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Microbial transformations of S-naproxen by *Aspergillus niger* ATCC 9142

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Aspergillus niger ATCC 9142 was used to catalyze the biotransformation of S(−)-naproxen (**1**) to three major metabolites that were isolated by solvent extraction, purified chromatographically, and characterized by mass spectrometry and NMR spectroscopy. Metabolites were identified as O-desmethylnaproxen (**2**), 7-hydroxynaproxen (**3**) and 7-hydroxy-O-desmethylnaproxen (**4**). The kinetics of naproxen biotransformation to **2** and **4** was established over an 84 h period to show that naproxen was completely metabolized at 36 h, the major metabolite was O-desmethylnaproxen at 24 h, and the 7-hydroxy-O-desmethylnaproxen that was formed after 24 h.

1. Introduction

The nonsteroidal anti-inflammatory drug (NSAID), naproxen, (S)-6-methoxy-α-methyl-2-naphthylacetic acid (**1**), has been widely prescribed for treatment of painful and inflammatory rheumatic and nonrheumatic conditions. It is primarily metabolized in humans and rats by direct acyl glucuronidation to form naproxen acyl glucuronide and by O-dealkylation to produce 6-O-desmethylnaproxen (**2**). Desmethylnaproxen is further metabolized to acyl glucuronide and sulfate conjugates [1, 2]. Structures of minor metabolites known as hydroxynaproxen and hydroxydesmethylnaproxen have not yet been identified [3].

Microbial models were first proposed as adjuncts to the study mammalian drug metabolism in our laboratory [4]. Microorganisms contain nearly every type of phase-I and phase-II metabolic system, thus permitting biotransformations of drug substances that very closely parallel drug metabolism observed in mammals. One of the advantages of the microbial models approach is the potential for obtaining relatively large quantities of drug metabolites via fermentation scale-up techniques. The availability of drug metabolites in sizable amounts enables the direct identification of metabolite structures and provides analytical standards of value in searching for similar metabolites in mammals. The value and use of microbial models in mammalian metabolism was extensively reviewed in this journal in work describing microbial transformation of **1** by *Cunninghamella elegans* [5]. *C. elegans* afforded the known mammalian metabolite, 6-O-desmethylnaproxen (**2**).

Aspergillus niger ATCC 9142 catalyzes a wide array of interesting metabolic transformation reactions including aromatic hydroxylation [6], reduction of carboxylic acids [7], and O-demethylations [8]. In connection with our interests in the metabolic capabilities of *A. niger*, we conducted biotransformations of naproxen and obtained three different phenolic metabolites. We describe the formation, isolation and characterization of these compounds.

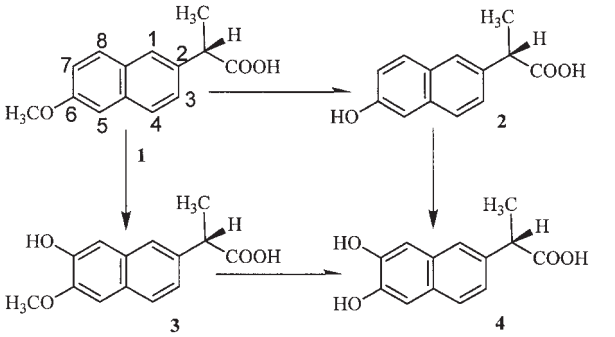
2. Investigations, results and discussion

Microbial reductions of aromatic carboxylic acids are widely observed in bacteria [9–11] and fungi [3, 12]. Species of *Nocardia* [9–10] and *Aspergillus* [3] contain enzyme systems that require ATP, Mg^{++} and NADPH as cofactors to catalyze reductions of carboxylic acids to their corresponding aldehydes, and subsequently to alcohols [13–14]. Ibuprofen was efficiently reduced to aldehyde and alcohol metabolites in stereoselective fashion by a species of *Nocardia* [15].

Screening studies using growing cultures of *A. niger* revealed that this microorganism reproducibly transformed naproxen to metabolites, albeit in variable and low yields. Cultures containing naproxen often produced dark particulate matter assumed to contain polymerization products of possibly oxygenated naproxen metabolites. Growing *A. niger* cultures also produced relatively large quantities of organic acids driving incubation media to pH 3, a condition deleterious to oxidative biotransformations. Resting cell suspensions of *A. niger* cells in pH 6.5, 0.1 M phosphate buffer containing glucose as an energy source were used to control incubation pH, and to produce metabolites in good yield. Three metabolites, none of which were the products of carboxylic acid reduction were chromatographically observed, isolated and characterized from 36 h old, preparative-scale incubations. We report the identification of O-desmethylnaproxen (**2**) as the major known metabolite, and two previously unknown naproxen metabolites, 7-hydroxynaproxen (**3**) and 7-hydroxy-O-desmethylnaproxen (**4**).

Metabolite **2** was obtained in 30% yield. The high resolution EIMS of **2** gave a molecular ion at *m/z* 216.0776 for

Scheme 1



$C_{13}H_{12}O_3$, 14 mass units less than that for naproxen. A base fragment peak of *m/z* 171 $(M\text{-}COOH)^+$ in EIMS indicated the presence of an intact carboxyl moiety. ^1H NMR spectroscopy showed that the δ 3.88 signal for $O-CH_3$ in naproxen was absent in **2**. These results indicated that **2** was 6-O-desmethylnaproxen, the known major phase I metabolite in humans [16–18] and a known naproxen microbial metabolite obtained from *Cunninghamella elegans* [5] (Scheme).

Metabolite **3** was obtained in approximately 12% yield. High resolution EIMS of **3** gave a molecular ion at *m/z* 246.0890 for $C_{14}H_{14}O_4$, for a metabolite that contained one additional oxygen atom vs. naproxen. The ^1H NMR spectrum of **3** contained all signals of naproxen, except that the AB system typically observed for protons at positions 7 and 8 was replaced by a new singlet signal at δ 7.18 for H-8. The presence of a singlet signal at δ 6.81 for H-5 ruled out the presence of a C-5 hydroxyl group, and confirmed that the metabolite was 7-hydroxynaproxen (**3**).

Metabolite **4** was obtained in 10% yield. The high resolution EIMS of **4** gave a molecular ion at *m/z* 232.0736 $C_{13}H_{12}O_4$, for a metabolite formed by loss of CH_3 and by the addition of a hydroxyl group. As with **3**, metabolite **4** contained singlet signals for protons at H5 (δ 6.85) and H-8 (δ 7.22) indicating the same oxygenation pattern for both metabolites. The absence of the methyl-group signal in **4** observed at δ 3.88 in the ^1H NMR spectrum of naproxen indicated that structure of metabolite **4** was 7-hydroxy-O-desmethylnaproxen.

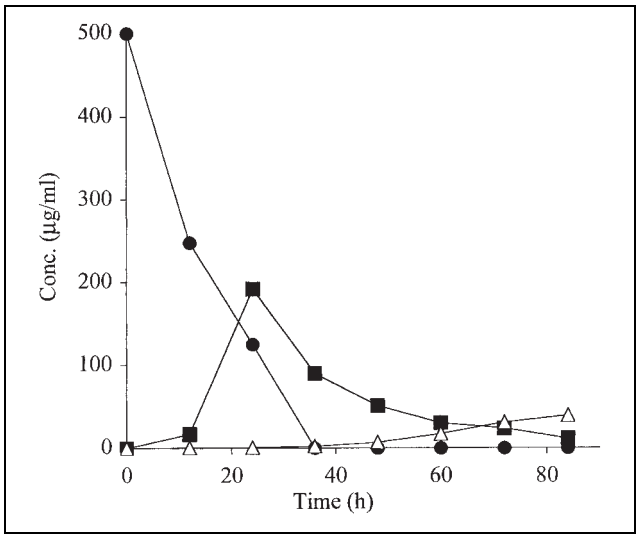


Fig: Rate of *A. niger* biotransformation of naproxen (●) to O-desmethylnaproxen (■) and 7-hydroxy-O-desmethylnaproxen (□)

Using resting cell incubations of *A. niger* in buffer, and characterized metabolites as analytical standards, rates of naproxen biotransformation to metabolites **2**, and **4** were examined versus time (Fig.). Naproxen was rapidly transformed being completely consumed by 36 h. O-Desmethylnaproxen (**2**) was formed within 12 h, and reached maximum levels of approximately 200 μg/ml (40% yield) in 24 h after which time, **2** was gradually consumed. 7-Hydroxy-O-desmethylnaproxen (**4**) was formed after 24 h gradually increasing to a maximum level of 40 μg/ml as **2** was consumed. In this experiment, only traces of **3** were obtained. In preparative scale naproxen biotransformations, yields of the minor metabolites were inexplicably variable requiring separate preparative-scale reactions to obtain sufficient 7-hydroxy-O-desmethylnaproxen for characterization.

A. niger has been widely exploited in the biotransformation of other types of substrates including steroids [19], terpenes [20] and alkaloids [21]. Hydroxylation is a common reaction catalyzed by CYP-450 enzyme systems in this organism [6]. *A. niger* also reduces ketones to alcohols, as was observed in the reduction of the 6′-ketone of zearalenone [22]. Unlike microbial transformations of ibuprofen by *Nocardia*, *A. niger* failed to reduce the naproxen COOH moiety. As in mammals, O-demethylation was the major metabolic transformation reaction of naproxen with *A. niger.*

3. Experimental

3.1. General

Melting points were determined in open capillary tubes with a Mel-Temp apparatus (Laboratories devices) and were uncorrected. Optical rotations at the sodium D-line were measured with a JASCO P-1020 digital polarimeter. High-resolution mass spectra were obtained by using a ZAB-HF mass spectrometer (VG Analytical Inc.). EIMS was performed at an ionization voltage of 70 ev. For direct inlet probe analysis, the probe temperature was set at 30 °C for 1 min, raised to 300 °C at 150 °C/min, and held at 300 °C for 10 min for analysis. NMR spectra were obtained with a Bruker WM 360-MHz high-field spectrometer equipped with an IBM Aspect-2000 processor. Tetramethylsilane was used as the internal standard. Chemical shifts are reported in parts per million (δ) and the coupling constants (J values) are reported in Hertz. Abbreviations for NMR spectra are: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; q, quartet.

3.2. Chromatography

TLC analyses were carried out on silica gel GF_{254} plates (E. Merck, Darmstadt, Germany). Developed chromatograms were directly visualized under 254 nm UV light to observe fluorescence quenching. Plates were then sprayed with phosphomolybdic acid visualization reagent (12 g phosphomolybdic acid in 250 ml ethanol) before being heated with a heat gun to develop colors from brown to dark green. With methylene chloride-methanol-formic acid (95 : 5 : 1 [v/v/v]), R_f values for naproxen (**1**), O-desmethylnaproxen (**2**), 7-hydroxynaproxen (**3**) and 7-hydroxy-O-desmethylnaproxen (**4**) were 0.42, 0.34, 0.31 and 0.15, respectively. CC was carried out with silica gel (Baker 3404; 40 μm) flash chromatography packing. HPLC was performed with a Shimadzu SCL-6B system controller, two LC-6A pumps, and a variable-wavelength UV-detector. For quantitation of naproxen biotransformations, separations were carried out under isocratic conditions over an Econosil C_{18} column (250 mm × 4.6 mm, 10 μm, Alltech, Deerfield, IL) with a mixture of methanol-water-formic acid (48 : 52 : 0.2 [v/v/v]) at a flow rate of 1 ml/min. Standard curves of various substrates were prepared with naproxen and its metabolites purified from *A. niger* preparative biotransformations. Eluted peaks were detected at 254 nm and retention times for **1**, **2** and **4** were 31.2, 9.6 and 6.1 min, respectively.

3.3. Chemicals

S(−)-Naproxen sodium was purchased from Sigma Chemical Co. (St. Louis, MO).

3.4. Incubation procedures

A. niger ATCC 9142 is maintained in the University of Iowa, College of Pharmacy culture collection and is grown and maintained on slants of Po-

tato Dextrose agar. Cultures were grown by our standard two-stage fermentation protocol [23] in 200 ml of medium held in stainless-steel capped 1 l DeLong culture flasks. The medium contained (w/vol) 2% Dextrose, 1% sucrose, 1% beef extract, 0.5% peptone, 0.2% yeast extract, 0.3% NaCl, 0.6% K_2HPO_4, 0.1% $MgSO_4 \cdot 7\,H_2O$ and 0.1% corn starch in double distilled H_2O and was adjusted to pH 7.0 with 6N HCl before being autoclaved at 121 °C for 20 min. Cultures were incubated by shaking at 250 rpm at 28 °C on an Innova 5000 Gyrotory Tier Shaker (New Brunswick Scientific, Edison, NJ). A 10% inoculum derived from a 72-h-old stage I culture was used to initiate stage II cultures, which were incubated as described above. Cultures were harvested by passing through 4-folds of cheesecloth after 24 h incubation in stage II. Cells were washed three times before use.

3.5. Biotransformation of naproxen with A. niger resting cells

For analytical reactions, 1.5 g of *A. niger* cells (wet weight) were suspended in 25 ml of pH 6.5, 0.1 M phosphate buffer containing 1% dextrose, and naproxen was added to a final concentration of 0.5 mg/ml of culture medium. The substrate-containing cell suspension was incubated with shaking as above. Samples (1 ml) of substrate-containing cultures were removed, at various time intervals, adjusted to pH 2 with 6N HCl and extracted with an equal volume of ethyl acetate. The organic and aqueous layers were separated by centrifugation for 3 min in a desktop centrifuge, the organic layer was removed, evaporated to dryness, redissolved in 0.5 ml of methanol, and spotted on TLC plates for analysis.

For preparative scale biotransformation, 9 g of cells (wet weight) were suspended in 200 ml of pH 6.5, 0.1 M phosphate buffer containing 1% dextrose and 0.5 mg/ml of S(−)-naproxen sodium. The cell suspension was incubated with shaking at 250 rpm at 28 °C for 36 h, at which time the reaction was stopped, and the suspension was filtered through cheesecloth. The filtrate was acidified to pH 2.0 with 6 N HCl and then extracted three times each with equal volumes of ethyl acetate. The organic layers were combined and concentrated by rotary evaporation to obtain about 200 mg of viscous residue. For purification of naproxen metabolites, the viscous residue was resolved over a 6 g silica gel column that had been slurry packed in n-hexane by elution with 150 ml of n-hexane-ethyl acetate-formic acid (90 : 10 : 0.025 [vol/vol/vol]). A second 4 g silica gel column slurry packed with methylene chloride and eluted with 100 ml of a mixture of methylene chloride−methanol−formic acid (98 : 2 : 1 [v/v/v]) (100 ml) was used to separate O-desmethylnaproxen (**2**) (32 mg) and 7-hydroxynaproxen (**3**) (12 mg) from a 55 mg combined fraction from the first column. 7-Hydroxy-O-desmethylnaproxen (10 mg) was obtained from a separate preparative scale reaction of *A. niger* with naproxen by eluting a 6 g silica gel column with 200 ml of a mixture of n-hexane-ethyl acetate-formic acid (86 : 14 : 0.04 [v/v/v]).

O-Desmethylnaproxen (**2**): Colorless needles (methanol), m.p. 186–188 °C. $[\alpha]_D^{25}$ 60° [c 1 methanol], high resolution EIMS *m/z* 216.0776 for $C_{13}H_{12}O_3$ (calculated 216.0786), low resolution EIMS, *m/z* (percent relative abundance), 216 (36.45% M^+), 171 (100% $[-COOH]^+$), 360 MHz (CD_3COCD_3): δ_H 1.51 (3 H, d, J = 7.2 Hz $-C\underline{H}_3$), 3.37 (1 H, q, J = 7.2 Hz $-C\underline{H}-$), 7.04 (1 H, dd, J = 2.5, 8.6 Hz, H-7), 7.06 (1 H, d, J = 2.5 Hz, H-5), 7.34 (1 H, d, J = 8.3 Hz, H-3), 7.59 (1 H, d, J = 8.3 Hz, H-4), 7.63 (1 H, s, H-1), 7.66 (1 H, d, J = 8.6 Hz, H-8). These data were in agreement with literature values [5, 22].

7-Hydroxynaproxen (**3**): Brown amorphous solid, $[\alpha]_D^{25}$ 60° [c 0.25 methanol], high resolution EIMS *m/z* 246.0890 for $C_{14}H_{14}O_4$ (calculated 246.0892), low resolution EIMS, *m/z* (percent relative abundance), 246 (43.85% M^+), 231 (100% $[M-COOH]^+$), 360 MHz (CD_3OD): δ_H 1.48 (3 H, d, J = 7.2 Hz $-C\underline{H}_3$), 3.73 (1 H, q, J = 7.2 Hz $-C\underline{H}-$), 3.88 (3 H, s, $-OCH_3$), 6.81 (1 H, s, H-5), 7.06 (1 H, dd, J = 2.5, 9.0 Hz, H-3), 7.18 (1 H, s, H-8), 7.46 (1 H, d, J = 2.5 Hz, H-1), 7.62 (1 H, d, J = 9.0 Hz, H-4).

7-Hydroxy-O-desmethylnaproxen (**4**): Brown-orange oil, $[\alpha]_D^{25}$ 65° [c 0.2 methanol], high resolution EIMS *m/z* 232.0736 for $C_{13}H_{12}O_4$ (calculated 232.0736), low resolution EIMS, *m/z* (percent relative abundance), 232 (65.60% M^+), 187 (100% $[-COOH]^+$), 360 MHz (CD_3COCD_3): δ_H 1.45 (3 H, d, J = 7.2 Hz $-C\underline{H}_3$), 3.74 (1 H, q, J = 7.2 Hz $-C\underline{H}-$), 6.85 (1 H, s, H-5), 7.09 (1 H, dd, J = 2.4, 9.0 Hz, H-3), 7.22 (1 H, s, H-8), 7.51 (1 H, d, J = 2.4 Hz, H-1), 7.62 (1 H, d, J = 9.0 Hz, H-4).

3.6. Rates of biotransformation of naproxen by resting cells

Triplicate 1.5 g suspensions of *A. niger* cells in 25 ml of pH 6.5, 0.1 M phosphate buffer containing 1% dextrose and 0.5 mg/ml naproxen sodium were incubated with shaking at 250 rpm at 28 °C for up to 84 h. Whole culture samples (1 ml) were taken at various time intervals, 10 µl of 6 N HCl and 1 ml methanol were added before pelleting cells by centrifugation at 20,000 × g for 5 min in a Microfuge. The resulting supernatants were removed, subjected to 0.1 microfiltration, and 20 µl samples of the microfiltrates were injected for HPLC analysis.

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