 sodium salt.

Appearance	White powder
MP (dec)	220 (°C)
$[\alpha]_D^{25}$ (*c* 1.0, H_2O)	+32°
Molecular formula	$C_{19}H_{19}N_2O_8Na$
Elemental analysis	
Calcd for $C_{19}H_{19}N_2O_8Na$:	C 53.52, H 4.49, N 6.57, Na 5.39
Found:	C 53.00, H 4.63, N 6.39, Na 5.61
FAB-MS	*m/z* 427 $(M+Na)^+$
UV $\lambda_{max}^{H_2O}$ nm (ε)	282 (4,900)
λ_{max}^{HCl} nm	282
λ_{max}^{NaOH} nm	313 (6,870)

Table 3. ^{13}C NMR data for WF-2421.

Position	δ_C[a] (major)	Multiplicity	δ_C (minor)	$\Delta\delta=\delta_{major-minor}$	Position	δ_C[a] (major)	Multiplicity	δ_C (minor)	$\Delta\delta=\delta_{major-minor}$
1	127.96	Q	128.10	−0.14	1′	128.28	Q	128.38	−0.10
2	132.74	CH	132.95	−0.21	2′	155.78	Q	Unidentified	—
3	134.21	Q	133.82	0.39	3′	119.13	CH	Unidentified	—
4	130.64	CH	130.79	−0.15	4′	130.11	CH	Unidentified	—
5	118.94	CH	Unidentified	—	5′	135.95	Q	135.53	0.42
6	155.94	Q	Unidentified	—	6′	132.36	CH	Unidentified	—
7	76.26	CH	76.41	−0.15	7′	74.22	CH	75.06	−0.84
8	61.86	CH	65.99	−4.13	8′	47.32	CH_2	51.46	−4.14
8-NHCHO	166.70	CH	169.84	−3.14	8′-NHCHO	167.15	CH	170.69	−3.54
8-COOH	178.34	Q	178.04	0.30					

[a] 100 MHz in D_2O.

† In the ^{1}H NMR spectrum of *N*-formyl-L-tyrosine, the formly proton of the major isomer resonated at δ 8.04, while the corresponding proton of the minor isomer was observed at δ 7.61 (ratio, *ca.* 9 : 1 in D_2O).

Fig. 5. Partial structures A, B, C and D for WF-2421 (1).

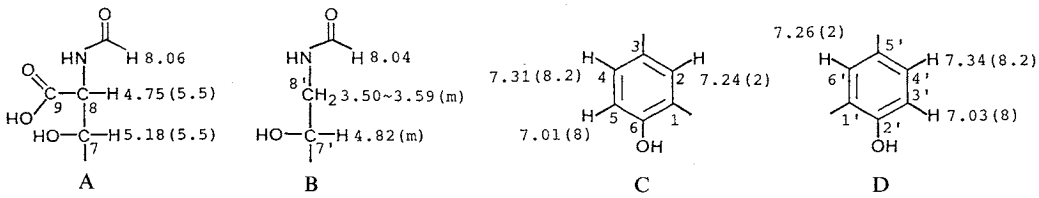


Fig. 6. Relationships of NOE's and ^{13}C-^{1}H long-range couplings in **I** and **II**.

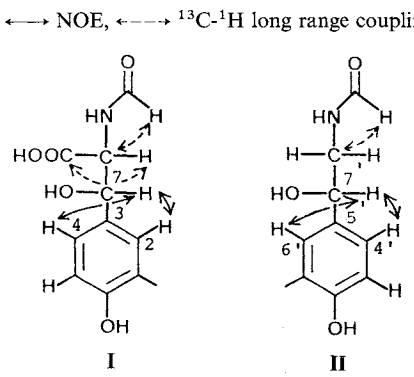


Table 4. Inhibition of aldose reductase by WF-2421 and sorbinil.

Inhibitor	IC_{50} value (M)[a]
WF-2421	3.0×10^{-8}
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.

^{1}H-^{1}H spin coupling relationships are shown in these structures.

The connection of these structural elements was investigated by using the correlation *via* long range coupling (COLOC) technique which showed long-range couplings between one of the formyl protons (δ 8.06) and C-8, 8-H and C-9, and 7-H and C-3 as shown in Fig. 6 leading to partial structure **I**.

Scheme 1. Tentative structure for WF-2421.

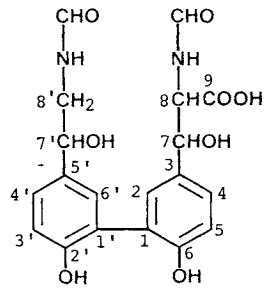
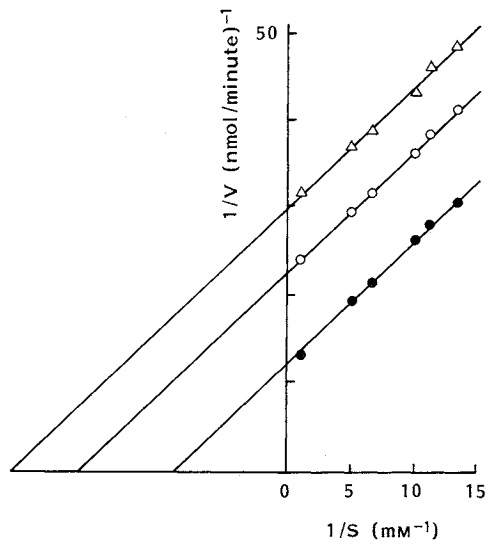


Fig. 7. Lineweaver-Burk plot of inhibition of rabbit lens aldose reductase by WF-2421.

WF-2421 concentrations: ● No inhibitor, ○ 2.3×10^{-8} M, △ 6.9×10^{-8} M.



The COLOC experiment further revealed couplings of the other formyl H (δ 8.04) to C-8' and of 7'-H to C-5', indicating the presence of partial structure **II**. The connection of A and C through C-3 and C-7 was further corroborated by the NOESY spectrum, which revealed NOE's between 7-H and 4-H, and between 7-H and 2-H. The spectrum also showed NOE's between 7'-H and 4'-H, and between 7'-H and 6'-H, confirming the connection of B and D through C-5' and C-7'.

The above spectral evidence thus indicates that the partial structures **I** and **II** are bonded to each

other through C-1 and C-1′, leading to **1** for the full structure of WF-2421†.

In comparison of the ^{13}C NMR signals of the major and minor isomers, the most significant differences in chemical shift were observed at C-8 and the 8-formamide C in partial structure **I**, and at C-8′ and the 8′-formamide C in partial structure **II**.

In the major isomer, C-8 and C-8′ resonated by 4.13~4.14 ppm to higher fields than those in the minor isomer, while the 8- and 8′-formamide carbons were also observed by 3.14~3.54 ppm to higher fields. These facts suggest that in the major isomer the amide bonds are in *cis* configuration[8].

Biological Properties

WF-2421 sodium salt inhibited aldose reductase in a dose dependent manner and its IC_{50} value was 3.0×10^{-8} M (Table 4). The IC_{50} value for sorbinil was 4.2×10^{-7} M in the same experiment.

The kinetic study of WF-2421 sodium salt was performed using a Lineweaver-Burk plot for aldose reductase inhibition. As shown in Fig. 7. WF-2421 inhibited aldose reductase uncompetitively with *dl*-glyceraldehyde as substrate.

WF-2421 sodium salt revealed no antimicrobial activity at the concentration of 1 mg/ml by the pulp assay method against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Trichophyton asteroides* and *Candida albicans*.

The LD_{50} value of WF-2421 sodium salt in *dd*Y (male, 8 weeks old) mice was greater than 1 g/kg iv.

Experimental

FAB-MS spectra were obtained with a VG ZAB-SE. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were measured at ambient temperature on a Bruker AM400wb and chemical shifts are given in ppm referenced to 3-(trimethylsilyl)propionic acid-d_4 sodium salt (TSP) (δ_H 0.0 and δ_C 0.0) as internal standard. NOESY spectra (phase-sensitive mode) were obtained with a Bruker AM500. Pulse programs of the standard Bruker software library were used for a series of 2D NMR experiments.

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† The high-field shifted aromatic carbons, C-5 and C-3′, are located at *ortho*-position of the respective phenol -OH. Starting from unambiguous assignment of 5-H and 3′-H, the other three biphenyl substitution patterns 4,4′; 3,4′; 4,5′ could be excluded.