# Biotransformation of Physalin H and Leishmanicidal Activity of Its Transformed Products

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**The transformation of physalin H (1) with *Rhizopus stolonifer* and *Cunninghamella elegans* has afforded two new physalins, 6,7-dehydrophysalin H (2) and 6-deoxyphysalin H (3), along with a known isophysalin B (4). Their structures were elucidated by spectroscopic analysis. All of these compounds have shown potent leishmanicidal activity with $IC_{50}$ values in the range of 6.03—13.80 $\mu$M.**

**Key words** microbial transformation; physalin; *Rhizopus stolonifer*; *Cunninghamella elegans*; antileishmanial

Biocatalysis play an important role in the organic synthesis. Biotransformation with whole cell cultures is extensively employed as an industrial method to synthesize many steroidal drugs, chiral building blocks, and for modifications in natural products with potent biological activities. Physalin H (**1**) is a steroidal lactone, isolated from *Physalis angulata* (Solanaceae).[1,2] Previous studies have shown that the steroidal lactones (withanolides and physalins) exhibit a range of biological activities, including anti-tumor,[3] immunomodulatory,[4] antimycobacterial,[5] anti-fungal, anti-inflammatory,[6,7] insect repellant,[8] and immunostimulating properties.[9] Compound **1** has also shown moderate cytotoxic activity against HeLa cells.[2]

During recent years, our research group has focused efforts on the structural modifications of bioactive natural products by using various microorganisms, in order to obtain biologically potent compounds with diverse structures.[10—16]

Recently we have investigated the metabolism of physalin H (**1**), by *Rhizopus stolonifer*, and *Cunninghamella elegans*, targeting the leishmanicidal activity of transformed metabolites. Leishmniasis is a parasitic disease caused by protozoa parasites of genus *Leishmania*. Genus *Leishmania* is comprises on biologically diverse group of flagellate parasites of the Tryanosomatidae family which can be differentiated by genetic, biochemical, and immunological studies. Leishmaniasis is distributed in the old world around the Mediterranean Sea, in East and West Africa, Afghanistan, Pakistan, India, Nepal, Bangladesh, and in China. In the new world, this disease is found in the southern part of the United States to the northern parts of Argentina and Paraguay. The domestic and wild animals are the main reservoirs of *Leishmania* parasites, while the female flying insects of the genera *Phlebotomus* and *Lutzomya* are the vectors of Leishmaniasis. The secondary metabolites of plants, such as alkaloids, quinine, and terpenes have been used to cure protozoan parasitic diseases. The use of quinine and emetine are best examples of the natural products used for the treatment of parasitic diseases such as malaria and amoebiasis.[17]

## Results and Discussion

Incubation of physalin H (**1**), $C_{28}H_{31}ClO_{10}$, with *Rhizopus stolonifer* yielded a new dehydrated metabolite **2**. The HR-EI-MS of metabolite **2** exhibited the molecular ion ($M^+$) at *m/z* 544.9762, corresponding to the formula $C_{28}H_{29}ClO_9$ (Calcd 544.9771). This indicated that a molecule of water was eliminated from the substrate during the fermentation. The UV spectrum showed an absorption maximum of 226 nm, suggesting the presence of an $\alpha,\beta$-unsaturated ketone moiety.[18] The IR spectrum showed absorptions for hydroxyl (3436 $cm^{-1}$), five-membered lactone (1775 $cm^{-1}$), and $\alpha,\beta$-unsaturated ketone (1668 $cm^{-1}$) functionalities.[18—20] The comparison of the $^1$H-NMR spectra (Table 1) of **2** with the substrate **1**, showed a new pair of resonances appeared at $\delta$ 5.69 (d, $J_{6,7}$=9.4 Hz) and 5.75 (br s), along with their corresponding carbons resonating at $\delta$ 125.7 and 127.7, respectively in the $^{13}$C-NMR spectrum (Table 2). These signals were assigned to the C-6/C-7 olefinic moiety. This assignment was based on the HMBC correlations of H-6 ($\delta$ 5.69) with C-5 ($\delta$ 80.4) and C-7 ($\delta$ 127.7), and correlations of H-7 ($\delta$ 5.75) with C-6 ($\delta$ 125.7) and C-8 ($\delta$ 37.8) (Fig. 1). In the COSY-45° spectrum, the aforementioned C-7 methine proton ($\delta$ 5.75) showed correlations with the C-6 ($\delta$ 5.69) and C-8 ($\delta$ 2.12) methine protons. The above spectral data indicated that the new metabolite **2** has a structure of 6,7-dehydrophysalin H.

The incubation of **1** (200 mg) with the *Cunninghamella elegans* for twelve days yielded a new metabolite **3** and a known compound **4**. The HR-EI-MS of compound **3** showed an [$M^+$] at *m/z* 546.9910, in accordance with the formula $C_{28}H_{31}ClO_9$ (Calcd 546.9930). The UV absorption appeared at 220 nm, indicated the presence of an $\alpha,\beta$-unsaturated ketone functionality in the molecule which was further supported by a strong IR absorption at 1668 $cm^{-1}$. The IR spectrum also showed absorptions for hydroxyl (3434 $cm^{-1}$), and five-membered lactone (1779 $cm^{-1}$) functionalities. Comparison of the $^1$H-NMR data (Table 1) of **3** with the physalin H (**1**) indicated the disappearance of the signal for the hydroxy-substituted C-6 methine proton ($\delta$ 3.82). The deoxygenation of C-6 was further supported by the appearance of a methylene carbon signal at $\delta$ 42.2 in the $^{13}$C-NMR spectrum (Table 2). The long-range $^1$H/$^{13}$C interactions of H-6 with C-5 ($\delta$ 78.4) and C-7 ($\delta$ 29.6) in the HMBC spectrum, further supported the deoxygenation at C-6 (Fig. 2). On the basis of the above spectroscopic data, the structure of metabolite **3** was deduced to be 6-deoxyphysalin H. The stereochemical assignments in compounds **2** and **3** based on spectral comparison with the substrate **1**. The X-ray structure of compound **1** was reported by us.[20]

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Table 1. $^1$H-NMR (400 MHz) Spectral Data of Compounds **1**—**4**

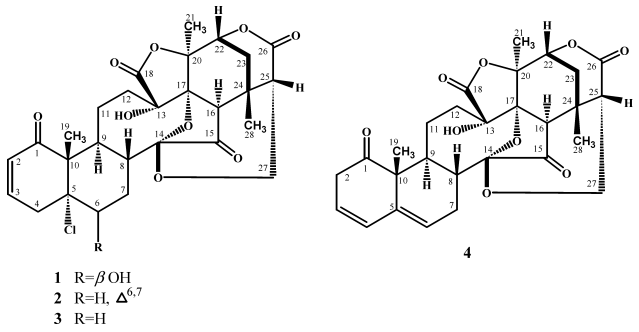
| H. No. | **1**[a] $\delta$ ($J$=Hz) | **2**[b] $\delta$ ($J$=Hz) | **3**[b] $\delta$ ($J$=Hz) | **4**[b] $\delta$ ($J$=Hz) |
|---|---|---|---|---|
| 2 | 5.62 (dd, $J_{2,3}$=10.1, $J_{2,4\beta}$=2.6) | 5.62 (dd, $J_{2,3}$=10.1, $J_{2,4\beta}$=2.6) | 5.98 (dd, $J_{2,3}$=10.1, $J_{2,4\beta}$=2.2) | 2.79 (m) |
| 3 | 6.52 (ddd, $J_{3,2}$=10.1, $J_{3,4a}$=4.9, $J_{3,4\beta}$=2.5) | 6.66 (ddd, $J_{3,2}$=10.2, $J_{3,4a}$=4.8, $J_{3,4\beta}$=2.5) | 6.84 (ddd, $J_{3,2}$=10.0, $J_{3,4a}$=5.2, $J_{3,4\beta}$=2.1) | 5.55 (m) |
| 4 | 2.42 (m) | 2.52 (m) | 2.15 (m) | 6.0 (dd, $J_{4,3}$=10.5, $J_{4,2\beta}$=2.6) |
| 6 | 3.82 (m) | 5.69 (d, $J_{6,7}$=9.4) | 2.82 (dd, $J_{6,7\beta}$=13.8, $J_{6,7\beta}$=2.9) | 5.66 (d, $J_{6,7\beta}$=3.4) |
| 7 | 2.28 (m) | 5.75 br s | 1.25 (m) | 2.45 (m) |
| 8 | 2.32 (d, $J_{8,9}$=8.3) | 2.12 (m) | 2.22 (m) | 2.22 (m) |
| 9 | 2.92 (m) | 2.89 (m) | 3.22 (m) | 2.99 (m) |
| 11 | 2.12 (m) | 1.29 (m) | 2.59 (m) | 1.28 (m) |
| 12 | 1.48 (m) | 1.50 m | 2.12 (m) | 1.58 (m) |
| 16 | 3.21 (s) | 2.20 (s) | 3.21 (s) | 2.20 (s) |
| 19 | 1.26 (s) | 1.23 (s) | 1.28 (s) | 1.22 (s) |
| 21 | 1.82 (s) | 1.86 (s) | 1.92 (s) | 1.96 (s) |
| 22 | 4.35 (t, $J_{22,23}$=2.3) | 4.38 (t, $J_{22,23}$=3.5) | 4.55 (d, $J_{22,23}$=3.6) | 4.50 (d, $J_{22\alpha,23\alpha}$=4.6) |
| 23 | 2.42 (m) | 2.0 (m) | 2.10 (m) | 2.22, m |
| 25 | 2.58 (m) | 2.41 (m) | 2.44 (m) | 2.43, m |
| 27 | 3.75 (d, $J$=14.5) | 3.73 (d, $J$=13.5) | 3.72 (d, $J$=3.6) | 3.77 (d, $J_{27,27}$=4.5) |
| | 4.33 (dd, $J$=14.9, $J_{27,25}$=4.4) | 4.48 (dd, $J$=13.5, $J_{27,25}$=4.5) | 4.48 (dd, $J$=13.4, $J_{27,25}$=4.5) | 4.55 (dd, $J_{27,27}$=14.5, $J_{27,25}$=4.6) |
| 28 | 1.12 (s) | 1.02 (s) | 1.23 (s) | 1.30 (s) |

All assignments are based on COSY and HMQC, *a*) In $CD_3OD$, *b*) In $CDCl_3$.

Table 2. $^{13}$C-NMR (100 MHz)[a] Spectral Data of Compounds **1**—**4**

| C-Atoms | **1**[a] ($\delta$) | **2**[b] ($\delta$) | **3**[b] ($\delta$) | **4**[b] ($\delta$) |
|---|---|---|---|---|
| 1 | 204.1 | 203.4 | 207.4 | 208.2 |
| 2 | 128.6 | 130.2 | 127.7 | 39.5 |
| 3 | 144.4 | 141.2 | 146.3 | 121.2 |
| 4 | 38.3 | 36.8 | 36.6 | 126.5 |
| 5 | 81.5 | 80.4 | 78.4 | 139.6 |
| 6 | 74.4 | 125.7 | 42.2 | 128.8 |
| 7 | 37.2 | 127.7 | 29.6 | 25.9 |
| 8 | 39.9 | 37.8 | 37.3 | 39.2 |
| 9 | 32.4 | 31.0 | 34.2 | 32.0 |
| 10 | 56.1 | 54.0 | 50.0 | 55.1 |
| 11 | 27.7 | 27.0 | 24.9 | 24.6 |
| 12 | 25.9 | 25.8 | 23.5 | 25.3 |
| 13 | 78.0 | 78.4 | 80.1 | 79.9 |
| 14 | 108.4 | 106.6 | 107.0 | 107.6 |
| 15 | 210.2 | 209.8 | 210.2 | 213.9 |
| 16 | 56.4 | 53.8 | 56.2 | 56.3 |
| 17 | 80.6 | 80.6 | 80.0 | 81.0 |
| 18 | 170.0 | 171.8 | 170.1 | 172.1 |
| 19 | 22.0 | 21.6 | 21.5 | 26.5 |
| 20 | 82.1 | 81.7 | 80.3 | 80.3 |
| 21 | 25.9 | 26.7 | 26.5 | 21.4 |
| 22 | 78.7 | 76.3 | 76.8 | 76.9 |
| 23 | 33.2 | 31.2 | 33.0 | 33.0 |
| 24 | 31.9 | 30.4 | 31.0 | 31.2 |
| 25 | 51.4 | 53.8 | 50.8 | 50.9 |
| 26 | 174.0 | 167.2 | 166.9 | 166.5 |
| 27 | 61.7 | 60.4 | 60.6 | 60.7 |
| 28 | 15.0 | 13.6 | 19.2 | 19.2 |

All assignments are based on HMQC and HMBC, *a*) In $CD_3OD$, *b*) In $CDCl_3$.



**1** R=$\beta$OH
**2** R=H, $\Delta^{6,7}$
**3** R=H

**4**

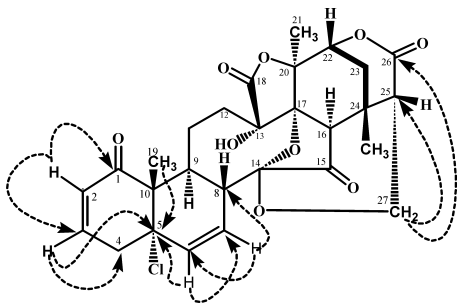


Fig. 1. Key HMBC Interactions of **2**

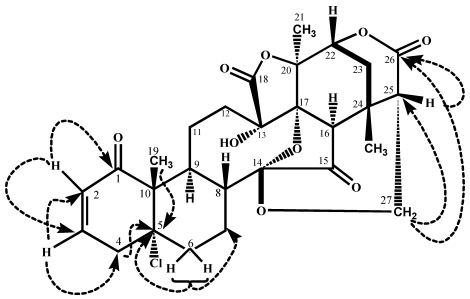


Fig. 2. Key HMBC Interactions of **3**

The HR-EI-MS of compound **4** showed an [$M^+$] at $m/z$ 510.5319, in agreement with the formula $C_{28}H_{30}O_9$ (Calcd 510.5324). The UV absorption appeared at 227 nm. The IR spectrum showed absorptions at 3415 and 1769 $cm^{-1}$ for hydroxyl and five-membered lactone ring, respectively. The spectral data of metabolite **4** was identical to the data reported for isophysalin B, which was previously reported from *P. alkekengi*.[21]

Compounds **1**—**4** were screened for their anti-leishmanial activity in an *in vitro* assay. Compounds **1**—**4** were found to have potent leishmanicidal activities. Amphotericin B ($IC_{50}$ 0.129 $\mu$M) was used as a standard drug. Physalin H (**1**) was found to be the most active in the series with an $IC_{50}$ value of

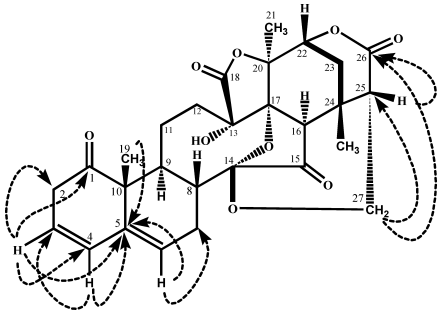


Fig. 3. Key HMBC Interactions of **4**

Table 3. *In Vitro* Anti-leishmanial Activities of Compounds **1**—**4**

| Compounds | $IC_{50}$ ($\mu$M)±S.D.[a)] |
|---|---|
| **1** | 6.03±0.005 |
| **2** | 7.74±0.015 |
| **3** | 6.34±0.03 |
| **4** | 13.8±0.05 |
| Amphotericin B[b)] | 0.129±0.105 |

*a*) Standard deviations. *b*) Standard drug.

6.03 $\mu$M (Table 3). Compound **4** was appeared least active in the series.

## Experimental

**Experimental Procedures** The UV spectra were measured on a Hitachi U-3200 spectrophotometer. The IR spectra were recorded on a FT-IR-8900 spectrophotometer. Optical rotations were measured on a Schmidt+Haensch Polartronic D polarimeter. The $^1$H-NMR spectra were recorded on Bruker Avance-400 and 500 NMR spectrometers, while the $^{13}$C-NMR spectra were recorded at 100 and 125 MHz on the same instruments, using $CDCl_3$ and $CD_3OD$ as solvents. The HR-EI-MS [ion source energy 70 eV, ion source temperature 250 °C] were recorded on Jeol JMS 600 and HX 110 mass spectrometers with the data system DA 5000. Column chromatography (CC) was performed on the silica gel (70—230 mesh size).

**Fungus and Culture Conditions** Fungal cultures of the *Rhizopus stolonifer* (TSY-0471), and *Cunninghamella elegans* (TSY 0865) were grown on Sabouraud-4% glucose-agar (Merck) at 25 °C and stored at 4 °C. Media was prepared for *Rhizopus stolonifer* by adding glucose (100 g), peptone (25 g), $KH_2PO_4$ (25 g), and yeast extract (15 g) into distilled water (5 l) and pH was maintained at 5.6. The media for *Cunninghamella elegans* were prepared by mixing the following ingredients into distilled $H_2O$ (2.5 l): glucose (25.0 g), glycerol (25 ml), peptone (12.5 g), yeast extract (12.5 g), $KH_2PO_4$ (5 g), and NaCl (5.0 g).

**General Fermentation and Extraction Conditions** The fungal media was transferred into conical flasks (100 ml each) and autoclaved at 121 °C. Seed flasks were prepared from 3-d old slant and fermentation was allowed for 2 d on a shaker at 25 °C. The remaining flasks were inoculated from seed flasks. After 2 d, physalin H (**1**) was dissolved in acetone and transferred in each flask and flasks were placed on a shaker (128 rpm) at 22 °C for fermentation period. The time course study was carried out by harvesting one flask with the interval of 2 d and the content was analyzed by TLC. The culture media were filtrated and mycelium was washed with EtOAc and the filtrate was extracted with EtOAc. A negative control containing fungi, and positive control containing compound **1**, were also prepared in order to check the chemical changes due to fungal metabolites and media. The organic extract was dried over anhydrous $Na_2SO_4$, evaporated under the reduced pressures, and the resulting brown gummy material was seperated by column chromatography on silica gel (70—230 mesh size) by using increasing polarities of pet. ether and EtOAc.

**Fermentation of Physalin H (1) with *Rhizopus stolonifer* (TSY-0471)** Physalin H (**1**) (400 mg) was dissolved in 50 ml acetone and finally distributed among 50 flasks (8 mg/ml) for fermentation. The fungal media after 12 d incubation, along with control flasks, were filtered. The filtrate were extracted with ethyl acetate and evaporated to afford a brown gummy material (approx. 0.96 g) and by column chromatography, the transformed metabolites were isolated from this gummy crude. Metabolite **2** (8.5 mg) was eluted with the pet. ether and EtOAc (50 : 50).

6,7-Dehydrophysalin H (**2**): Yellowish gum (8.5 mg, 2.12% yield); $[\alpha]_D^{25}=-26°$ ($c$=1.8, MeOH); UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$)=226 (2.56); IR ($CHCl_3$) cm$^{-1}$=3436, 1775, 1668. EI-MS $m/z$ (rel. int. %): 544 (7.7, $M^+$), 526 (24.6), 508 (67.2), 482 (83.3), 454 (42.5), 375 (33.9), 362 (25.2), 322 (31.5), 221 (17), 91 (69), 79 (42.5), 67 (35.4), 55 (75.8). HR-EI-MS: $m/z$ 544.9762 ($C_{28}H_{29}ClO_9$, Calcd 544.9771). $^1$H-NMR: see Table 1. $^{13}$C-NMR: see Table 2.

**Fermentation of Physalin H (1) with *Cunninghamella elegans* (TSY 0865)** Compound **1** (200 mg) was dissolved in 25 ml acetone and finally distributed among 25 flasks (8 mg/ml). Fermentation was continued upto 12 d and then filtrate was extracted with EtOAc and evaporated to afford a brown gummy crude material (0.65 g). Column chromatographic techniques were used for the separation of metabolites **3** and **4** from *Cunninghamella elegans* crude extract. Compound **3** (8.2 mg) was eluted from the column (silica gel) with pet. ether and EtOAc (40 : 60), while the compound **4** (10.5) was eluted with the pet. ether and EtOAc (38 : 62).

6-Deoxyphysalin H (**3**): White amorphous powder (8.2 mg, 4.1% yield); $[\alpha]_D^{25}=-111°$ ($c$=3.8, MeOH); UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$) 220 (2.56); IR ($CHCl_3$) cm$^{-1}$; 3434, 1779, 1668; EI-MS $m/z$ (rel. int. %) 546 (5.7, $M^+$), 528 (25.6), 500 (15.2), 482 (83.3), 454 (42.5), 91 (69), 79 (42.5), 67 (35.4), 55 (75.8). HR-EI-MS: $m/z$ 546.9910, ($C_{28}H_{31}ClO_9$, Calcd 546.9930). $^1$H-NMR: see Table 1; $^{13}$C-NMR: see Table 2.

Isophysalin B (**4**): White amorphous powder (10.5 mg, 5.25% yield); $[\alpha]_D^{25}=-85°$ ($c$=3.5, $CH_2Cl_2$). UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$) 227 (2.8); IR ($CHCl_3$) cm$^{-1}$: 3415, 1769; EI-MS $m/z$ (rel. int. %): 510 (69.2, $M^+$), 492 (90.3), 464 (88.7), 171 (47.2), 131 (80.2) 105 (44.5), 131 (80.2), 105 (44.5), 91 (76.5), 67 (44.2); HR-EI-MS $m/z$ 510.5319 ($C_{28}H_{30}O_9$, Calcd 510.5324). $^1$H-NMR see Table 1; $^{13}$C-NMR see Table 2.

**Leishmanicidal Activity (*in Vitro*)** *Leishmania major* (DESTO) promastigotes were grown at 22—25 °C in RPMI-1640 medium (Sigma) containing 10% heat-inactivated (56 °C for 30 min) foetal bovin serum. Promastigote culture in the logarithmic phase of growth were maintained and the final concentration of parasites were adjusted to $2\times10^6$ parasites/ml. The test compound (1 mg) was dissolved in 50 $\mu$l DMSO. Then the volume was adjusted to 1 ml with complete media. 20 $\mu$l of test compound dilution was added in first well, which contained 180 $\mu$l of media, then serially it was diluted. A total of 100 $\mu$l of parasite suspension was added into each well of the 96-well plates and incubated at 21—22 °C for 72 h, in the presence and absence of amphotericin B (as a positive and negative control, respectively). The experiments were carried out in duplicates and the numbers of surviving parasites were counted in Neubauer chamber. The 50% inhibitory concentrations ($IC_{50}$) were determined by a windows based EZ-Fit 5 Perrella scientific software.

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