Appl Biochem Biotechnol (2008) 144:201–212
DOI 10.1007/s12010-007-8102-2

Biotransformation of Valdecoxib by Plant Cell Cultures

V. Molmoori · K. Srisailam · V. Ciddi

Received: 15 December 2006 / Accepted: 16 November 2007 /
Published online: 18 January 2008

Abstract Valdecoxib is a new anti-inflammatory drug that is highly selective for inhibition of the inducible form of cyclooxygenase (COX-2). In the present study, biotransformation of valdecoxib was investigated in cell cultures of five medicinal plants, viz., *Catharanthus roseus*, *Azadirachta indica*, *Capsicum annuum*, *Ervatamia heyneana*, and *Nicotiana tabacum*. Identification of the biotransformed products was carried out by using high-performance liquid chromatography coupled with diode array detection and liquid chromatography–tandem mass spectrometry analysis. All the cultures transformed valdecoxib into more polar compounds, and *C. roseus* also produced one unknown compound that is less polar than the substrate. The reactions performed by these plant cell cultures include hydroxylation, methylation, and demethylation. Optimization studies were performed to investigate the effect of the day of extraction and substrate concentration on biotransformation.

Keywords Valdecoxib · Biotransformation · Plant cell cultures · *Catharanthus roseus* · *Azadirachta indica* · *Capsicum annuum* · *Ervatamia heyneana* · *Nicotiana tabacum*

Introduction

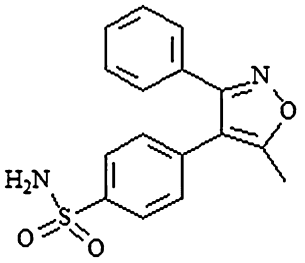
The cultured cells of plants have the capacity to specifically convert low-cost and plentiful substrates into rare and expensive substances. Biotransformation of foreign substrates by plant cell cultures produces many novel compounds, depending on the structure and functionality of the starting material. Biotransformation reactions performed by plant cell cultures are more stereospecific and regioselective when compared to chemical reactions. Because of factors such as difficulty of maintaining culture sterility and slow growth rate, plant cell cultures cannot compete with microbial systems for the same biotransformation reaction. However, the plants are genetically very diverse and possess a rich repertory of enzymes, and the industrial utility of biotransformation using plant cell culture systems will

V. Molmoori · K. Srisailam · V. Ciddi (✉)
University College of Pharmaceutical Sciences, Kakatiya University,
Warangal 506 009 Andhra Pradesh, India
e-mail: ciddiveeresham@yahoo.co.in

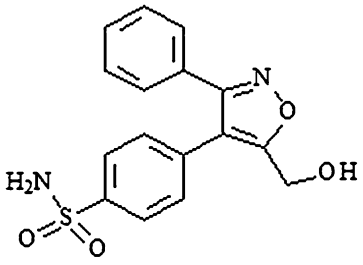
be possible if the reaction is unique to plant cells and the product has a high market value. Considerable progress has been achieved during the last 25years concerning the biotransformation of exogenous substrates by cultured plant cells [1].

Valdecoxib, 4-(5-methyl-3-phenyl-4-isoxazolyl) benzenesulfonamide (Fig. 1), is a potent and specific inhibitor of cyclooxygenase-2 (COX-2) used in the treatment of rheumatoid arthritis, osteoarthritis, and primary dysmenorrhea. A hydroxylated metabolite of valdecoxib was also found to be a COX-2 inhibitor in rodents and dogs [2]. The phenyl sulfonamide group of valdecoxib interacts with protein residues of COX-2 enzyme to elicit its action. In animals, valdecoxib will primarily be metabolized by hepatic microsomal enzymes (CYP 2C9 and CYP 3A4) that form hydroxylated and carboxylic acid metabolites (Fig. 1) [3].

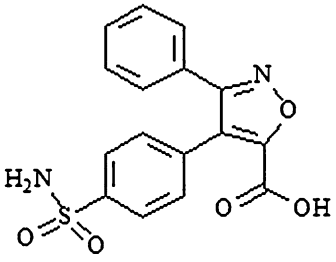
The present study was aimed at producing analogs of valdecoxib by feeding the drug to various plant cell cultures having biotransformation capability. The plants selected for biotransformation study include *Catharanthus roseus*, *Azadirachta indica*, *Capsicum annuum*, *Ervatamia heyneana*, and *Nicotiana tabacum*. All these plants produced transformation of a good number of substrates according to the previous reports [4–10]. Callus cultures of the selected plants were established from which the cell suspension cultures were established. After two subcultures, the cell cultures were added with valdecoxib and incubated for 48h. The cultures were extracted and analyzed by high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) for presence of transformed compounds. The structures of the transformed compounds were elucidated by liquid chromatography–tandem mass spectrometry (LC-MS-MS) analysis. The transformation was optimized with reference to time of incubation and substrate concentration.



Valdecoxib



Hydroxylated Metabolite



Carboxylic acid Metabolite of Valdecoxib

Fig. 1 Structures of valdecoxib and its transformed products

Materials and Methods

Materials

Young leaves of *A. indica* and *C. roseus* and stems of *E. heyneana* were collected from plants located in the Medicinal Plant Garden of University College of Pharmaceutical Sciences, Kakatiya University, Warangal. Seeds of *C. annuum* were procured from the local market and seeds of *N. tabacum* were generously given by Dr. A.V.S.R. Swamy, Senior Scientist, Central Tobacco Research Institute, Guntur. The plants were authenticated by Prof. V.S. Raju, Botanist, Kakatiya University, Warangal. Plant Growth Regulators were purchased from Sigma-Aldrich Chemicals, Bangalore, India. Valdecoxib was kindly given by Mepro Pharmaceuticals, Ahmedabad, India. HPLC solvents were purchased from Ranbaxy Fine Chemicals, New Delhi, India, and all other chemicals were purchased from HiMedia, Mumbai, India.

Initiation of Callus Cultures

The leaves were directly used for callus initiation; however, the seeds were aseptically germinated and the hypocotyls portions were used for callus initiation. All the explant materials were surface sterilized using 0.1% mercuric chloride solution for 5–6 min. The explants were cut into small pieces and transferred onto MS medium with sucrose (3% w/v), agar (1.1% w/v), and required hormones (Table 1) and incubated at 27 ± 2°C in a biochemical oxygen demand incubator. The callus cultures were maintained by subculturing onto fresh media of the same composition at an interval of 4 weeks.

Initiation of Cell Cultures

About 5% w/v of callus after four passages was used to initiate cell cultures. The medium used was the same as that of callus cultures except for the addition of agar. The cell cultures were incubated at 25 ± 2°C in a refrigerated shaker incubator at 120 rpm. The cell cultures were maintained by subculturing the fine suspension into the same medium at an interval of 2 weeks.

Biotransformation of Valdecoxib

After two passages, the cell cultures were subcultured into fresh medium with 50% as inoculum. Ten milliliters of cell culture was aseptically transferred into each of the presterilized conical flasks (50 ml capacity). Valdecoxib, 2 mg in 100 μl of methanol, was added aseptically and incubated at 25 ± 2°C in a refrigerated shaker incubator at 120 rpm. Each subject was studied in triplicate with suitable controls.

Table 1 Media used for initiation and maintenance of callus cultures.

Plant	Hormone concentration (mg/l)	Reference
A. indica	NAA (1) and Kinetin (0.5)	Balaji et al. [13]
C. annuum	2,4-D (2) and Kinetin (1)	Ravi Gopal [14]
C. roseus	NAA (0.5) and BA (0.5)	Min et al. [15]
E. heyneana	Picloram (0.2) and BA (0.2)	Sumalatha [16]
N. tabacum	IAA (2), 2,4-D (0.25) and Kinetin (0.5)	Eikert et al. [6]

Extraction of Valdecoxib and Transformed Compounds

The cultures, after 48h of incubation with valdecoxib, were taken out and extracted with 3 × 10 ml of ethyl acetate by vortex mixing for 1 min. The organic phase was separated and evaporated under reduced pressure followed by drying in a vacuum oven. The dried samples were reconstituted in 1.5 ml each of HPLC-grade methanol by vortex mixing for 1 min. The samples were then centrifuged at 10,000 rpm and 20°C for 20 min. The supernatants were taken out and used for HPLC analysis.

HPLC-DAD Analysis

The HPLC analysis was performed on LC-10AT (Shimadzu, Kyoto, Japan) system by injecting 20 μl of sample following the conditions quite close to the method described previously by Keshetty et al. [11] with a modification. The column used was Luna C18, 5 μm, 250 × 4.6 mm i.d. (Phenomenex, Torrance, CA, USA). The mobile phase consisted of a mixture of acetonitrile–water, pH adjusted to 6.2 with orthophosphoric acid in 40:60. The mobile phase was degassed using an ultrasonic bath. The analysis was performed isocratically at a flow rate of 1 ml/min, and the analytes were detected using diode array detector (Shimadzu SPD M10Avp model) at a wavelength of 240nm. The UV absorption spectrum of the transformed products was compared with that of valdecoxib.

LC-MS-MS Analysis

Masses of the transformed products and their fragmentation ions were recorded by LC-MS-MS using Perkin Elmer (Waltham, MA, USA) SCIEX system with API positive mode. The column used was Inertsil ODS-2, RP, C18 of 250 × 4.6 mm dimension, 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 microliters of the sample was injected using an autosampler device. The transformed products were identified based on a pattern of UV spectra in HPLC-DAD and masses of the fragmentation products obtained in LC-MS-MS analysis.

Time Course of Biotransformation of Valdecoxib

Transformed products of interest were studied for optimizing the day of extraction for their highest yield. The cell cultures, after three passages, were subcultured into fresh medium with 50% inoculum. Ten milliliters of cell culture was taken into a conical flask (50 ml capacity) aseptically. Valdecoxib, 2 mg in 100 μl of methanol, was added and incubated at 25 ± 2°C in a refrigerated shaker incubator at 120 rpm. The cultures were withdrawn at the end of days 1, 3, 5, and 7, extracted, and analyzed for valdecoxib and its transformed products. Each subject was studied in triplicate with suitable controls. The percent of transformation products formed was calculated and the results were expressed in mean ± SD.

Optimization of Valdecoxib Concentration

Transformed products of interest were studied for optimizing the substrate concentration for their highest yield. The cell cultures, after three passages, were subcultured into fresh medium with 50% inoculum. Ten milliliters of cell culture was taken into a conical flask (50 ml capacity) aseptically. Valdecoxib, 0.25, 0.5, 1, 2, and 5 mg in 100 μl of methanol was added and incubated at 25 ± 2°C in a refrigerated shaker incubator at 120 rpm. The cultures

were withdrawn at the end of the day on which maximum transformation was observed, i.e., day 1 for *A. indica*; day 3 for *E. heyneana*; and day 5 for *C. roseus*, *C. annuum*, and *N. tabacum* (based on the results obtained in the time course of biotransformation), extracted, and analyzed for valdecoxib and its transformed products. Each subject was studied in triplicate with suitable controls. The percent of transformation products formed was calculated by assuming the total of peak areas of valdecoxib and its transformed products as 100%. The results were expressed in mean ± SD.

Results and Discussion

Establishment of Cultures

The calli obtained from *A. indica*, *C. annuum*, and *E. heyneana* were creamy in color, whereas *C. roseus* and *N. tabacum* calli were light green in color. The calli obtained from *A. indica*, *C. annuum*, and *C. roseus* were fragile in nature, whereas *E. heyneana* and *N. tabacum* calli were compact in nature. Complete callus formation was observed in 4 weeks in all the plants studied, and every time after this period, the calli were subcultured for maintenance.

The cell suspension cultures initiated from four-passage-old callus were fine in nature with few large clumps of cells. After two-passage maintenance, each once in 15 days, the total suspension was turned fine. All the plants studied had shown similar natures of cells.

Biotransformation of Valdecoxib

In all the media controls studied, none has shown any additional peaks in HPLC analysis. This indicates that the substrate valdecoxib was stable in the media used for different plants. The recoveries of valdecoxib from these media were >85.8%.

In preliminary study, HPLC analysis of the extracts of all the plants shows that the cell cultures were able to transform valdecoxib into one or more compounds. Some of the transformed compounds were produced by more than one plant. Valdecoxib was eluted at 15min and the new compounds produced in the cultures were designated as M1 (4.9 min), M2 (6.5 min), M3 (7.6 min), and M4 (9.5 min). All the plants produce compounds that are polar than the substrate evidenced from their retention times (Fig. 2a–e). *Catharanthus roseus* also produced a less polar metabolite (M5) at a retention time of 17.2 min. The products of interest were identified in HPLC based on the similarity in UV spectrum in the diode array detector. Substrate and transformed products, with slightly modified structures, were shown to have similar UV spectral patterns. However, the HPLC peak pertaining to the metabolite M5 has shown much less peak area and was found to contain an impurity that is coeluted with the metabolite. Hence, this metabolite was not investigated for quantification and structure elucidation.

Masses of the substrate and transformed products and their fragmentation ions were recorded by LC-MS-MS analysis. The structures of the transformed products were proposed from the masses of the fragmentation ions and retention times. M3 and M4 gave the same protonated molecular ion at m/z 331, that is, 16 higher than valdecoxib. This suggests that there might be hydroxylation products of valdecoxib. However, their ionization spectra were different. M3 showed a major characteristic fragment ion at m/z 198. This indicates that there was a loss of $C_6H_5CNOCH_2$ via a five-membered ring rearrangement. 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 above fragment, respectively. Based on this assumption, M3 was identified as 4-[5-hydroxymethyl-3-

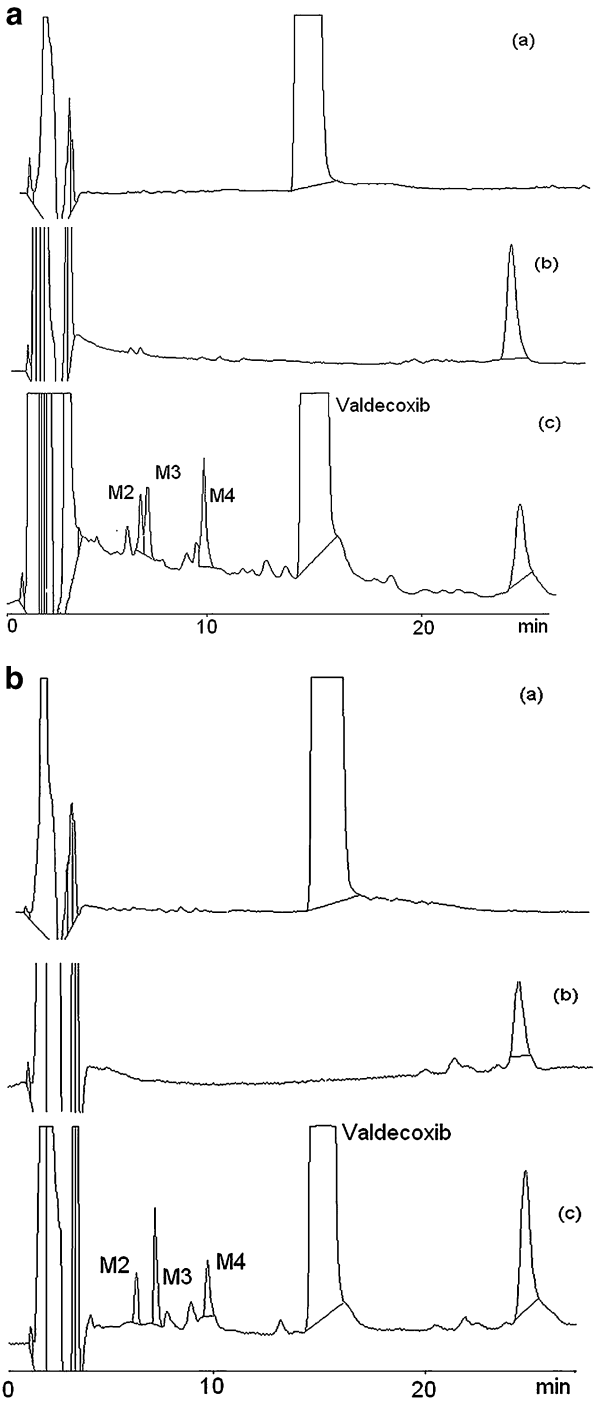


Fig. 2 **a** HPLC chromatograms showing (*a*) drug control (*b*) culture control, and (*c*) valdecoxib and its transformed products M2, M3, and M4 peaks detected in cell cultures of *A. indica*. **b** HPLC chromatograms showing (*a*) drug control (*b*) culture control, and (*c*) valdecoxib and its transformed products M2, M3, and M4 peaks detected in cell cultures of *C. annuum*. **c** HPLC chromatograms showing (*a*) drug control (*b*) culture control, and (*c*) valdecoxib and its transformed products M1, M2, M4, and M5 peaks detected in cell cultures of *C. roseus*. **d** HPLC chromatograms showing (*a*) drug control (*b*) culture control, and (*c*) valdecoxib and its transformed products M1, M2, and M4 peaks detected in cell cultures of *E. heyneana*. **e** HPLC chromatograms showing (*a*) drug control (*b*) culture control, and (*c*) valdecoxib and its transformed products M2, M3, and M4 peaks detected in cell cultures of *N. tabacum*

Fig. 2 (continued)

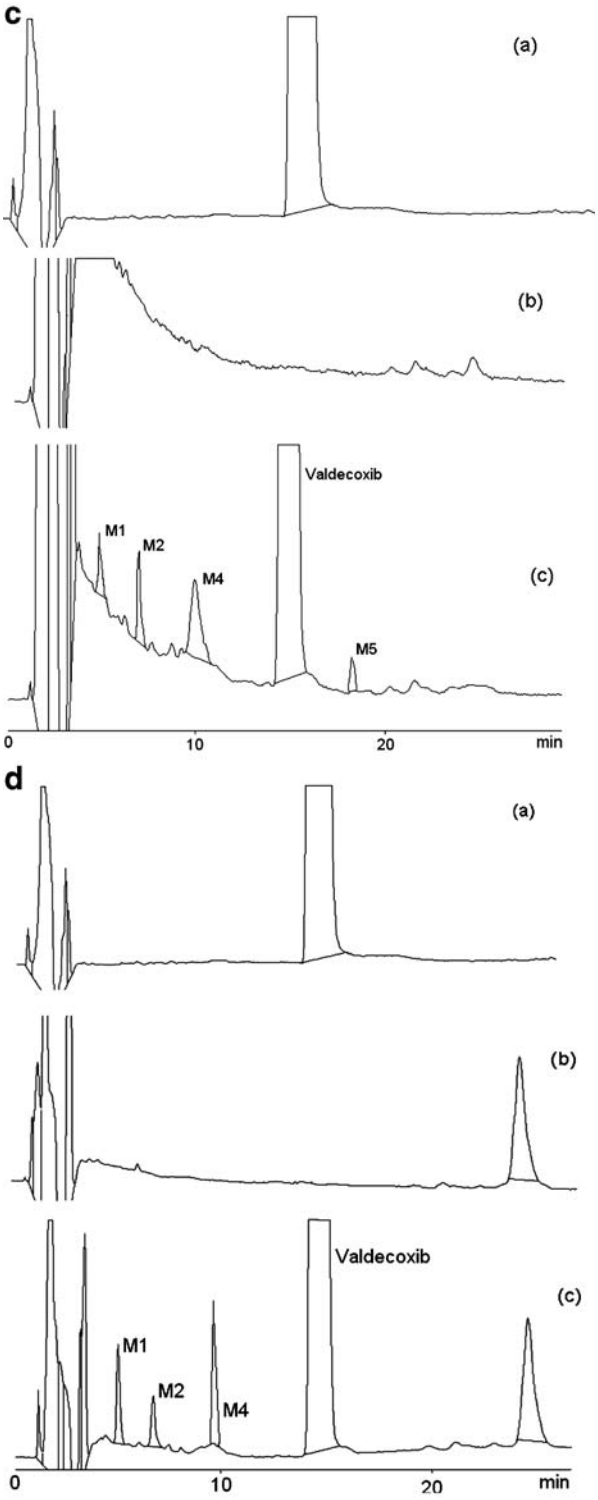
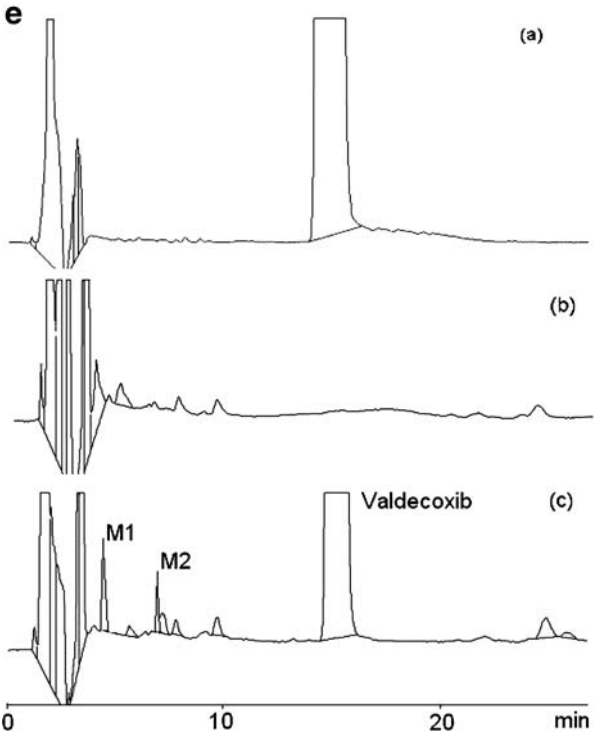


Fig. 2 (continued)



phenylisoxazol-4-yl] benzenesulfonamide. M4 showed characteristic fragment 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 fragment ions of mass 224, 208, and 160, respectively. The pattern of this mass fragmentation depicts that the substrate underwent hydroxylation of 3-phenyl ring, and thus, the product was identified as 4-[3-(4-hydroxyphenyl)-5-methyl-4-isoxazolyl] benzenesulfonamide. These products were also formed as metabolites of valdecoxib in mice [12]. M3 was also formed in humans in a disposition study performed by Yuan et al. [3]. In the similar fragmentation studies, M1 (m/z, 302) and M2 (m/z, 345) were found to be demethylated and carboxylic acid derivatives of valdecoxib. The fragmentation pattern of valdecoxib and its metabolites M2 and M3 is shown in Fig. 3. M1 was found to be 4-(3-phenyl-4-isoxazolyl) benzenesulfonamide, and M2 was derived from M3 and was designated as 4-[5-carboxy-3-phenylisoxazol-4-yl] benzenesulfonamide. The results revealed that the extracts derived from the cultures contained demethylated, hydroxylated, and carboxylic acid derivatives of valdecoxib. *Catharanthus roseus* also produced a new transformed product, which is less polar than the parent substrate. The identification of this product was not possible because of its coelution with impurity peak(s) in chromatography. The possible reactions involved in the biotransformation of valdecoxib are depicted in Fig. 4. The transformation products of cell cultures of various plants were shown in Table 2. The enzymes present in the cell cultures of the plants might be responsible for the transformation.

All the plants employed in the present investigation have shown positive reactions of biotransformation of a good number of substrates. The cultured cells of *N. tabacum* were

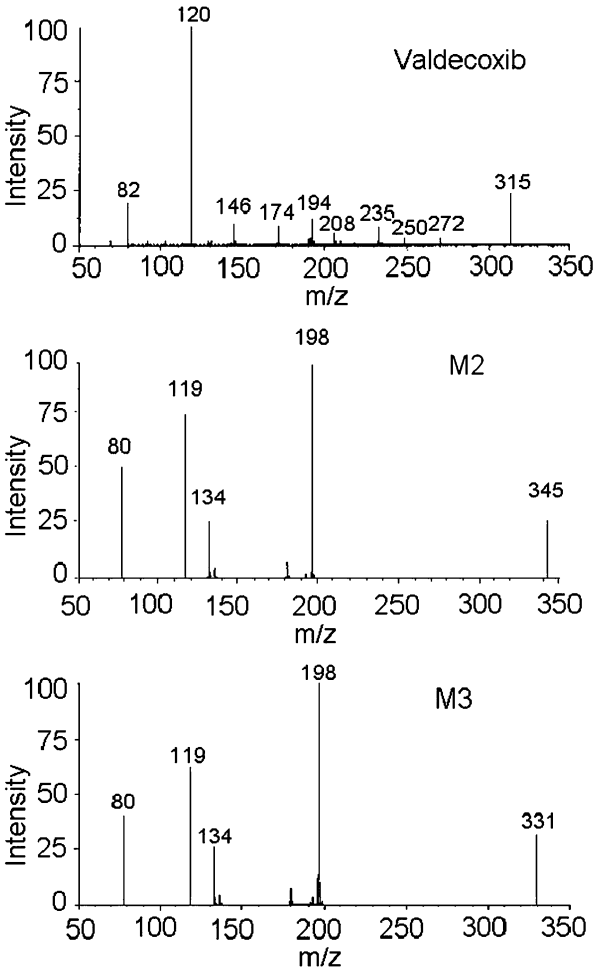


Fig. 3 Mass spectral fragmentation of valdecoxib and its metabolites M2 and M3

found to carry out hydroxylation at the sixth position of (4R)-(+)-enantiomer in α-terpineol [4]. Hirata et al. [5] reported on the biotransformation of monoterpene hydrocarbons 3-carene and 2-pinene by cell suspension cultures of *N. tabacum* and *C. roseus*, which were found to possess the ability to regio- and enantioselectively introduce the oxygenated functional groups into C=C bond and the allylic positions. Eikert et al. [6] reported that biotransformation of tryptophan and tyrosine by suspension cultures of *N. tabacum* and *C. roseus* produced hydroxylation products mainly and in minor quantities of methylation, acetylation, decarboxylation, transamination, and nonoxidative deamination products. Hao and Yeoman [7] reported that *N. tabacum* was able perform oxidative N-demethylation of nicotine into nornicotine. Cell suspension cultures of *C. roseus* reduced the carbonyl group of warfarin to the corresponding alcohols and regioselectively hydroxylated warfarin [8]. Yagen et al. [9] reported that the cell cultures of *Capsicum frutescens* metabolized progesterone to Δ-4-pregnene-20 beta-ol-3one and Δ-4-pregnene-14 alpha-ol-3, 20-dione. Dagnino et al. [10] found that the cell cultures of *Tabernaemontana divaricata* were able to transform conopharyngine, coronaridine, vobasine, and tabersonine.

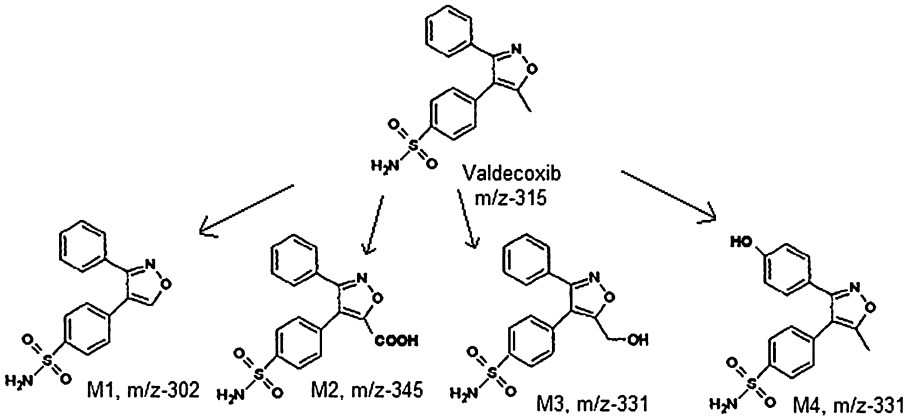


Fig. 4 Scheme showing the biotransformation of valdecoxib in plant cell cultures

Time Course of Biotransformation of Valdecoxib

Transformation products obtained in preliminary studies were further investigated for optimizing the day of extraction for their highest formation. Table 3 shows the percent of different transformed products when the cultures were extracted on 1, 3, 5, and 7 days of incubation.

Optimization of Valdecoxib Concentration

Transformation products obtained in preliminary studies were also investigated for optimizing the concentration of substrate to be added for their highest yield when the cultures were extracted on optimum days of incubation for the products in respective plants. Table 4 shows the percent of different products formed when the cultures were incubated with various concentrations of substrate and extracted after the optimum day for the respective plant.

Conclusion

The cell cultures of the various plants studied have exhibited the ability to biotransform the substrate valdecoxib. All the cultures have been found to produce compounds that are more polar than the parent compound valdecoxib. *Catharanthus roseus* has also produced an

Table 2 The transformation products of cell cultures of various plants.

Plant	Product
A. indica	M2, M3, M4
C. annuum	M2, M3, M4
C. roseus	M1, M2, M4, M5
E. heyneana	M1, M2, M4
N. tabacum	M1, M2

Table 3 Optimization of time course for highest yield of transformed products by cell cultures of various plants.

Plant	Product	Day of extraction			
		1	3	5	7
A. indica	% M2	**0.027±0.0042**	0.026±0.0040	ND	ND
	% M3	**0.034±0.0028**	0.003±0.0004	ND	ND
	% M4	**0.096±0.0038**	ND	ND	ND
C. annuum	% M2	0.012±0.0002	0.013±0.0004	**0.015±0.0005**	0.014±0.0022
	% M3	0.021±0.0031	0.016±0.0023	**0.032±0.0046**	0.019±0.0070
	% M4	0.008±0.0008	0.007±0.0005	**0.014±0.0002**	0.004±0.0058
C. roseus	% M1	ND	ND	**0.009±0.0003**	ND
	% M2	ND	0.010±0.0012	**0.012±0.0009**	0.010±0.0003
	% M4	ND	0.010±0.0031	**0.012±0.0005**	0.009±0.0006
E. heyneana	% M1	ND	**0.039±0.0083**	0.019±0.0027	ND
	% M2	ND	**0.022±0.0087**	0.019±0.0092	ND
	% M4	0.013±0.0067	**0.089±0.0106**	0.074±0.0061	ND
N. tabacum	% M1	ND	0.086±0.0502	**1.231±0.5200**	0.238±0.0205
	% M2	ND	0.093±0.0132	**0.410±0.0535**	0.303±0.1338

Values in bold face indicate the maximum yield of that particular product

ND = not detected

unknown polar metabolite that is less polar than the substrate. The plant cell cultures were found to perform hydroxylation and demethylation reactions. The day of extraction and substrate concentration has influenced the amount of product formation. To summarize, the plant cell cultures can be used as potential biocatalysts for producing novel compounds from the added substrates.

Table 4 Optimization of valdecoxib concentration for highest yield of transformed products by cell cultures of various plants.

Plant	Product	Valdecoxib concentration (mg/10 ml culture)				
		0.25	0.5	1.0	2.0	5.0
A. indica	% M2	ND	**0.043±0.0154**	0.034±0.0155	0.034±0.0230	0.033±0.0270
	% M3	**0.105±0.0011**	0.084±0.0074	0.044±0.0039	0.035±0.0040	0.005±0.0001
	% M4	ND	**0.199±0.010**	0.114±0.0085	0.061±0.0064	0.021±0.0048
C. annuum	% M2	ND	0.010±0.0058	0.024±0.0003	**0.033±0.0003**	0.026±0.0004
	% M3	ND	ND	ND	**0.030±0.0005**	0.027±0.0009
	% M4	ND	ND	ND	**0.017±0.0001**	ND
C. roseus	% M1	ND	0.010±0.0036	0.030±0.0079	**0.034±0.0037**	0.021±0.0012
	% M2	ND	0.022±0.0350	0.035±0.0200	**0.317±0.0157**	0.180±0.0053
	% M4	ND	ND	ND	**0.018±0.0261**	0.008±0.0006
E. heyneana	% M1	0.090±0.0017	0.193±0.0029	**0.253±0.0240**	0.146±0.0158	0.021±0.0246
	% M2	0.038±0.0539	0.106±0.0337	**0.128±0.0665**	0.015±0.0002	0.011±0.0149
	% M4	ND	ND	**0.078±0.0071**	0.050±0.0004	0.017±0.0185
N. tabacum	% M1	**0.481±0.0094**	0.465±0.1440	0.233±0.1479	0.080±0.0131	0.034±0.0099
	% M2	**0.485±0.0189**	0.394±0.0683	0.180±0.1694	0.062±0.0062	0.034±0.0099

Values in bold face indicate the maximum yield of that particular product

ND = not detected

Acknowledgements The authors are thankful to Dr. Ramesh Mullangi and Mr. Raj Kallem, Discovery Research, Dr. Reddy's Laboratories, Hyderabad, for recording LC-MS-MS spectra. The authors are also thankful to Dr. A.V.S.R. Swamy, Senior Scientist, Central Tobacco Research Institute, Guntur, for providing the seeds of *N. tabacum* and Mepro Pharmaceuticals, Ahmedabad, for providing the valdecoxib gift sample. One of the authors, Molmoori, is thankful to All India Council for Technical Education, New Delhi, India, for providing fellowship.

References

1. Ishihara, K., Hamada, H., Hirata, T., & Nakajima, N. (2003). *Journal of Molecular Catalysis. B, Enzymatic*, *23*, 145–170.
2. Talley, J. J., Brown, D. L., Carter, J. S., Graneto, M. J., Koboldt, C. M., Masferrer, J. L., et al. (2000). *Journal of Medicinal Chemistry*, *43*, 775–777.
3. Yuan, J. J., Yang, D., Zhang, J. Y., Bible Jr., R., Karim, A., & Findlay, J. W. A. (2002). *Drug Metabolism and Disposition*, *30*, 1013–1021.
4. Suga, T., Hirata, T., & Lee, Y. S. (1982). *Chemistry Letters*, *11*, 1595–1597.
5. Hirata, T., Yoshihiro, I., Izumi, S., Shimoda, K., Hamada, H., & Kawamura, T. (1994). *Phytochemistry*, *37*, 401–403.
6. Eikert, H., Karasinska, T., & Kohlnunzer, S. (1983). *Herba Hungarica*, *22*, 29–32.
7. Hao, D. H., & Yeoman, M. M. (1998). *Journal of Plant Physiology*, *152*, 420–426.
8. Hamada, H., Fuchikami, Y., Jansinj, R. L., & Kaminsky, L. S. (1993). *Phytochemistry*, *33*, 599–600.
9. Yagen, B., Gallili, G. E., & Matcles, R. I. (1978). *Applied and Environmental Microbiology*, *36*, 213–216.
10. Dagnino, D., Schripsema, J., & Verpoorte, R. (1994). *Phytochemistry*, *35*, 671–676.
11. Keshetty, S., Venisetty, R. K., Molmoori, V., & Ciddi, V. (2006). *Pharmazie*, *61*, 245–246.
12. Zhang, J. Y., Yuan, J. J., Wang, Y., Bible Jr., R. H., & Breau, A. P. (2003). *Drug Metabolism and Disposition*, *31*, 491–501.
13. Balaji, K., Veeresham, C., Srisilam, K., & Kokate, C. K. (2003). *Journal of Plant Biotechnology*, *5*, 121–129.
14. Ravi Gopal, N. (1998). M. Pharm Thesis, Kakatiya University, Warangal, India.
15. Min, Y., Lili, N., Jixun, Z., Hongzhu, G., & Dean, G. (2003). *Journal of Molecular Catalysis. B, Enzymatic*, *22*, 89–95.
16. Sumalatha, D. V. (2001). M. Pharm Thesis, Kakatiya University, Warangal, India.