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Tobacco Chemistry 76.* Biotransformations of Tobacco Isoprenoids using Plant Cell Cultures of *Tripterygium Wilfordii*

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A detailed study involving the biotransformation of two abundant tobacco constituents, (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**1**) and the 4*R* isomer (**2**), by incubation with plant cell cultures of *Tripterygium wilfordii*, an important Chinese herbal plant, is presented. The experiments involve incubation of **1** and **2** with growing as well as resuspended cells of *T. wilfordii* for varying time periods and utilizing cells of different ages. Major products are the known epoxides resulting from enzymatic epoxidation at the 11,12 double bond, while enzymatic hydroxylation at C-10, C-12 and C-13 is less predominant and affords several triols, one of which so far has not been reported present in *Nicotiana* species.

The major cembranoid components of tobacco are (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**1**) and the 4*R*-epimer (**2**) which possess growth- and tumor-inhibiting as well as insect-repelling properties. Biomimetic studies of these and extensive chemical exploration of the isoprenoids of tobacco have disclosed that they can be regarded as the predominant precursors of nearly sixty C_{20} cembranoids.[1] Some of these, in turn, give rise to a large number of degradation compounds comprising 12, 13, 14, 15, 18 or 19 carbon atoms, several of which are important aroma constituents. The predominant cleavage of the cembrane skeleton required to account for these compounds are indicated in Fig. 1. These reactions are probably initiated by enzymatic and/or singlet oxygen attack which can generate primary precursors that on further degradation furnish the host of degraded cembranoids encountered in tobacco.

Since this picture emerges from chemical investigations based exclusively on isolation, structure elucidation and chemical transformations, it was of considerable interest to examine whether similar interconversions could be executed, more regiospecifically and hopefully in higher yield, by enzymatic processes. In particular, if it were possible to accomplish direct enzymatic cleavage at the 4,5- and 11,12-positions of the 2,7,11-cembratriene-4,6-diols (**1** and **2**), it would yield lower molecular weight compounds of potential use in the area of aroma and fragrance.

The major reason for selecting the plant cell culture line of *Tripterygium wilfordii*, coded as TRP4a, for these biotransformation experiments was its well established and well characterized ability to generate peroxidase (oxidase) activity.[2,3] A total of 32 experiments were conducted—four with diol **2** and 28 with the more readily available diol **1**. In these experiments, different reaction conditions were utilized, with such parameters as cell culture age, pH of the medium, incubation time and cell culture:substrate ratio being varied. Two types of experiment were conducted and these are discussed in parts A and B, respectively.

(A) Biotransformation of **1** *and* **2** *using resuspended cells.* The TRP4a cells used here were grown in the appropriate growth medium[2,3] for varying time periods (16–19 days), and subsequently removed by filtration and resuspended in either Tris HCl (0.05 M, pH 7.5 or 9.0) or phosphate (0.1 M, pH 6.3) buffer. The substrates, dissolved in ethanol, were then added in one batch, and allowed to interact with the cells for different reaction

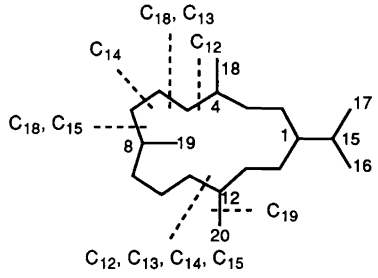

Fig. 1.

* For part 75, see Ref. 6.
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Table 1. Biotransformation of (1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (**2**) with resuspended cells of TRP4a.

Exp. No.	SM[a] (mg)	Cell age/d	Buffer[b] pH	Incubation time/h	Suspension (vol./ml)	SM rec.[c] **2**(%)	Product yield, **3**(%)
1	25	19	T, 9.0	144	500	30	37
2	50	19	T, 7.5	72	500	17	46
3	50	19	T, 7.5	144	500	0	53
4	25	19	P, 6.3	72	500	0	54

[a] SM = starting material. [b] T = Tris HCl, P = phosphate. [c] SM rec = starting material recovered.

times (72–240 h) at varying substrate:cell suspension ratios. Extraction of broth and cells with ethyl acetate was followed by chromatographic separation of the products.

The data obtained with diol **2** are summarized in Table 1, which shows that the major component of the reaction mixture, obtained in approximately 50% yield, could be identified (MS, NMR) as the known[4] 11*S*,12*S*-epoxide (**3**) (Scheme 1). However, a lower yield was obtained when the pH was higher.

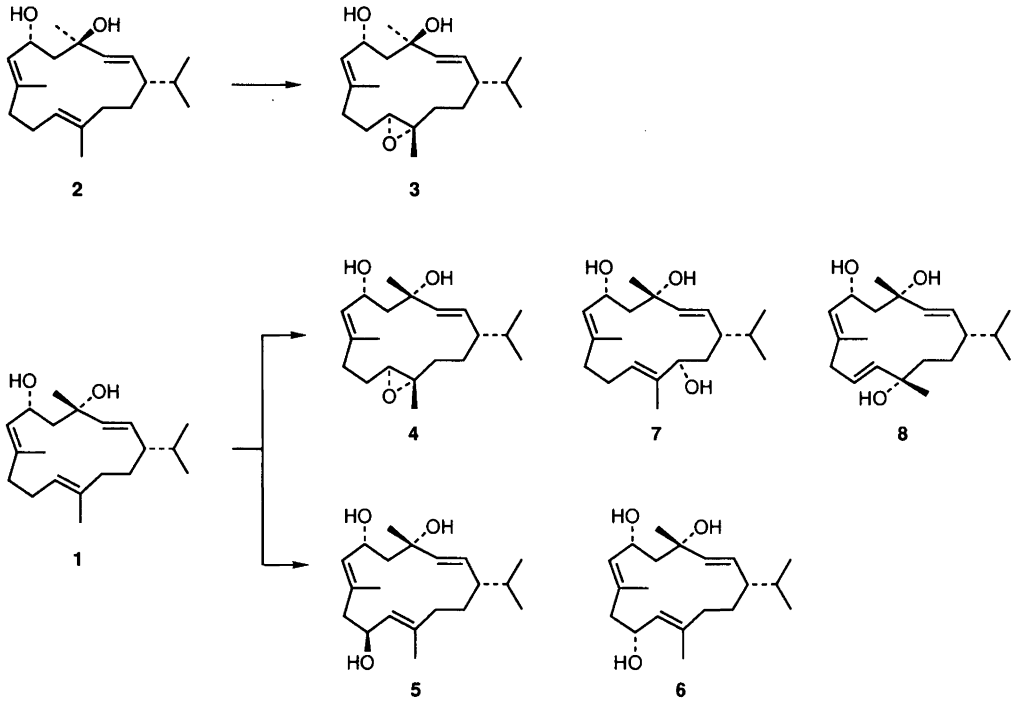
The other fractions of the chromatographic separation constituted approximately 20% of the original mixture, and contained a complex mixture of compounds, showing nearly identical retention times on thin layer chromatographic plates. Repeated attempts to separate this mixture met with failure and, eventually, compound decomposition.

The results obtained and the conditions used when the diol **1** was exposed to the resuspended cells of *T. wilfordii* are detailed in Table 2. By analogy with the 4*R*-epimer (**2**), the 4*S*-diol (**1**) was also enzymatically attacked at the 11,12 position to afford as the predominant product the known 11*S*,12*S*-epoxide (**4**) readily identified on the basis of published spectral data.[4]

Also, three minor components generated in this biotransformation could be isolated and characterized. ^1H and ^{13}C NMR spectral and mass spectrometric examination of these disclosed that they were oxygenated at either of the allylic carbons C-10 or C-13. Comparison with unpublished data, established that they were identical with three compounds most recently prepared and/or isolated from *N. tabacum* in one of our laboratories, possessing structures **5**, **6**, and **7**.[5,6]

In summary, cells resuspended in buffers can attack the diol **1** regioselectively to afford the products **4**–**7**. With 16–19 days old cells, in which the enzyme systems are properly developed, relatively efficient bioconversions can be achieved and yields exceeding 50% are observed in most cases.

(B) Biotransformation of **1** *with 'growing'* cells*: (i) single batch addition.* Biotransformation of diol **1** with 'growing' cells of the TRP4a cell line, i.e., addition of the substrate



Scheme. 1. Biotransformation of diols **1** and **2** by TRP4a cell culture.

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 resuspended cells of TRP4a.

Exp. No.	SM[a] (mg)	Cell age/d	Buffer[b] pH	Incubation time/h	Suspension (vol./ml)	SM rec.[c] **1**(%)	Prod. yield (%) **4**	**5**	**6**	**7**
5	25	19	T, 9.0	144	500	36	30	[d]	20	8
6	120	19	T, 7.5	120	1500	21	34	[d]	13	12
7	314	16	T, 7.5	168	3000	22	29	9	4	9
8	23	19	P, 6.3	72	500	0	50	[d]	24	13
9	400	16	P, 6.3	144	2000	5	35	14	5	12
10	1100	18	P, 6.3	168	2500	0	39	4	9	9
11	600	19	P, 6.3	216	1000	3	29	11	9	9
12	800	19	P, 6.3	144	1000	23	27	4	7	7
13	1000	18	P, 6.3	240	1000	18	23	5	4	4

[a]SM = starting material. [b]T = Tris HCl, P = phosphate. [c]SM rec. = starting material recovered. [d]Not determined.

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 growing cells of TRP4a (single batch addition).

Exp. No.	SM[a] (mg)	Cell age/d	Medium pH	Incubation time/h	Suspension (vol./ml)	SM rec.[b] **1**(%)	Prod. yield (%) **4**	**5**	**6**	**7**	**8**
14	50	5	4.90	24	500	32	27	4	7	7	9
15	50	5	5.12	24	500	28	30	3	8	8	8
16	100	5	5.09	24	500	52	16	2	3	3	5
17	200	5	5.12	24	500	70	7	1	1	1	2
18	50	13	5.20	24	500	24	33	4	8	8	10
19	50	13	5.25	42	500	13	33	4	10	10	15

[a]SM = starting material. In these experiments, the diol **1** dissolved in ethanol (10 mg ml^{-1}) was added in one batch. [b]SM rec. = starting material recovered.

Table 4. 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 TRP4a (batch-wise addition).

Exp. No.	SM[a] (mg)	Cell age/d	Medium pH	Incubation time/h	Suspension (vol./ml)	SM rec.[b] **1**(%)	Prod. yield (%) **4**	**5**	**6**	**7**	**8**
20	50	5	4.92	72	500	15	30	3	7	7	12
21	250	6	5.81	72	500	39	23	2	4	4	14
22	50	12	5.12	72	500	17	23	2	6	6	11
23	250	12	5.12	72	500	31	21	2	5	5	8

[a]SM = starting material. In these experiments, the diol **1** dissolved in ethanol (10 mg ml^{-1} in exp. 20 and 22, 25 mg ml^{-1} in exp. 21 and 23) was divided into five parts and added batch-wise twice a day. [b]SM rec. = starting material recovered.

Table 5. 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 TRP4a (variable addition time).

Exp. No.	SM[a] (mg)	Cell age/d	Buffer pH	Incubation time/h	Suspension (vol./ml)	SM rec.[b] **1**(%)	Prod. yield (%) **4**+**5**	**6**+**7**	**8**
24	60	12	5.12	24	500	25	28	18	15
25	60	12	5.61	48	500	20	22	15	10
26	60	12	5.11	72	500	15	20	15	12
27	60	12	5.10	96	500	18	25	20	17

[a]SM = starting material. In these experiments, the diol **1** dissolved in ethanol (10 mg ml^{-1}) was added to the cell suspension as follows: exp. 24 in one batch at time 0, exp. 25 in four equal batches during the first 24 h, exp. 26 in five equal batches during the first 48 h and exp. 27 in seven equal batches during the first 72 h. [b]SM rec. = starting material recovered.

Table 6. 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 TRP4a (continual addition).

Exp. No.	SM[a] (mg)	Cell age/d	Medium pH	Incubation time/h	Suspension (vol./ml)	SM rec.[b] **1**(%)	Prod. yield (%)				
							4	**5**	**6**	**7**	**8**
28	100	2	5.60	48	500	14	21	—	—	3	2
29	100	6	5.02	48	500	34	27	3	6	6	19
30	100	6	4.95	48	1000	23	33	1	2	8	4
31	100	12	5.33	48	500	35	24	7	9	4	6
32	100	12	5.36	48	1000	27	18	3	4	7	5

[a]SM = starting material. In these experiments, the diol **1** dissolved in ethanol (10 mg ml^{-1}) was added to the cell suspension at a rate of about 0.5 ml h^{-1} during the first 24 h. [b]SM rec. = starting material recovered.

directly to the nutrient medium at varying ages of the growing cells, is summerized in Table 3. It was felt that this approach may afford different modes of attack because of differences in the enzymatic set-up. In these experiments, the diol **1**, dissolved in ethanol, was added in one batch and incubated for relatively short time periods and with fairly young cells. Entries 18 and 19 reveal that, at a low substrate:cell suspension ratio, efficient biotransformation to five compounds could be achieved in a relatively short time using cells 13 days old, rather than 16–19 days old, cf. Table 2. Very young cells (entries 14–17), in which the enzyme systems are not so well developed, appear still capable of achieving bioconversion provided that low concentrations of substrate are involved. The lower yields seen in entries 14–18 (Table 3) when compared with the best results in Table 2, may, however, also relate to the longer incubation periods used in the studies summarized in Table 2.

Since, in addition to compounds **4**–**7** obtained earlier, the known[7,8] triol **8** was now generated in reasonable yield as a biotransformation product in the studies involving 'growing' cells, it appears that a further enzyme system is active, which can accomplish reactions analogous to those of singlet oxygen, i.e., $^1O_2$ oxidation of the diol **1** followed by reduction of the intermediate 12-hydroperoxide furnishes the triol **8**.[8]

(ii) Batch-wise addition. The biotransformation of the diol **1** summarized in Tables 4 and 5 also involves 'growing' cells but differs from those shown in Table 3, in that the substrate was added 'batch-wise'. It was felt that this approach may reduce any inhibition of cell growth and, in turn, enzyme production, if a large concentration of substrate proved toxic to the cells. Comparison of data with those of Table 3 does not reveal any improvement in terms of bioconversion yields, i.e., comparison of entry 19 (Table 3) with entry 22 (Table 4) indicates a superior result in the former experiment, although it involves a significantly shorter incubation time.

(iii) Semi-continual addition. Finally, a study involving carefully controlled semi-continual addition of the diol **1**, by means of a peristaltic pump, to the 'growing' cells was completed. Table 6 summarizes these results. Very young cells (2 days old, entry 28) already possess the enzyme system for epoxidation but seem incapable of producing any significant amounts of the alcohols **5**–**8**.

In summary, the careful control of addition appears to allow higher substrate to suspension culture ratios although the overall benefits in terms of bioconversion yields are marginal.

In conclusion, the above studies have provided evidence that the TRP4a cell line, originally propagated for the production of di- and tri-terpenes as part of a study relating to Chinese herbal medicine,[2,3] can tolerate the cembrane diols as 'foreign' substrates, unrelated in structure to those normally produced by this *T. wilfordii* cell line and/or living plant. It is clear that the enzymatic attack involves the 11,12-double bond of the diol **1** to afford the epoxide **4** as the major product. Of the minor products, the triols **5**–**7** are the result of hydroxylations at the allylic positions C-10 and C-13, while the triol **8** is apparently formed by a reaction analogous to the ene-type of $^1O_2$ oxidation.[8]

Experimental

Melting points were determined using a Reichert melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin Elmer 710 spectrometer using sodium chloride cells in chloroform solution (0.1 mm path length). Optical rotations were recorded on a Perkin-Elmer 141 polarimeter at ambient temperature using a quartz cell of 10 cm path length with the solvent and concentration (in g per 100 ml) given in parentheses. ^1H NMR spectra were recorded on a Bruker WH-400, a Bruker AC 200 or a Varian XL-300 spectrometer. Chemical shift values are reported in ppm relative to tetramethylsilane as an internal standard. ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer at 75.3 MHz or a Bruker AC-200 spectrometer at 50.2 MHz. Mass spectra were recorded on an AEI-MS-902 (low resolution) or Kratos-MS-50 (high resolution) spectrometer. Elemental analyses were performed by the Microanalysis Laboratory, University of British Columbia. Column chromatography was performed using silica gel (230–400 mesh), while thin layer chromatography was performed using commercial aluminium-backed silica gel plates. Visualization was accomplished by spraying the plates with a solution of vanillin in concentrated sulfuric

acid (0.5%) in ethanol followed by heating in the oven at 120°C for 1 min.

T. wilfordii cell line TRP4a suspensions were maintained in PRL-4 broth[9] supplemented with 2,4-dichlorophenoxyacetic acid (2.0 mg l^{-1}) and coconut milk (100 ml l^{-1}), referred to as PRDCo. Stock cultures were subcultured at 10% (v/v) each 17 days. The stationary phase coincided with a minimal refractive index for the filtered broth at 1.3333 at 25°C. All incubations were done in darkness at 26°C on a rotary shaker (135 rpm) except where specified otherwise. For details of TRP4a initiation, see Ref. 3. Cell homogenization, as required to liberate compounds within the cells, was accomplished with an IKA Ultra-Turrax Disperser T-25 fitted with an S25N-25F rotor/stator (Jankie and Kunkel GmbH and Co. KG) at 20,000 rpm.

Preparation of re-suspension medium. Two kinds of buffer solution were used as a resuspension medium for the biotransformation experiments (Tables 1 and 2). Both solutions contained sucrose (8%) as an osmotic balancing agent. The volumes of buffer employed in the various experiments (1–13) are indicated in Tables 1 and 2.

Tris HCl (0.05 M; pH 7.5). Trizma® (6.06 g, 0.05 mol) was dissolved in distilled water (800 ml) and then sucrose (80 g) was added. The volume of the solution was made up to 1 l with distilled water while the pH of the solution was adjusted to 7.5 by adding 5% hydrochloric acid. Aliquots of this standard solution were then utilized.

Phosphate (0.1 M; pH 6.3). Dibasic K_2HPO_4 (4.1 g, 0.0235 mol), monobasic KH_2PO_4 (10.44 g, 0.0765 mol) and sucrose (80 g) were dissolved in 800 ml of distilled water and the volume of the solution was made up to 1 l with distilled water. Aliquots of this standard solution were then utilized.

General procedures for experiments 1 to 13 (resuspended cells). All culture manipulations including cell resuspensions and biotransformations were performed under aseptic conditions. Either of the two substrates (**1** or **2**) was added to the resuspended culture as described below.

Typical procedure for biotransformation of the diol **2** *(entry 3; Table 1).* The diol **2** (50 mg) was dissolved in 95% ethanol (5 ml) and added to an Erlenmeyer flask containing 19 day old cells (211 g, wet weight of cells) resuspended in Tris HCl buffer (500 ml). The mixture was incubated on the shaker at 125 rpm for a period of 144 h. Filtration through Miracloth (Calbiochem Corp.) provided a filtrate which was extracted with ethyl acetate (150 ml). Drying of this over anhydrous magnesium sulfate and removal of solvent *in vacuo*, furnished a broth extract (45 mg).

Ethyl acetate (100 ml) was added to the cell material and the mixture was homogenized for 5 min. The resulting homogenate was then filtered through Miracloth and the filtrate washed with water and brine solution. Subsequent drying ($MgSO_4$) and removal of the solvent *in vacuo* furnished the cell extract (25 mg).

The combined extracts were chromatographed on silica gel (25 g) using ethyl acetate as the eluent. The initial fractions afforded the known[4] epoxide (**3**, 26 mg, 53%) while the final fractions (total weight, 12 mg) contained an inseparable mixture of several compounds which were not studied further.

Typical procedure for biotransformation of the diol **1** *(Entry 9; Table 2).* The cells from four flasks of suspension culture of the TRP4a cell line (4 × 500 ml, 16 days old) were filtered through Miracloth, washed with phosphate buffer (400 ml) and subjected to suction to remove excess water. The resulting cells (800 g) were resuspended in phosphate buffer (pH 6.3, 2000 ml) in an Erlenmeyer flask and to this mixture was added the diol **1** (400 mg) dissolved in ethanol (40 ml). The mixture was then placed on a rotary shaker for 144 h. Filtration of the entire mixture through Miracloth provided a filtrate which, in turn, was extracted with ethyl acetate (600 ml). After washing of the latter with water (200 ml) and brine solution (200 ml), the extract was dried ($MgSO_4$) and concentrated *in vacuo* to afford a crude mixture of products (318 mg).

Ethyl acetate (300 ml) was added to the cell material and the resulting suspension was homogenized with an Ultra-Turrax T-25 disperser at 20000 rpm for 5 min. The resulting homogenate was then filtered through Miracloth, and the filtrate washed with water (200 ml) and brine (200 ml). Drying ($MgSO_4$) and concentration *in vacuo* afforded a crude mixture of products (105 mg). The combined extracts (423 mg) were chromatographed on silica gel (200 g) using ethyl acetate as the eluting solvent. The resulting fractions were separated into three portions (A, 35 mg; B, 239 mg; C, 89 mg). Portion A was purified by chromatography on silica gel (25 g) using an ethyl acetate–hexane mixture (2 : 1) as the eluent to give diol **1** (20 mg, 5%). Portion B was purified similarly (silica gel, 100 g) using an acetone–hexane mixture (1 : 2) as the eluent to give the known[4] expoxide **4** (140.0 mg, 35%) and allylic alcohol **5** (55.2 mg, 14%). Chromatography of portion C (silica gel, 40 g) using an acetone–hexane mixture (1 : 1) as the eluent furnished the C-10 alcohol **6** (20.0 mg, 5%) and C-13 alcohol **7** (49.0 mg, 12%). The physical properties of the novel compounds are as follows.

(*1*S,*2*E,*4*S,*6*R,*7*E,*10*S,*11*E)-*2,7,11-Cembratriene-4,6,10-triol* (**5**).[5] M.p. 41–42°C; $[\alpha]_D^{25} + 51.3°$ (*c* 0.60, $CHCl_3$); IR ν_{max} ($CHCl_3$): 3625, 3420 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 0.80 (d, *J* 7 Hz, 3 H)/0.85 (d, *J* 7 Hz, 3 H) (H-16/H-17), 1.25 (s, 3 H, H-20), 1.58 (s, 3 H, H-18), 1.60 (s, 3 H, H-19), 4.48 (t, *J* 7 Hz, 1 H, H-6), 4.54 (dt, *J* 2 and 8 Hz, 1 H, H-10), 5.16 (d, *J* 8 Hz, 1 H, H-7), 5.30 (d, *J* 8 Hz, 1 H, H-11), 5.40 (d, *J* 15 Hz, 1 H, H-3), 5.50 (dd, *J* 8 and 15 Hz, 1 H, H-2); ^{13}C NMR ($CDCl_3$) δ: 16.8 (C-19), 19.0

(C-20), 19.2, 20.4 (C-16, C-17), 28.8 (C-14), 30.9 (C-18), 34.0 (C-15), 39.2 (C-13), 45.6 (C-9), 47.5 (C-1), 50.0 (C-5), 66.8 (C-6), 67.2 (C-10), 72.8 (C-4), 127.7 (C-2), 128.4 (C-11), 129.7 (C-7), 134.5 (C-12), 137.8 (C-3), 138.6 (C-8); MS [*m/z* (%)]: 304 ($[M-18]^+$, 0.4), 286 (4.5), 268 (2.4), 243 (2.6), 223 (10.7), 203 (6.4), 177 (6.0), 165 (19.1), 136 (21.4), 123 (24.7), 109 (25.7), 97 (51.5), 81 (62.8), 69 (57.0), 55 (41.9) and 43 (100). High resolution molecular weight determination: calcd. for $C_{20}H_{32}O_2$: 304.2402 $[M-18]^+$, found: 304.2406. Anal. calcd. for $C_{20}H_{34}O_3$: C 74.49, H 10.63; found: C 74.50, H 10.58.

(*1*S,*2*E,*4*S,*6*R,*7*E,*10*R,*11*E)-*2,7,11-Cembratriene-4,6,10-triol* (**6**).[5] M.p. 63–64°C; $[\alpha]_D^{25}+74.5°$ (*c* 0.51, $CHCl_3$); IR ν_{max} ($CHCl_3$): 3620, 3410 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 0.80 (d, *J* 7 Hz, 3 H)/0.85 (d, *J* 7 Hz, 3 H) (H-16/H-17), 1.37 (s, 3 H, H-18), 1.69 (s, 6 H, H-19 and H-20), 2.55 (dd, *J* 7 and 14 Hz, 1 H, H-9), 4.43 (dt, *J* 2 and 8 Hz, 1 H, H-6), 4.54 (m, 1 H, H-10), 5.22 (d, *J* 8 Hz, 1 H, H-11), 5.30–5.37 (m, overlapped, 2 H, H-2 and H-3), 5.43 (d, *J* 8 Hz, 1 H, H-7); ^{13}C NMR ($CDCl_3$) δ: 14.7 (C-20), 16.6 (C-19), 19.4, 20.5 (C-16, C-17), 27.2 (C-14), 30.1 (C-18), 33.0 (C-15), 36.3 (C-13), 46.3 (C-1), 48.3 (C-9), 52.0 (C-5), 65.0 (C-10), 65.8 (C-6), 72.4 (C-4), 127.7 (C-2), 128.0 (C-11), 132.6 (C-7), 133.6 (C-12), 137.4 (C-3), 139.9 (C-8); MS [*m/z*: (%)]: 322 (0.1), 304 (0.4), 286 (1.1), 268 (2.7), 243 (1.8), 223 (8.1), 205 (5.7), 177 (4.7), 165 (10.9), 123 (19.5), 112 (41.4), 97 (32.4), 81 (35.7), 69 (29.6), 59 (100), 55 (43.0) and 43 (53.2). High resolution molecular weight determination: calcd. for $C_{20}H_{34}O_3$: 322.2508, found: 322.2498. Anal. calcd. for $C_{20}H_{34}O_3$: C 74.49, H 10.63; found: C 74.40, H 10.39.

(*1*S,*2*E,*4*S,*6*R,*7*E,*11*E,*13*R)-*2,7,11-Cembratriene-4,6,13-triol* (**7**).[6] M.p. 66–67°C; $[\alpha]_D^{25}+25.6°$ (*c* 0.18, $CHCl_3$); IR ν_{max} ($CHCl_3$): 3620, 3420 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 0.80 (d, *J* 7 Hz, 3 H)/0.85 (d, *J* 7 Hz, 3 H) (H-16/H-17), 1.35 (s, 3 H, H-18), 1.54 (s, 3 H, H-19), 1.66 (s, 3 H, H-20), 3.98 (dd, *J* 8 and 14 Hz, 1 H, H-13), 4.45 (t, 8 Hz, 1 H, H-6), 5.20 (d, *J* 8 Hz, 1 H, H-3), 5.30 (m, overlapped, 2 H, H-7 and H-11), 5.40 (dd, *J* 8 and 15 Hz, 1 H, H-3); ^{13}C NMR ($CDCl_3$) δ: 9.6 (C-20), 15.9 (C-19), 19.5, 20.4 (C-16, C-17), 22.9 (C-10), 30.1 (C-18), 33.2 (C-15), 35.7 (C-14), 38.7 (C-9), 46.2 (C-1), 52.7 (C-5), 66.1 (C-6), 72.4 (C-4), 77.4 (C-12), 127.5 (C-2), 128.1 (C-11), 131.3 (C-7), 135.2 (C-12), 136.2 (C-8), 137.4 (C-3); MS [*m/z* (%)]: 304 ($[M-18]^+$, 0.5), 260 (2.8), 243 (1.1), 227 (4.2), 203 (1.1), 177 (6.0), 165 (3.1), 140 (34.6), 123 (20.2), 109 (21.9), 95 (29.5), 81 (34.9), 69 (35.6), 55 (49.3) and 43 (100). High resolution molecular weight determination: calcd. for $C_{20}H_{32}O_2$: 304.2403 $[M-18]^+$, found: 304.2401. Anal. calcd. for $C_{20}H_{34}O_3$: C 74.49, H 10.63; found: C 74.38, H 10.36.

General procedures for experiments 14–32 ('growing' cells). In all instances, the diol **1** was dissolved in ethanol and the solution added to the growing cells in Erlenmeyer flasks shaken on a rotary shaker for the incubation times indicated in Tables 3–6. All other parameters (pH, age of culture, etc.) are indicated in the tables.

Typical procedure for biotransformation of the diol **1**: *single batch or batch-wise addition (entry 18, Table 3).* Diol **1** (50 mg in 5 ml of ethanol) was added to a 13 day old culture (500 ml) in an Erlenmeyer flask and incubated on the shaker for 24 h. Filtration and extraction of broth and cells (after homogenization) was performed with ethyl acetate as described above, to afford a combined crude extract (67 mg). Chromatographic separation of the crude extract on silica gel (25 g) with ethyl acetate as the eluting solvent afforded, in order of polarity, the starting diol **1** (12 mg, 24%), the epoxide **4** (16.5 mg, 33%), and the triols **5** (2 mg, 4%), **6** (3.7 mg, 8%), **7** (3.9 mg, 8%) and **8** (5.2 mg, 10%).

Typical procedure for biotransformation of the diol **1**: *continual addition with a peristaltic pump (entry 30, Table 6).* Six day old cell culture (500 ml) was transferred to a 1 l bioreactor, aerated through a sintered glass disk at 200 ml l^{-1} min^{-1}, and kept at room temperature (21–24°C). Each hour over the next 24 h, a timer-controlled peristaltic pump added 0.5 ml of a solution of **1** (100 mg in 12 ml ethanol). Incubation was continued for an additional 24 h. Work-up, as before, afforded a combined crude extract (132 mg). Chromatographic separation of the crude extract on silica gel (50 g) afforded the starting diol **1** (22.8 g, 23%), the epoxide **4** (23.1 mg, 33%), and the triols **5** (1.1 mg, 1%), **6** (8.3 mg, 8%), **7** (1.6 mg, 2%) and **8** (4.3 mg, 4%).

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References

1. Wahlberg, I. and Enzell, C. R. *Nat. Prod. Rep.*, Royal Society of Chemistry, *237* (1987), and references cited therein.
2. Kutney, J. P., Hewitt, G. M., Kurihara, T., Salisbury, P. J., Sindelar, R. D., Stuart, K. L., Townsley, P. M., Chalmers, W. T. and Jacoli, G. G. *Can. J. Chem. 59* (1981) 2677.
3. Kutney, J. P., Choi, L. S. L., Duffin, R., Hewitt, G., Kawamura, N., Kurihara, T., Salisbury, P., Sindelar, R., Stuart, K. L., Townsley, P. M., Chalmers, W. T., Webster, F. and Jacoli, G. G. *Planta Med. 48* (1983) 158.
4. Behr, D., Wahlberg, I., Nishida, T., Enzell, C. R., Berg, J. E., and Pillotti, A. M. *Acta Chem. Scand., Ser. B 34* (1980) 195.
5. Vogt, C., Berg, J.-E. and Wahlberg, I. *Acta Chem. Scand. 46* (1992) 789.
6. Forsblom, I., Berg, J.-E. and Wahlberg, I. *Acta Chem. Scand. 47* (1993) 80.
7. Wahlberg, I., Wallin, I., Narbonne, C., Nishida, T., Enzell, C. R. and Berg, J. E. *Acta Chem. Scand., Ser. B 36* (1982) 147.
8. Wahlberg, I., Arndt, R., Wallin, I., Vogt, C., Nishida, T. and Enzell, C. R. *Acta Chem. Scand., Ser. B 38* (1984) 21.
9. Gamborg, O. L. and Eveleigh, D. E. *Can. J. Biochem. 46* (1968) 417.

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