Biotransformation of (−)-Ambrox by Cell Suspension Cultures of *Actinidia deliciosa*

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Biotransformation of (−)-ambrox (**1**) with cell suspension cultures of *Actinidia deliciosa* (Kiwifruit) yielded the regio- and stereospecific oxygenated products 3-oxoambrox (**2**), 3β-hydroxyambrox (**3**), 1α-hydroxyambrox (**4**), 3β,6β-dihydroxyambrox (**5**), 1α,6β-dihydroxyambrox (**6**), and 1α,3β-dihydroxyambrox (**7**). Metabolites **6** and **7** were found to be new compounds. These metabolites were structurally characterized on the basis of spectroscopic studies. The structure of compound **6** was unambiguously deduced by single-crystal X-ray diffraction techniques. Metabolites **2**−**7** were evaluated for in vitro inhibitory activity against the thymidine phosphorylase enzyme.

Use of plant cell suspension cultures for structural modification is a new approach in biotransformation research. Very few natural products have been transformed through different plant cell cultures.[1] This paper describes the biotransformation of (−)-ambrox (**1**) using cell suspension culture of *Actinidia deliciosa* (A. Chev) C.F. Liang et A.R. Ferguson (family Actinidiaceae).

Actinidia deliciosa (Kiwifruit)[2] is the most nutrient-rich among the top 26 fruits consumed in the world today.[3] It is an excellent source of vitamin C and dietary fiber. Kiwifruit has been shown to contain an antimutagenic component, which can prevent the mutations of genes and onset of cancer.[4−6] (−)-Ambrox (**1**), a sesquiterpene, is a highly fragrant constituent of Ambergris, a metabolite of the sperm whale. Compound **1** possesses an amber-like odor and is a valuable animal-based perfume.[7,8] In continuation of our biotransformation studies on bioactive compounds, incubation of (−)-ambrox (**1**) with an *A. deliciosa* cell suspension culture afforded six oxidative metabolites (**2**−**7**). Compounds **2**−**5** were previously reported as metabolites of compound **1**,[9−12] while compounds **6** and **7** are new. Compounds **1**−**7** were evaluated for their inhibitory potential against thymidine phoshorylase (TP). TP is an angiogenic enzyme, which has been shown to stimulate endothelial cell migration in vitro and proliferation in vivo. TP overexpression also has been observed in diabetic retinopathy and chronic inflammatory diseases such as rheumatoid arthritis and psoriasis.[13]

Incubation of compound **1** with the cell suspension culture of *A. deliciosa* for 15 days yielded six oxidative metabolites (**2**−**7**). The structures of known metabolites **2**−**5** were identified through comparison with their reported data,[9−12] while the structures of new metabolites **6** and **7** were elucidated through spectroscopic studies, with single-crystal X-ray diffraction used to confirm the structure of metabolite **6**.

Compound **6** was obtained as a white crystalline solid. The HREIMS showed a M^+ peak at *m*/*z* 268.2064, corresponding to the formula $C_{16}H_{28}O_3$ (calcd 268.2038), indicating the incorporation of two hydroxyl groups in the molecule. Other fragment ions were detected at *m*/*z* 232 [$M^+ - 2H_2O$] and 217 [$M^+ - 2H_2O + Me$], which supported the formation of a dihydroxy derivative of ambrox (**1**). The presence of hydroxyl groups was also inferred from the IR absorption at 3371 cm^{-1}. The ^1H NMR spectrum of compound **6** showed signals for two methine protons, geminal to hydroxyl groups, at δ 3.41 (t, $J_{1eq,2eq,ax} = 2.7$ Hz) and 4.59 (dt, $J_{6eq,5ax,7ax} = 5.2$ Hz, $J_{6ax,7eq} = 3.0$ Hz). The splitting pattern and coupling constants of these signals suggested axial orientations of the OH groups at the C-1 and C-6 positions. The ^{13}C NMR (broad band decoupled and DEPT) spectra of compound **6** showed the disappearance of the C-1 and C-6 methylene carbons and the appearance of two additional hydroxyl-bearing methine signals at δ 73.5 and 72.3, when compared with compound **1** (Table 1). The C-1 proton (δ 3.41) showed interactions with H_2-2 (δ 2.21 and 1.62), while the C-6 proton (δ 4.59) showed interactions with the C-7 methylenes (δ 1.95 and 1.71) and the C-5 methine proton (δ 1.53) in the COSY-45 spectrum of compound **6**. The C-6 proton also showed heteronuclear interactions (HMBC) with C-5 (δ 49.7), C-8 (δ 80.7), and C-10 (41.4), while H-1 showed J_2 correlations with C-2 (δ 24.7) and C-10. These observations further supported the placement of new hydroxyl groups at C-1 and C-6. Metabolite **6** may be formed by the sequential hydroxylation of compound **4** (Scheme 1). An X-ray diffraction study was used to establish the absolute structure of compound **6** (Figure 1). All the bond angles and bond lengths were found to be in the normal range. The newly formed hydroxyl groups at the C-1 and C-6 positions were α- and β-oriented, respectively. The six-membered rings A and B are in chair conformations, while the five-membered ring C is in a half-chair conformation.

The HREIMS of **7** showed a M^+ peak at *m*/*z* 268.2041 ($C_{16}H_{28}O_3$, calcd 268.2038), indicating two new OH functions in the molecule as compared to substrate **1**. The IR absorption at 3329 cm^{-1} also indicated the presence of OH groups. The ^1H NMR spectrum displayed two methine signals at δ 3.52 (t, $J_{1eq,2ax,eq} = 2.8$ Hz) and 3.71 (dd, $J_{3ax,4ax} = 11.8$ Hz, $J_{3ax,4eq} = 4.5$ Hz), characteristic of methine protons geminal to OH groups. The ^{13}C NMR spectrum of **7** exhibited two downfield methine signals at δ 71.6 (C-l) and 72.7 (C-3). The new downfield methine protons at δ 3.52 and 3.71 showed mutual couplings with C-2 methylene protons (δ 2.51, 2.37) in the COSY-45 spectrum and were thus assigned to the C-1 and C-3 protons, respectively. Furthermore, H-3 also demonstrated a *W*-interaction with H-5 (δ 1.61). The HMBC spectrum of **7** showed interactions between H_2-2 (δ 2.51, 2.37)/C-3 (δ 72.7); H-3 (δ 3.71)/C-2 (δ 35.1), C-4 (δ 39.7); H_2-2 (δ 2.51, 2.37)/C-l (δ 71.6); and H-1 (δ 3.52)/C-2 (δ 35.1), C-10 (δ 36.7), which further supported the formation of 1α,3β-dihydroxyambrox (**7**). The 1α,3β-configurations of the newly introduced hydroxyl groups were established on the basis of coupling constant values of the geminal H-1β ($J = 3.1$ Hz) and H-3α ($J = 11.8$ Hz). This compound might have been formed by the monohydroxylation of compound **3** at C-1 or the monohydroxylation of compound **4** at C-3 (Scheme 1).

Very few natural inhibitors of thymidine phosphorylase (TP) have been reported so far. None of the compounds from the present study exhibited substantial potency in this assay, with the two most active compounds being **5** and **6** (IC_{50} values of 81.4 ± 1.2 and 72.0 ±

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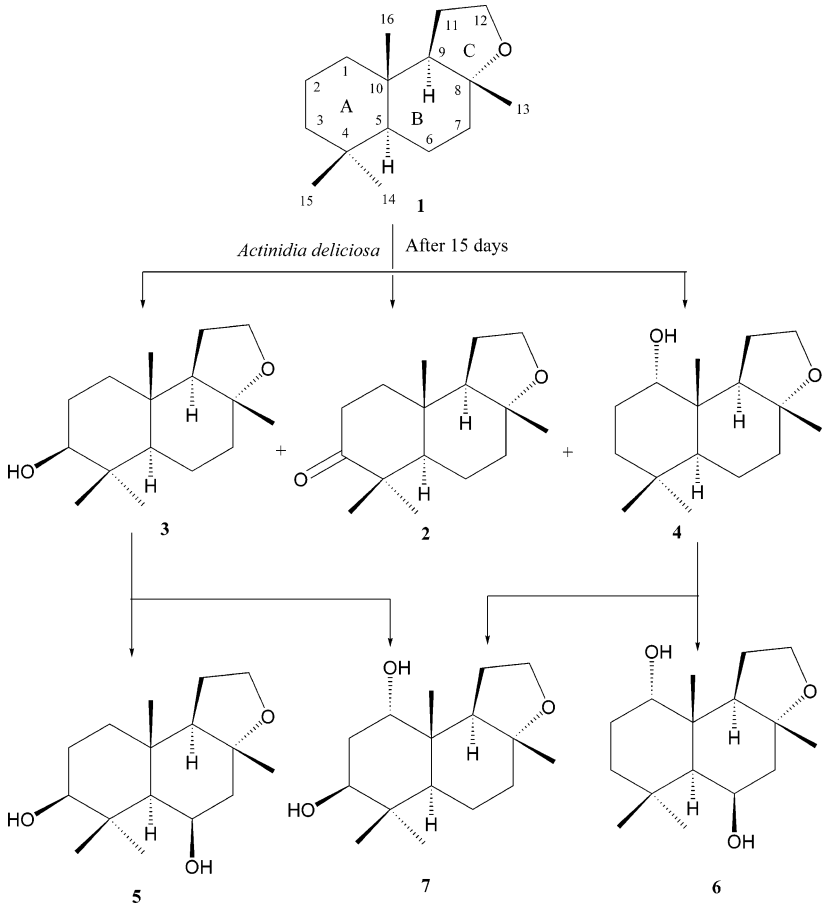
Table 1. NMR Spectroscopic Data of Metabolites **6** and **7** in $CDCl_3$

	6			**7**		
position	δ_H (mult., *J* in Hz)[a]	δ_C[b]	multiplicity	δ_H (mult., *J* in Hz)[a]	δ_C[b]	multiplicity
1	3.41 (t, 2.7)	73.5	CH	3.52 (t, 2.8)	71.6	CH
2	2.21, 1.62 (m)	24.7	CH_2	2.51, 2.37 (m)	35.1	CH_2
3	1.64, 1.28 (m)	38.2	CH_2	3.71 (dd, 11.8, 4.5)	72.7	CH
4		33.6	C		39.7	C
5	1.53 (d, 5.2)	49.3	CH	1.61 (m)	46.9	CH
6	4.59 (dt, 5.2, 3.0)	72.3	CH	1.91, 1.74 (m)	20.3	CH_2
7	1.95, 1.71 (m)	47.4	CH_2	1.85, 1.57 (m)	38.3	CH_2
8		82.6	C		81.2	C
9	1.81 (m)	53.8	CH	1.77 (m)	51.4	CH
10		40.7	C		36.5	C
11	1.68, 1.57 (m)	27.3	CH_2	1.69, 1.51 (m)	26.6	CH_2
12	3.93 (dd, 12.0, 7.8), 3.81 (dd, 16.0, 7.9)	64.4	CH_2	3.91 (dd, 12.0, 8.0), 3.84 (dd, 16.0, 8.0)	64.2	CH_2
13	1.31 (s)	22.7	CH_3	1.32 (s)	21.3	CH_3
14	1.22 (s)	25.7	CH_3	1.14 (s)	27.7	CH_3
15	1.15 (s)	21.6	CH_3	1.18 (s)	15.6	CH_3
16	1.02 (s)	17.8	CH_3	1.05 (s)	15.1	CH_3

[a] ^{1}H NMR data, 500 MHz. [b] ^{13}C NMR data, 125 MHz.

Scheme 1. Biotransformation of Compound **1** by *Actinidia deliciosa*



0.4 μM, respectively). The IC_{50} value for a positive control, deazaxanthine, was 36.6 ± 4.4 μM.

Experimental Section

General Experimental Procedures. (−)-Ambrox (**1**) was purchased from Sigma Aldrich. Melting point: Buchi-535 melting-point apparatus. Optical rotations: JASCO-DIP-360 digital polarimeter. IR spectra: FTIR-8900 spectrophotometer, in cm^{-1}. ^{1}H and ^{13}C NMR spectra: Bruker-Avance 500 NMR at 500 and 125 MHz, respectively, 2D experiments with $CDCl_3$, and the same instrument; chemical shifts (δ) in ppm relation to $SiMe_4$ as an internal standard; coupling constants *J* in Hz. EIMS and HREIMS: JEOL JMS-600H mass spectrometer, in *m*/*z* (rel %). TLC: silica gel precoated plates (Merck, PF_{254}; 20 × 20, 0.25 mm). Column chromatography: silica gel (70-230 mesh, Merck). The compounds were detected on TLC with vanillin spray reagent.

Culture Media and Biotransformation Protocol. Plant material of *Actinidia deliciosa* (Kiwifruit) was collected from the greenhouse of H. E. J. Research Institute of Chemistry, University of Karachi, in April 2004 and identified by Mr. Tahir Ali (taxonomist at the Department of Botany, University of Karachi, Pakistan). A voucher specimen (KU#193661) was deposited in the Herbarium of the University of Karachi, and their calli were maintained. The callus culture of the plant was derived from young leaves, cultivated in 100 mL jars each having 25 mL of Murashige and Skoog medium[14] solidified by agar (8 g/L) at 25 ± 1 °C under complete darkness. The cultures were maintained on the same medium at 25 ± 1 °C in the dark by subculturing after every four weeks. Two-week-old friable calli were used to initiate cell suspension culture in the Murashige and Skoog medium containing 1 mg/L IBA (indole-3-butyric acid) and 0.5 mg/L BAP (6-benzylaminopurine) with 2.5% sucrose. The cells were grown

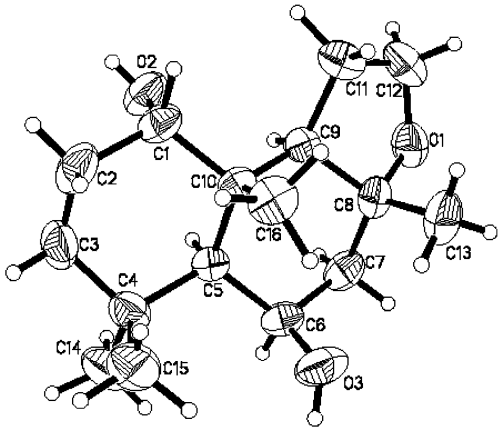


Figure 1. Computer-generated ORTEP diagram of the final X-ray model of compound **6**.

on shaking at 110 rpm for 15 days at 25 °C under a 16 h photoperiod. After 15 days, the cells were harvested and introduced onto the above-mentioned freshly prepared medium.

After 4 days of transfer to fresh media, the substrate **1** (200 mg) was dissolved in DMSO (10 mL) and evenly distributed among 10 culture flasks under aseptic conditions, which were kept for 15 days under the same conditions. During the fermentation period, aliquots from culture were taken out daily and analyzed by TLC in order to determine the degree of transformation of substrate. In all experiments, one control flask, without cell culture (for checking substrate stability), and another flask, without exogenous substrate (for checking endogenous metabolite), were used.

Extraction and Isolation. The culture media and cells were separated by filtration after 15 days. The cells were washed with CH_2Cl_2 (1 L), and the filtrate was extracted with CH_2Cl_2 (2 × 2 L). The combined organic extract was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to obtain a brown gum (1.27 g). This extract after repeated column chromatography (petroleum ether−AcOEt gradient) yielded compounds **2** (16.4 mg; 8.2% yield; with petroleum ether−AcOEt, 78:22), **3** (8.2 mg; 4.1% yield; with petroleum ether−AcOEt, 61:39), **4** (19.1 mg; 9.5% yield; with petroleum ether−AcOEt, 57:43), **5** (21.4 mg; 10.5% yield; with petroleum ether−EtOAc, 49:51), **6** (21.1 mg; 10.1% yield; with petroleum ether−EtOAc, 44:56), and **7** (31.8 mg; 15.7% yield; with petroleum ether−EtOAc, 40:60).

1α,6β-Dihydroxyambrox (6): white crystalline solid; mp 128−129 °C; $[\alpha]^{25}_D$ −32.8 (*c* 0.5, MeOH); IR ($CHCl_3$) ν_{max} 3371, 2947, 2868 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) see Table 1; EIMS *m*/*z* 268 ([M^+], 12), 253 ([M − Me]$^+$, 100), 235 ([M − Me − H_2O]$^+$, 51), 232 ([M − $2H_2O$]$^+$, 26), 217 ([M − $2H_2O$ + Me]$^+$, 15) 163 (17), 217 (12), 191 (33), 167 (11), 139 (14), 111 (22), 81 (23), 55 (62); HREIMS *m*/*z* 268.2064 ($C_{16}H_{28}O_3$, calcd for 268.2038).

1α,3β-Dihydroxyambrox (7): white crystalline solid; mp 141−142 °C; $[\alpha]^{25}_D$ −57.4 (*c* 0.5, MeOH); IR ($CHCl_3$) ν_{max} 3329, 2941, 2863, 1135 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1; EIMS *m*/*z* 268 ([M^+], 3), 253 ([M − Me]$^+$, 100), 235 ([M − Me − H_2O]$^+$, 27), 232 ([M − $2H_2O$]$^+$, 17), 217 ([M − $2H_2O$ + Me]$^+$, 11), 163 (4), 217 (17), 191 (15), 167 (8), 139 (21), 111 (16), 81 (27), 55 (69); HREIMS *m*/*z* 268.2041 ($C_{16}H_{28}O_3$, calcd for 268.2038).

Crystallographic Data of Compound 6.[15] The structure of compound **6** was determined unambiguously by single-crystal X-ray diffraction. A suitable crystal was obtained by recrystallization from CH_2Cl_2−MeOH (1:1). A colorless crystal with dimensions 0.46 × 0.16 × 0.11 mm^3 was selected for the crystallographic measurements. $C_{16}H_{28}O_3$: M_r 268.38; orthorhombic, a = 7.2694(12) Å, b = 12.823-(2) Å, c = 16.107(3) Å, $\alpha = \beta = \gamma = 90.0°$, V = 1501.4(4) $Å^3$, space group = $P2(1)2(1)2(1)$, Z = 4, D_{calc} = 1.187 mg/m^3, $F(000)$ = 592. Unit cell dimensions were determined by least-squares fit of 3362 reflections measured at 293(2) K using Mo Kα (λ = 0.71073 Å) radiation on a SMART diffractometer.[16] The intensity data within a (θ) range of 2.03−28.17° were collected at 293(2) K. A total of 7461 reflections were collected, of which 2620 reflections were judged observed on the basis of $I > 2\sigma(I)$. The structure was solved by the direct methods and expanded using Fourier transformation techniques.[17] The structure was refined by a full-matrix least-squares calculation on F^2 with the program SHELXTL.[18] The final R and R_w factors were measured as 0.0381 and 0.1047, respectively. The figures were plotted with the SHELXTL program.[18]

In Vitro Thymidine Phosphorylase Inhibition Assay. Thymidine phosphorylase (*Escherichia coli* origin), thymidine, and all other required chemicals were purchased from Sigma. The specific inhibitor of TP was synthesized as previously described.[19] The TP inhibition activity was assayed by a modification of the previously described method.[20] Briefly, 200 mM potassium phosphate buffer, pH 7.00, 245 μL, TP (0.068U/300 μL) 20 μL, and test samples in 5 μL of DMSO were mixed in 96-well microplates and preincubated for 10 min at 30 °C. The reaction was initiated by adding 30 μL of 1 mM thymidine as the substrate (dissolved in 200 mM potassium phosphate buffer, pH 7). The decrease in absorbance was monitored spectrophotometrically at 290 nm, with a 96-well microplate reader (Spectramax, Molecular Devices). The IC_{50} values are the average of at least three determinations performed in triplicate.

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