Biotransformation of a Dibenzylbutanolide to Podophyllotoxin Analogues by Shoot Cultures of *Haplophyllum patavinum*

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Received December 3, 2002; accepted April 3, 2003

A peroxidase from spent medium of shoot cultures from *Haplophyllum patavinum* (L.) G. DON catalyzes the biotransformation of a synthetic dibenzybutanolide into a podophyllotoxin analogue and a novel compound, derived by the opening of the lactone ring.

Key words biotransformation; dibenzybutanolide; shoot culture; *Haplophyllum patavinum*

During studies on *in vitro* regeneration of *Haplophyllum patavinum* (L.) G. DON FIL. (Rutaceae), an endangered herb with a punctiform relict disjointed range on the Euganean Hills (Padua, Italy), cell and tissue cultures were established.[1,2] The phytochemical analysis of shoot cultures revealed the presence of arylnaphthalene lignans such as justicidin B, diphyllin, tuberculatin, majidine, patavine and arabelline.[3]

Dibenzylbutanolides have been suggested[4] as precursors of aryltetralin lignans and the bioconversions to podophyllotoxin analogues have been performed with peroxidase from cell cultures of different species.[5]

We hypothesized that dibenzylbutanolides might represent early intermediates also in the biosynthetic pathway to the arylnaphthalene lignans[6] and, as a consequence, *H. patavinum* shoot cultures must contain a peroxidase able to promote the cyclization of dibenzylbutanolides. This study deals with biotransformation studies with *H. patavinum* shoot cultures, culminating in the isolation of a novel product (**3**).

The activity of the peroxidase enzymes present in the spent medium was evaluated during the growth cycle, and the possibility of producing podophyllotoxin analogues by ring closure of dibenzylbutanolides with peroxidase enzymes of shoot cultures was explored by a series of trials using precursor **1**. In the optimal experiment, compound **1** was completely biotransformed after 20 min of incubation. The major compound **2** was identical with the cyclization product 1,2,3,4-tetrahydro-4β,6-dihydroxy-7-isopropyloxy-3-hydroxymethyl-1-(4-hydroxy-3,5-dimethoxy phenyl)-2-naphthoic acid butanolide (co-TLC, EI-MS, ^{1}H- and ^{13}C-NMR spectra), obtained earlier from the same substrate by treatment with cell cultures of *Podophyllum peltatum*, *Nicotiana sylvestris* and *Cassia didymobotrya*.[5]

A molecular formula $C_{24}H_{30}O_9$ was determined for the minor compound **3** by a combination of ^{1}H- and ^{13}C-NMR spectral data (Table 1) with the molecular peak at *m/z* 462 in the EI-MS spectrum. The similar UV spectra of compounds **2** and **3** suggested an analogous skeleton. Comparison of ^{1}H-NMR spectra of compounds **2** and **3** (Table 1) revealed in the latter the presence of a new (3H) signal at δ 3.51 and the highfield shifts for the signals attributed to H-3 (δ 2.14) and to H_2-3a,3b (δ 3.58 and 3.80). A similar highfield shift for C-3a,b carbon (δ 67.3 in **2** to δ 62.5 in **3**) in the ^{13}C-NMR spectra suggested the passage CH_2OCO to CH_2OH, that is the hydrolysis of the lactone ring to the hydroxy acid. As a confirmation, the new signal in the ^{1}H-NMR spectrum of **3** was attributed to a carbomethoxyl group (δ 51.4 in the ^{13}C-NMR spectrum), derived from the esterification of the carboxylic function. The EI-MS spectrum was characterized by the losses of H_2O, CH_3OH (very likely from the carbomethoxy group), and C_3H_6 (isopropyl group) from the molecular ion and by a base peak coincident with the C ring. Therefore, compound **3** was assigned the structure 1-(4′-hydroxy-3′,5′-dimethoxyphenyl)-4β,6-dihydroxy-3-hydroxymethyl-7-isopropyloxy-1,2,3,4-tetrahydro-2-naphtoic acid methyl ester, a novel product among those obtained from precursor **1**.[5] The esterification of compound **3** was ascribed to the conditions (silica gel, methanol) used for the purification. Actually, the HPLC profile of the reaction mixture prior to the purification is characterized by the presence of two peaks, only the major corresponding to compound **2**.

The lactone ring opening was obtained by Kutney and coworkers[9] during experiments of biotransformation of a synthetic dibenzylbutanolide using cell free extracts of *Catharanthus roseus* cell cultures. The authors, however, suggested that the large excess of sodium borohydride, used to treat the

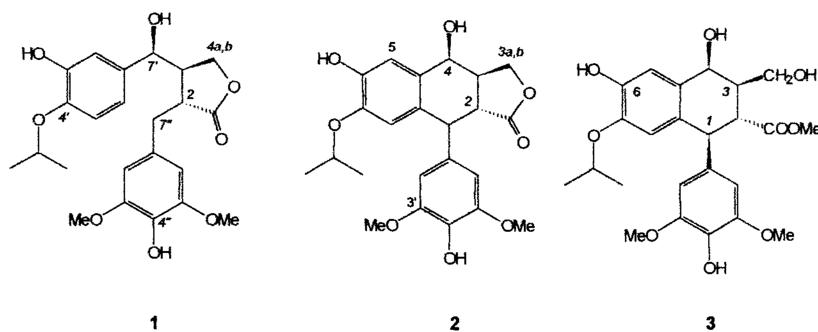

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of Arylteralins **2** and **3**

Position[a]	**2**		**3**	
	δ_C	δ_H (m, J in Hz)	δ_C	δ_H (m, J in Hz)
1	46.7	3.96 (d, 11.4)	46.0	4.10 (d, 11)
2	45.5	3.21 (dd, 11.4, 13.9)	48.0	3.14 (t, 11)
3	41.1	2.73 (dddd, 13.9, 10.5, 7.2, 2.7)	50.3	2.14 (dddd, 11, 8, 5, 3)
3a,b	66.0	4.41 (dd, 8.2, 7.2)	62.5	3.80 (dd, 10.5, 8)
		4.30 (dd, 10.5, 8.2)		3.58 (dd, 10.5, 5)
4	67.3	4.81 (d, 2.7)	67.8	4.84 (d, 3)
4a	132.5		132.5	
5	116.2	6.81 (s)	116.1	6.80 (s)
6	146.5		146.9	
7	145.5		145.8	
8	116.6	6.44 (s)	116.5	6.36 (s)
8a	131.7		130.6	
1′	135.4		135.9	
2′	107.7	6.58 (s)	107.4	6.46 (s)
3′	147.9		148.5	
4′	135.0		135.6	
5′	148.0		148.5	
6′	107.7	6.58 (s)	107.4	6.46 (s)
CO	175.9		175.6	
2×OMe	56.2	3.77 (s)	56.6	3.74 (s)
(CO)OMe			51.4	3.51 (s)
OCH	71.3	4.28 (m)	71.7	4.28 (m, 6)
Me_2	21.2	1.09 (d, 6)	21.7	1.10 (d, 6)
	21.6	1.20 (d, 6)	22.1	1.19 (d, 6)

a) 300 (^{1}H) and 75 (^{13}C) MHz, MeCO-d_6, TMS as int. stand. Protons showed the appropriate integrated intensity. Assignments were confirmed by NOE and INEPTL experiments.

ethyl acetate extract of the reaction mixture, might be responsible for the hydrolysis of the lactone to the hydroxy acid. On the other hand, the chemical opening of the lactone ring in **1** does not only require strong alkaline conditions, but also could lead to a rearrangement with the loss of the methylene group as formaldehyde.[10)]

To exclude the possibility of an acid hydrolysis during the purification, compound **2** was again submitted to such a procedure (see Experimental) as the reaction mixture, but compound **3** was not formed under these conditions. For the above reasons we believe that the opening of lactone ring in the compound **3** is the result of an enzymatic reaction and not an artefact due to the reaction conditions.

A number of investigations concerning the ability of plant cell cultures to perform biotrasformations of different precursors to suitable end products are reported in the literature.[11—13)] Our results further support the strategy of utilizing plant cell cultures or enzymes derived from within the cells as "reagents" in organic synthesis.

Experimental

General Procedures The following instruments were used: UV spectra, Perkin-Elmer mod. Lambda 5 spectrometer; ^{1}H- and ^{13}C-NMR spectra (in MeCO-d_6), Varian Gemini 300 MHz spectrometer; EI-MS spectra, VG7070EQ spectrometer.

HPLC analysis was performed using a ChromQuest (Thermoseparation, San Josè, CA, U.S.A.) instrument, with a P4000 pomp and UV6000 PDA rivelator; a Jupiter column (RP C18, 5μ, 300 Å, 250×4.60 mm, Phenomenex) was used with a small precolumn (C18, 4×3 mm I.D.). The flow rate was 1 ml/min, the volume of injection was 20 μl. Silica gel 60F_{254} (Merck) were used for TLC.

Tissue Cultures Shoot cultures were obtained by organogenesis experiments on *H. patavinum* cell cultures as previously reported,[7)] and maintained by subculturing on hormone-free fresh MS[8)] medium every third week.

General Biotransformation Procedure The activity of the peroxidase enzymes present in the spent medium was evaluated by UV absorptions at 420 nm of the oxidation products of pyrogallol. The text was performed at 25 °C in a reaction mixture containing 2 ml H_2O, 0.28 ml pyrogallol solution (4.7%), 0.14 ml H_2O_2 (0.5%), 0.42 ml K–phosphate buffer (pH 6.3) and 0.14 ml shoot cultures spent liquid medium. In the optimal experiment the synthetic dibenzylbutanolide **1** (45 mg) was incubated at 25 °C with the spent medium (30 ml) of shoot cultures collected at 30th day of subculturing (higher peroxidase activity, data not shown), K–phosphate buffer (30 ml, pH 6.3) and H_2O_2 0.5% (0.5 ml).

Extraction and Isolation After 20 min of incubation, compound **1** was completely biotransformed (TLC) and the reaction mixture was extracted with EtOAc. Preparative-TLC of the residue eluted with EtOAc/cyclohexane (3 : 1), and $CHCl_3$/MeOH (9 : 1) afforded products **2** (33 mg, biotransformation yield, 73%) and **3** (5 mg, 11%), respectively. The purity of compounds was checked by HPLC, monitoring the product elution at 280 nm; the multistep linear solvent gradient was performed using steadly 5% MeOH and varying the percentage of H_2O and CH_3CN: at time 0 min 10% CH_3CN; at time 8 min 35% CH_3CN; at time 20 min 60% CH_3CN; at time 22 min 70% CH_3CN. Retention times for **2** and **3** were 17.2 and 16.1 min, respectively. Notably, the HPLC chromatogram of the reaction mixture prior to purification showed two peaks corresponding to 17.2 and 15.2 min.

Stability of Compound 2 The cyclization product **2** (4.0 mg) was mixed to silica gel (600 mg) with $CHCl_3$–MeOH (4 : 1) and the solid was maintained under a solvent layer for 3 d. Elution with $CHCl_3$–MeOH (4 : 1) gave recovered **2** (3.9 mg), which did not show any extra spot (TLC) or peak (HPLC).

1-(4′-Hydroxy-3′,5′-dimethoxyphenyl)-4β,6-dihydroxy-3-hydroxymethyl-7-isopropyloxy-1,2,3,4-tetrahydro-2-naphtoic Acid Methyl Ester (**3**): An off-white amorphous powder; mp 130—132 °C; UV λ_{max} (MeOH): 281 nm; UV λ_{max} (MeOH/NaOH 0.1 N): 250, 281 nm; ^{1}H- and ^{13}C-NMR in Table 1; EI-MS m/z (%): 462 (14) $[M]^+$, 444 (7) $[M-H_2O]^+$, 430 (45) $[M-CH_3OH]^+$, 388 (50) $[430-C_3H_6]^+$, 367 (329), 325 (39), 238 (31), 224 (44), 154 (100) $[C\ ring]^+$.

Acknowledgements The authors wish to thank Prof. J. P. Kutney and Dr. N. M. Stoynov for their pratical help and constructive suggestions, and to acknowledge the financial support of CNR Target Project "Biotechnology and Bioinstrumentation."

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