Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.11 No.3, Issue of July 15, 2008

Received June 7, 2007 / Accepted May 22, 2008
DOI: 10.2225/vol11-issue3-fulltext-10
RESEARCH ARTICLE

Biotransformation of 3-hydroxydibenzo-α-pyrone into 3,8 dihydroxydibenzo-α-pyrone and aminoacyl conjugates by *Aspergillus niger* isolated from native"shilajit"

Aminul Islam
Research and Development Centre
Natreon Inc.
Cl-18A, Sector -II, Salt Lake City
Kolkata 700091, India
Tel: 033 23216459
Fax: 033 23371910
E-mail: natr1910@dataone.in

Runa Ghosh*
Research and Development Centre
Natreon Inc.
Cl-18A, Sector -II, Salt Lake City
Kolkata 700091, India
Tel: 033 23216459
Fax: 033 23371910
E-mail: natr1910@dataone.in

Dipankar Banerjee
Research and Development Centre
Natreon Inc.
Cl-18A, Sector –II, Salt Lake City
Kolkata 700091, India
Tel: 033 23216459
Fax: 033 23371910
E-mail: natr1910@dataone.in

Piali Nath
Research and Development Centre
Natreon Inc.
Cl-18A, Sector –II, Salt Lake City
Kolkata 700091, India
Tel: 033 23216459
Fax: 033 23371910
E-mail: natr1910@dataone.in

Upal Kanti Mazumder
Department of Pharmaceutical Technology
Jadvapur University
Kolkata-700032, India
Tel: 033 23216459
Fax: 033 23371910
E-mail: mazumderu@yahoo.co.in

Shibnath Ghosal[#]
Research and Development Centre
Natreon Inc.
Cl-18A, Sector -II, Salt Lake City
Kolkata 700091, India
Tel: 033 23216459
Fax: 033 23371910
E-mail: natr1910@dataone.in; vishnu20024@rediffmail.com

Financial support: Natreon Inc, USA.

Keywords: Coenzyme Q_{10} reduction, electron transport chain humification, paleohumus.

[#]**Present address:** Research Adviser, R&D Centre, Indian Herbs Ltd. Saharanpur-247001 (U.P), India.

*Corresponding author

Abbreviations: $3,8(OH)_2$-DBP: 3,8 Dihydroxydibenzo-α-pyrone
3-OH-DBP: 3-Hydroxydibenzo-α-pyrone
AUC: area under the curve
BSFTA: N, *O*-bis (Trimehylysislyl)-triflouro-acetamide
CD: Czapek Dox
DBPs: dibenzo-α-pyrones
FM: fermentation medium
GC-MS: gas chromatography-mass spectrometry
HPFC: high performance flash chromatography
HPLC: high performance liquid chromatography
TMS: trimethylsilyl

"Shilajit" is a panacea in Ayurveda, the Indian traditional system of medicine. The major bioactives of "shilajit" have been identified as dibenzo-α-pyrones (DBPs), its oligomers and aminoacyl conjugated derivatives. These bioactive compounds play a crucial role in energy metabolism in all animal cells including those of man. 3-hydroxydibenzo-α-pyrone (3-OH-DBP), a key DBP component of "shilajit" is converted, among other products, to another active DBP derivative, viz. 3,8-hydroxydibenzo-α-pyrone, $3,8(OH)_2$-DBP, *in vivo*, when its precursor is ingested. $3,8(OH)_2$-DBP is then involved in energy synthesis in the mitochondria in the reduction and stabilization of coenzyme Q in the electron transport chain. As the chemical synthesis of $3,8(OH)_2$-DBP is a complex, multi-step process and economically not readily viable, we envisioned the development of a process using microorganisms for bioconversion of 3-OH-DBP to $3,8(OH)_2$-DBP. In this study, the biotransformation of 3-OH-DBP is achieved using *Aspergillus niger*, which was involved in the humification process on sedimentary rocks leading to "shilajit" formation. A 60% bioconversion of 3-OH-DBP to $3,8(OH)_2$-DBP and to its aminoacyl derivatives was achieved. The products were characterized and estimated by high performance liquid chromatography (HPLC), high performance flash chromatography (HPFC) and gas chromatography-mass spectrometry (GC-MS) analyses. Among the *Aspergillus species* isolated and identified from native "shilajit", *A. niger* was found to be the most efficient for this bioconversion.

"Shilajit", a panacea of oriental medicine, is an organic exudation, comprising humic substances, from steep rocks (1200-5000 m) of different formations found throughout the world (Phillips, 1997; Ghosal, 2002). "Shilajit" is used as a rasayana (rejuvenator) in Ayurveda (Tiwari et al. 2001) to arrest aging and for augmenting energy synthesis. "Shilajit" constitutes fresh and modified remnants of rock humus admixed with rock minerals (Ghosal et al. 1997) and other organic substances occurring in the "shilajit" bearing rock rhizospheres. On interaction with microorganisms, isolated from the "shilajit" surface and by geochemical and geothermal reactions the organic ingredients of sedimentary rocks are transformed into humic substances (Ghosal, 2006). The major fungi isolated from the "shilajit" (paleohumus) rhizosphere are *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus ydowii*, *Aspergillus usfres, Fusarium moniliformae and Trichotehecium roseum*. Among these fungi, the percent incidence and intensity of the fungus *Aspergillus niger* is the maximum in "shilajit" (Ghosal, 2006).

Biochemical transformation of phenolic organic compounds to generate new compounds by specific type of fungus and bacteria (*e.g. Aspergillus niger*, *Fusarium moniliforme*, *Thiobacillus sp*.) is known in literature (Roberts et al. 1995). The chemical process involves oxidation, reduction, hydrolysis and/or conjugation (Holland, 1998; Lima et al. 2006). Microbial transformation for synthesis of new therapeutic agents is now an emerging subject also in pharmaceutical industry (Wackett and Hershberger, 2001).

The central bioactive compounds of "shilajit" are 3-hydroxy-dibenzo-α-pyrone, 3,8-dihydroxydibenzo-α-pyrone and their aminoacyl conjugates which constitute the core nucleus of "shilajit" (rock)-humus-paleohumus. The biological effects (immunomodulatory and adaptogenic) of "shilajit" are believed to be due to these two distinct bioactive compounds (Ghosal et al. 1989; Ghosal, 2002). One of the chemical sequences for eliciting such biological effects involves transformation of 3-OH-DBP into $3,8(OH)_2$-DBP and their aminoacyl conjugates. $3,8(OH)_2$-DBP is involved in energy synthesis in the mitochondria of animals by stabilizing and facilitating the function of Coenzyme Q_{10} (Ghosal, 2006). However, synthesis of $3,8(OH)_2$-DBP is quite an involving process (Ghosal et al. 1989). Conceivably, the use of microorganisms constitutes an interesting alternative to study this type of transformation. Additionally, microbial oxidations have advantages over classical organic synthetic procedures (Schmid et al. 2001; Boaventura et al. 2004), since they are carried out in environment friendly and mild conditions and use biodegradable reagents and they are generally stereo- and regio- selective (Venisetty and Ciddi, 2003; de Carvalho and da Fonseca, 2006).

In this study, 3-OH-DBP has been converted into $3,8(OH)_2$-DBP and its aminoacyl conjugates by use of *A. niger* occurring in "shilajit" bearing rhizosphere by fermentation process. The details of the chemistry and mechanism of transformation constitute the subject of this paper.

MATERIALS AND METHODS

Reagents

3-OH-DBP was prepared synthetically in the laboratory and was >95% pure (Ghosal et al. 1989) (λ_{max} 209.7, 275.7, 300.6, 336.3 nm). Reagents for preparation of the fermentation medium were of analytical reagent grade procured from SRL, India. The water used throughout the study was purified with a Millipore system (Millipore, Bedford, USA). Acetonitrile and *o*-phosphoric acid were of HPLC grade obtained from E-Merck, India. All solvents used for HPFC were of GR grade and purchased from Merck, India.

The silylating reagent for GC-MS analysis, BSFTA was procured from Pierce, USA; Cat. No.38830.

Fungal strain

A native *Aspergillus niger* strain was isolated from crude "shilajit" collected from the Kumaon regions of the Himalayas. It was identified by Microbiology Department, Calcutta University. It was subcultured in Czapek Dox (CD) agar medium and maintained at 29 ± 5ºC. This parent strain was preserved in our microbial collection at -4ºC.

Biotransformations

Biotransformation of a synthetic 3-OH-DBP (>95% pure, Ghosal et al. 1989) was carried out by fermentation by adopting the following optimized conditions.

Conditions adopted for biotransformation. The following conditions of biotransformation were found to be suitable, by trial and error, for optimum production of the products.

Aspergillus niger strain was cultured in CD agar medium and incubated for 5 days at 29-30ºC. The cells were washed with 5.0 ml sterilized distilled water and 2.0 ml of the spore suspension containing 1 x 10^9 spores/ml was used to inoculate the fermentation medium for biotransformation. The fermentation medium with the following composition: KH_2PO_4, 0.01%, $MgSO_4.7H_2O$, 0.01%, KCl 0.01%; $NaNO_3$ 0.05% and trace quantity of $FeSO_4.7H_2O$ in 100 ml of double distilled de-ionized water, pH 5.0 was sterilized in Erlenmeyer flasks in an autoclave. 0.1 mg/ml of synthetic 3-OH-DBP (total 10 mg/100 ml of the fermentation medium (FM)) was added to these flasks. These were incubated at 30ºC in a Biological oxygen demand incubator for 7 days and the products of biotransformation were analyzed.

In another set of fermentation experiments, a low concentration of 3-OH-DBP (0.001 mg/ml~0.1 mg / 100 ml of FM) was added to the FM. Cell growth and products formed were analyzed.

Cell growth in the fermentation media was determined by estimating the dry cell weight after the fermentation period of 7 days. The culture broth was filtered (Whatman 1) and the mycelial mat was added to pre-weighed aluminium foils and dried overnight at 100ºC. The difference in initial and final weights gave the dry weights of the mycelium. Experiments were performed in triplicates.

Isolation of transformed metabolites. The mycelia were separated from the FM after 7 days of fermentation. The cells were washed with distilled water and then dried in hot air oven and powdered by a homogenizer. Thereafter, the mycelia were extracted with ethyl acetate. The marc was again suspended in bligh and dyer solvent ($CHCl_3$- MeOH-H_2O, 1:2:0.8, v/v/v) and disintegrated by an ultrasonicator. The suspensions were centrifuged for 10 min at 5000 rpm. The organic layer was dried over anhydrous sodium sulphate, filtered and the solvent was removed under reduced pressure. The residue was dissolved in methanol. The chemical metabolites present in the methanolic extracts were characterized and detected initially by HPLC (Hawari et al. 1998). For further separation and for obtaining pure end-products and the residual substrate (if any), the extracts were subjected to HPFC. Each of the fractions thus obtained, were analyzed by HPLC for identification of the desired products and the residual substrate, by comparing with authentic standards. To determine the amounts of generated products and the residual substrate the peak areas obtained from the HPLC chromatogram were plotted in standard curves of these products. For reconfirmation of the end-products and the residual substrate, the pure HPFC fractions were further analyzed by GC-MS as trimethylsilyl (TMS) derivatives.

The FM after 7 days of fermentation was separately extracted with ethyl acetate and the extract was dried over anhydrous sodium sulphate. The solvent was removed and the residue was reconstituted in methanol for HPLC, HPFC and GC-MS analyses as before.

To detect the presence of amino acids, the end-products were subjected to acid hydrolysis with 0.2 (N) HCl, and the hydrolysate was subjected to TLC followed by spraying with ninhydrin reagent for detection and identification of amino-acids.

All operations were performed in triplicates.

Isolation, characterization and quantitation of obtained products. HPLC was carried out in a WATERS HPLC system with PDA detector and isocratic mobile phase consisting of acetonitrile- *ortho*-phosphoric acid- water (32:1:67) with a flow rate of 0.6 ml/min using a C_{18} Novapak reverse-phase column attached with a guard column for separation. The injection volume was 20 µl in water. The photodiode array detector wavelength was set 240 nm.

The end products were further purified using HPFC Model SP1 (Biotage, Sweden), equipped with normal phase cartridge silica column (Flash 12+M). Mobile phase used was A) chloroform and B) methanol with a gradient run 0-100% B in 66 min. Flow rate was 5 ml/min. Collection and monitor wavelength was 240 and 305 nm respectively. All 55 fractions were collected and analyzed by HPLC for search of pure compounds.

Quantitative determination of the end-products and unreacted 3-OH-DBP was done by comparing the area under the curve (AUC) values of the end-products with the AUC values obtained from standard curve of the components determined in the HPLC chromatogram. The regression equation of 3-OH- DBP is Y = 6470.60X + 11379 (R^2 = 0.9972) and of 3,8-$(OH)_2$-DBP is Y = 5281X + 2958.6 (R^2 = 0.9998).

GC-MS was carried out on a Varian GC-MS, Model: Saturn 2200, GC 3800; equipped with a VF-5 ms column (5% phenyl)- methyl polysiloxane (30 m x 0.25 mm i.d.). Carrier gas used was ultra pure helium with constant flow rate: 1.2 ml / min. The GC oven temperature was programmed as follows: first step: initial temperature was 75ºC and hold time for 3 min; second step: final temperature was 130ºC with an increment of 10ºC/min and hold time for 1 min; third step: final temperature was 200ºC with an increment of 12ºC/min and hold time for 3 min; fourth step: final temperature was 260ºC with an increment of 15ºC/min and hold time for 5 min; fifth step: final temperature was 280ºC with an increment of 20ºC/min and hold time for 5 min. The injection port (No. 1079) temperature was kept at 260ºC. The samples were injected using split ratio 1:20. The transfer line temperature was 260ºC and the injection volume was 0.5 µL. The conditions for Mass Spectrometer were as follows: Mass range was 50-650. Ionization potential: 70 eV. Emission current: 10 micro amps. Ion trap temperature: 180ºC. Manifold temperature: 40ºC. Background mass: 45 *m/z*. RF dump value: 650 *m/z*.

The samples were derivatized by reaction with *N, O*-bis (Trimehylysislyl)-triflouro-acetamide at 70ºC for 30 min to form TMS derivatives (Yu et al. 1998; Nam et al. 2006).

All the analytical data of GC-MS analysis were based on Varian MS workstation software.

RESULTS AND DISCUSSION

The HPLC chromatogram of the extractives of the mycelia and FM (when pooled together, since their analytical profiles were similar) showed the presence of polar metabolites which were identified as 3,8-$(OH)_2$-DBP (t_R 4.684 min; λ_{max} 219.1, 237.9, 280.4, 305.4, 355.4 nm) and its 3-*O*-aminoacylconjugates (t_R 3.53 min; λ_{max} 205, 245.5, 290.5, 335 nm, t_R at 3.81 min λ_{max} at 221.5, 255.8, 293.0 nm) and also some unreacted 3-OH-DBP (t_R 11.41 min; λ_{max} 209.7, 275.7, 300.6, 336.3 nm). The PDA spectrum of the product 3,8-$(OH)_2$-DBP was also identical with that of standard 3,8-$(OH)_2$-DBP. The products in the t_R regions 3.5 and 3.8 min, were the 3-*O*-aminoacylconjugates of 3,8-$(OH)_2$-DBP. On subsequent acid hydrolysis of these products, two amino acids viz. glycine and arginine were detected. The unreacted 3-OH-DBP which was detected in the extractives after biotransformation had a spectrum indistinguishable from that of standard 3-OH-DBP.

When the organic solvent extractives of mycelia, after biotransformation, were separated by HPFC (Figure 1) and further analyzed by HPLC (Figure 2 and Figure 3), fractions 6-7 of HPFC were identified to be pure 3-OH-DBP (Figure 2) and fractions 18-19 and 23-24 of HPFC when pooled together were identified to be pure 3,8-$(OH)_2$-DBP (t_R: 4.37 min) and its aminoacyl conjugates (t_R: 3.53 min) (Figure 3). For determination of the amounts of the products, the total areas obtained from the HPLCs were plotted in the standard curves of these compounds.

The amount of unreacted 3-OH-DBP present in the mycelial extract and FM was determined to be 3.6 mg. The amounts of 3,8-$(OH)_2$-DBP and its aminoacyl conjugates were determined to be 4.9 mg and 0.85 mg respectively. Thus, the percentage of conversion of 3-OH-DBP to its derivatives, viz. 3,8-$(OH)_2$-DBP and its 3-*O*-aminoacylconjugates, was observed to be about 60% after following the abovementioned optimized biotransformation conditions and using 0.1 mg/ml of 3-OH-DBP as the substrate in each unit operation.

No metabolite of DBP was detected in the blank culture control after 7 days of incubation, which proves that *Aspergillus niger* by itself does not have the ability to synthesize DBP.

To confirm the structures of the above mentioned compounds, the end- products were analyzed by GC-MS using corresponding compounds as synthetic markers (Figure 4, Figure 5 and Figure 6). The gas chromatogram of the reaction products showed residual 3-OH-DBP (1), as its silyl derivative, at t_R 15.860 min with *m/z* values of 284(M+) and fragment ion peaks at 269$(M\text{-}CH_3)^+$, 241$[M\text{-}(CH_3\text{+}CO)]^+$ and the desired 3,8-$(OH)_2$-DBP, as its silyl derivative (2), at t_R 19.615 with *m/z* values of 372(M+) and fragment ion peaks at 357$(M\text{-}CH_3)^+$, 329$[M\text{-}(CH_3\text{+}CO)]^+$.

3-*O*-Glycyl-8-hydroxydibenzo-α-pyrone (HPLC:t_R 3.8 min) (6), as TMS derivative, was subjected to EI-MS analysis. The mass spectrum showed molecular-ion (M+) peak at *m/z* 429 and fragment ion peaks at *m/z* 299 (due to 3-*O*-8-*O*-trimethylsilyl dibenzo-α-pyrone moiety) and at *m/z* 130 (trimethylsilyl glycyl moiety). On mild acid hydrolysis (dil. HCl), the aminoacyl dibenzo-α-pyrone produced 3,8-$(OH)_2$-DBP and glycine (co-TLC, HPTLC using authentic markers).

3-*O*-Arginyl-8- hydroxydibenzo-α-pyrone (HPLC: t_R 3.35 min) as TMS derivative, was subjected to EI-MS analysis.

The compound fragmented before exhibiting any molecular ion peak (M+). The fragment-ions showed the presence of 3-*O*-8-*O* trimethylsilyl dibenzo-α-pyrone, *m/z* 299. On mild acidic hydrolysis the aminoacyl compound produced arginine and 3,8-$(OH)_2$-DBP. The identities of these products were established by (co-TLC, HPTLC) using markers (Ghosal, 2006).

On analyses of the dry weight of the mycelium after 7 days of fermentation with 3-OH-DBP (0.1 mg/ml) as the substrate, it was observed that the dry cell weight was less than that of the blank culture control. 3-OH-DBP in this concentration seems to thwart the growth of the microorganism. This postulate finds support from the fact that the microorganisms found in the "shilajit" rhizosphere are present only at the periphery and not in the core of the "shilajit" exudates (Ghosal, 2006). However, in fermentation experiments with low DBP concentration (0.001 mg/ml), the cell growth was even higher than that of the blank operation. At low concentration, "shilajit" conceivably acts as a nutrient to microorganisms and, as expected, in fermentation experiments with this low concentration of DBP, the yield of the desired end-product was poor.

Most of the filamentous fungi possess machinery for biotransformation (metabolism) of substances toxic to their physiology (Makovec and Breskvar, 2002). It was shown in earlier reports, that the enzymatic mechanisms involved in biotransformation by fungi include oxidation (Lacki and Duvnjak, 1998; Santos et al. 2003), reduction or hydrolysis; formation of conjugates with products of intermediary metabolism such as acetic acid, cysteine, glucuronic acid, glycine and ornithine (Carey et al. 2006). These mechanisms are two-phased, Phase I - metabolism (*e.g.* cytochrome-P-450 reductase and epoxide hydrolase), which primarily act by hydroxylation (Uzura et al. 2001), and Phase II - conjugation (*e.g.* Glutathione-S transferase, UDP-glucuronosyl transferase and UDP glycosyl transferase), which form conjugates with compounds like glucuronic acid, glutathione and amino acids (Bezalel et al. 1997).

Based on these observations, a probable mechanism of biotransformation of 3-OH-DBP by *Aspergillus niger* into 3,8$(OH)_2$DBP and 3-*O*-glycyl-8-hydroxy- DBP is envisaged (Figure 7). In the first phase of the reaction, *Aspergillus niger* generates hydroxyl radicals which attack the hydroxyl group at C-3 position of 3-OH-DBP (1) and causes abstraction of $H^{\cdot}$ leading to the formation of a semiquinone radical (2) which is resonance stabilized with 3(2↔3). Attack by ·OH radical, generated by *Aspergillus niger*, at the C-8 site of DBP forms (4). Rearrangement of (4) leads to formation of 3,8 $(OH)_2$-DBP (5) which is then transformed by aminoacylation, *e.g.* with glycine, to produce3-*O*-glycyl-8- hydroxy- DBP (6) which is more water soluble. The nutrient when provided in homogenous phase would be readily bioavailable than when present in a heterogeneous form of mixture (solid-liquid).

CONCLUDING REMARKS

The optimized conversion of 3-OH-DBP into another highly bioactive form 3,8$(OH)_2$-DBP is a novel reaction catalyzed by the filamentous fungi *Aspergillus niger*. The DBP-converting factor is perhaps a metabolism related enzyme which by oxidation forms 3,8$(OH)_2$-DBP; which acts as a substrate for further enzymatic reactions like conjugation with amino acids to form aminoacyl conjugates and render these compounds more water-soluble and hence more bioavailable. In certain cases, these mechanisms are operative in the fungi not only to detoxify the toxic substance, but also to make them beneficial for the organism (Venisetty and Ciddi, 2003).

These DBPs are essential for the therapeutic activity of "shilajit". In summary, it can be stated that the mechanism by which dibenzo-α-pyrones are produced in "shilajit", can be simulated *in situ* using *Aspergillus niger*. In view of this, scaling up of the operation was also carried out when up to 1 g of substrate material produced desired results. Further scaling-up of this process is in progress.

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APPENDIX
FIGURES

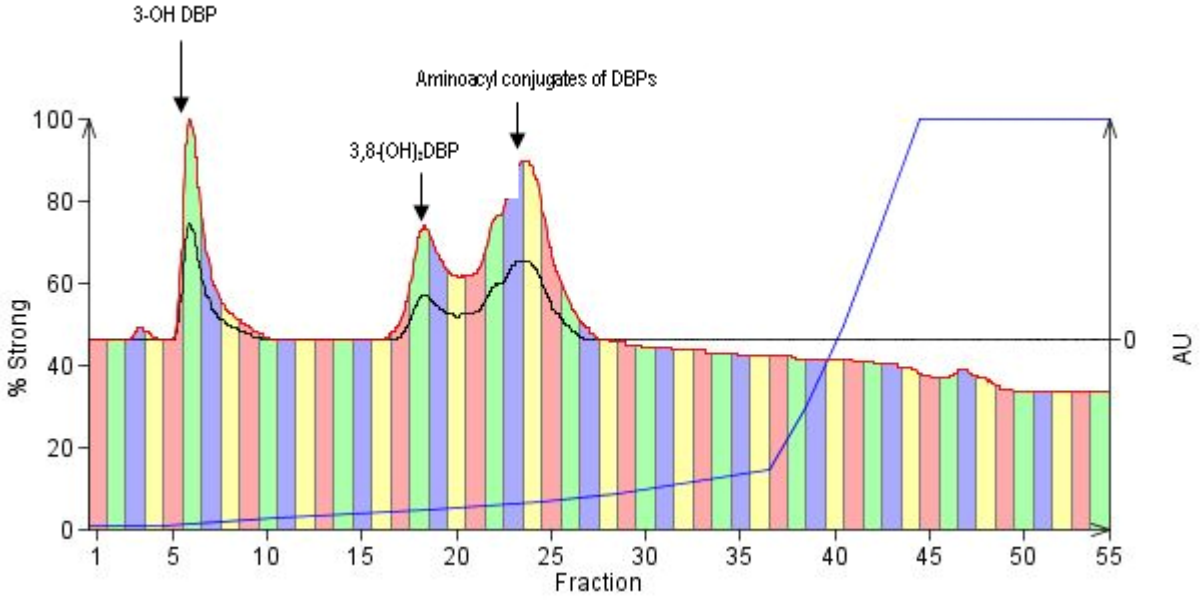

Figure 1. HPFC chromatogram of extractives of mycelium and FM (pooled together) after the biotransformation with *A. niger* showing areas of 3-OH-DBP, 3,8 $(OH)_2$-DBP and aminoacyl conjugates of 3,8$(OH)_2$-DBP as identified by HPLC.

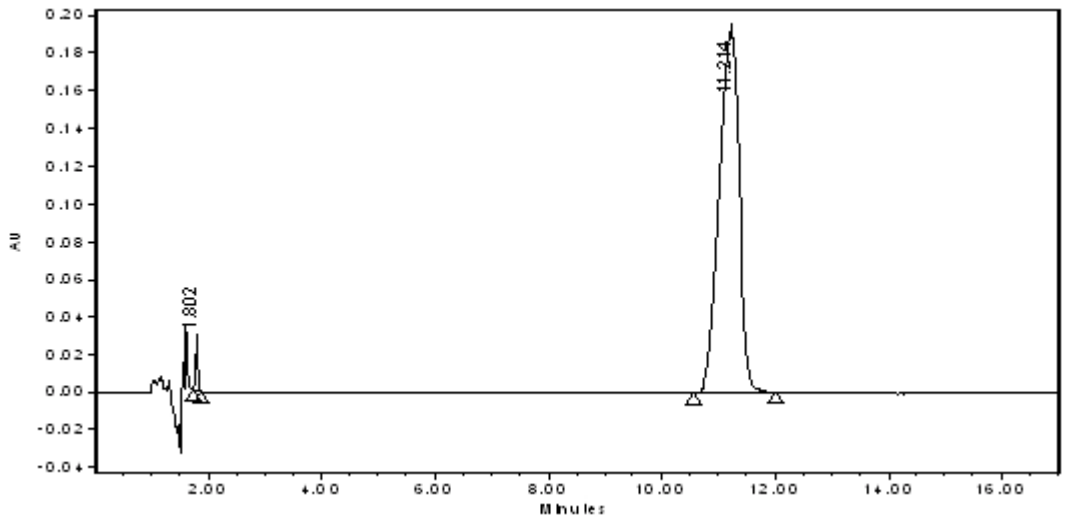

Figure 2. HPLC chromatogram of fraction 6-7 of HPFC showing pure unreacted 3-OH-DBP.

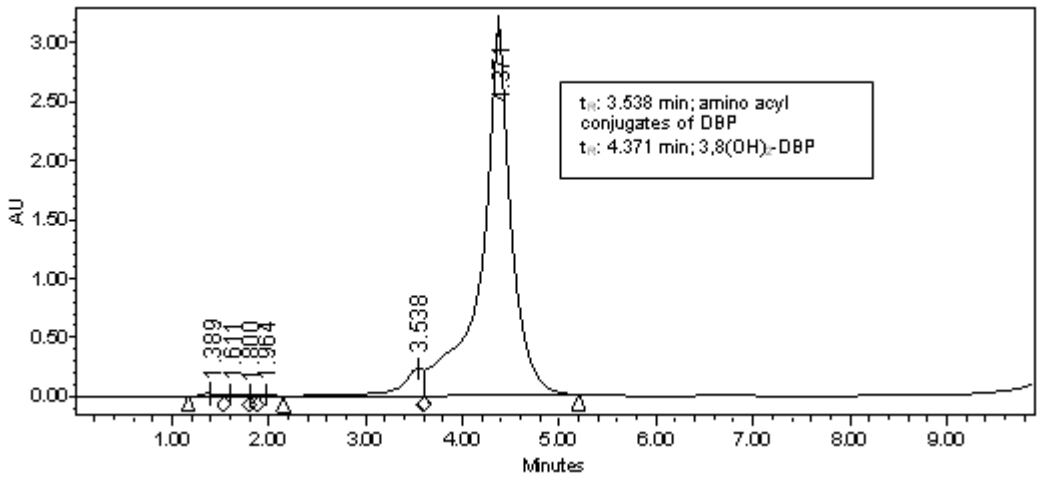

Figure 3. HPLC chromatogram of fractions 18-19 and 23-24 of HPFC showing 3,8(OH)$_2$-DBP and its aminoacyl conjugates.

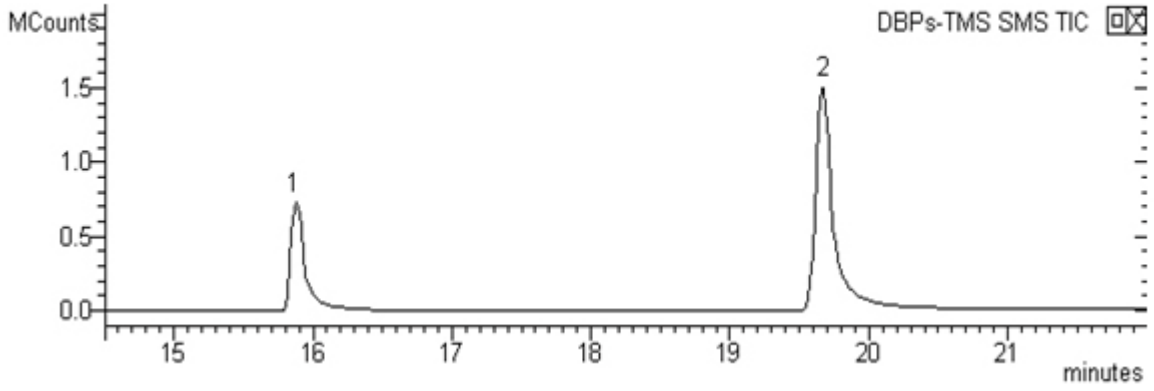

Figure 4. GC chromatogram of DBPs, analyzed as TMS derivatives; Peak- 1) 3-OH-DBP, t_R: 15.86 min, Peak- 2) 3,8(OH)$_2$-DBP, t_R: 19.615 min.

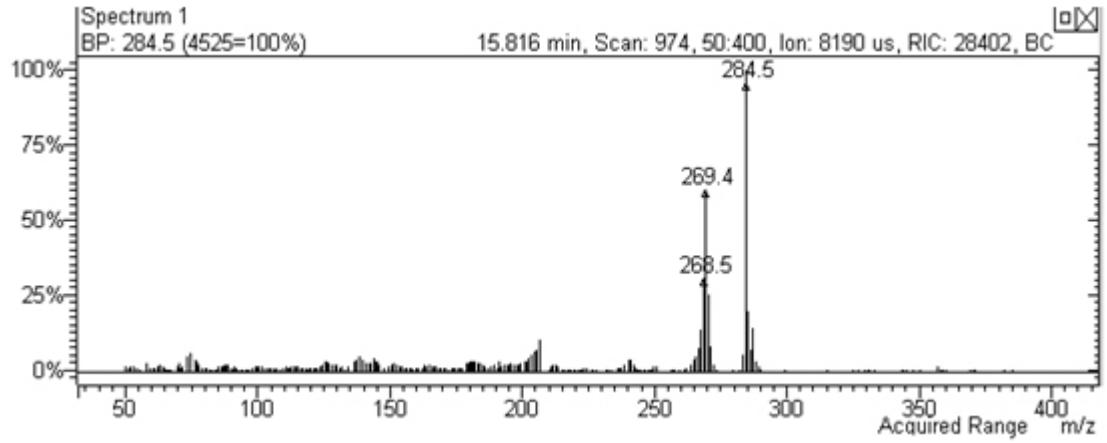

Figure 5. Mass spectrum of 3-OH-DBP.

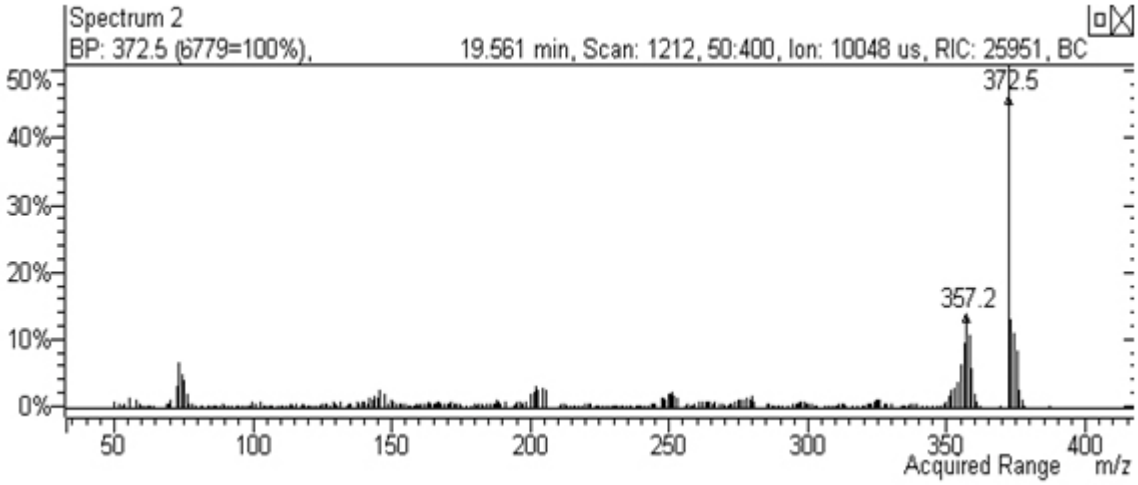

Figure 6. Mass spectrum of 3,8$(OH)_2$-DBP.

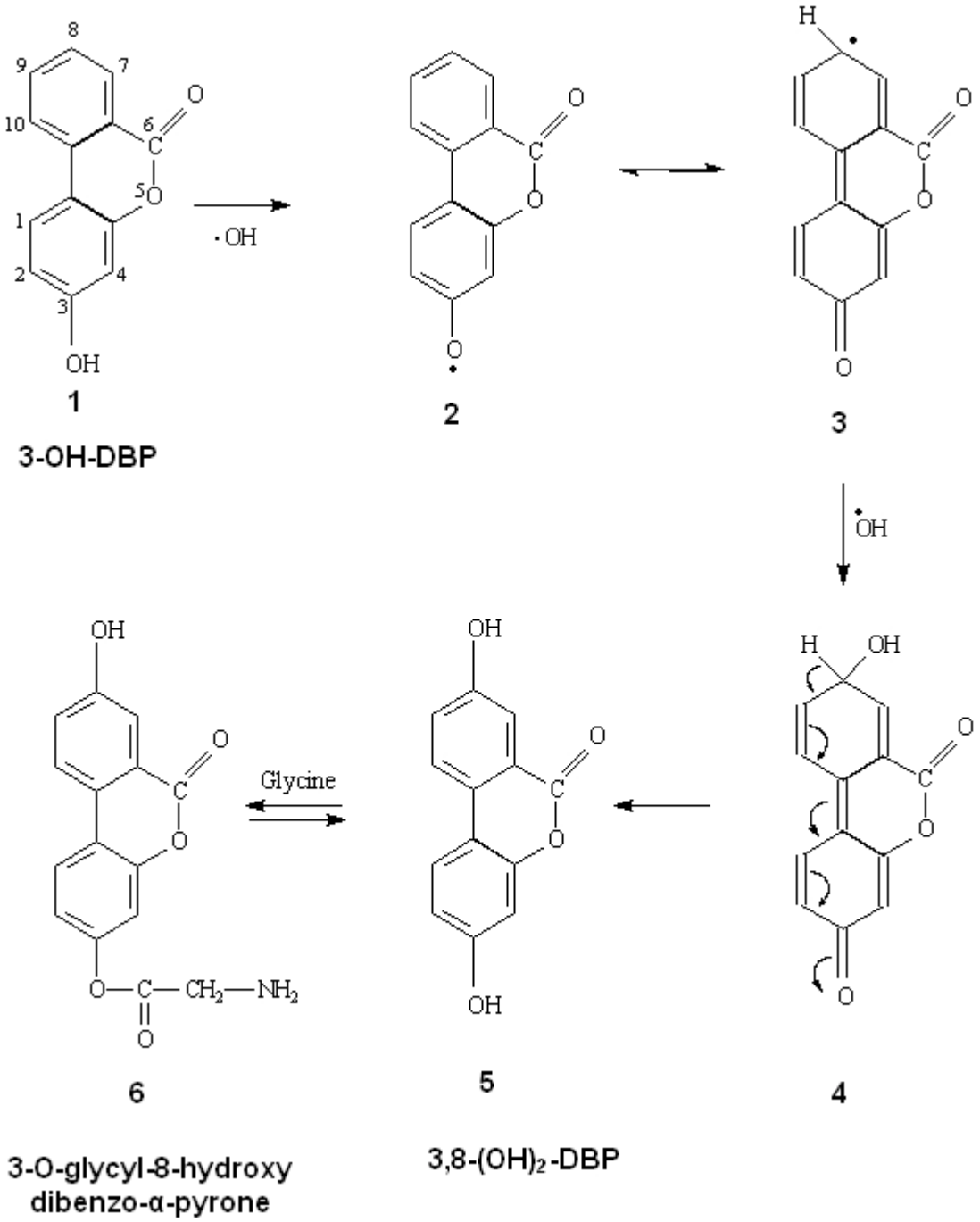

Figure 7. Proposed mechanism of biotransformation of 3-OH- DBP into 3,8-$(OH)_2$ DBP and its amino-acyl conjugate(s) by *Aspergillus niger*.