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Biotransformation of Valdecoxib by Microbial Cultures

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Microbial biotransformations can be used to predict mammalian drug metabolism. The present investigation deals with microbial biotransformation of valdecoxib using microbial cultures. Thirty-nine bacterial, fungal, and yeast cultures were used to elucidate the biotransformation pathway of valdecoxib. A number of microorganisms metabolized valdecoxib to various levels to yield nine metabolites, which were identified by HPLC–DAD and LC–MS–MS analyses. HPLC analysis of biotransformed products indicated that a majority of the metabolites are more polar than the substrate valdecoxib. Basing on LC–MS–MS analysis, the major metabolite was identified as a hydroxymethyl metabolite of valdecoxib, whereas the remaining metabolites were produced by carboxylation, demethylation, ring hydroxylation, *N*-acetylation, or a combination of these reactions. The hydroxymethyl and carboxylic acid metabolites were known to be produced in metabolism by mammals. From the results, it can be concluded that microbial cultures, particularly fungi, can be used to predict mammalian drug metabolism.

Keywords: Biotransformation, microbial, valdecoxib, metabolites, HPLC, LC–MS–MS

Valdecoxib, 4-(5-methyl-3-phenyl-4-isoxazolyl) benzenesulfonamide, used in the treatment of rheumatoid arthritis, osteoarthritis, and primary dysmenorrhea, has shown to be a highly selective and potent inhibitor of COX-2 [23]. Since valdecoxib is lipophilic in nature, it should be eliminated predominantly by metabolism and hence the study of metabolic pathways is important. Valdecoxib is metabolized predominantly by CYP3A4 [3] to produce an active hydroxylated metabolite that also possesses good selectivity towards COX-2. In human subjects, valdecoxib produced nine phase I metabolites where the drug primarily is metabolized by hepatic microsomal enzymes (CYP2C9 and CYP3A4) that form hydroxylated and carboxylic acid metabolites [28]. The hydroxylated metabolite was also found in rodents and dogs [23]. Zhang *et al*. [30] identified 16 metabolites after administration of valdecoxib in mouse plasma, red blood cells, urine, and feces.

Apart from the anti-inflammatory effects, valdecoxib was also found to possess antiproliferative effect in prostate cancer cell proliferation and carbonic anhydrase inhibitory and antimigraine effects. Harris *et al*. [16] found that addition of a tertiary alcohol to the terminal carbon of the alkoxy chain of A-282904, a 6-membered analog of valdecoxib, yielded ABT-963, a potent and selective COX-2 inhibitor that had significantly improved solubility, improved selectivity in whole blood assays, and improved the pharmacokinetic profile. These varied activities and studies indicate that the analogs of valdecoxib might be interesting from therapeutic point of view. Placebo-controlled studies conducted in 2005 reveal that valdecoxib posed cardiovascular adverse effects in moderate to high risk individuals.

Microbial biotransformations are important steps to introduce functional groups into inaccessible sites of the molecules and thereby to produce rare structures. The reactions also involve high degrees of regio- and stereo selectivities and require mild reaction conditions. Microbial cultures, particularly some fungi, were found to possess cytochrome P450 enzyme systems and metabolize drugs in a manner similar to mammalian systems. There are several reports on the production of drug metabolites using microbial biotransformations [2, 4, 5].

The aim of the present work is to study the metabolites of valdecoxib formed by microorganisms and to compare them with those produced in mammals. This will help in the production of large quantities of metabolites for further pharmacological and toxicological evaluations apart from structure elucidation. Moreover, the hydroxymethyl metabolite of valdecoxib has shown some COX-2 inhibitory activity, and there are chances of producing new metabolites with fungi that possess COX-2 inhibitory or any other interesting

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activity. The advent of cardiovascular adverse effects adds to the aim of the present investigation with a viewpoint that analogs and derivatives of valdecoxib with lesser or no side effects can be obtained with microbial transformation.

Material and Methods

Materials

Valdecoxib was kindly gifted by Mepro Pharmaceuticals Pvt. Ltd., Ahmedabad, India. The microbial cultures were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India or National Collection of Industrial Microorganisms (NCIM), Pune, India.

Culture Procedure

The bacterial cultures were grown in nutrient broth containing (per liter) beef extract 10 g, sodium chloride 5 g, and peptone 10 g (final pH adjusted to 7.4). For screening experiments, a two-stage fermentation protocol was used. The first-stage culture was initiated in 50-ml conical flasks containing 10 ml of sterile liquid medium, inoculated with a loop of sample scratched from a freshly grown agar slant, and orbital shaken at 180 rpm and 30°C in a refrigerated shaker incubator. For second-stage cultures, 50-ml culture flasks containing 10 ml of the same media were inoculated with 500 µl of 48 h grown first stage culture and incubated under similar conditions for 48 h. Similarly, fungal cultures were grown in liquid medium containing (per liter) dextrose 20 g, yeast extract 5 g, peptone 5 g, sodium chloride 5 g, and KH_2PO_4 5 g supplemented with 0.02% Triton X 100 [25], and yeast cultures were grown in MGYP broth containing (per liter) malt extract 3 g, glucose 10 g, yeast extract 3 g, and peptone 5 g (final pH adjusted to 6.8).

Biotransformation

The second-stage cultures were added with 2 mg each of valdecoxib (in 100 µl of methanol) to obtain a final drug concentration of 0.2 g/l. Each culture was studied in quadruplicate while running suitable controls. Culture controls consisted of culture blanks in which the organisms were grown under identical conditions but without adding the substrate. The culture controls were added with 100 µl of methanol, the solvent used to dissolve the drug. Drug controls were composed of the sterile medium to which the same amount of the drug was added and incubated without microorganisms. The incubation was continued under similar conditions for 10 days, and the flasks were then taken out, and the culture extracted and analyzed.

Extraction and Sample Preparation of Valdecoxib and Metabolites

The cultures were extracted with 3 volumes of ethyl acetate. The combined organic phases were evaporated under reduced pressure and the dried samples were reconstituted in 1.5 ml each of HPLC-grade methanol. The samples were then centrifuged at 12,000 ×*g* and 20°C for 20 min in a refrigerated micro centrifuge. The supernatants were used for HPLC–DAD and LC–MS–MS analysis.

HPLC–DAD Analysis

The samples were analyzed by an isocratic HPLC method for the presence of metabolites. The HPLC analysis was performed on an LC-10AT system by injecting 20 µl of sample into a syringe loading sample injector following the conditions described by Keshetty *et al*. [18]. The column used was a Luna C18, 5 µ and 250×4.6 mm i.d. and the mobile phase consisted of a mixture of acetonitrile:water, pH adjusted to 6.2 with orthophosphoric acid, in 40:60 ratio. The analysis was performed at a flow rate of 1 ml/min and the drug and its metabolites were detected using a diode array detector (SPD M10Avp model; Shimadzu Corporation, Kyoto, Japan) at a wavelength of 240 nm. The data analysis was performed by Class M10 software. The UV absorption spectrum of the metabolites was compared with that of valdecoxib. The metabolites were quantified based on the peak areas and expressed as percentages of metabolites formed. The calculations were performed with respect to the total area of drug and metabolites together taken as 100%.

LC–MS–MS Analysis

The *m/z* values of drug and metabolites and their fragmentation ions were recorded by LC–MS–MS using a Perkin Elmer SCIEX API mass spectrometer set in positive mode. The API-4000 LC–MS–MS was operated under the multiple reaction monitoring mode (MRM). The column used was an Inertsil ODS-2, RP, C18, 250×4.6 mm and the mobile phase, acetonitrile:water (pH adjusted to 3.2 with formic acid) (35:65), was pumped at a flow rate of 1 ml/min. Fifty µl of the sample was injected using an autosampler device. The vaporizer temperature and the discharge current were set at 300°C and 10 µA, respectively. The fragments were scanned in the *m/z* range of 100 to 500 and the data were processed with Sciex Analyst software. The metabolites were identified basing on the patterns of UV spectra in HPLC–DAD and *m/z* values of the fragmentation products obtained in LC–MS–MS analysis.

Results and Discussion

HPLC analysis of the extracts of the cultures showed that 24 out of 39 cultures were able to metabolize valdecoxib to produce one or more metabolites (Table 1), evidenced from the absence of these new peaks in the drug control as well as culture controls. Representative HPLC chromatograms are depicted in Fig. 1. Only two organisms produced a single metabolite. Most of the organisms studied produced the same range of metabolites, but in different relative amounts. The production of metabolites by microbial cultures may be due to the presence of enzymes expressed naturally or induced by the drug or media component(s). Most of the metabolites produced were eluted before the drug, which denotes that they are more polar than valdecoxib. The metabolite peaks were identified in HPLC based on the similarity in UV spectral patterns in the diode array detector.

Valdecoxib was eluted at 16 min and the metabolites produced were designated as M_1 (4.5 min), M_2 (4.8 min), M_3 (7 min), M_4 (7.2 min), M_5 (7.8 min), M_6 (8.4 min), M_7 (10 min), M_8 (18 min), and M_9 (20 min). The results pertaining to the metabolite production is shown in Table. 1. A large variation was observed in the quantitative metabolite production among various cultures. M_3 was the

Table 1. Metabolites of valdecoxib produced by microbial cultures.

Culture	M_1 4.5 min	M_2 4.8 min	M_3 7 min	M_4 7.2 min	M_5 7.8 min	M_6 8.4 min	M_7 10 min	M_8 18 min	M_9 20 min
Absidia coerulea MTCC 1335	-	-	26.4	0.92	-	-	-	0.11	-
Absidia glauca MTCC 982	-	-	-	-	-	-	-	-	-
Aspergillus flavipes NCIM 1209	-	-	0.72	-	-	-	-	-	-
Aspergillus flavus NCIM 554	-	-	4.39	-	-	-	-	-	-
Aspergillus flavus NCIM 557	-	-	-	-	-	-	-	-	-
Aspergillus niger NCIM 1006	-	-	-	-	-	-	-	-	-
Aspergillus niger NCIM 589	-	-	-	-	-	-	-	-	-
Aspergillus niger NCIM 620	-	-	2.65	0.32	-	-	-	-	-
Aspergillus ochraceous NCIM 1140	-	-	-	-	-	-	-	-	-
Aspergillus parasiticus NCIM 898	-	-	1.55	-	0.11	-	-	-	-
Bacillus subtilis MTCC 619	-	-	0.12	0.04	0.03	-	0.02	-	-
Beauveria bassiana NCIM 1216	-	-	1.12	0.13	-	-	-	-	-
Cunninghamella blakesleana NCIM 687	-	-	10.19	16.91	3.52	-	0.03	-	-
Cunninghamella blakesleana NCIM 688	-	-	22.36	4.35	2.65	-	0.03	-	-
Cunninghamella echinulata NCIM 691	-	-	65.94	15.32	2.37	0.79	0.31	0.82	-
Cunninghamella echinulata NCIM 693	-	-	35.04	6.4	1.81	0.1	0.32	0.07	-
Cunninghamella elegans NCIM 689	-	-	0.27	0.04	-	-	-	-	-
Cunninghamella elegans NCIM 690	-	-	11.22	5.39	0.94	0.05	0.05	0.08	0.36
Cunninghamella sp. NCIM 1184	-	-	68.76	18.12	2.8	-	0.04	-	-
Curvularia lunata NCIM 716	-	-	1.04	0.53	-	-	-	-	-
Escherichia coli MTCC 118	-	-	-	-	-	-	-	-	-
Fusarium oxysporum NCIM 1008	-	-	-	-	-	-	-	-	-
Mucor plumbeus NCIM 984	-	-	-	-	-	-	-	-	-
Mucor rouxii MTCC 386	-	-	5.23	5.28	7.85	-	4.37	-	-
Pencillium brevicompactum MTCC 549	-	-	-	-	-	-	-	-	-
Pencillium chrysogenum NCIM 733	-	-	0.15	0.76	0.22	-	-	-	-
Pencillium chrysogenum NCIM 738	-	-	1.3	0.24	0.46	-	-	-	-
Pseudomonas putida NCIM 2782	-	-	-	-	-	-	-	-	-
Rhizopus arrhizus NCIM 997	-	-	19.6	3.09	1.28	-	-	-	-
Rhizopus stolonifer NCIM 880	-	-	3.51	0.75	0.49	-	-	-	-
Rhodotorula rubra NCIM 3172	-	-	-	-	-	-	-	-	-
Saccharomyces cerevesiae NCIM 3090	-	-	-	-	-	-	-	-	-
Streptomyces griseus NCIM 2622	-	-	9.98	1.23	0.27	-	-	-	-
Streptomyces griseus NCIM 2623	-	-	1.38	0.22	0.95	-	0.01	-	-
Streptomyces lavendulae NCIM 2827	-	-	-	-	-	-	-	-	-
Streptomyces rimosus NCIM 2213	6.17	4.06	83.86	-	0.69	-	0.31	1.84	-
Streptomyces sp. NCIM 2214	-	-	0.79	0.95	0.25	-	0.08	-	-
Thamnostylum piriforme NCIM 974	-	-	-	-	-	-	-	-	-
Trichothecium roseum NCIM 1147	-	-	-	-	-	-	-	-	-

[a]Values indicate the percentage of metabolites' area in HPLC analysis. The value is the percentage of a metabolite with respect to the total area of drug and all the metabolites formed. M_1-Ring-*p*-hydroxylation and Methyl hydroxylation. M_2-Demethylation. M_3-Methyl hydroxylation. M_4-Methyl carboxylation. M_5-Ring-*p*-hydroxylation. M_6-Ring-*m*-hydroxylation. M_7-Ring-*o*-hydroxylation. M_8-Ring-*p*-hydroxylation and N^4 acetylation. M_9-N^4 acetylation.

metabolite found in most of the cultures. *Streptomyces rimosus* NCIM 2213 produced 83.86% of this metabolite whereas *Bacillus subtilis* MTCC 619 produced only 0.12% of this metabolite. The low production of M_3 in *B. subtilis* MTCC 619 may be due to several reasons, namely, low levels of enzyme expression required for this reaction, substrate/product inhibited biotransformation, *etc*.

The structure elucidation of the metabolites was carried out from the fragmentation results obtained in LC–MS–MS analysis. The LC–MS spectra and fragmentation pattern for valdecoxib and its major metabolites M_3 and M_4 are shown in Fig. 2 and 3. M_3, M_5, M_6, and M_7 gave the same protonated molecular ion at *m/z* 331; that is, 16 higher than valdecoxib. This suggests that these might be hydroxylation products of valdecoxib. However, their ionization spectra were different. Table 2 gives the retention times, *m/z* values of parent ions $(M+H)^+$, and predicted molecular formulae and metabolic reactions for various metabolites.

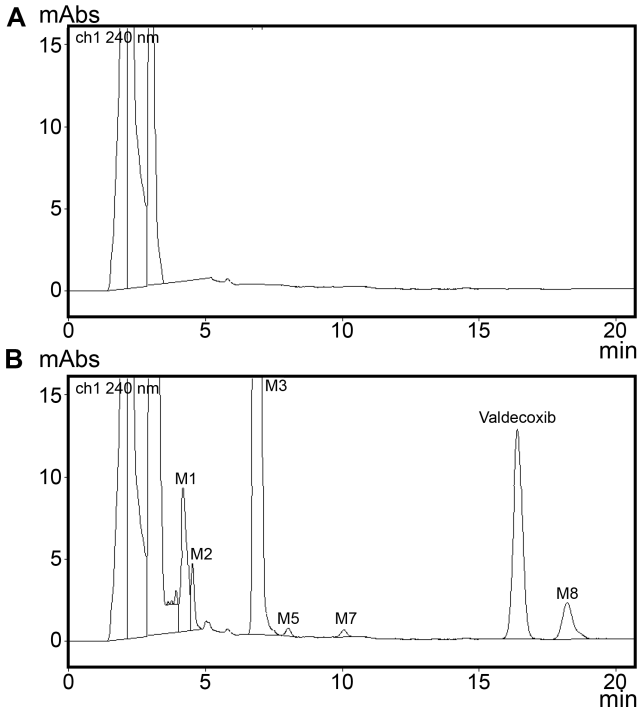

Fig. 1. HPLC chromatograms showing (**A**) culture control and (**B**) valdecoxib and its metabolites peaks detected in *Streptomyces rimosus* NCIM 2213.

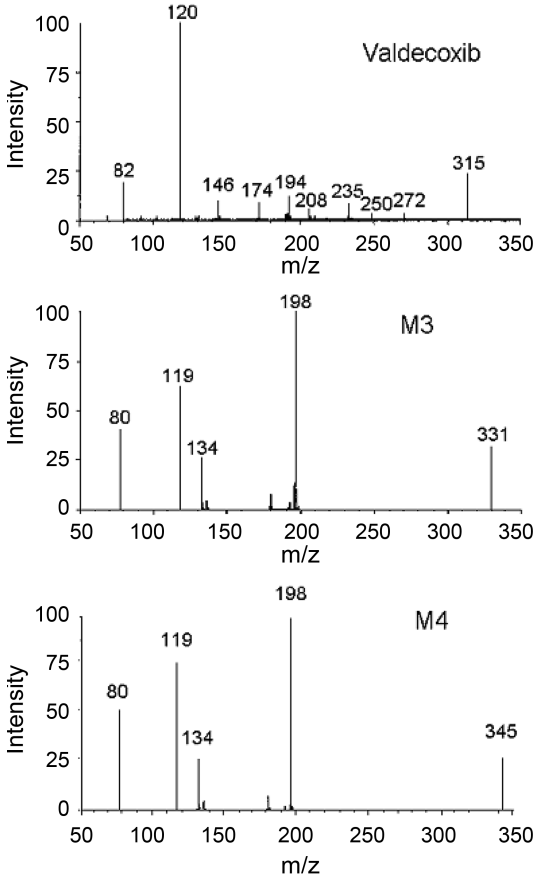

Fig. 3. Mass fragmentation of valdecoxib and its metabolites M_3 and M_4.

M_3 showed a major characteristic product ion at *m/z* 198. This indicates that there was a loss of $C_6H_5CNOCH_2$ *via* five-membered ring rearrangement on the isoxazole ring. The loss of $C_6H_5CNOCH_2$ corresponds to the 3-phenyl ring (C_6H_5), the attached CN group on the isoxazole ring, and the 5-methylene oxide (CH_2O). Also produced were the fragment ions at *m/z* 134 and 119, which might be generated from the loss of SO_2 (64) and $NHSO_2$ (79) from the fragment with *m/z* 198, respectively. Based on this assumption, M_3 was identified as 4-[5-hydroxymethyl-3-phenylisoxazol-4-yl] benzenesulfonamide. This metabolite was also formed in humans [28] and mice [30]. Similar hydroxylation of the terminal methyl group was demonstrated for triprolidine [15], *rac*-mexiletine [12], and tolbutamide [17] in previous studies on microorganisms.

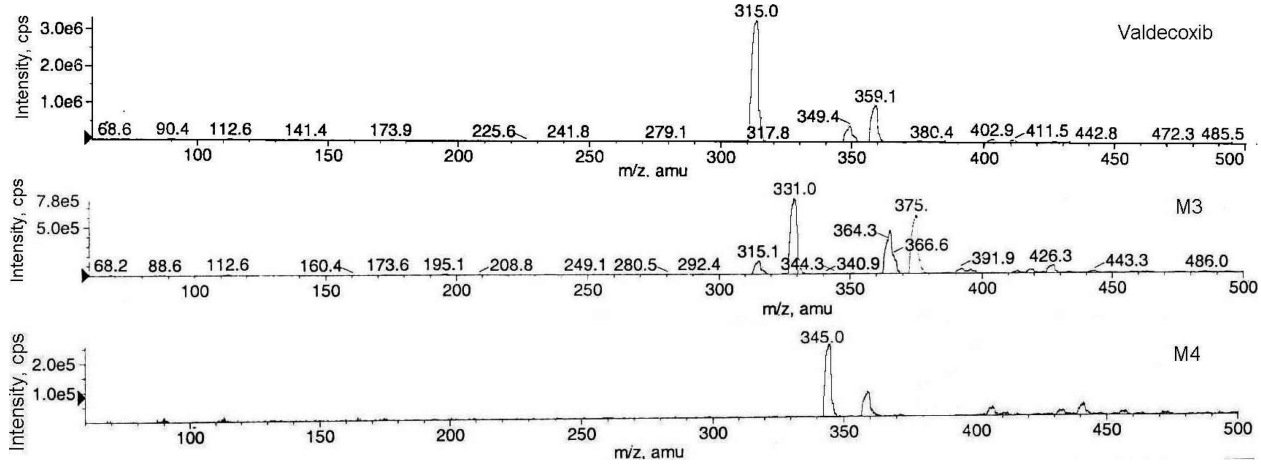

Fig. 2. LC–MS spectra of valdecoxib and its metabolites M_3 and M_4.

Table 2. Retention times, *m/z* values, and predicted reactions involved in the biotransformation of valdecoxib by microbial cultures.

Metabolite	R_t	$[M+H]^+$	Predicted molecular formulae	Predicted reaction
Valdecoxib	15	315	$C_{16}H_{14}N_2O_3S$	-
M_1	4.5	347	$C_{16}H_{14}N_2O_5S$	Aromatic ring-*p*-hydroxylation and methyl hydroxylation
M_2	4.8	301	$C_{15}H_{12}N_2O_3S$	Demethylation
M_3	7	331	$C_{16}H_{14}N_2O_4S$	Methyl hydroxylation
M_4	7.2	345	$C_{16}H_{12}N_2O_5S$	Methyl carboxylation
M_5	7.8	331	$C_{16}H_{14}N_2O_4S$	Aromatic ring-*p*-hydroxylation
M_6	8.4	331	$C_{16}H_{14}N_2O_4S$	Aromatic ring-*m*-hydroxylation
M_7	10	331	$C_{16}H_{14}N_2O_4S$	Aromatic ring-*o*-hydroxylation
M_8	18	373	$C_{18}H_{16}N_2O_5S$	Aromatic ring-*p*-hydroxylation and N^4 acetylation
M_9	20	357	$C_{18}H_{16}N_2O_4S$	N^4 acetylation

M_5 showed characteristic product ions at 288, 251, and 239, which were formed by the loss of $COCH_3$ (43), SO_2NH_2 (80), and C_6H_4O (92) from *m/z* 331, respectively. The further loss of SO_2 (64), $COCH_3$ (43), and SO_2NH (79) from these fragments produced fragments ions at m/z 224, 208, and 160, respectively. The pattern of this mass fragmentation depicts that the substrate had undergone hydroxylation at the *para* position of the 3-phenyl ring, and thus the product was identified as 4-[3-(4-hydroxyphenyl)-5-methyl-4-isoxazolyl] benzenesulfonamide. The formation of this metabolite might take place *via* an epoxide intermediate. This metabolite was also formed in mice [30]. *p*-Hydroxylations by microbial cultures were previously reported for anisole [8], warfarin [27], and *rac*-mexiletine [12].

M_6 showed characteristic product ions at *m/z* 251 and 239, which were formed by the loss of SO_2NH (79) and C_6H_4O (92) from *m/z* 331, respectively. The fragment ion with *m/z* 239 had undergone further loss of SO_2NH (79) to form a fragment ion with *m/z* 160. Another fragment ion was observed at *m/z* 120 and this may be attributed to the OC_6H_4CN group. This pattern of mass spectral fragmentation reveals that the hydroxylation has occurred on the *meta* position of the 3-phenyl ring. With these data, the metabolite M_6 can be assumed as 4-[3-(3-hydroxyphenyl)-5-methyl-4-isoxazolyl] benzenesulfonamide. The formation of this metabolite might take place *via* an epoxide intermediate. This metabolite was also formed in humans [28] and mice [30]. Similar *m*-hydroxylations of organic compounds by microbial cultures were reported for methoxyphenamine [9] and warfarin [27]. The metabolites M_5 and M_6 might be formed *via* the same epoxide intermediate. In mice, the *meta* and *para* metabolites were found to be formed *via* the same epoxide intermediate in the study by Zhang *et al*. [30].

M_1 showed a protonated molecular ion at *m/z* 347, which was 32 higher than the parent drug, giving a clue that hydroxylation took place twice. The compound formed three characteristic fragmentation ions at *m/z* 288, 267, and 255, which were formed by the loss of $COCH_2OH$ (59), SO_2NH_2 (80), and C_6H_5O (92) from *m/z* 347, respectively. The fragment ion at *m/z* 255 underwent further loss of H_2O (18) to form another fragment ion at *m/z* 237. The fragment with *m/z* 267 produced fragments with *m/z* 222 and 197 with subsequent loss of CH_2ONH (45) and COCON (70), respectively. Another fragment ion was observed at *m/z* 120 and this may be attributed to the OC_6H_4CN group. Based on these data, the metabolite was assumed to be obtained by ring *p*-hydroxylation and terminal methyl hydroxylation. The one hydroxylation was took place on the 3-phenyl group and second one on the 5-methyl group on the isoxazole ring. Formation of this metabolite took place either from M_3 or M_5, one of which underwent hydroxylation again to produce the dihydroxylated metabolite M_1. This dihydroxylated metabolite was also reported in mice [30]. Vigne *et al*. [26] reported this type of double regiospecific hydroxylation of exo-3-pinanyl-*N*-phenyl carbamate and exo-3-pinanyl-*N*,*N*-methylphenyl carbamate where one hydroxylation occurs on the terminal methyl group of the pinanyl moiety and a second hydroxylation takes place on the *para* position of the aromatic ring. Their study also reported that, when a compound possesses an aromatic ring and an alkyl group, the hydroxylation first occurs on the alkyl group, and that the alcohol thus formed serves as a substrate to undergo second hydroxylation on the aromatic ring leading to the dihydroxylated product. Hence, in the present study, it can be assumed that the formation of M_1 might take place from M_3. Vigne *et al*. [26] reported that the rate of second hydroxylation depends on the lipophilicity of the alkyl groups, where less lipophilic groups (lower number of carbons) will produce slower rates of the second hydroxylation reaction and form less amount of the metabolites. This might be the reason for production of low levels of M_1 in the present study.

In similar fragmentation studies, M_2 (*m/z*, 302) was found to be a demethylated derivative of valdecoxib, which was 4-(3-phenyl-4-isoxazolyl) benzenesulfonamide. Abel *et al*. [1] reported that the filamentous fungus *Cunninghamella*

echinulata NRRL 1384 was able to demethylate a thebaine derivative to give the intermediates for the synthesis of buprenorphine. M_4 (*m/z*, 345) was found to be a carboxylic acid derivative of valdecoxib. M_4 has shown a protonated molecular ion at *m/z* 345 and formed a characteristic product ion at *m/z* 198, formed by the loss of C_6H_5CNCOO *via* the five-membered ring rearrangement mechanism on the isoxazole ring. M_4 was thought to be derived from M_3 by subsequent oxidation and was designated as 4-[5-carboxy-3-phenylisoxazol-4-yl] benzenesulfonamide. The carboxylic acid metabolite was also formed in humans [28] and mice [30] and it was assumed that the same enzymes (*i.e*., CYP2C9 and CYP3A4) were responsible for the sequential formation of hydroxylated and carboxy metabolites. Similar sequential reactions (*i.e*., $-CH_3$ to $-CH_2OH$ to -COOH) were found in microbial oxidation of ebastine and terfenadine [19, 20], triprolidine [15], and muraglitazar [29].

In similar fragmentation studies, M_7 and M_9 were assumed to be the *o*-hydroxylation and N^4-acetylation products, respectively. Ferris *et al*. [8] reported the *ortho*-hydroxylation of anisole by fungi previously. *N*-Acetylation of phenelzine and pheniprazine [10], tranylcypromine [11] and protryptiline [7] was reported previously using microbial cultures. In the present investigation, a few microbial cultures produced some promising metabolites for valdecoxib (*i.e*., by *N*-acetylation of the sulfonamido group). Acetylation of the *para* amino group of the sulfonamides is a prime example of acetylation in mammalian metabolism reactions. Procainamide and isoniazid also undergo *N*-acetylation at the $-NH_2$ group to form the corresponding *N*-acetyl derivatives. In mammals, the *N*-acetylation takes place in many tissues including liver, GIT, *etc*. However, the acetylated metabolites were not detected in any of the mammalian system for the metabolism of valdecoxib. Since *N*-acetylated metabolites are more hydrophobic, they might reabsorb back in the mammalian system to undergo further metabolism. In the present valdecoxib N^4-acetylation, the microbial enzymes transfer the acetyl group to NH_2 in the sulfonamide moiety to form *N*-acetylated metabolite. The acetylation enzymes utilize acetyl-coenzyme A to form an acetylated enzyme intermediate that transfers the acetyl group to an acceptor, usually an amine or hydrazine group. The amine group is thought to be the acceptor of an acetyl group in the valdecoxib metabolism by microorganisms. During the discovery of valdecoxib, Talley [22] reported this *N*-acetylated analog of valdecoxib with improved aqueous solubility (44 mg/ml) and found that this *N*-acetylated compound can be converted to valdecoxib in 2 h in the human body and thus act as a prodrug of valdecoxib. Talley *et al*. [24] reported that still better prodrugs of valdecoxib could be prepared by replacing the $N\text{-}COCH_3$ with $N\text{-}COCH_2CH_3$ or $N\text{-}COCH_2CH_2CH_3$, where the compound with $N\text{-}COCH_2CH_3$ was denoted as parecoxib and was used in clinics as a parenteral COX-2 inhibitor with improved water solubility.

Similarly, M_8 was assumed to be the product of ring *p*-hydroxylation and N^4-acetylation. This metabolite might be obtained by successive reactions either from M_5 or M_9. Usually, *N*-acetylation produces more hydrophobic molecules that are difficult to excrete from the organism, and hence it is assumed that, in the present study, the *N*-acetylated metabolite M_9 had undergone ring *p*-hydroxylation to form the metabolite M_8. Generally, nonpolar molecules are not excreted from the mammalian systems and are usually reabsorbed into the renal tubule, undergo further metabolism to form hydrophilic or more polar molecules, and then excreted. However, in the cultures employed in the present investigation, M_9 was produced in only one culture, *Cunninghamella elegans* NCIM 690, which also produced M_5. The remaining cultures did not shown the presence of M_9, indicating that the metabolite M_9 is instantaneously and completely converted to M_8 as soon as it is produced in the cultures. Even in the case of *C. elegans* NCIM 690, it is assumed that M_9 is converting into M_8 because of two reasons: one is that, generally, the nonpolar metabolite M_9 has to undergo further metabolism in order to be excreted from the organism; the second one is that, the metabolite M_5, being a polar one, does not undergo uptake by the microorganism, but remains in the media and do not undergo further metabolism.

The formation of *o*-, *m*-, and *p*-hydroxylation products from valdecoxib in the present study is in agreement with earlier studies reported by Dodge *et al*. [6], Golbeck *et al*. [14], Smith *et al*. [21], and Gessel *et al*. [13], that the genera of the filamentous fungi *Absidia*, *Aspergillus*, *Cunninghamella*, *Gliocladium*, and *Helicostylum* were able to transform biphenyl to produce 2-, 3-, and 4-hydroxylbiphenyl, indicating that the hydroxylation by microorganisms will take place at any position on the benzene ring.

The results of the present investigation revealed that the extracts derived from the cultures produced demethylated, hydroxylated, carboxylic acid, and acetylated metabolites of valdecoxib. Some of the metabolites were already reported in mice [30] and humans [28]. With valdecoxib, the microbial cultures extended their repertoire for these hydroxylation, demethylation, and acetylation reactions. The present study produced many hydroxylated products of valdecoxib, indicating the existence of multiplicity of CYP. In this context, the question of CYP multiplicity will be of practical relevance, since the formation of isomers of valdecoxib metabolites was observed in the present biotransformation study. Regiospecific (*o*-, *m*-, and *p*-) hydroxylation of aromatic rings is a very difficult task in synthetic organic chemistry, whereas the enzymatic (especially CYP) hydroxylations are simple to perform to produce

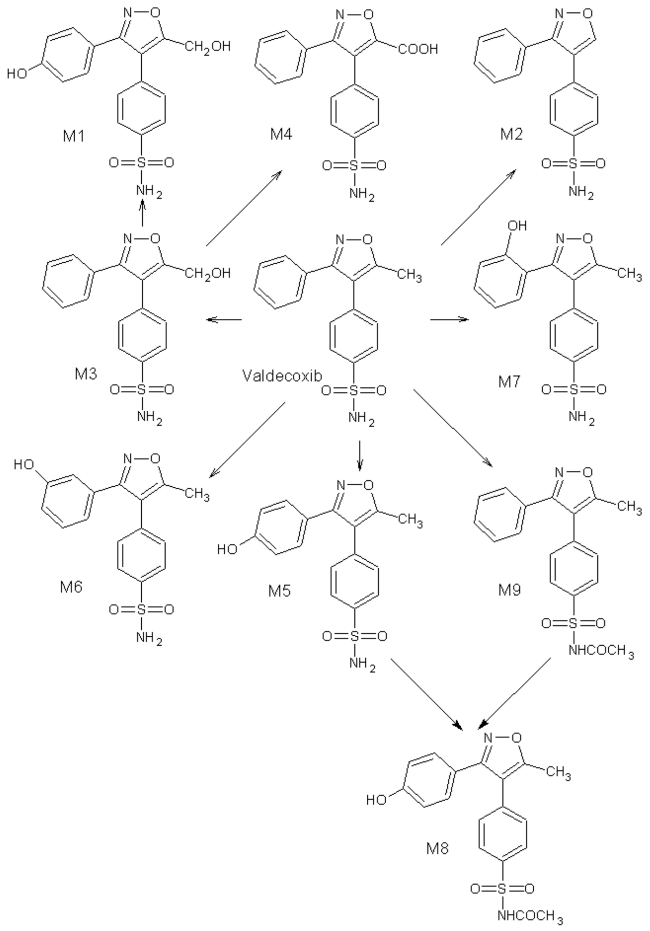

Fig. 4. Proposed biotransformation pathways of valdecoxib in microbial cultures.

this regiospecificity [26]. The reactions involved in the biotransformation of valdecoxib by various cultures are depicted in Fig. 4.

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