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Tobacco Chemistry 77.* Biotransformations of a Major Tobacco Cembratrienediol using Plant Cell Cultures of *Nicotiana Sylvestris*

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A detailed study of the biotransformation of a major tobacco constituent. (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**1**), by incubation with plant cell cultures of *Nicotiana sylvestris*, is presented. Growing cells of *N. sylvestris*, as well as cell homogenates, cell-free extracts and cell pellets thereof were employed and the bioconversion of the diol **1** into five products was evaluated in terms of varying incubation times and the age of the cell cultures. The major product, resulting from regioselective enzymatic attack at the 11,12 double bond of **1** is the known 11*S*,12*S*-epoxide with minor components being formed from enzymatic hydroxylation at C-10, C-12 and C-13. The outcome of the present enzyme-catalyzed processes is compared with earlier chemical conversions and hypothetical biosynthetic schemes for the large family of natural cembranoids found in *Nicotiana* species.

In the accompanying publication,[1] we have presented our results on the biotransformation of (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**1**) and the corresponding 4*R*-epimer when incubated with cell cultures of *Triptery-gium wilfordii*. While these studies were underway, we succeeded in developing a cell culture of *Nicotiana sylvestris*, a species in which both these diols and many other cembranoids have been encountered. It was therefore of interest to compare the biotransformation of **1**, the more abundant of these diols, when exposed to the two different types of cell culture. The present studies include incubation of **1** with 'growing' cells, cell homogenates and cell free extracts (Scheme 2) of an *N. sylvestris* cell line designated T-43-T and the results are discussed below under corresponding headings.

Biotransformation with 'growing' cells. The influence of the age of an *N. sylvestris* culture on its growth, as measured by its ability to lower the sugar concentration in the medium in the presence of starting material, is illustrated in Fig. 1. Here the refractive index (RI) of the medium, reflecting its sugar concentration, is plotted against the age of the cell culture (in days). The precursor is added for the first time when the cells are respectively 0, 4 or 12 days old, and further additions are made if and when there is a substantial drop in the RI. In the experiments involving the 0- and 4-day-old cell cultures 10 mg were added on each occasion, but 15 mg in the experiments involving the 12-day-old cell culture. It is readily seen that the older the cell culture the less its growth is affected by the addition of the precursor. It

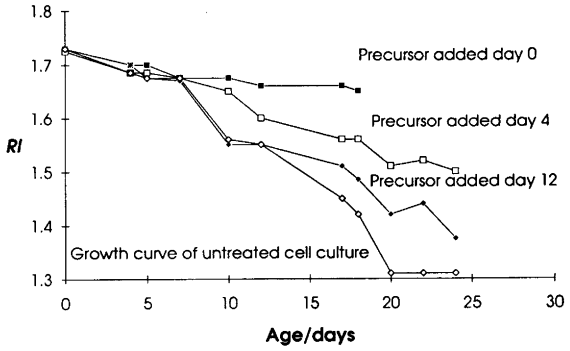

Fig. 1. The influence of the age of an *N. sylvestris* culture on its growth, as measured by its ability to lower the sugar concentration in the medium in the presence of starting material. The refractive index (RI) of the medium, reflecting its sugar concentration, is plotted against the age of the cell culture in days. The precursor was added for the first time when the cells were, respectively, 0, 4 or 12 days old, and further additions were made if and when there was a substantial drop in the RI value. In the experiments involving the 0- and 4-day-old cell cultures 10 mg precursor were added on each occasion; 15 mg were added in the experiments involving the 12-day-old cell culture.

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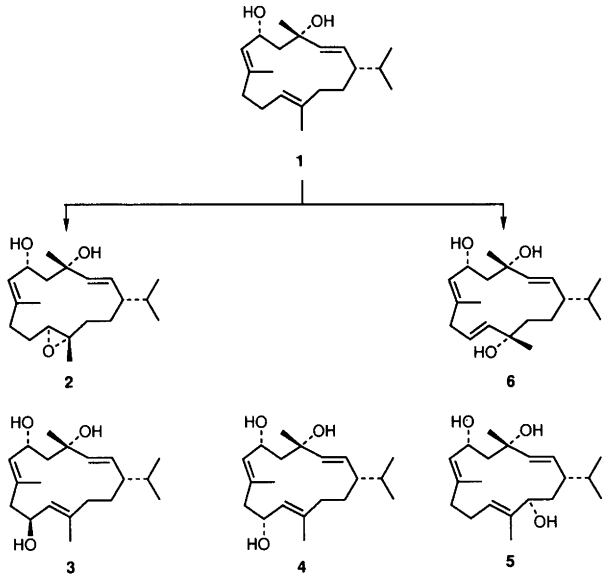
Table 1. Biotransformation of (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**1**) with growing cells of T-43-T cell line.

Exp.[a] No.	SM[b] (mg)	Medium pH[c]	Number of doses	Duration of add./h	Incubation time/h[d]	SM rec.[e] **1** (%)	Prod. yield (%) **2**	**3**	**4**	**5**	**6**
1	90	5.38–5.11	1	0	24	60	6	—	2	—	13
2	90	5.35–5.75	5	36	60	37	13	3	5	—	10
3	90	5.35–5.75	5	48	72	42	3	1	12	—	15
4	90	5.28–5.60	8	79	126	31	20	—	21	—	14
5	90	5.25–5.81	8	91	158	9	12	7	29	—	20
6	90	5.78–5.78	8	91	180	7	8	5	15	—	15
7	90	5.50–5.89	6	216	288	5	16	3	12	—	5
8	90	5.25–5.80	8	91	300	1	9	6	17	—	16
9	200	5.31–5.65	5	36	60	63	3	—	4	—	4

[a] In all experiments 12-day-old cells and a suspension volume of 500 ml were used. [b] SM = starting material. In these experiments, the diol **1** dissolved in methanol (7.5 mg ml^{-1}) was added batch-wise as indicated by the number of doses and time between first and last addition in the table, e.g., in exp 2, 5 additions of 18 mg each in intervals of approximately 9 h were carried out over a 36 h period. [c] Medium pH varies in the range indicated during incubation time. [d] Total incubation time from the first addition of SM. [e] SM rec. = starting material recovered.

should be recognized that the biomass increases with age so the 'apparent' observation of lower retardation may be due to the fact that the precursor is being added to a larger biomass.

In these experiments, the substrate (**1**) dissolved in alcohol, was added in portions to a suspension culture of the T-43-T cell line grown in Erlenmeyer flasks on a rotary shaker and under the conditions detailed in Table 1. As shown here, the diol **1** is incubated with 12-day-old cells, and the ratio substrate:suspension volume is held constant in experiments 1–8, while incubation and addition times are varied. Based on the recovery of substrate and overall yield of the biotransformation products **2**–**6**, the longer incubation time is preferable. Since these products (Scheme 1) were the same as those encountered in the previous study with *T. wilfordii* cells, their identity was readily ascertained. It is interesting to note that the epoxide **2**, being the major product in the experiments involving 'growing' cells of *T. wilfordii*,[1] is a minor component in the present study. In contrast, the cells of the T-43-T cell line obviously effect hydroxylation at C-10 and C-12 to afford the products **4** and **6** more efficiently. These results are illustrated graphically in Fig. 2.



Scheme 1. Biotransformation of the diol **1** by T-43-T cell culture.

When a higher ratio of substrate to suspension culture is employed (Table 1, entry 9), substantial recovery of starting material and very little biotransformation is encountered. It follows, as shown in separate experiments (see Experimental), that **1** is obviously toxic to the cells.

With this in mind it was of interest to perform experiments in which a solution of **1** was added slowly in a controlled semicontinual manner by means of a peristaltic pump. The results were, however, not sufficiently encouraging to justify further studies using this technique.

When compared with the results obtained with whole cells of *T. wilfordii*,[1] it was clear that the bioconversion yields were much lower. Studies with cell homogenates were therefore initiated and the procedures employed

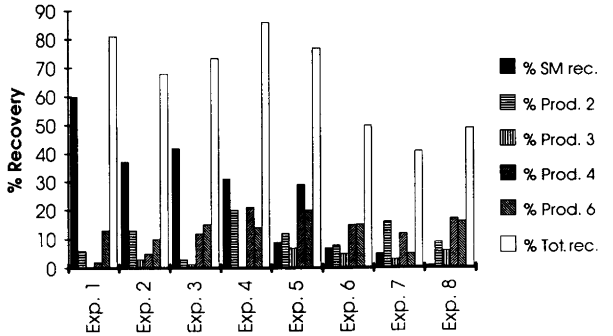


Fig. 2. Relative yields of products and recovered starting material (SM) on exposure of (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**1**) (90 mg) to a suspension (500 ml) of cells of *N. sylvestris* (12 days old) related to the incubation time.

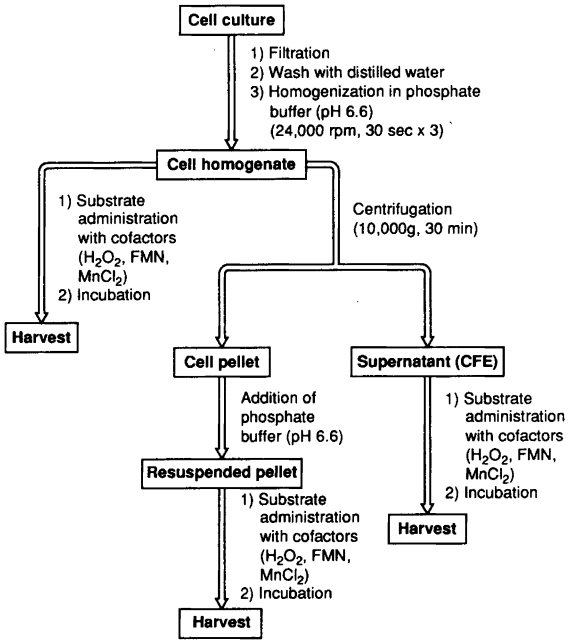
Table 2. Biotransformation of (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**1**) with cell homogenate of T-43-T cell line.

Exp.[a] No.	SM[b] (mg)	Cell age/d	Homogenate pH[c]	Incubation time/h	Homogenate vol./ml	SM rec.[d] **1** (%)	Prod. yield (%) **2**	**3**	**4**	**5**	**6**
10	50	12	6.30	48	80	30	58	—	—	—	—
11	50	14	6.45	120	100	5	71	1	9	—	—
12	500	14	6.40	120	850	6	72	—	5	—	—
13[e]	100[f]	16	6.40	120	530	13	58	2	3	1	2
14	25	18	6.35	120	190	6	70	—[g]	—[g]	—[g]	—

[a] In all experiments, H_2O_2 (2.16 mol equiv., unless otherwise stated), FMN (0.5 mol equiv.) and $MnCl_2$ (0.5 mol equiv.), were added to the cell homogenate as cofactors. [b] SM = starting material. In these experiments, the diol **1** dissolved in ethanol (5 mg ml^{-1}) was added in one batch at time zero, unless otherwise stated. [c] In all cases, phosphate buffer was used. The value shown is the initial pH of homogenate prior to addition of SM. [d] SM rec. = starting material recovered. [e] In this experiment a 4.0 mol equiv. of H_2O_2 were used. [f] A concentration of 10 mg ml^{-1} of **1** in ethanol was used. [g] A mixture (10% yield) of compounds **3**, **4** and **5** was obtained but not separated.

to obtain the cell homogenate, supernatant (cell-free extract), cell pellet, etc. are outlined in Scheme 2.

Biotransformation with cell homogenates. The results of the five experiments employing the homogenates of cells of the T-43-T line supplemented by the cofactors H_2O_2, FMN and $MnCl_2$ are summarized in Table 2. The outcome of these experiments is dramatically different from those obtained with whole cells (Table 1). The epoxide **2** is now the major product and the yields exceed those obtained with *T. wilfordii* cells. Clearly, epoxidation is favored over allylic hydroxylation at positions 10 and 13, possibly because epoxidizing enzymes are liberated and hydroxylating enzymes are being partly destroyed during the homogenization procedure. Although the toxicity of **1**, in terms of inhibition of cell growth, is a problem at higher substrate concentration (entry 9, Table 1), the enzymes, once liberated from the cells, are able to tolerate reasonably high concentrations of **1** in the cell homogenate experiments and carry out biotransformations in respectable yields. In order to gain additional information about the nature of the enzyme systems and, perhaps, to obtain still higher yields of the epoxide **2**, the cell homogenate was subjected to further purification, cf. Scheme 2.



Scheme 2. Preparation of cell homogenate, cell-free extract and resuspended pellet.

Table 3. Biotransformation of (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**1**) with cell free extract (CFE) or resuspended pellet of T-43-T cell line.

Exp.[a] No.	SM[b] (mg)	Cell age/d	CFE pH	Medium pH	Incubation time/h	CFE vol./ml	Buffer vol./ml	SM rec.[c] **1** (%)	Product[d] yield, **2** (%)
15	10	2	6.55		24	8.0		95	—
16	10	7	6.53		24	6.3		96	—
17	20	11	6.55		24	6.0		91	1
18[e]	50	12	6.35		48	80		70	18
19[e]	50	12		6.55[f]	48		80	28	62

[a] In all experiments, H_2O_2 (2.16 mol equiv.) was added as cofactor. [b] SM = starting material. In these experiments, the diol **1** dissolved in ethanol (5 mg ml^{-1}) was added in one batch at time zero. [c] SM rec. = starting material recovered. [d] This was the only product detected in these experiments. [e] In these experiments H_2O_2 (2.16 mol equiv.), FMN (0.5 mol equiv.) and $MnCl_2$ (0.5 mol equiv.) were added as cofactors. [f] This indicates the initial pH at time of addition of SM.

Biotransformation with cell-free extracts. The four experiments carried out with the cell-free extract (CFE or supernatant, Scheme 2) are summarized in Table 3. It is clear that centrifugation of the cell homogenate at 10000*g* to afford the CFE, eliminates essentially all of the relevant enzymes required to biotransform **1** into the products **2–6**. However, the enzyme capable of generating epoxide **2** seems to be present in low concentration in the supernatant, since when the CFE to substrate ratio is increased (entry 18, Table 3), a low yield of epoxide **2** was noted.

In agreement with expectation incubation with a resuspension of the pellet in phosphate buffer shows that the enzyme capable of biotransforming **1** into the epoxide **2** is predominantly located here (entry 19, Table 3).

In conclusion, the studies with the cell cultures of *N. sylvestris* reveal the presence of enzymes which, in their attack of the diol **1**, resemble $^1O_2$ in that the epoxide **2** as well as the triol **6** are produced, but differ in that they are also capable of attacking the allylic C-10 and C-13 positions with the generation of the triols **3–5**. Although many *seco*-cembranoids have been encountered in tobacco,[2] no such products were detected, which seems to suggest that none of the enzymes capable of cleaving the 14-membered ring are present in the cell cultures examined.

Experimental

The general procedures for isolation and characterization of the biotransformation products **2–6** obtained in these studies are already described in the accompanying publication.[1] Details concerning the development of the T-43-T cell line of *Nicotiana sylvestris* and the specific procedures relating to the studies summarized in Tables 1–3 are presented below.

Cultivation of T-43-T cell line. Tobacco seeds of *Nicotiana sylvestris* were obtained from Bergianska Botanical Garden, Stockholm University, Stockholm, Sweden. Leaf-derived tobacco calli were cultured on solidified agar (8 g l^{-1}) Murashige–Skoog medium (MS)[3] containing sucrose (3 g l^{-1}) and 2,4-D (1 mg l^{-1}) and were kept at 25 °C with a cycle of 8 h darkness and 16 h light. Calli were subcultured every 3–4 weeks. Suspension cultures (500 ml in 1 l Erlenmeyer flasks) were inoculated at a rate of 1.5 g dry cell mass l^{-1} of liquid MS medium. All suspension cultures were agitated on a rotary shaker (135 rpm, 1″ throw) at 26 °C without illumination.

Indicators of growth inhibition of T-43-T suspension cultures by cembranoids. (1) The diol **1** was added (1 mg ml^{-1}) to a 10-day suspension (10 ml) of T-43-T which was incubated for 24 h and 48 h before addition to fresh MS broth (90 ml). The culture did not grow whereas a methanol–treated control did. The diol-treated cells were 100% dead as determined with Evan's blue stain.[4] (2) Low concentrations of the diol **1** (20 μg ml^{-1}) added to freshly subcultured suspensions of T-43-T severely retarded growth as measured by refractive index monitoring of filtered culture broth. (3) Addition of the diol **1** (1 mg ml^{-1}) to 12-day suspension cultures retarded growth as indicated by a reduced rate in the decrease in the refractive index of the filtered broth. (Note: RI is directly proportional to the residual sugar concentration in the broth and inversely proportional to the biomass). (4) Stationary-phase T-43-T cells resuspended in phosphate buffer turned from pale green to dark green within 20 min when the diol **1** (1 mg ml^{-1}) was included in the buffer. There were no viable cells as determined by Evan's blue stain.[4]

General procedure for experiments 1 to 9 (batchwise addition of the diol **1** *to the growing T-43-T cell suspension culture).* The suspension culture of T-43-T was grown in MS medium for 12 days. Portions of an alcoholic solution of **1** were added at the time intervals indicated in the Table 1. After the addition of the last batch of substrate, the incubation was generally allowed to continue for a further 24 h or longer. The resulting biotransformation mixture was harvested by extraction with ethyl acetate and the products were separated by chromatography on silica gel using ethyl acetate as the eluent.

Typical procedure for biotransformation of the diol **1** *(entry 9, Table 1).* The diol **1** (200 mg) was dissolved in ethanol (25 ml), and the solution was divided into 5 portions, then added to the cell suspension at time intervals of 0, 12, 7, 5, and 12 h. After the last addition, the incubation was allowed to continue for 24 h. The biotransformation mixture was then filtered through Miracloth and the filtrate was extracted with ethyl acetate (3 × 400 ml). The extract was washed with water (200 ml) and brine (200 ml), and dried over $MgSO_4$. Ethyl acetate (300 ml) was added to the cell material and the resulting suspension was homogenized with an IKA Ultra-Turrax Disperser T-25 fitted with an S25N-25F rotar/stator (Jankie and Kunkel GmbH and Co. KG) at 20000 rpm for 5 min. The homogenate was then filtered through Miracloth, the filtrate was washed with water (200 ml) and brine (200 ml), and dried over magnesium sulfate. The extracts were combined and the solvent removed *in vacuo* to provide the product mixture (278 mg). Chromatography on silica gel (85 g) using ethyl acetate as the eluent gave, successively, the recovered diol **1** (125 mg, 63%), the triol **6** (8 mg, 4%), the epoxide **2** (6 mg, 3%) and the triol **4** (7.2 mg, 4%). The identity of these compounds was established by spectral comparison (NMR, MS, IR) and melting-point determinations with authentic samples obtained in our earlier studies.[1]

General procedures for experiments involving semi-continual addition of substrate via a peristaltic pump. The suspension culture was transferred to a 500 or 1000 ml benchtop bioreactor (depending on culture volume), aerated through a sintered glass disk at 200 ml l^{-1} min^{-1} and kept at 21–24 °C. Every hour over the next 24 h, a timer-controlled peristaltic pump added 0.25–0.5 ml of an

alcoholic solution of **1**. Incubation was then continued for a further 24 h. Work-up as described above, afforded the product mixture in yields similar to those given in Table 1.

General procedure for the preparation of cell homogenate, resuspended pellet and cell-free extracts (CFE). All procedures were performed at 0–4 °C. The procedures for such preparations are outlined in Scheme 2. T-43-T cell suspension culture was harvested by filtration through a Büchner funnel equipped with Miracloth and the filtrate was collected for pH and refractive index measurements. The cells were washed with distilled water and allowed to dry by suction before their fresh weight was determined.

Phosphate buffer (0.1 M, pH 6.6, 140 ml) was added to the cells (100 g fresh weight) and the resulting suspension was then homogenized with an IKA Ultra-Turrax Disperser T-25 fitted with an S25N-25F rotor/stator (Jankie and Kunkel GmbH and Co. KG) at 24000 rpm for 30 s. The same procedure was repeated three times. In order to avoid overheating the suspension, a 1 min break was allowed between each operation.

The homogenate thus obtained was divided into two portions. One portion of the homogenate was used directly in the biotransformation experiment. The remaining portion of the homogenate was then centrifuged at 10000*g* (8000 rpm) for 30 min. The supernatant was collected as CFE for biotransformation experiments and the pellet was resuspended in the same phosphate buffer (pH 6.6, 40 ml/10 g wet weight) and also used directly in biotransformation experiments. The peroxidase activity and protein concentration were measured for the cell homogenate, CFE and the resuspended pellet according to the following procedures.

Bio-Rad protein assay.[5] This assay procedure is available from Bio-Rad Chemical Division, Bio-Rad Protein Assay, Bio-Rad, Richmond California. The protein concentration was done according to Bio-Rad instructions.

Peroxidase activity assay.[6] In order to measure the peroxidase activity of CFE, it was necessary initially to utilize a 'blank' solution prepared in the following manner. A mixture of 5% aqueous pyrogallol solution (2 ml), 0.1 M phosphate buffer (3 ml, pH 6.6), freshly prepared 0.5% H_2O_2 solution (1 ml) and distilled water (14 ml) was extracted with ether (50 ml). Several ml of ether extract placed in a W cell were then used to adjust the spectrometer reading at 420 nm to zero. A standard curve of absorbance at 420 nm was derived by measuring absorbance at this wavelength by means of a set of standard solutions of purpurogallin (0.5–3.5 mg 50 ml^{-1} of ether). CFE (1 ml) was added to a 50 ml Erlenmeyer flask containing 5% aqueous pyrogallol solution (2 ml), 0.1 M phosphate buffer (2 ml, pH 6.6), freshly prepared 0.5% H_2O_2 solution (1 ml) and distilled water (14 ml) at 20 °C. This mixture was allowed to stand for 20 s at 20 °C, then sulfuric acid (2 M, 1 ml) was added to quench the reaction and the mixture was then extracted with ether (2 × 25 ml). An aliquot (4 ml) of this ether solution was placed in the W cell, and its absorbance measured at 420 nm and the data compared with the standard curve.

General procedures for biotransformations using cell homogenate, CFE and resuspended pellet. The CFE solution, cell homogenate or resuspended pellet (prepared as shown in Scheme 2) was added to an Erlenmeyer flask containing precursor dissolved in ethanol, distilled water, phosphate buffer (0.1 M, pH 6.6) and the cofactors H_2O_2, FMN and $MnCl_2$. After being stirred at room temperature for an appropriate interval (incubation time, see Tables 2 and 3), the products were isolated by extraction with ethyl acetate as described below.

Typical procedure for biotransformation of diol **1** *using cell homogenate (entry 11, Table 2).* The cell homogenate (100 ml, containing 439 units of peroxidase and 170 mg of protein) prepared from a cell suspension culture (14 days old) was added to an Erlenmeyer flask containing the diol **1** (50 mg/10 ml EtOH), distilled water (75 ml), phosphate buffer (0.1 M, pH 6.6, 175 ml), H_2O_2 (2.16 equiv.), FMN (0.5 equiv.) and $MnCl_2$ (0.5 equiv.). The mixture was stirred at room temperature for 120 h, after which ethyl acetate (125 ml) was added and the mixture was allowed to stir for another 5 min. The resulting mixture was then filtered through Celite and the filtrate was extracted with ethyl acetate (3 × 250 ml). The Celite was sonicated with ethyl acetate (150 ml) for 30 min and then filtered. The combined organic extracts were washed with water (400 ml) and brine (400 ml), and dried over $MgSO_4$. Concentration *in vacuo* afforded a crude mixture of products (89 mg). Chromatography over silica gel (25 g) gave recovered diol **1** (2.7 mg, 5%), the epoxide **2** (35.3 mg, 71%) and the triols **3** (0.4 mg, 1%), and **4** (3.4 mg, 9%).

Typical procedure for biotransformation of the diol **1** *using CFE (entry 18, Table 3).* The cell-free extract (80 ml, containing 329 units of peroxidase and 77 mg of protein) prepared from cell suspension culture (12 days old) was added to an Erlenmeyer flask containing the diol **1** (50 mg/10 ml EtOH), distilled water (75 ml), phosphate buffer (0.1 M, pH 6.6, 175 ml), H_2O_2 (2.16 equiv.), FMN (0.5 equiv.) and $MnCl_2$ (0.5 equiv.). After being stirred at room temperature for 48 h, the reaction mixture was worked up as described above and a crude mixture of products (59 mg) was obtained. Chromatography over silica gel (25 g) using ethyl acetate as the eluent gave the recovered diol **1** (35.0 mg, 70%) and the epoxide **2** (9.2 mg, 18%).

Typical procedure for biotransformation of the diol **1** *using resuspended pellet (entry 19, Table 3).* The resuspended pellet (80 mg, containing 175 units of peroxidase and 100 mg of protein) prepared from cell suspension culture (12 days old) was added to an Erlenmeyer flask con-

taining the diol **1** (50 mg/10 ml EtOH), distilled water (75 ml), phosphate buffer (0.1 M, pH 6.6, 175 ml), H_2O_2 (2.16 equiv.), FMN (0.5 equiv.) and $MnCl_2$ (0.5 equiv.). After being stirred at room temperature for 48 h, the reaction mixture was worked up as described above and a crude mixture of products (87.5 mg) was obtained. Chromatography over silica gel (25 g) using ethyl acetate as the eluent gave the recovered diol **1** (14.1 mg, 28%) and the epoxide **2** (50.9 mg, 62%).

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