Oxidation of meloxicam by *Streptomyces griseus*

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Abstract
The aim of the present investigation was to biotransform the anti-inflammatory compound meloxicam by enzymes present in whole cells of five actinomycete cultures to produce novel bioactive derivatives. Among the actinomycetes screened, *Streptomyces griseus* NCIM 2622 was found to possess the enzyme system(s) that oxidize meloxicam into two metabolites whereas that present in *S. griseus* NCIM 2623 could oxidize meloxicam to only one metabolite in significant quantities. The formation of enzymatic metabolites was monitored and confirmed by high-performance liquid chromatography (HPLC) analysis. The structures were elucidated based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) data and previous reports as 5-hydroxymethyl meloxicam and 5-carboxy meloxicam. From the results obtained in this study, it can be concluded that *S. griseus* NCIM 2622 possesses oxidizing enzymes, which can be employed to oxidize meloxicam.

***Keywords*:** Biotransformation; Meloxicam; 5-hydroxymethyl meloxicam; 5-carboxy meloxicam *Streptomyces griseus*

INTRODUCTION

Biotransformations are the preferred pathways for the hydroxylation of aliphatic alicyclic, aromatic and heterocyclic compounds, particularly at positions different from pre-existing positions and inaccessible sites that are difficult by chemical means. The ability of microorganisms to convert alicyclic compounds into related alcohols by regio- and stereo-controlled hydroxylation at positions distinct from region- and stereo- directing functional groups was used extensively in the modification of steroids (Devis *et al*., 1989). Hydroxylation of progesterone with *Rhizopus* sp. or *Aspergillus* sp. furnished the oxidized product forming a key step in a highly efficient pathway towards the formation of anti-inflammatory steroids such as Betnovate (Price and Roberts, 1985). Other complex alicyclic natural products and closely related compounds e.g. taxanes (Hu *et al*., 1996; 1997) have been selectively hydroxylated using some of the more easily handled organisms such as *Mucor* sp., *Absidia* sp. and *Cunninghamella* sp. The hydroxylation of aromatic compounds using microorganisms is more predictable and a number of processes have been adapted to large scale, for example the preparation of 6-hydroxynicotinic acid (Lonza, 1992; Torimura *et al*., 1998) and (R)-2-(4-hydroxy-phenoxy) propanoic acid (Dingler *et al*., 1996) that are important intermediates to pesticides and herbicides, respectively.

Bioconversion processes involve enzymatic or microbial biocatalysts; when compared to their chemical counterparts, offer the advantages of high selectivity and mild operating conditions. Use of biocatalysts also minimizes the problems of isomerization, racemization, epimerization, and rearrangement that are common in chemical processes (Patel, 2000). From a pharmaceutical point of view, hydroxylations and glycosylations (Alarcon *et al*., 2005; Azerad 1999) are considered to be particularly useful bioconversions. They can yield new drugs and existing drugs can be improved with regard to increased activity and decreased toxicity. Side-effects can be reduced and the

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stability increased by modification of the parent drug.

Meloxicam is a non-steroidal anti-inflammatory drug (NSAID) effective in the treatment of rheumatoid arthritis (Reginster *et al*., 1996), osteoarthritis (Hosie *et al*., 1996) and appears to be well tolerated due to its preferential inhibition of cyclooxygenase (Cox-2) (Engelhardt *et al*., 1995b). Some of the reported side effects of the meloxicam include signs of bleeding, signs of an allergic reaction, blurred vision, change in the amount of urine passed, difficulty when swallowing, severe heart burn, pain in throat, pain or difficulty in passing urine, stomach pain or cramps, swelling of feet or ankles, unexplained weight gain or edema, yellowing of eyes, diarrhea, dizziness, gas or heart burn, nausea or vomiting (Mobic, Boehringer Ingelheim, Taiwan). Meloxicam is practically insoluble in water. The poor solubility and wettability of meloxicam leads to poor dissolution and thereby variation in bioavailability (Guruswamy *et al*., 2006).

Streptomyces griseus catalyzes a wide array of oxidative biotransformation reactions with simple aromatic compounds (Smith and Rosazza, 1974), alkaloids (Rosazza *et al*., 1977) and terpene substrates (Chien and Rosazza, 1981). Reactions common for *S. griseus* include O- and N-dealkylations, aromatic hydroxylation, epoxidation, and carbon-carbon bond fission.

In the present study, *Streptomyces* species were employed to oxidize the anti-inflammatory compound meloxicam, as this approach could be a simple and efficient method to oxidize organic compounds when compared to the chemical method.

MATERIALS AND METHODS

Microorganisms: Five actinomycete cultures were selected based on the previous reports (Azerad, 1999; Venisetty and Ciddi, 2003). The cultures *Streptomyces griseus* NCIM 2622, *S. griseus* NCIM 2623, *Streptomyces lavendulae* NCIM 2827, *Streptomyces rimosus* NCIM 2213 NCIM 2213 and *Streptomyces sps.* NCIM 2214 were procured from the National Collection of Industrial Microorganisms (NCIM), Pune, India. Stock cultures were maintained on potato dextrose agar (PDA) slants at 4°C and subcultured every 3 months.

Chemicals: Meloxicam was gifted by Unichem laboratories, Mumbai, India. Methanol and acetonitrile were of HPLC grade obtained from Ranbaxy, New Delhi, India. Peptone, yeast extract, PDA, glucose and all other chemicals of the highest available purity were obtained from Himedia, Mumbai, India.

Biotransformation: Biotransformation was performed using a two-stage fermentation protocol. In the first stage, fermentation was initiated by inoculating a 250 ml culture flask consisting of 50 ml of liquid broth. The liquid broth used contained (per litre) glucose (20 g), peptone (5 g), yeast extract (5 g), K_2HPO_4 (5 g) and NaCl (5 g). The pH of the broth was adjusted to 6.0 with 0.1 N HCl or 0.1 N NaOH. The prepared media were autoclaved and cooled at room temperature. The media were then inoculated with a loopful of culture obtained from freshly grown PDA slants. The flasks were incubated at 120 rev/min and 28°C for 72 h. Second stage cultures were initiated by transferring 1 ml of the first stage culture into 20 ml of the same media in 100 ml culture flasks. The second stage cultures were incubated for 24 h at 28°C to which the substrate meloxicam (2 mg) dissolved in dimethylformamide (200 µl) was added to give a final concentration of 100 mg/l. The flasks were incubated under similar conditions for 5 days. Culture controls consisted of a fermentation blank in which the microorganism was grown under identical conditions in the absence of substrate. Substrate controls comprised of meloxicam added to the sterile medium were incubated under similar conditions. Each culture was studied in triplicate. The cultures were extracted with three volumes of ethyl acetate and the combined organic extracts were evaporated using a rotary vacuum evaporator and dried over a bed of Na_2SO_3 The resultant residues were analysed by HPLC and LC-MS/MS for identification of metabolites.

Analysis: HPLC analysis was performed according to the method described by Elbary *et al*. (2001), with a slight modification. The samples were analysed using an LC-10AT system (Shimadzu, Japan) by injecting 20 µl of sample into the syringe-loading sample injector (Model 7725i, Rheodyne, USA). The column used was Wakosil II, C18, 250×4.6 mm and 5 µm (SGE, Australia). The mobile phase consisted of a mixture of methanol-water (pH adjusted to 3.0 with orthophosphoric acid) in the ratio of 60:40. The analysis was performed isocratically at a flow rate of 1 ml/min and the

Table 1. Biotransformation of meloxicam by actinomycetes. Quantitative metabolite formation of meloxicam in culture broth of Actinomycetes.

Actinomycetes	Percentage of metabolites (%)		
	5-carboxy meloxicam	5-hydroxy methyl 5-meloxicam	Meloxicam remaining
S. griseus NCIM 2622	0.67	40.56	58.77
S. griseus NCIM 2623	0.00	4.37	95.63
S. lavendulae NCIM 2827	0.00	0.00	100
S. rimosus NCIM 2213	0.00	0.00	100
Streptomyces sps NCIM 2214	0.00	0.00	100

analytes were detected at 360 nm using a photodiode array detector (Model SPD M10Avp, Shimadzu, Japan). The metabolite peaks were identified based on similarity with UV spectra. LC-MS/MS analysis was carried out using a Waters system; XTerra C18 column (250×4.6 mm, 5 µm) and a mobile phase consisting of methanol and water (pH adjusted to 3.0 with formic acid) at a 60:40 ratio. The Electrospray Ionization (ESI) detection was set to positive mode. A temperature of 300°C and a scan range of 50-500 were set for the analysis. The transformed compounds were identified from the masses of the fragmentation products obtained.

RESULTS

Screening of cultures: In the present investigation, five actinomycetes cultures were screened for the ability to oxidize the anti-inflammatory compound meloxicam. Among the cultures screened, *S. griseus* NCIM 2622 was found to transform meloxicam into two metabolites (Table 1), whereas *S. griseus* NCIM 2623 was found to transform meloxicam to only one metabolite. Rest of the cultures, *S. lavendulae* NCIM 2827, *S. rimosus* NCIM 2213 and *Streptomyces* sp NCIM 2214 employed in the study could tolerate and grow in the medium containing meloxicam without performing any transformation.
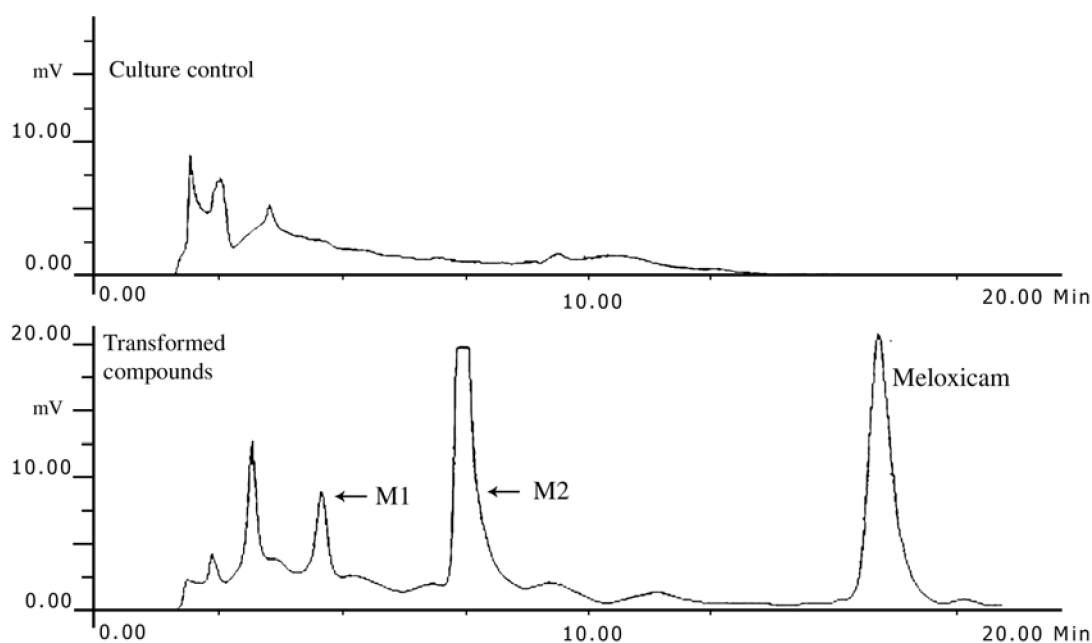

Figure 1. Metabolite peaks observed in HPLC chromatograms of the control culture and the culture showing biotransformation of meloxicam by *Streptomyces griseus* NCIM 2622. (X-axis: time in minutes, Y-axis: millivolts (absorbance), HPLC: high performance liquid chromatography).

Identification of metabolites: The metabolites formed were identified based on observation of new peaks in the test samples (albendazole fed) compared to the culture control samples (without addition of albendazole) by HPLC (Fig. 1). On the basis of LC-MS-MS data and previous reports (Busch *et al*., 1998), the metabolites were predicted to be 5-hydroxymethyl meloxicam (M_2) and 5-carboxy meloxicam (M_1). The molecular weight of meloxicam was mass charge ratio (m/z)=351. Mass spectrometric analysis of the metabolite M_2 showed a molecular ion at m/z 368 (an increase of 16 units), indicating addition of single oxygen atom to meloxicam which results in the formation of 5-hydroxymethyl meloxicam (M_2). Another molecular ion was found at m/z 382 (an increase of 14 units

and loss of 2 mass units) indicating addition of one oxygen atom to 5-hydroxy methyl meloxicam and loss of two hydrogen atoms, which results in the formation of 5-carboxy meloxicam (M_1) (Fig. 2). Hence, the fragmentation of the metabolites and previous reports (Busch *et al*., 1998) led to the identification of the transformed compounds.

These analyses indicated that the metabolites were 5-hydroxymethyl meloxicam (M_2, eluting at 7.0 min), 5-carboxy meloxicam (M_1, eluting at 5.0 min), whereas the substrate meloxicam was eluted at 19.0 min. The pathway of metabolite formation is shown in Figure 3. The metabolites were quantified based on the peak areas obtained by HPLC analysis, whereby the drug and metabolites' peak areas were taken together as 100%.
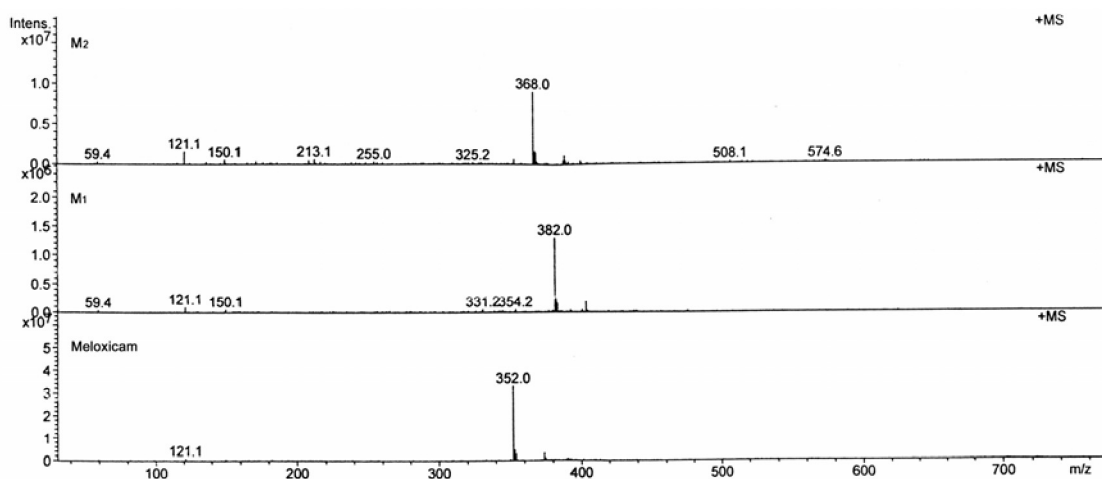

Figure 2. LC-MS/MS spectra showing m/z values of metabolites detected in the meloxicam-fed *Streptomyces griseus* NCIM 2622 culture broth. (X-axis: mass, Y-axis: intensity, LC-MS/MS: liquid chromatography-mass spectrometry/mass spectrometry).
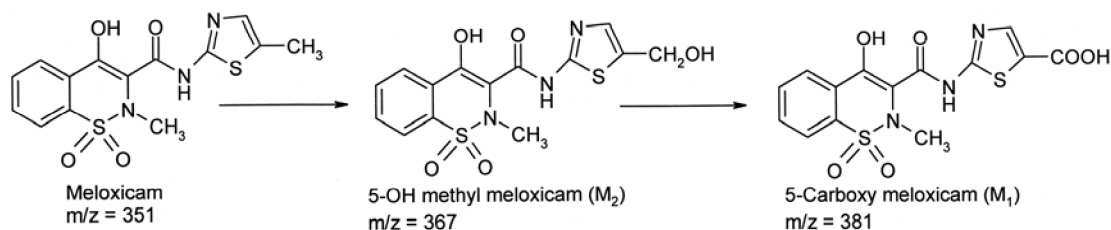

Figure 3. Metabolic pathway of meloxicam in *Streptomyces griseus* NCIM 2622 showing the formation of 5-hydroxy methyl and 5-carboxy meloxicam.

DISCUSSION

This study showed that *S. griseus* NCIM 2622 could produce nearly 41% of 5-hydroxymethyl meloxicam (M_2) and trace amounts of 5-carboxy meloxicam (M_1) from meloxicam. While the other strain NCIM 2623 could transform meloxicam to M_2 only in trace amounts. Such oxidative transformations of different organic substances by *S. griseus* have also been reported by many researchers. Sariaslani and Rosazza (1985) have reported the oxidation of 1, 2-dihydro rotenone by *S. griseus*. Oxidation of precocene II to *cis-* and *trans*-precocene-II-3, 4 dihydro diols and (+) 3-chloromenol by *S. griseus* has been reported by Sariaslani *et al.* (1987). Similarly, *S. griseus* has been found to oxidize (+) camphor to 6-endo-hydroxy camphor and other minor metabolites (Sariaslani *et al.*, 1990). This investigation showed that *S. lavendulae* NCIM 2827, *S. rimosus* NCIM 2213 and *Streptomyces* sp. NCIM 2214 could grow in culture medium without performing any transformation. This may be due to non-toxic environmental conditions in the culture flask despite the addition of meloxicam.

The transformed compounds of meloxicam in the present work were produced from oxidative reactions. The metabolites formed by oxidative biotransformations are found to be biologically more active than the parent compound and such oxidation reactions have been reported by many researchers (Mazier *et al.*, 2004; Kunz *et al.*, 1985; Johnson and Milne, 1981).

To provide comprehensive profiles and to determine which animal species exhibit a profile most closely resembling the pharmacokinetic profile in humans, the pharmacokinetics of meloxicam has been investigated in a number of animal species, including mice, rats, dogs, mini-pigs and baboons (Busch *et al.*,1998). Among all the animals that have been studied, the main metabolites formed are 5-hydroxymethyl meloxicam and 5-carboxy meloxicam. Interestingly, in the present investigation *S. grieus* NCIM 2622 could also produce 5-hydroxymethyl meloxicam and 5-carboxy meloxicam upon oxidation of meloxicam.

Streptomyces has also been reported to catalyse oxidative transformation of chemicals such as alkaloids, coumarins, retinoids and other complex xenobiotics. Cytochrome P450 enzymes (P540) may have key roles in these biosynthetic and biotransformation reactions (Chun *et al.*, 2006). Meloxicam is metabolized to 5-hydroxymethyl metabolite, which is further converted to a 5-carboxy metabolite (Schmid *et al.*, 1995a; Schmid *et al.*, 1995b).

The 5-hydroxylation of meloxicam is predominantly catalyzed by cytochrome P450 2C9 subtype (CYP 2C9) and with a minor contribution by cytochrome P450 3A4 (CYP 3A4) (Chesne *et al.*, 1998). Similarly *Streptomyces* P450s are involved in polyketide biosynthesis by catalysing the stereo-and regio-specific oxidative modification of macrolide antibiotics such as erythromycin, tylosin and oleandomycin (Gaisser *et al.*, 2002).

CONCLUSION

Generally, the transformation of biologically active compounds will either be aimed at widening the spectrum of activity or acquiring additional properties of economical importance with regard to the product. Meloxicam is an established anti-inflammatory agent with some side effects. Furthermore, there are no reports of other biological activities of meloxicam or its metabolites, which can be exploited in such fields. There are a number of new drugs of anti-inflammatory character which are safe and more effective. Therefore, the testing for other activities of meloxicam and its metabolites required to sustain its market potential is currently in progress in our laboratory.

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