VOL. 56 NO. 2, FEB. 2003 THE JOURNAL OF ANTIBIOTICS pp. 173 — 176

Asperaldin, a New Aldose Reductase Inhibitor from *Aspergillus niger* CFR-1046

I. Fermentation, Isolation and Characterization

K. C. SEKHAR RAO, S. DIVAKAR, M. SRINIVAS, K. NAVEEN BABU, N. G. KARANTH and A. P. SATTUR*

Fermentation Technology and Bioengineering Department, Central Food Technological Research Institute, Mysore 5700013, India

(Received for publication September 2, 2002)

Aldose reductase (EC 1.1.1.21) catalyzes the conversion of glucose to sorbitol and promotes the accumulation of sorbitol in various tissues under the condition of hyperglycemia such as diabetes mellitus. The accumulated intracellular sorbitol causes development of diabetic complications such as cataracts, neuropathy, retinopathy and nephropathy[1]. 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. It has been reported that inhibitors of aldose reductase reduce the tissue sorbitol content in diabetic animals and are useful as therapeutic agents for diabetic complications[2].

In our screening programme on bioactive molecules through the fermentation route[3~7], we screened 15 different strains of *Aspergillus* sp. for potential inhibitors against rat lens aldose reductase (RLAR). We found that, *Aspergillus niger* CFR-1046 produced a compound, termed by us as asperaldin, which exhibits potent inhibitory activity against RLAR. The present paper describes the fermentation, isolation, physico-chemical properties and biological activities of asperaldin.

The organism used in this study *Aspergillus niger* CFR 1046 was obtained from CFTRI culture collection. Submerged fermentation was carried out in a potato dextrose (soluble starch 0.4% and glucose 2%) medium. A portion of the mature agar slant was inoculated into 100 ml of potato dextrose broth in a 500 ml Erlenmeyer flask and incubated at 30°C on a rotary shaker at 250 rpm for 8 days. The fermented broth (3 liters) was treated with ethyl acetate (2 liters) for two hours followed by cheesecloth filtration to remove the biomass; the organic extract was separated and dried over anhydrous sodium sulfate and concentrated *in vacuo* to yield a crude solid (0.42 g). The residue was subjected to silica gel column chromatography. Elution of the crude extract was carried out using hexane, chloroform, ethyl acetate and methanol in different combinations. Two bed volumes were taken as a fraction. The fractions were analyzed by qualitative TLC (benzene - methanol, 3 : 2) and enzyme assay according to the method described by HAYMAN & KINOSHITA 1965. The active compound was eluted as a deep yellow band in the chloroform - ethylacetate (9 : 1) and the solvent was evaporated *in vacuo* to obtain 25 mg of amorphous red powder. The compound was redissolved in ethyl acetate and washed with 5% sodium bicarbonate solution. The carbonate washings were collected, acidified and reextracted into ethyl acetate to give 15 mg of red compound which was further purified by preparative TLC using benzene - methanol - acetic acid (3 : 2 : 0.1) as the mobile phase.

Asperaldin is an amorphous red powder. It is soluble in methanol, ethyl acetate, dimethyl sulphoxide, diethyl ether, sodium bicarbonate, sodium carbonate and sodium hydroxide, slightly soluble in chloroform and hexane, but insoluble in water. The Physico-chemical properties are summarized in Table 1. The EI-MS spectra of the compound showed the molecular ions at *m/z* 205, based on the mass spectra the molecular formula of compound was established as $C_{16}H_{18}O_5$.

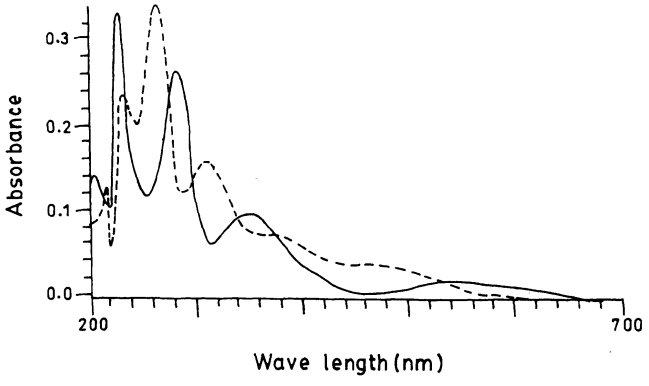
The compound exhibits UV absorption at 232 nm, 265 nm and 310 nm at pH 3.8. However at pH 9.0, while the λ_{max} at 232 nm showed a hypochromic shift to 226 nm both the 265 nm peak and 310 nm peak showed a bathochromic shift to 286 nm and 355 nm respectively (Figure 1). This indicates that there are ionizable phenolic groups on aromatic rings. The broad IR absorption at 3437 cm^{-1} indicates –OH stretching frequencies. The peak at 2995 cm^{-1}, 2912 cm^{-1} indicate aromatic C–H stretching. A broad ketonic absorption band at 1660 cm^{-1} indicate probably hydrogen bonded keto group. The structure was further confirmed by 2DHMQCT and GC-EIMS. The corresponding nuclear magnetic resonence signals are; ^1H NMR (500 MHz, DMSO-d_6) δ 0.8 (16 CH_3), 1.0 (11 CH_2), 1.2 (12, 13, 14 CH_2), 1.5 (15 CH_2), 6.0 (H-7), 6.5 (H-3), 6.95 (H-5). ^{13}C NMR (500 MHz, DMSO-d_6) δ 29.9[a] (16

* Corresponding author: ferm@cscftri.ren.nic.in

Table. 1. Physico-chemical properties of asperaldin.

Appearance	red amorphous powder
Melting Point	decomposes at 170 - 175 ^{0}C
UV Spectrum	
λ_{max} nm (ε) at pH 3.8	232 (8,800), 265 (12,700), 310 (5,900)
λ_{max} nm (ε) at pH 9.0	226 (21,600), 286 (15,400), 355 (5,800)
IR	3437, 2995, 2912 and 1660 cm^{-1}
Molecular formula	$C_{16}H_{18}O_5$
EI-MS m/z	205 ^{M} (290 – 85) [GC-EIMS]
HPLC (Rt)	4.5 min (column: RP-C18, 25 X 4.6mm, mobile phase: methanol, 0.5ml/min, 265 nm)
GC (Rt)	21.4 min (column: HP-5, Conditions: 50^{0}C, 2 min, 10^{0}C/min; 220^{0}C, 2min, 15^{0}C/min; 270^{0}C, 20min.)
TLC (Rf)	0.5 (normal phase silica gel plate, benzene-methanol-acetic acid (3:2:0.1) as mobile phase.)

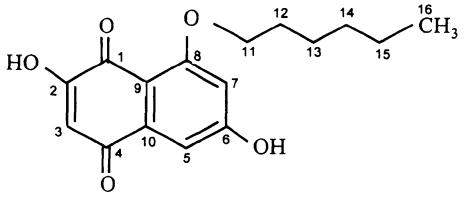
Fig. 1. UV Spectrum of asperaldin at pH 3.8 (– – –) and at pH 9.0 (——).



CH_3), 52.2 (11 OCH_2), 39.9 to 40.9 (–CH_2), 111.1 (H-7), 108.9 (H-3), 108.2 (H-5), 190.83[a] (4-CO), 181.7[a] (1-CO), 164.4[a] (6-C_{Ar}-OH), 163.6[a] (2-C_{Ar}-OH), 160.9[a] (8-C_{Ar}-O) [[a]Carbon-13 assignments are interchangeable]. ^{1}H NMR (500 MHz, methanol-d_4) δ 0.95 (16-CH_3), 1.31 to 1.37 (11-, 12-, 13-, 14-CH_2), 1.67 (15-CH_2), 6.55 (6-OH), 7.06 (7-H), 7.65 (3-H), 7.74 (5-H).

An aliphatic group corresponding to six carbons was observed at 0.8 (CH_3) and 1.0 to 1.5 (5-CH_2 groups) ppm. One of the –CH_2 (1.0 ppm) might have come from –OCH_2 group of hexyl moiety being subjected to ring current effect of the aromatic group present. This was confirmed by recording the ^{1}H NMR spectrum in a second solvent namely d_4-methanol, where the signal at 1.0 ppm was not detected. It might have probably merged with the –CH_2 envelope at 1.31 to 1.67 ppm. Three separate single signals were seen at 6.0, 6.5 and 6.95 ppm, indicating aromatic protons. EI-MS analysis showed a parent peak at 205 (M^+) and other fragment peaks like 178 (M^+-CO), 150 (178–CO), 108 and 81 clearly indicating the aromatic group of the molecule. However a parent ion peak was distinctly missing. Based on EI-MS and 2DHMQCT analysis the proposed structure is shown in Figure 2 and chemically

Fig. 2. Chemical structure of asperaldin.



named as 2,6-dihydroxy-8-hexyl-oxy-1,4-naphthaquinone.

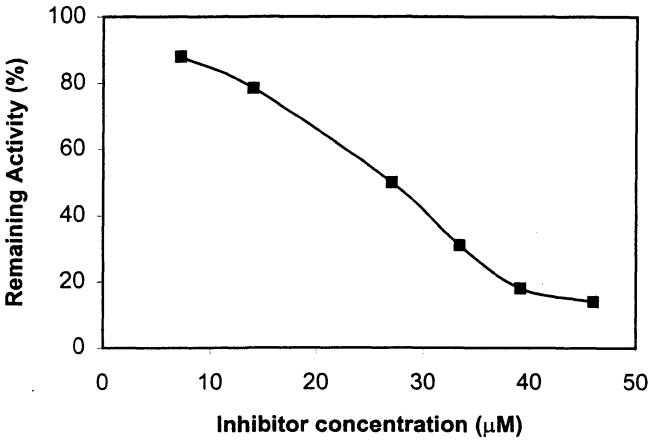
The isolated compound exhibited a dose-dependent aldose reductase inhibition at an IC_{50} of 27 μM (Figure 3). Due to the novel inhibitory nature and new structure, the compound is henceforth designated as asperaldin. Asperaldin compares better with reported RLAR inhibitors such as YUA001, produced by *Corynebacterium* sp. YUA-25, which has an IC_{50} of 1.8 mM[9,10], and is moderate when compared to thiazocin A, produced by *Actinosynnema* sp. c-304, which has an IC_{50} of 0.45 μM[11]. Other RLAR inhibitors, salfredins[12], thermorubins and 2-hydroxy phenyl acetic acid[13] have also been reported. In contrast, chemically synthesized tolrestat, alrestatin and sorbinil have IC_{50} of 0.035 μM, 2.7 μM and 1.5 μM, respectively[14].

Naphthaquinones have a versatile range of biological properties, including antifungal and antibacterial activites[15~19]. Asperaldin was also tested for inhibition activity against commerecial soybean 15-lipoxygenase (LOX-1)[20], commercial porcine pancreas lipase (PPL)[21], crude rat brain acetylcholinesterase (AChE)[22] and crude rat lens aldose reductase[8]. Interestingly, asperaldin upto 300 μM did not show any inhibition against LOX-1, PPL and AChE. Also, asperaldin revealed no antimicrobial property upto a concentration of 200 μg by disk plate method against *Bacillus subtilis*, *B. pumilis*, *E. coli*, *Pseudomonas aeruginosa*, *Penicillium notatum*, *Aspergillus niger*, *Saccharomyces cerevisiae* and *Candida utilis*.

Acknowledgements

The authors thank the Director, CFTRI, Mysore, for providing facilities and encouragement. K. C. SEKHAR RAO and K. NAVEEN BABU are thankful to the Council of Scientific and Industrial Research, India, for the award of Senior Research Fellowship.

Fig. 3. Concentration dependent inhibition of rat lens aldose reductase inhibitor using asperaldin.



References

1) WILLIAMSON, J.; C. KILO & R. G. TILTON: Mechanisms of glucose and diabetes-induced vascular dysfunction. *In* Hyperglycemia, diabetes and vascular disease. pp. 107~132, *Eds.* N. RUDERMAN, J. WILLIAMSON & M. BROWNLEE: American physiological society, New York, 1992
2) KADOR, P. F.; W. G. ROBINSON & J. H. KINOSHITA: The pharmacology of aldose reductase inhibitors. Ann. Rev. Pharmacol. Toxicol. 25: 691~714, 1985
3) RAO, K. C. S.; S. DIVAKAR, N. G. KARANTH & A. P. SATTUR: 14-(2′,3′,5′-Trihydroxyphenyl) tetradecan-2-ol, a novel acetylcholinesterase inhibitor from *Chrysosporium* sp. J. Antibiotics 54: 848~849, 2001
4) RAO, K. C. S.; S. DIVAKAR, A. G. A. RAO, N. G. KARANTH & A. P. SATTUR: A lipoxygenase inhibitor from *Aspergillus niger*. Appl. Microbiol. Biotechnol. 58: 539~542, 2002
5) RAO, K. C. S.; S. DIVAKAR, A. G. A. RAO, N. G. KARANTH & A. P. SATTUR: A lipoxygenase inhibitor from *Lactobacillus casei*. Biotechnol. Lett. 24: 511~513, 2002
6) RAO, K. C. S.; S. DIVAKAR, A. G. A. RAO, N. G. KARANTH, W. J. SUNEETA, T. P. KRISHNAKANTHA & A. P. SATTUR: Asperenone: a 15-lipoxygenase inhibitor with human platelet aggregation inhibition from *Aspergillus niger*. Biotechnol. Lett. 24: 1967~1970, 2002
7) RAO, K. C. S.; S. DIVAKAR, K. N. BABU, A. G. A. RAO, N. G. KARANTH & A. P. SATTUR: Nigerloxin, a novel inhibitor of aldose reductase and lipoxygenase with free radical scavenging activity from *Aspergillus niger* CFR-W-105. J. Antibiotics 55: 789~793, 2002
8) HAYMAN, S. & J. H. KINOSHITA: Isolation and properties of lens aldose reductase. J. Biol. Chem. 240: 877~882, 1965
9) BAHN, Y.; J. PARK, D. BAI, TAKASE & J. YU: YUA001, a novel aldose reductase inhibitor isolated from

alkalophilic *Corynebacterium* sp. YUA25. J. Antibiotics 51: 902~907, 1998

10) SUN, W.; H. LEE, J. PARK, S. KIM, J. YU & J. KIM: YUA001, a novel aldose reductase inhibitor isolated from alkalophilic *Corynebacterium* sp. YUA25. II. Chemical modification and biological activity. J. Antibiotics 54: 827~830, 2001
11) OZASA, T.; T. YONEDA, M. HIRASAWA, K. SUZUKI, K. TANAKA, S. KADOTA & M. IWANAMI: Thiozocins, New aldose reductase inihibitors from *Actinosynnema* sp. J. Antibiotics 44: 768~773, 1991
12) MATSUMOTO, K.; K. NAGASHIMA, T. KAMIGAUCHI, Y. KAWAMURA, Y. YASUDA, K. ISHII, N. UOTANI, T. SATO, H. NAKAI, Y. TERUI, J. KIKUCHI, Y. IKENISI, T. YASHIDA, T. KATO & H. ITAZAKI: Salfredins, new aldose reductase inhibitors produced by *Crucibulum* sp. RF-3817. I. Fermentation, isolation and structures of salfredins. J. Antibiotics 48: 439~446, 1995
13) HAYASHI, K.; M. DOMBOU, M. SEKIYA, H. NAKAJIMA, T. FUJITA & M NAKAYAMA: Thermorubin and 2-hydroxyphenyl acetic acid, aldose reductase inhibitors. J. Antibiotics 48: 1345~1346, 1995
14) SHOWALTER, H. D. H.; J. L. JOHNSON, L. M. WERBEL, W. R. LEOPOLD, R. C. JACKSON & E. F. ELSLAGER: Tolrestat, a potent, orally active aldose reductase inhibitor. J. Med. Chem. 27: 255~256, 1984
15) FUKUDA, D. S.; J. S. MYNDERSE, P. J. BAKER, D. M. BERRY, L. D. BOECK, R. C. YAO, F. P. MERTZ, W. M. NAKATSUKASA, J. MABE & J. OTT: A80915, a new antibiotic complex produced by *Streptomyces aculeolatus*. Discovery, taxonomy, fermentation, isolation, characterization, and antibacterial evaluation. J. Antibiotics 43: 623~633, 1990
16) AMMAR, M. S.; N. N. GERBER & L. E. MCDANIEL: New antibiotic pigments related to fusarubin from *Fusarium solani* (Mart.) Sacc. I. Fermentation, isolation, and antimicrobial activities. J. Antibiotics 32: 679~684, 1979
17) RAO, K. V.; K. SREERAMULU, D. GUNASEKAR & D. RAMESH: Two new sesquiterpene lactones from *Ceiba pentandra*. J. Nat. Prod. 56: 2041~2045, 1993
18) KADKOL, M. V.; K. S. GOPALKRISHNAN & N. NARASIMHACHARI: Isolation and characterization of naphthaquinone pigments from *Torula herbarum* (Pers.). Herbarin and dehydroherbarin. J. Antibiotics 24: 245~248, 1971
19) BURR, R. G.: Inhibition of urease by miscellaneous ions and compounds. Implications for the therapy of infection-induced urolithiasis. Invest. Urol. 15: 180~182, 1977
20) AXELROD, B.; T. M. CHEESBROUGH & S. LAAKSO: Lipoxygenase from soybeans. Methods Enzymol. 71: 441~451, 1981
21) QUINN D, M.; K. SHIRAI, R. L. JAKSON & J. K. HARMONY: Lipoprotein lipase catalyzed hydrolysis of water soluble *p*-nitrophenyl esters. Inhibition by apolipoprotein C-II. Biochemistry 21: 6872~6879, 1982
22) ELLMAN, G. L.; K. D. COURTNEY, V. ANDRES, JR. & R. M. FEATHERSTONE: A new and rapid colourimetric determination of acetylcholinesterase activity. Biochemical Pharmacol. 7: 88~95, 1961