VOL. 55 NO. 9, SEPT. 2002 THE JOURNAL OF ANTIBIOTICS pp. 789 — 793

Nigerloxin, a Novel Inhibitor of Aldose Reductase and Lipoxygenase with Free Radical Scavenging Activity from *Aspergillus niger* CFR-W-105

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(Received for publication March 25, 2002)

An enzyme inhibitor, nigerloxin, with inhibition against soy bean lipoxygenase-I (LOX-1), rat lens aldose reductase (RLAR) as well as free radical scavenging activity was isolated from the fermented wheat bran using *Aspergillus niger* CFR-W-105. Its chemical structure was identified as 2-amido-3-hydroxy-6-methoxy-5-methyl-4-(prop-1′-enyl) benzoic acid by NMR and GCEIMS data. The IC_{50} values against LOX-1 and RLAR were found to be 79 μM and 69 μM and ED_{50} against 1,1-diphenyl-2-picrylhydrazyl (DPPH) was 66 μM.

Lipoxygenases are a family of non-heme iron-containing dioxygenases distributed widely throughout the plant and animal kingdoms. They catalyze the oxygenation of polyunsaturated fatty acids, containing the *cis,cis*-1,4-pentadiene moiety.[1] Mammalian lipoxygenases have been implicated in the pathogenesis of several anti-inflammatory conditions such as, asthma mad rheumatoid arthritis.[2,3] These enzymes are designated as 5-, 12-, 15-LOX on the basis of their ability to oxygenate arachidonic acid at carbons 5, 12 and 15 respectively. In plants, lipoxygenases favor germination and participate in the synthesis of traumatin and jasmonic acid and in the response to abiotic stress.[4] Soybean (*Glycine max*), a rich source of lipoxygenases in plants, has three isoenzymes, LOX-1, LOX-2 and LOX-3 which have optimum pH values of 9.0, 6.1 and 6.5 respectively.[5] LOX-1, which is the dominant component among the three isoenzymes, catalyzes the addition of molecular oxygen to unsaturated fatty acids such as arachidonic acid at the 15th carbon. Inhibitors against these enzymes are hence called 15-LOX inhibitors. This enzyme has been implicated in the causation of food deterioration. Hence, inhibitors against this enzyme have a great potential in the preservation of food. Soybean lipoxygenase-1 (LOX-1) widely used as a prototype for studying the homologous family of lipoxygenases from tissues of different species, both in structural and in kinetic investigations.[6~8] Moreover, Allgayer *et al* have reported that, an enzyme inhibition assay of soybean lipoxygenase can be used as an *in vitro* biochemical model for the anti-inflammatory action of certain drugs used in the treatment of ulcerative colitis.[9]

In a search for novel enzyme inhibitors from fungi,[10,11] we screened various *Aspergillus* strains, grown on potato dextrose broth and wheat bran, for lipoxygenase inhibitors, using LOX-1 as the target enzyme. Crude ethyl acetate extracts of these cultures were tested for inhibition. We found one of the cultures, *Aspergillus niger* CFR-W-105 produce an efficient inhibitor of LOX-1 when grown on wheat bran as a substrate. Isolation of the active component was achieved by enzyme assay-guided purification to yield a novel metabolite, nigerloxin. This report describes the fermentation, isolation, chemical structure and inhibitory activity against LOX-1 by nigerloxin.

Experimental

General Experimental Procedures

IR absorption spectra were obtained with a Perkin Elmer model 2000 Infrared Fourier-transform spectrophotometer

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using an attenuated total reflectance cell on neat 2 μg samples. UV absorption spectra were measured with a Spectronic Genesys-5 spectrophotometer.

NMR spectra were recorded at 500 MHz on a Brüker DRX-500 MHz spectrometer (500.13 MHz proton and 125 MHz carbon frequencies) at 27°C. Proton and carbon 90° pulse width were 11.2 and 8.8 μs respectively. About 10 mg of the solid sample dissolved in DMSO-d_6 was used for recording the spectra.

Two-dimentional heteronuclear multiple quantum coherence transfer spectra (2DHMQCT) were recorded in magnitude mode with the sinusoidal shaped z gradients of strength 25.7, 15.42 and 20.56 G/cm in the ratio of 5 : 3 : 4 were applied for duration of 1 ms each with a gradient recovery delay of 100 μs to defocus unwanted coherences. The t_1 was incremented in 256 steps. The size of the computer memory used to accumulate the 2D data was 4K. The spectra were processed using unshifted and π/4 shifted sine bell window function in F1 and F2 dimensions respectively.

GCEIMS data was generated using VG Auto Spec M mass spectrometer equipped with HP 5890 series II gas chromatograph under GC/EIMS conditions. For GC, an HP-5 capillary column was used with the following temperature program: 80°C, 5 minutes; 10°C/minute; 220°C, 15 minutes. Injection temperature was at 275°C. For MS conditions, the source temperature was 25°C at an electrical energy of 70 eV and a trap current of 200 μl.

Fungal Material, Fermentation and Extraction

Seed Culture

Aspergillus niger CFR-W-105 was propagated on Potato Dextrose Agar (Hi Media Mumbai, India) slant comprising soluble starch 0.4% and glucose 2%. After incubation for 4 days at 30°C, a portion of the mature agar slant was inoculated into 100 ml of a seed liquid medium of the same medium composition in a 500 ml Erlenmeyer flask and incubated at 30°C on a rotary shaker at 250 rpm.

Flask Fermentation

A 5-ml of the seed culture was transferred into 250-ml Erlenmeyer flasks each containing 10 g of wheat bran, 10 ml of 0.2 N HCl comprising 2.1 mg each of ferrous sulfate, zinc sulfate and copper sulfate and 5 ml distilled water. The inoculated flasks were incubated for 5 days at 30°C.

Soybean Lipoxygenase Assay

Soybean lipoxygenase enzyme was assayed by monitoring the appearance of conjugated diene at 234 nm.[5] The fraction containing the inhibitor was dissolved in a minimum quantity of DMSO, which was found to have no effect on enzyme activity at less than 0.1% concentration. For purification of the inhibitor, a crude enzyme preparation (0.14 Units/mg protein) was used. For dose dependency experiments using the purified inhibitor and caffeic acid, the assay was conducted using purified Lipoxygenase-1 from the Hardee variety of soybean.[5] The semi-purified enzyme which was used in the present study had a specific activity of 33 units/mg of protein. The assay was initiated by the addition of the enzyme to the reaction mixture. The enzyme concentration was determined by measuring the absorbance at 280 nm and using a value of 0.7 mg of protein/ml per absorbance unit.[12] One unit of the enzyme activity is defined as the amount of the enzyme required forming 1 μmol of the product per minute at 30°C under the assay conditions.[5] Inhibition was expressed as a percentage relative to solvent control. All activities were carried out in duplicates and the average has been reported. The relative activity was expressed as percentage ratio of enzyme activity in the presence of inhibitors to the enzyme activity in the absence of enzyme inhibitors at the end of 3 minutes of enzyme reaction time.

Rat Lens Aldose Reductase (RLAR) Assay

Lens were collected from rat eyes and homogenized with sodium, potassium phosphate buffer (0.135 M, pH 7.0) containing 0.5 mM of phenyl methyl sulphonyl fluoride and 10 mM of β-mercaptoethanol. The homogenate was centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant was taken as enzyme source. Enzyme reaction was carried out at 25°C in a quartz cuvette with a 1 cm light path. The assay mixture contained 0.32 mM nicotinamide adenine dinucleotide phosphate tetra sodium salt, 5.5 mM DL-glyceraldehyde, 10 μl of inhibitor dissolved in dimethyl sulphoxide and sodium-potassium-phosphate buffer (0.135 M, pH 7.0).[13] The reaction was initiated by the addition of the enzyme solution (0.083 units/ml homogenate) and monitored by the decrease in absorbance at 340 nm. One unit of the enzyme activity is defined as the amount of the enzyme required forming 1 μmol of the product per minute at 30°C under the assay conditions. Inhibition was expressed as a percentage relative to solvent control. All activities were carried out in duplicates and the average has been reported. The relative activity was expressed as percentage ratio of enzyme activity in the presence of inhibitors to the enzyme activity in the absence of enzyme inhibitors at the end of 3 minutes of enzyme reaction time.

Free Radical Scavenging Activity

An ethanolic solution of the inhibitor sample (2 ml) was mixed with a 0.5 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) ethanol solution (1 ml) and 0.1 M acetate buffer (pH 5.5; 2 ml). After standing for 30 minutes, the absorbance of the mixture at 517 nm was measured.[14] The ED_{50} value was determined as the concentration of each sample required to give 50% of the absorbance shown by a blank test.

Isolation and Purification

At the end of the fermentation, the wheat bran (450 g) was treated with ethyl acetate (1 liter) for two hours. The organic extract was separated from the wheat bran by cheesecloth filtration. This extract was dried over anhydrous sodium sulfate and concentrated *in vacuo* to yield a crude solid (1.36 gm). The residue was resuspended in chloroform (25 ml) and gently centrifuged (2000 rpm, 20 minutes, 27°C). The residue was dried to afford an orange colored active fraction (230 mg). This was dissolved in ethanol (50 ml) by gentle warming and treated with activated charcoal (200 mg) filtered (Whatman No. 1) while warm. The filtrate was concentrated under vacuum to obtain 30 mg of yellow amorphous powder. Structure determination was accomplished using this compound.

Results and Discussion

Physico-chemical Properties of Inhibitor

Obtained as yellow amorphous powder. It is soluble in ethanol, methanol, ethyl acetate, dimethyl sulphoxide, sodium bicarbonate, sodium carbonate and sodium hydroxide solutions, slightly soluble in chloroform and hexane, but insoluble in water. Decomposes at 253°C; λ_{max} nm (ε) in methanol: 235 (20,700), 292 (11,600), 358 (4,400); IR ν_{max} 3499 cm^{-1}, 1657 cm^{-1}, 2994 cm^{-1}; The molecular formula of the compound was calculated as $C_{13}H_{15}NO_5$, based on the mass spectra and ^1H and ^{13}C NMR spectra. Nuclear Magnetic Resonance Spectroscopy; ^1H NMR (500 MHz, DMSO-d_6) δ 2.04 (3H, d, 6.6, CH_3–CH=CH–), 6.61 (1H, dq, 16.4, 6.9, CH_3–CH=C), 6.69 (1H, d, 16.4, HC=CH-Ar), 2.02 (S) (3H, S, Ar-CH_3), 3.43 (S) (3H, S, Ar-OCH_3), 10.3 (Ar-OH), 11.5 (Ar-COOH). ^{13}C NMR (500 MHz, DMSO-d_6) δ 19.5 (CH_3), 119.4 (=CH), 133.7 (=CH), 32.7 (C_{Ar}–CH_3), 114.6 (C_{Ar}–CH_3), 163.4 (C_{Ar}), 166.0[b] (=C–C_{Ar}), 159.2[b] (–COOH), 147.1[a] (C_{Ar}–O–CH_3), 145.3[a] (C_{Ar}–OH), 168.6 ($CONH_2$) [[a,b] assignments are interchangeable]. Mass Spectrometry; EI-MS (*m/z*): 265 [M^+, 0%], 263 [M^+-2H, 60%], 235 [M^+-28 (CO), 45%], 207 [235−28 (CH_3–CH=), 30%], 163 (207−44 (CO_2), 49%], 161 [100%], 99 [45%], 81 [37%].[15]

Structure Determination

The compound exhibited UV absorption maxima at 235, 292 and 358 nm, of these the 292 nm band corresponds to the π–π^* transition of phenolic absorption. The 358 nm band indicates extended conjugation of the phenolic ring. The broad IR absorption at 3499 cm^{-1} indicates OH/NH2 stretching frequencies. Similarly the 1657 cm^{-1} band indicates the carbonyl (–CO–) stretching frequency of an acid. The –CH– aromatic stretching is indicated by the 2994 cm^{-1} band. Further confirmation of the structure was obtained from 2DHMQCT. The signals at 2.04 (doublet) and 2.02 (singlet) ppm indicates CH_3 attached to an olefinic double bond and an aromatic ring, respectively. The olefinic protons at 6.61 and 6.69 which corresponds to only two protons possess characteristic splitting of an olefinic group attached to an aromatic ring and a methyl group. The signal at 6.69 ppm which is a doublet with 16.4 Hz coupling constant indicates the *trans* proton of an olifinic group. Correspondingly the signal at 6.61 ppm (doublet of a quartet) with 16.4 and 6.90 Hz coupling indicate that the trans proton is coupled to the olefinic proton at 6.61 ppm and to a $–CH_3$ group attached to the same carbon. These characteristics indicate the presence of a CH_3CH=CH– group. The presence of olefinic double bond was further confirmed by decoloration of bromine-water. A singlet at 3.43 ppm indicate a $–OCH_3$ group. A –OH signal at 10.3 ppm and carboxyl proton at 11.5 ppm indicates that these groups are present on the aromatic ring. The Lassaigne's, sodium test indicate the presence of nitrogen which is probably in the form of an amide, which was further confirmed by detecting the evolution of ammonia, upon heating with sodium hydroxide solution. 2DHMQCT spectra also gave corresponding carbon signals wherever protons were attached. The structure of compound, arrived from spectroscopic data, was further confirmed by GC-EIMS analysis. A parent ion peak at 263 (M^+-2H) was observed. Other fragmented peaks like 235 [M^+−(CO)], 207 (235–CH_3CH=), 163 (207–COO^-), 161, 99 and 81 also confirmed the proposed structure of Inhibitor **1**. Based on this data the proposed structure of compound is given as Figure 1 and chemically named as 2-amido-3-hydroxy-6-methoxy-5-methyl-4-(prop-1′-enyl) benzoic acid.

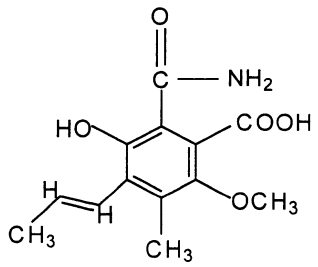
An HPLC analysis of **1** shows a retention time of 19 minutes in a RP-C18 column with a mobile phase of 0.01 M sodium phosphate buffer, pH 8.0 operating at a flow rate of

1 ml/minute and the UV detector fixed at 235 nm.

The compound was tested for inhibition against purified LOX-1. It showed a dose-dependent inhibition against the enzyme and an IC_{50} of 79 μM. This compares well with a standard LOX-1 inhibitor, caffeic acid.[16] which shows an IC_{50} of 207 μM (Figure 2). Due to the novel inhibitory nature and new structure, the compound is henceforth designated as nigerloxin.

As some lipoxygenase inhibitors have been reported to possess free radical scavenging activity,[17] nigerloxin was also tested for the scavenging ability against DPPH. The results indicate that this inhibitor shows an ED_{50} of 66 μM as against standard antioxidants, butyl-hydroxy-anisole (BHA) and butyl-hydroxy-toluene (BHT), which possess an ED_{50} of 8.8 and 42 μM, respectively.

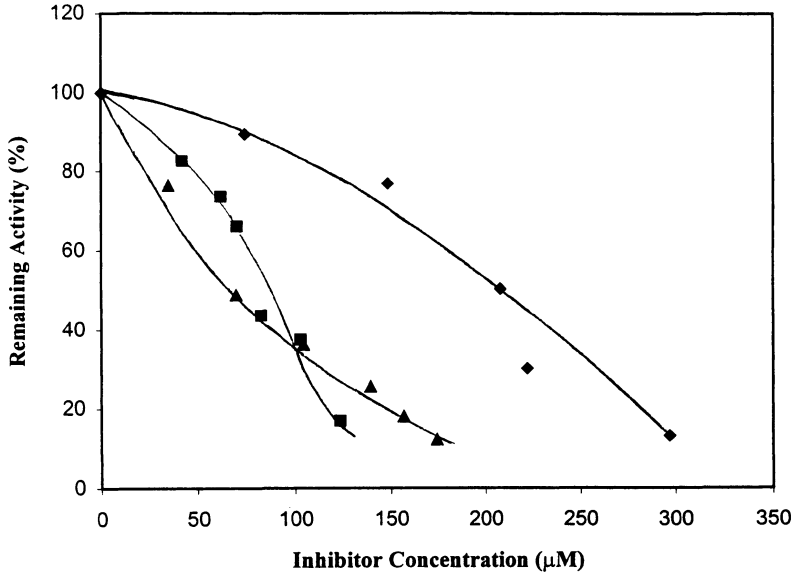
Fig. 1. Chemical structure of **1** (nigerloxin).



This purified inhibitor was also tested against other mammalian enzymes such as rat brain acetylcholine esterase, porcine pancreatic lipase and RLAR.[18~20] Aldose reductase (EC 1.1.1.21) catalyzes the conversion of glucose to sorbitol and causes 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.[21~25] 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. Interestingly, nigerloxin did not show any inhibition against lipase and brain acetylcholine esterase but exhibited a dose-dependent aldose reductase inhibition at an IC_{50} of 69 μM (Figure 2). This compares well with reported RLAR inhibitors such as YUA001, produced by *Corynebacterium* sp. YUA-25, which has an IC_{50} of 1.8 mM,[26] and is moderate when compared to thiazocin A, produced by *Actinosynnema* sp. c-304, which has an IC_{50} of 0.45 μM.[27]

Nigerloxin, the novel metabolite from *Aspergillus niger* shows potent activity against two dissimilar enzymes, LOX-1 and RLAR. This dual active inhibitor shows interesting possibilities of utilizing the same functional groups to develop more powerful analogues against these two enzymes and a free radical scavenger.

Fig. 2. Concentration dependent inhibition of purified LOX-1 from soybean using nigerloxin from *Aspergillus niger* CFR-W-105 (—■—), caffeic acid (—◆—) and inhibition against rat lens aldose reductase (RLAR) by nigerloxin (—▲—).



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.

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