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Atropa belladonna Hairy Roots: Orchestration of Concurrent Oxidation and Reduction Reactions for Biotransformation of Carbonyl Compounds

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Abstract The biotransformation potential of a selected *Atropa belladonna* hairy root clone (AB-09) had been evaluated with regard to three different aromatic carbonyl compounds, i.e., 3,4,5-trimethoxybenzaldehyde (*1*), 3,4,5-trimethoxyacetophenone (*2*), and 3,4,5-trimethoxy benzoic acid (*3*). The results demonstrated for the first time the untapped potentials of the selected hairy root clone to perform simultaneous oxidation (34.49%) and reduction (32.68%) of 3,4,5-trimethoxy benzaldehyde (*1*) into 3,4,5-trimethoxy benzoic acid (*3*), and 3,4,5-trimethoxy benzyl alcohol (*4*), respectively, without any intermediate separation or addition of reagents. The same hairy root clone also demonstrated reduction (<5%) of a 3,4,5-trimethoxyacetophenone (*2*) into a secondary alcohol, i.e., 1-(3,4,5-trimethoxyphenyl) ethanol (*5*), while in the case of aromatic carboxylic acid substrate (*3*), no biotransformation could be obtained under the similar conditions. The current observations revealed oxidation and reduction of the formyl group of the aromatic ring, and only reduction of the carbonyl group of acetophenone through the specific hairy root clone. The concurrent oxidation and reduction reactions by the selected hairy root clone highlight the importance of this study, which, as per our observations, is the first of its kind relating the hairy root culture of *A. belladonna*.

Keywords *Atropa belladonna* · Hairy roots · Biotransformation · Aromatic carbonyl compounds · Oxidation · Reduction

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Introduction

Biotransformation has lately attained the status of applied research in generating pharmaceutical lead compounds by stimulating chemical transformations aided through biological systems. Plant enzymes offer exceptional opportunities to regio- and stereo-specifically transform exogenous substrates to analogous compounds that are difficult to achieve by synthetic chemistry [1]. The biotransformed molecules can unearth notable efficacies by acquiring improved physico-chemical/therapeutic properties, bioavailability, and lower toxicity [2].

A wide array of chemical compounds has been effectively transformed by plant cells and organ cultures [3]. Currently, *Agrobacterium rhizogenes*-mediated hairy root cultures are preferred over plant cells/callus cultures as biocatalyst due to their genetic/biochemical stability, hormone autotrophy, and multi-enzyme biosynthetic potentials [4]. Moreover, its low-cost cultural requirements and independency over seasonal fluctuations further emphasize its superiority. Additionally, the hairy root cultures of diverse medicinal plant species have demonstrated bio-catalytic ability to specifically convert administered foreign compounds into useful analogs [4]. Essentially, the inherent biosynthetic potentials of hairy roots are determined by the functional metabolic pathway of the source plant [3, 5]. It is therefore absolutely realistic to utilize the operating pathway-related enzymes of specific plant-based hairy root cultures for biotransformation mediated diversification of targeted chemical moiety. The reported hairy root mediated biotransformation reactions include oxidation, reduction, hydroxylation, esterification, methylation, isomerization, and glycosylation [4]. However, attaining reaction sequences involving reduction followed by oxidation steps or vice versa in "one pot" in a sequential or simultaneous manner is hitherto a challenge due to the requirement of divergent reaction conditions [6].

The present investigation was undertaken to explore the biotransformation ability of a pre-established fast-growing hairy root clone (AB-09) of *Atropa belladonna* [7] with regard to three different aromatic carbonyl compounds (ACC), i.e., 3,4,5-trimethoxy benzaldehyde (1), 3,4,5-trimethoxy acetophenone (2), and 3,4,5-trimethoxy benzoic acid (3). The selection of these substrates were based on their commonality of possessing the trimethoxy phenyl unit which had already been noted to be one of the salient factors in rendering anti-cancerous activity to many well-established anticancer molecules, such as podophyllotoxin, etoposide, teniposide, combretastatin, colchicin, etc. [8]. Besides this, these molecules also possessed antimicrobial activities [9, 10]. The overall objective of biotransformation of these molecules was to generate analogs of these compounds with improved selectivity/toxicity profiles and better/novel pharmacokinetic potentials.

This study reports the simultaneous oxidation and reduction potential of the AB-09 hairy root clone for (*1*) into its corresponding oxidized (3) and reduced (*4*) products without any intermediate separation or addition of reagents. It further reveals reduction of the ketone (*2*) into alcohol derivative (*5*). The biotransformation potential of the *A. belladonna* hairy root clone for concurrent oxidation and reduction in a single flask highlights the importance of this study [6], which, as per our observations, is the first of its kind involving the hairy root culture of *A. belladonna*.

Materials and Methods

General Experimentation

All spectrophotometric studies were carried out using Bruker Avance 300 DRX spectrometer (USA). Electrospray ionization (ESI) mass spectra were recorded on Shimadzu LC-MS

(Japan), and high performance thin layer chromatography (HPTLC) was done in CAMAG (Switzerland). Optical rotation was measured in HORIBA high sensitive polarimeter (Japan). IR spectra were generated by Spectrum BX Perkin Elmer (USA).

Hairy Root Culture

Pre-selected hairy roots clone of *A. belladonna* (AB-09) [7], cultured in a half-strength liquid MS [11] medium and incubated on a rotary shaker (80 rpm) at 25±2 °C under dark conditions, was used in the present study.

Biotransformation Experiments

All biotransformation experiments were carried out in 250-ml Erlenmeyer flasks. The substrates (*1*), (*2*), and (*3*) were dissolved in DMSO (330 mg/ml), and 0.3 ml of each solution was added separately to 50 ml of half-strength MS (3% sucrose) liquid media at the concentration of 2 mg substrate/ml of media. These feeded media were dispensed in 4 weeks old hairy root cultures (approx. 1.0 g) which were subsequently incubated at 25±1 °C on a rotary shaker (80 rpm). Two controls were also established, one of which was substrate control, containing the same concentration of the individual substrates dissolved in DMSO and added to the medium devoid of hairy roots, and the other was culture control containing the same concentration of DMSO (0.6%) without the substrates in the medium containing the hairy roots.

Extraction of Substrate and Products

Cultures were harvested after 3, 6, 24, 48, 72, and 144 h of feeding, and the hairy roots and media from each experiment were separated by vacuum filtration. Hairy roots were washed thoroughly with distilled water and oven-dried. The dried powders (100 mg) of hairy roots were soaked in methanol followed by ultrasonication for an hour and kept overnight. The MeOH extracts were filtered and concentrated in vacuo (Buchi Rotavapour R-144). Filtered media (50 ml) were fractionated with ethyl acetate (4×50 ml). The final solution was dried over anhydrous sodium sulfate and concentrated in vacuo. The residual extract of media and hairy roots was dissolved in 1 ml of MeOH for TLC and HPTLC analysis.

Isolation and Identification of Biotransformed Products

The biotransformed products were isolated through preparative TLC (silica gel 60 F_{254} plate) by using the optimized mobile phases, i.e., chloroform/ethanol/acetic acid (18:1:1) for (*1* and *3*) and ethyl acetate/hexane (1:1) for (*2*). Isolated biotransformed products were characterized by NMR, ESI mass, and IR spectrometry.

HPTLC Analysis—Quantification of Products

HPTLC was performed in twin trough glass tank that had been pre-saturated with the optimized mobile phase (as described above) for 30 min. Loading of the samples was done through automatic sampler by using ATS 4 (CAMAG, Switzerland); 5 μl of the standards (50 ng) was loaded as reference. After the development of HPTLC plates, the substrates and products were quantified using a CAMAG TLC scanner model 3 equipped with Camag Wincats software. The following scan conditions were applied: slit width, 6.0 mm;

wavelength, 250 and 254 nm; absorption–reflection scan mode. In order to prepare calibration curves, stock solutions of 1, 5, 10, and 20 mg were prepared, and various volumes of these solutions were analyzed by HPTLC as described above. Actual concentration vs. observed concentration was prepared; the r^2 value of 0.995 is obtained from the curve. The yield of the products was calculated using the calibration curve.

Results and Discussion

The selected hairy root clone of *A. belladonna* (AB-09) proved to be a competent system to carry out bioconversion of the exogenously supplied substrates in comparison to no reactions in the substrate-control and culture-control experiments. Interestingly, the maximum biotransformed products could be recovered from the media which corroborated previously reported observations involving a similar kind of products [12].

Oxidation and Reduction of 3,4,5-Trimethoxy Benzaldehyde (1)

The *A. belladonna* hairy root clone (AB-09) demonstrated more than 67% bioconversion of (*1*) which resulted to the formation of the corresponding oxidized and reduced products, i.e., 3,4,5-trimethoxy benzoic acid (*3*) and 3,4,5-trimethoxy benzyl alcohol (*4*), respectively (Fig. 1). The R_f values of compounds (*1*), (*3*), and (*4*) were noted to be 0.75, 0.60, and 0.54, respectively, in TLC with the optimized solvent system (i.e., chloroform/ethanol/acetic acid (18:1:1, by vol.). The selected hairy root clone demonstrated a notable potential to execute simultaneous oxidation and reduction reactions targeting the formyl group of the first substrate, i.e., 3,4,5-trimethoxy benzaldehyde (*1*).

A time course study demonstrated substantial oxidation and reduction reactions starting at 3 h of the substrate feeding (Fig. 2). The maximum oxidation of (*1*) to (*3*) could be recorded at the 6 h of feeding both in the media (27.46%, 29.72 mg) as well as in the root tissues (7.03%, 7.6 mg) (Fig. 2). The reduction reaction, on the other hand, demonstrated a reasonably perpetual trend from the 24 h onward, and the maximum bioconversion of (*1*)

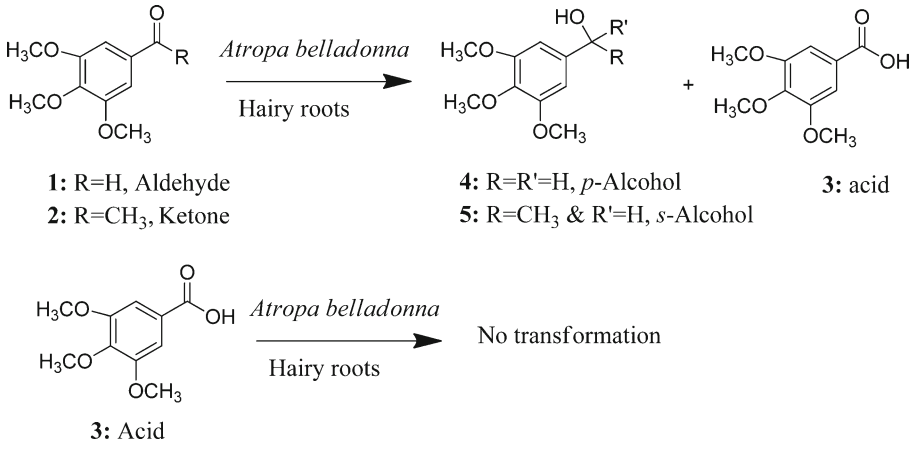

Fig. 1 Hairy root cultures of *A. belladonna* transformed (*1*) into its corresponding oxidized product (*3*) and reduced product (*4*). Compound (*2*) was transformed into its alcohol derivative (*5*); no transformation of (*3*)

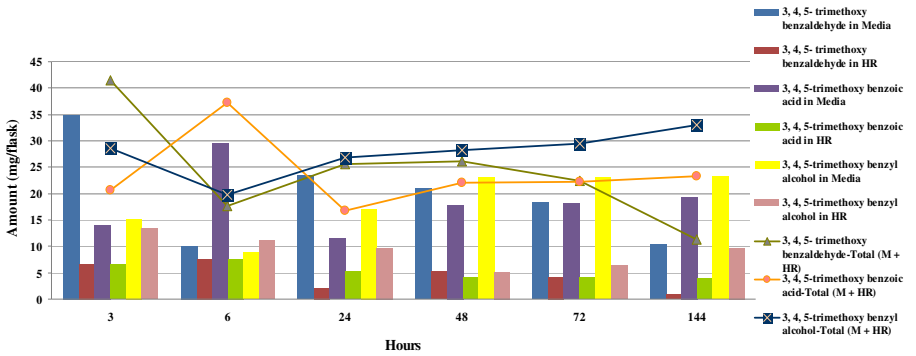

Fig. 2 Time course studies of biotransformation of 3,4,5-trimethoxy benzaldehyde by *A. belladonna* hairy root clone

to (*4*) could be noted at the 144 h of feeding when the total content of (*4*) attained 32.68% conversion, i.e., 33.0 mg (Fig. 2).

Compound 3

^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.77 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 6.54 (s, 2H, 2 & 6 CH of aromatic ring). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 61.19, 61.19, 65.49, 104.24, 107.55, 123.0, 146.52, 153.24, 153.66, 170.38. ESI mass (MeOH): 212 $[M]^+$, 213 $[M+H]^+$. IR (CCl_4, per centimeter): 3,019; 1,703; 1,509; and 1,216.

The ^{1}H NMR spectra of compound (*3*) (creamish white solid, 37.32 mg) showed three distinct singlets that appeared at δ 3.77, 3.80, and 3.83 indicating the presence of three methoxy groups. Two aromatic protons were present at δ 6.54 as singlet clearly indicating that there is no substitution in the aromatic ring. Further, ^{13}C NMR and DEPT-135 experiments exhibited the presence of three methoxyls (OCH_3), two methines (aromatic), and four quaternary carbons (one COOH). Its IR spectrum at 3,019 (hydroxyl of the COOH group), 1,703 (carbonyl of COOH), 1,509 (aromatic), and 1,216 cm^{-1} (ether) showed the presence of various functional groups. All these spectral data endorsed (*3*) as 3,4,5-trimethoxy benzoic acid with the molecular formula of $C_{10}H_{12}O_5$.

Compound 4

^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.72 (s, 3H, OCH_3), 3.95 (s, 6H, $2XOCH_3$), 4.49 (s, 2H, CH2-O-), 6.53 (s, 2H, 2&6 CH of aromatic ring), ^{13}C NMR ($CDCl_3$, 75 MHz): δ 56.31, 56.31, 61.01, 64.86, 104.01, 104.01, 137.11, 37.91, 137.91, 153.42. ESI Mass (MeOH): 198 $[M]^+$, 199 $[M+1]^+$. IR (CCl_4, per centimeter): 3,400; 2,938; 1,592; and 1,131.

The ^{1}H NMR spectrum of compound (*4*) (yellowish sticky solid, 33.0 mg) represented three singlet at δ 3.72 (OCH_3) and 3.95 ($2\times OCH_3$) suggesting the presence of three methoxy groups and a singlet at δ 4.49 of two protons which consequently justified the presence of the oxygenated CH_2 (OCH_2) group. The presence of another singlet at δ 6.53 for two aromatic protons further specified the existence of an intact aromatic ring. The ^{13}C NMR and DEPT 135 experiments endorsed the presence of three methyl (OCH_3), one methylene (OCH_2), two methines (CH, aromatic), and four quaternary carbons (three oxygenated). ^{13}C NMR and ESI MS suggested its molecular formula as $C_{10}H_{12}O_4$. IR spectra at 3,400 (OH), 2,939 (aliphatic), 1,592 (aromatic), and 1,131 (p-alcohol) confirmed the presence of

various functional groups. All these spectral data designated the transformed product (*4*) as 3,4,5-trimethoxy benzyl alcohol.

Oxidative biotransformation reactions of aromatic aldehydes (viz. 3,4,5-trimethoxy benzaldehyde, vanillin, *p*-anisaldehyde, pyridoxal hydrochloride, etc.) had earlier been reported through the use of sulfate-reducing bacteria viz. *Desulfovibrio vulgaris* Marburg, "*Desulfovibrio simplex*" XVI, and *Desulfovibrio* sp. strain MP47 [13]. Although oxidation of phenol-like compounds had earlier been carried out by the hairy root systems of several plant species [14, 15], as far as oxidation of aromatic aldehyde is concerned, no study has yet been reported as per our knowledge.

Likewise, reduction and glucosylation reactions of several exogenous aromatic aldehydes (viz. 3,4,5-trimethoxy benzaldehyde, salicylaldehyde, 3,4-dihydroxy benzaldehyde, 3,4-dimethoxy benzaldehyde, and vanillin) had been reported earlier using the hairy root cultures of *Pharbitis nil* [12]. Besides this, only reduction reaction had been demonstrated involving p-hydroxy benzaldehyde into its corresponding alcohol using hairy roots of *Polygonum multiflorum* [16].

In the present study, we were enthralled to find the oxidation–reduction cascade occurring simultaneously through a single hairy root system without any addition or separation of compounds. This kind of simultaneous catalytic reactions through one biosystem is still a challenge which has gained noteworthy attention since they can effectively reduce operation time, costs, and environmental impact [6, 17, 18]. Furthermore, the novelty of this investigation lies in the fact that the presence of the oxidized and reduced products neither hampered the hairy root growth nor indulged any negative impact on the reaction drive.

Reduction of 3,4,5-trimethoxy acetophenone (2)

Biotransformation of (*2*) resulted in the formation of (*5*) after 144 h of incubation with the AB-09 hairy root clone. The final conversion percentage of (*2*) to (*5*) could never reach over 5% even after 144 h of incubation. The structure of the biotransformed product was determined as 1-(3, 4, 5- trimethoxyphenyl) ethanol (*5*) based on their ^{1}H NMR and ESI-MS spectra (Fig. 1).

Compound 5

^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.49 (d, 3H, *O*-C-CH_3), 3.84 (s, 3H, OCH_3), 3.86 (s,6H, 2X OCH_3), 4.85 (q, 1H, 1-CH), 6.61 (s, 2H, 2 & 6 aromatic protons).ESI MS (CH_3CN): 235 $[M+Na]^+$, 447 $[2\ M+Na]^+$. IR (CCl_4, per centimeter): 3,448; 2,850; 1,508; 1,560; and 1,096. $[\alpha]_D^{22}$=+3.10 (MeOH)

Compound (*5*) (creamish white solid, 5.3 mg) was a reduced product of (*2*), i.e., a secondary alcohol, where the reduction occurred in a stereo-specific manner at the C-2 position. The R_f values of compounds (*2*) and (*5*) were noted to be 0.52 and 0.29, respectively, in TLC in the optimized solvent system, i.e., ethyl acetate/hexane (1:1, v/v). The ^{1}H NMR spectrum of compound (*5*) exhibited three singlets at δ 3.84 (OCH_3) and 3.86 ($2\times OCH_3$) indicating the presence of three methoxy groups; a doublet of three aliphatic protons at δ 1.49, specifying the presence of a CH_3 group neighboring this CH group; a quartet at δ 4.85 of a single proton which confirmed a –CHOH–CH_3-type arrangement. Two aromatic protons were also intact at δ 6.61. This transformation was further confirmed by ESI mass peak at 235 $[M+Na]^+$ and 447 $[2\ M+Na]^+$. The IR spectrum at 3,448 (OH), 2,850 (aliphatic), 1,508, 1,560 (aromatic), and

1,096 (s-alcohol) confirmed the presence of various functional groups. These confirmed the reduction of substrate (*2*) into compound (*5*). ^{1}H NMR and ESI MS suggested its molecular formula as $C_{11}H_{16}O_4$, and the specific rotation of (*5*) indicated that one of the enantiomers was predominant over the other.

The present observations substantiate earlier findings related to ketone biotransformation mediated through *Daucas carota* [19] and *Raphanus sativus* [20] hairy root cultures along with that through several fungi (viz. *Penicillium miczynskii* Gc5, *Trichoderma* sp. Gc1, *Aspergillus sydowii* Gc12, *A. sydowii* Ce19, *A. sydowii* Ce15, *Bionectria* sp. Ce5, *P. miczynskii* Ce16) [21]. Pertinently, more than 35 patents had already been filed relating to ketone reductions, which signifies the commercial magnitude of such reactions [22].

No Transformation of 3,4,5-Trimethoxy Benzoic Acid (3)

No detectable biotransformation could be observed with the AB-09 hairy root clone of *A. belladonna* with respect to the 3,4,5-trimethoxy benzoic acid (*3*) even up to the maximum period of 144 h of incubation. In contrast, *Panax ginseng* hairy root cultures demonstrated the capability of glycosylation of two aromatic acids, i.e., 18 β-glycyrrhetinic acid [23] and *m*- and *p*-hydroxy benzoic acids [24]. Nevertheless, no modification could be obtained with another targeted aromatic acid, i.e., *o*-hydroxy benzoic acid by the same hairy root culture of *P. ginseng* [24], which clearly indicates the position-based biotransformation response of the biological enzyme systems. Moreover, Yan et al. [16] revealed the glucosylation potential of *P. multiflorum* hairy root culture with respect to 4-hydroxy benzoic acid as one of the substrates, but akin to the present study, the –COOH moiety in their case as well remained unaffected which was the solitary position accessible to the chemical reaction as the rest of the position, i.e., 3, 4 and 5, is protected by methoxyl groups [16].

Conclusions

On the basis of overall analysis, it can be stated that the enzymatic machinery of the selected *A. belladonna* hairy root clone simultaneously performed oxidation and reduction of the formyl group of the aromatic aldehyde resulting to the formation of the corresponding acid and alcohol. This hairy root clone further demonstrated reduction of the carbonyl group of aromatic ketone into a secondary alcohol. The redox potential of the *A. belladonna* hairy root clone for parallel oxidation and reduction reactions of single exogenous substrate highlights the importance of this study [6, 17] which, as per our observations, is the first of its kind involving the hairy root culture of *A. belladonna*. This unique biotransformation machinery can be utilized to mediate similar kinds of opposite reactions involving other target molecules requiring analogous types of modifications. Elucidations of the plausible enzymes, accountable to carry out such types of reactions, are currently under process.

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