Title: Aryl carboxylic acid reduction and further reactions with GABA and glucose promoted by whole cells of *Xylaria arbuscula*

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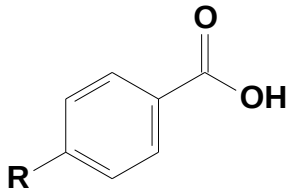

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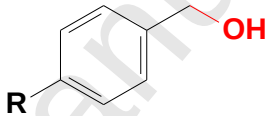
2. R=Cl
3. R=OH
4. R=NH_2
5. R= NO_2

Xylaria arbuscula



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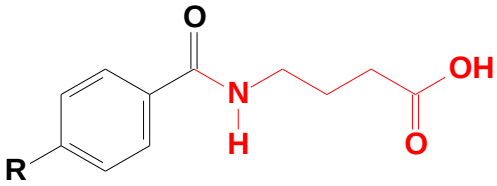
2a. R=Cl
5a. R=NO_2



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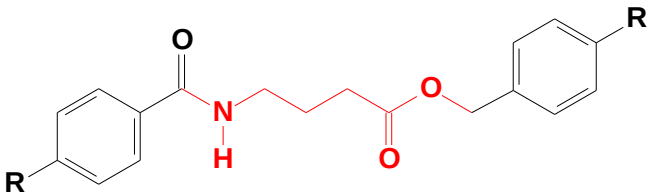

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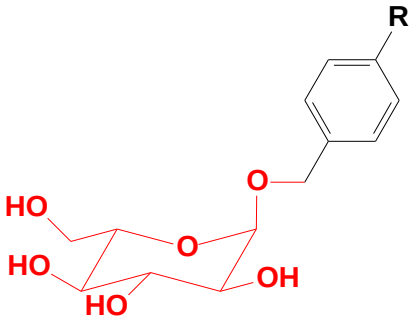

1d. R=Br
2d. R=Cl

- Screening of whole cell biotransformation of *p*-substituted benzoic acids by endophytic fungus *Xylaria arbuscula*.
- Aryl carboxylic acid reduction to alcohol, GABA incorporation and glycosylation.
- Bioreduction activity is dependent on substituents with respect to carboxylic acid in benzene ring.

Aryl carboxylic acid reduction and further reactions with GABA and glucose promoted by whole cells of *Xylaria arbuscula*

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Abstract

Xylaria arbuscula was collected from a "Cypress" tree, *Cupressus lusitanica*. The whole cells of this endophytic fungus were used in screening for microbial bioreduction of aryl acids. Different *p*-substituted benzoic acids were evaluated. However, only *p*-bromobenzoic acid, *p*-chlorobenzoic acid and *p*-nitrobenzoic acid were converted to their corresponding alcohols. Aryl acid metabolism of *X. arbuscula* included GABA incorporation and glycosylation when the substrates were *p*-bromobenzoic and *p*-chlorobenzoic acids were also observed. The substrates *p*-hydroxybenzoic and *p*-aminobenzoic acids were not transformed. These results could suggest that electron-withdrawing groups at *para* orientation activate the substrate for reduction.

Keywords: *Xylaria arbuscula*, aromatic carboxylic acid reduction, GABA, amino acid conjugation, glycosylation

1. Introduction

Whole cell microbial reductions of aromatic carboxylic acids into their corresponding alcohols have been described for some bacteria [1-8], archaea [9, 10], and fungi [3, 11-16]. The reduction of carboxylic acids into aldehydes, and subsequently to alcohols, is thermodynamically unfavorable and a complex biological process [17]. The biocatalysts that promote these reductions usually convert the substrate first into aldehyde and later into the correspondent alcohol [1, 3, 5-12, 15, 16] in a similar way of reductions performed by classical reducing reagents such as $LiAlH_4$, used in synthetic organic chemistry. Using enzymes instead of inorganic hydrides in carboxylic acid reduction reactions may result in greener processes, occasionally enhancing selectivity, and allows the use of water-soluble substrates.

Microbial reductions of carboxylic acids are closely related to the nature of the microorganism. It has been described that anaerobic microorganisms such as *Clostridium thermoaceticum*, *C. formicoaceticum*, and *Pyrococcus furious* catalyze carboxyl group reduction due to aldehyde oxidoreductases (AOR), highly oxygen-sensitive tungsten enzymes (W-AOR) [2, 5, 18-20]. *C. formicoaceticum* also contains a less oxygen-sensitive molybdenum aldehyde oxidoreductase (Mo-AOR) [21] and, in both cases, posterior reduction to the corresponding alcohols is catalyzed by NAD(P)H-dependent alcohol dehydrogenases. On the other hand, bioreductions of carboxylic acids in aerobic conditions have been investigated extensively by Rosazza and co-workers regarding the participation of the carboxylic acid reductase (CAR). The activity of this enzyme is dependent on ATP and NADPH [5-8, 16, 22], which imposes additional challenges related to in situ regeneration of the cofactors that are important for catalysis.

Furthermore, CAR enzymes have broad substrate specificity, since they accept a wide variety of aromatic and aliphatic acids [5-8, 23-26].

The aforementioned researches evince the need for further studies on diversity of the CAR biocatalyst in order to explore each of its good characteristics. The intimate relationships between endophytic microorganisms and their host plants may define a nature-inspired approach to great diversity of these CARs and other biocatalysts. In short, after a long period of adaptation these endophytes are used to handle the plant's chemical defense and elaborate enzymes capable of transforming functional groups in these defensive molecules. These kinds of ecological relationships have inspired our research trying to detect and rationalize interesting biochemical processes that may be useful in organic synthesis and biotransformations.

We evaluated the biotransformation capability of *Xylaria arbuscula*, an endophytic fungus isolated from leaves of *Cupressus lusitanica*, toward benzoic acids derivatives. *C. lusitanica* is a coniferous resinous tree that contains a variety of terpenoid acids [27]. We used aryl benzoic acids to describe the bioreduction of *p*-bromobenzoic acid (**1a**), GABA incorporation **(1b**, **1c**), and glycosylation (**1d**) of the substrate. The products were fully elucidated on the basis of 1D and 2D NMR, and LC-HRMS techniques. Furthermore, LC-HRMS analyses showed that *p*-chlorobenzoic followed the same transformation pattern, while *p*-nitrobenzoic acid gave only carboxyl reduction product.

2. Materials and Methods

2.1 Solvents and chemicals

p-Bromobenzoic acid, *p*-chlorobenzoic acid, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and *p*-nitrobenzoic acid were purchased from Sigma-Aldrich and used without

further purification. All solvents used in chromatographic procedures were of HPLC grade, while all other solvents were of analytical grade.

2.2 General experimental procedures

All 1D and 2D NMR spectra were recorded in CH_3OH-*d*4 and $CHCl_3$-*d* (Aldrich) on a Bruker Avance III spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C, and TMS was used as internal standard. LC-UV-MS was performed using a reversed-phase analytical Luna Phenyl Hexyl column (4.6 x 250 mm, 5 μm). The analytes were detected by a photodiode array detector (PDA) and by electrospray (ESI) mounted in a triple-quadrupole Micromass Quattro LC mass spectrometer. High-resolution mass spectral data were acquired in a Bruker Micro Tof-Q II mass spectrometer. Preparative reversed-phase HPLC separations were achieved using a Luna Phenyl Hexyl column (21.2 × 250 mm, 10 μm) mounted in a Shimadzu SIL-20AP VP system.

2.3 Plant and fungal material

Healthy leaves of *Cupressus lusitanica* were collected in São Carlos, state of São Paulo, Brazil. A voucher specimen (No. 7281) has been deposited in the Herbarium of the Botanic Department of Universidade Federal de São Carlos, Brazil.

The strain *Xylaria arbuscula* was isolated from healthy tissues of *C. lusitanica* leaves in accordance with the methodology used by Petrini et al. [28]. After isolation, the fungus was conserved in sterilized water, and deposited under the identification code LaBioMMi 445 at LaBioMMi (Laboratório de Bioquímica Micromolecular de Micro-organismos) of the Chemistry Department at Universidade Federal de São Carlos, Brazil. Working stocks were prepared with a commercial PDA (potato 200 g, glucose

20 g, agar 15 g and water 1000 mL) medium. The microorganism was identified through molecular techniques based on the analysis of actin gene [29].

2.4 Fermentation and extraction

The fungus was grown under static conditions at room temperature for 20 days in 6 Erlenmeyer flasks containing liquid medium (100 mL per flask) composed of glucose (26.7 g L^{-1}), $NaNO_3$ (3.0 g L^{-1}), K_2HPO_4 (1.0 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}), KCl (0.5 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (0.01 g L^{-1}), supplemented with 25 mg of each aryl-benzoic acid per flask. After growing, the mycelium was separated by filtration under reduced pressure and the liquid phase was partitioned with ethyl acetate (3 x 600 mL). The organic solvent was concentrated under vacuum to afford a crude extract. The extracts were analyzed by LC-MS using gradient elution with a mobile phase initially set as 30:70 v/v acetonitrile–water (both containing 0.1% formic acid), followed by a linear gradient to 100% acetonitrile over 45 min at a flow-rate of 200 µl min^{-1}. After LC-MS analysis the crude extract obtained from the culture medium supplemented with *p*-bromobenzoic acid (**1**) was subjected to preparative RP-HPLC (30-100% ACN/H_2O), and 25 fractions were collected. Further purification via reversed-phase chromatography yielded compounds **1a** (2.5 mg), **1b** (1.0 mg), **1c** (1.6 mg) and **1d** (3.5 mg).

3. Results and discussion

The *Xylaria* genus is an underexplored source for fungal whole cells biotransformations. Only few studies show the potential of Xylareaceous fungi for oxidation reactions in bioconversions [30-33]. The ability of *Xylaria arbuscula* to perform biocatalysis was investigated by administrating different *p*-substituted benzoic

acid derivatives. Following a period of incubation, several biotransformation products from halogenated and nitrobenzoic acids were detected by LC-HRMS, such as represented in Figure 1. The biotransformation products of *p*-bromobenzoic acid were isolated and purified, and their structure established based on NMR, HRMS and HRMS/MS data as described below.

The ^{1}H NMR spectrum of compound **1a** suggests that the enzymatic machinery of the fungus promotes the reduction of *p*-bromobenzoic acid to a correspondent primary alcohol. The structure of compound **1a** was confirmed by comparison of the ^{1}H and ^{13}C NMR data with those previously reported [34, 35]. However, the *full scan* mass spectra showed the protonated molecular ion at *m/z* 168.9649, indicating their dehydration and consequently formation of brominated tropylium ion (Figures S3 and S4). This result provides evidence that the fungus *Xylaria arbuscula* has carboxylic acid reductase (CAR) activity, since the biotransformation was carried out under aerobic conditions. That is the first evidence of CAR enzyme in the *Xylaria* genus, which could be broadly explored in green chemistry. Figure 2A suggests the catalytic cycle by CAR reduction of *p*-bromobenzoic acid to *p*-bromobenzyl alcohol. The catalytic cycle begins with the activation of bound *p*-bromobenzoic acid by ATP to give acyl-adenylate intermediate [16, 22]. The sulfhydryl moiety of the phosphopantetheinyl prosthetic group, represented by HS-CAR, reacts with the acyl adenylate intermediate, eliminating adenosyl monophosphate, and generating a thioester by which the *p*-bromobenzoic acid derivative is covalently bound to CAR. The thioester is reduced at the C-terminus of CAR by NADPH releasing the corresponding aldehyde product, and a free phosphopantetheine-sulfhydryl moiety available for another catalytic cycle [5, 7, 22]. Further reduction to the corresponding alcohol **1a** probably is catalyzed by an enzyme dependent of NAD(P)H.

Compound **1b** was obtained as a colorless amorphous solid, and its molecular formula was determined to be $C_{11}H_{12}BrNO_3$ by HRMS (*m/z* 286.0074 $[M+H]^+$, calcd. 286.0073). ^1H NMR spectrum of **1b** (Table S1) showed similarity with the precursor *p*-bromobenzoic acid, with the same substitution pattern of the aromatic ring δ 7.72 (*d*, 8.74, 2H), 7.62 (*d*, 8.74, 2H), and displayed three methylene groups H-6 δ 3.42 (*t*, 2H), H-7 δ 1.91 (*quint*, 2H), H-8 δ 2.38 (*t*, 2H). In the HMBC spectrum of **1b** correlations of H-6 (δ 3.42) with C-5 (δ 167.5), and of H-7 (δ 1.91) and H-8 (δ 2.38) with C-9 (δ 32.78), indicated the presence of an amide and carboxylic acid, respectively. The COSY spectrum showed correlations between H-7 and H-6, H-8 with typical spectral profile of γ-aminobutyric acid (GABA) [36]. GABA is produced by filamentous fungi and is responsible for several functions [37], although there are only few reports concerning secondary metabolites containing GABA [38 - 40]. The data above showed how the fungus is able to promote an amide bond formation between *p*-bromobenzoic acid and γ-aminobutyric acid. This is the first evidence of the γ-aminobutyric acid production by this genus. The proposed structure for **1b** was confirmed by HRMS/MS analysis. The product ions at *m/z* 267.9981 and 182.9449 resulting from $[M+H]^+$ at *m/z* 286.0074 are formed by losses of H_2O and GABA moiety (Figure S10). The accurate masses of the product ions are shown in Table 1.

Plants and mammals can transform aromatic carboxylic acids in several ways, and one of these mechanisms is through amino acid conjugation. In the first step, the carboxylic acid moiety is activated by ATP, which generates an acyl-adenylate (acyl-AMP) intermediate; this reaction is catalyzed by acyl-CoA synthetase (ACS). In the second step, the adenylate group is displaced by CoA resulting in a CoA conjugated carboxylic acid. Then, the CoA can be replaced by an amino acid, which is catalyzed by N-acyltransferases [41, 42]. Hence, the fungus must have promoted the combination of

glutamic acid followed by decarboxylation, leading to biotransformation product **1b**, as shown in Figure 2B.

Compound **1c** was obtained as a colorless amorphous solid. The molecular formula of **1c** was determined to be $C_{18}H_{17}Br_2NO_3$ by HRMS (*m/z* 453.9637 $[M+H]^+$, calcd. 453.9648) and NMR spectral analysis, requiring 10 degrees of unsaturation. ^{1}H NMR spectrum (Table S2) exhibited two *p*-substituted benzene rings [δ 7.63 (*d*, 8.6 Hz, 2H), 7.57 (*d*, 8.6 Hz, 2H) and δ 7.48 (*d*, 8.4 Hz, 2H), 7.21 (*d*, 8.4 Hz, 2H)]. NMR data also revealed the presence of four methylene groups H-7 δ 1.19 (*quint*, 7.1 Hz, 2H), H-8 δ 2.51 (*t*, 2H), two of which are heteroatom-bonded, H-6 δ 3.52 (*t*, 6.9 Hz, 2H), and H-10 δ 5.08 (*s*) and two carboxylic carbons. The COSY spectrum showed correlations between H-7 (δ 1.91) and H-6 (δ 3.52); H-8 (δ 2.51) and HMBC showed correlation of H-3 (δ 7.63) and H-6 (δ 3.52) to C-5 (δ 166.5) as in compound **1b**, indicating the presence of γ-aminobutyric acid. The deshielded chemical shift of H-10 (δ 5.08) indicates that it should be attached to an oxygen atom from an ester group, which was confirmed by HMBC, since H-10 (δ 5.08) showed correlation with the carboxylic carbon at C-10 (δ 173.5). Furthermore, H-10 showed HMBC correlations with C-11 (δ 134.5) and C-12 (δ 129.9) indicating it was also attached to the aromatic ring. Key COSY and HMBC correlations are represented in Figure 3. The HRMS/MS analysis confirmed the NMR data and the fragmentation patterns of $[M+H]^+$ at *m/z* 453.9637 for compound **1c**. The product ions at *m/z* 267.9955 and 168.9649 are formed from $[M+H]^+$ due to losses of *p*-bromobenzyl alcohol and **1b**. Through these spectroscopic data, it was possible to conclude that the microorganism used the compounds **1a** and **1b** as precursors of **1c**, a novel structure produced by *X. arbuscula*.

HRMS of **1d** gave a $[M+H]^+$ at *m/z* 349.0274 indicating a molecular formula $C_{13}H_{17}BrO_6$ (calcd. 349.0281). ^{1}H NMR spectrum indicated a *p*-substituted benzene

ring with hydrogens at δ 7.49 (*d*, 8.45, 2H) and δ 7.35 (*d*, 8.45, 2H). HSQC spectrum showed the correlation between the oxymethylenic hydrogens H-5a/H-5b at δ 4.69 (*d*, 12.2, 1H) and δ 4.50 (*d*, 12.2, 1H) with carbon at δ 69.52, and the HMBC correlations indicated these hydrogens were connected to the aromatic ring as in compound **1a**. The ^{13}C, HSQC and HMBC spectra (Table S3) exhibited 13 carbons, 6 of which could be attributed to a sugar with a six-membered ring at δ 99.4, 73.5, 75.0, 71.8, 74.0 and 62.3. The ^{1}H NMR spectrum of **1d** was typical for glucoside with sugar region showing one anomeric proton H-1' δ 4.80 (*d*, 3.8 Hz, 1H), with coupling constant characteristic of α anomers. The coupling constants observed for H-2' δ 3.32 (*dd*, 9.7, 3.8 Hz, 1H) indicated that protons H-2' (δ 3.32), H-3' (δ 3.57), H-4' (δ 3.20), and H-5' (δ 3.50) all occupied axial positions. Therefore, the sugar unit was assigned as α-glucopyranose. HMBC correlations for an H-1' (δ 4.80) to C5 (δ 69.4) and C-5' (δ 71.8) showed that the glucoside unit was attached to C-5. This result shows interesting enzymatic diversity, since the fungus was able to promote carboxylic acid reduction, GABA incorporation and also glycosylation. The yield of *p*-bromobenzoic acid into **1a**, **1b**, **1c** and **1d** was 10.8, 2.8, 1.4 and 8% respectively.

The microorganism biocatalytic capability was evaluated against other benzoic acids such as *p*-chlorobenzoic, *p*-hydroxybenzoic, *p*-aminobenzoic and *p*-nitrobenzoic acids. LC-HRMS analysis demonstrated that *p*-chlorobenzoic acid was converted into analogues of brominated transformed products and *p*-nitrobenzoic acid was reduced to its correspondent alcohol. In the fungal extract containing *p*-chlorobenzoic acid, compound **2a** was detected with $[M+H]^+$ at *m/z* 125.0154 corresponding to the chlorinated tropylium ion (calcd. 125.0152), with a MS spectrum pattern similar to that of **1a**. Furthermore, compounds **2b**, **2c** and **2d** were detected and their HRMS showed protonated molecular ions at *m/z* 242.0581 (calcd 242.0578), 366.0661 (calcd 366.0641)

and 305.0796 (calcd 305.0786), corresponding to the molecular formulas $C_{11}H_{12}ClNO_3$, $C_{18}H_{17}Cl_2NO_3$ and $C_{13}H_{17}ClNO_6$, respectively. In addition, HRMS/MS analysis also helped to identify chlorinated transformed products **2b** and **2c** since their fragmentation patterns are identical to those observed in brominated compounds. Table 1 summarizes the accurate masses of the product ions. On the other hand, in the fungal extract supplemented with *p*-nitrobenzoic acid, only compound **5a** was detected, with molecular formula determined to be $C_7H_7NO_3$ (*m/z* $[M+H]^+$ 154.0493, calcd. 154.0499), indicating the reduction of the substrate. The substrates *p*-hydroxybenzoic and *p*-aminobenzoic acids accumulated in the fermentation medium without transformation. The results demonstrate that biocatalytic activity is dependent on substituents with respect to carboxylic acid in the benzene ring, whereas the halogen atom and the nitro group have a withdrawing inductive effect and turn the carboxylic carbon susceptible to the observed reactions.

4. Conclusion

Xylaria arbuscula has an interesting enzymatic potential to promote biotransformations, mainly reduction of *p*-substituted benzoic acids derivatives containing withdrawing groups at *para* position to correspondent alcohol. This result offers evidence of the presence of carboxylic acid reductase activity in this fungus for the first time. Also, the fungus was capable of realizing γ-aminobutyric acid incorporation and glycosylation.

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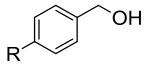
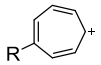
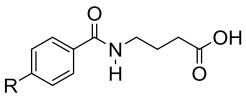
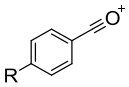
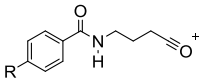
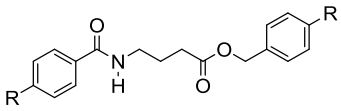
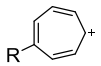
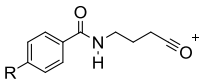
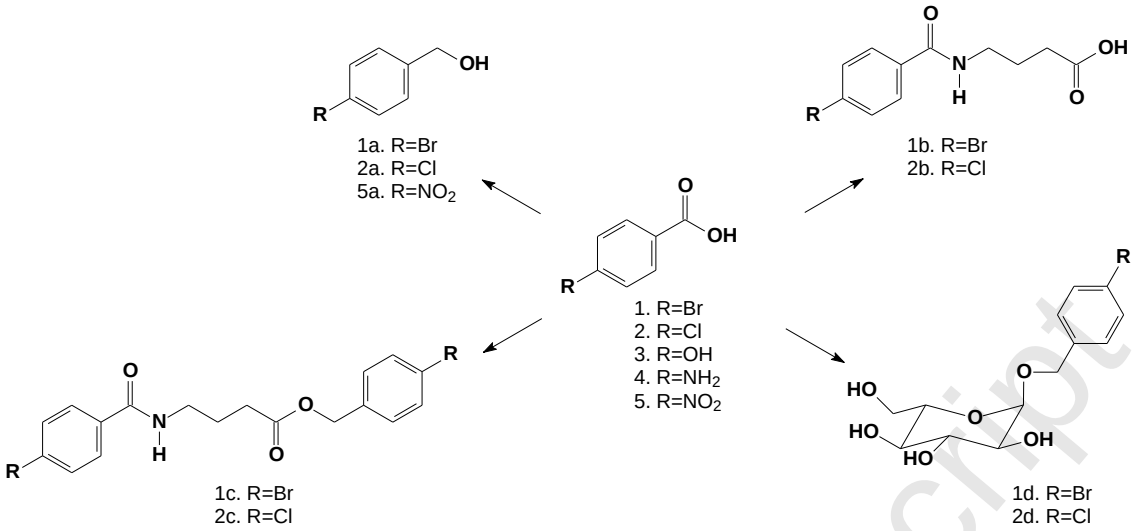
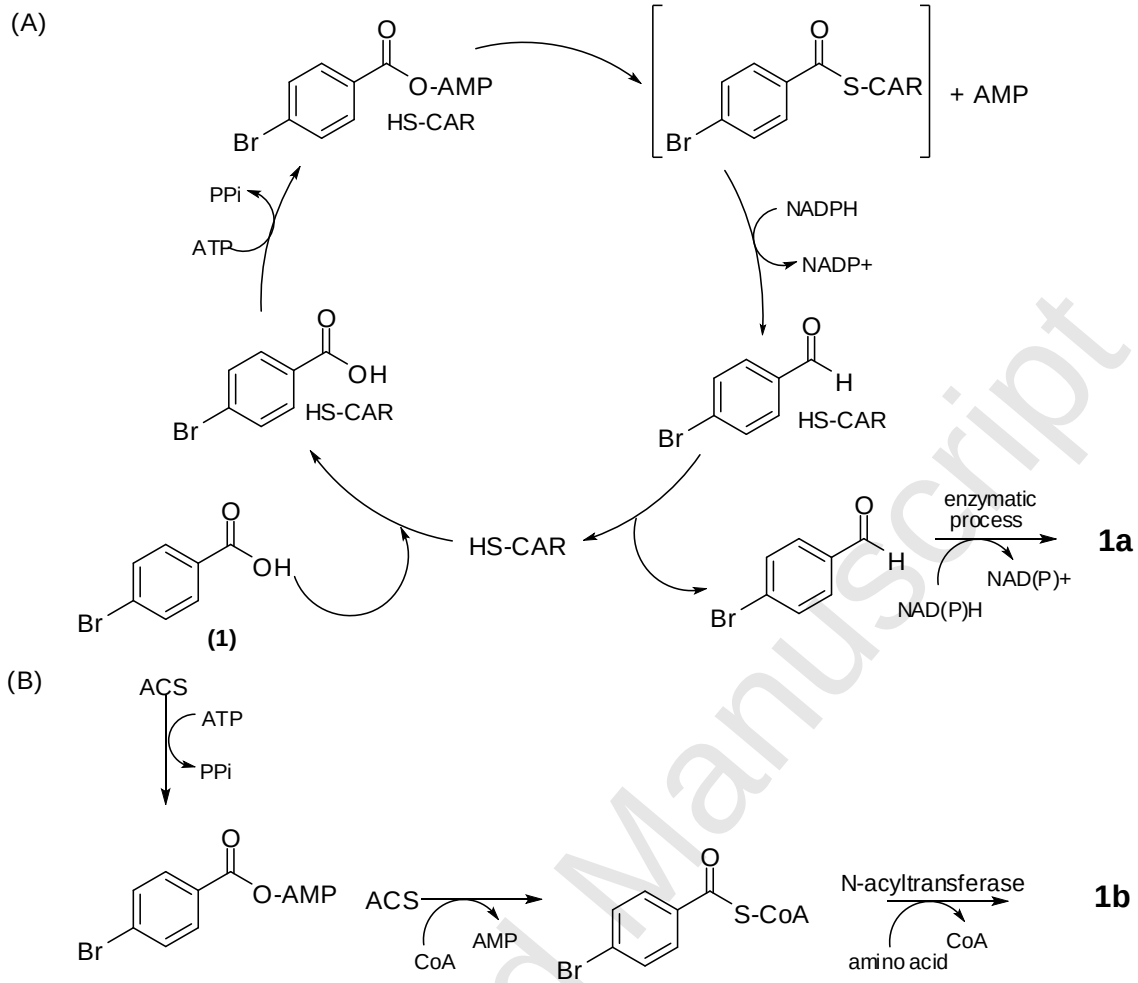
Table 1. Accurate masses of brominated and chlorinated product ions

Biotransformed product	Product ion	Substitution (R)	Observed (*m/z*)	Calculated (*m/z*)
		Br	168.9649	168.9647
		Cl	125.0154	125.0152
		Br	182.9450	182.9440
		Cl	138.9958	138.9945
		Br	267.9991	267.9968
		Cl	224.0472	224.0471
		Br	168.9649	168.9647
		Cl	125.0165	125.0153
		Br	267.9955	267.9968
		Cl	224.0493	224.0473





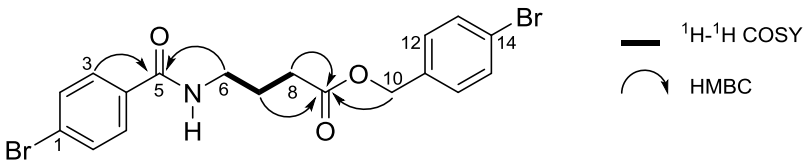


Figure Captions

Figure 1. Biotransformation capability of *Xylaria arbuscula* toward *p*-substituted benzoic acids derivatives and correspondent biotransformation products

Figure 2. (A): CAR cycle in the reducing of *p*-bromobenzoic acid to *p*-bromobenzaldehyde and further reduction to alcohol. HS-CAR represents phosphopantetheinylated holo-Car. (adapted from 7). (B): GABA conjugation with *p*-bromobenzoic acid: The first step is catalyzed by acyl-CoA synthetase (ACS) generating an acyl-adenylate (acyl-AMP) intermediate. The adenylate group is displaced by CoA resulting in a CoA conjugated with carboxylic acid. The third reaction is catalyzed by N-acyltransferases, the CoA is replaced by glutamic acid and following a decarboxylation leading compound **1b** (adapted from 41)

Figure 3. Key COSY and HMBC correlations of compound **1c**