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Whole-Cell Mediated 11β-Hydroxylation on the Basic Limonoid Skeleton by *Cunninghamella echinulata*

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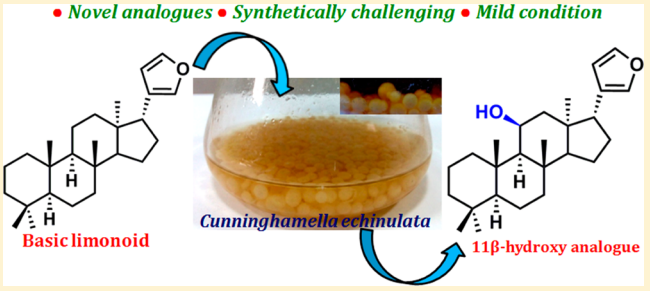
Supporting Information

ABSTRACT: Regio- and stereoselective 11β-hydroxylation was achieved on the basic limonoid skeleton through microbial transformation. Whole cells of *Cunninghamella echinulata* efficiently converted basic limonoids such as epoxyazadiradione, azadiradione, and gedunin to their 11β-hydroxy analogues as the sole metabolite. Fermentation conditions affecting the efficiency (96%) of biotransformation including substrate concentration, incubation period, pH, and temperature were optimized. The position and stereochemistry of hydroxyl functionality on the isolated metabolites were established through extensive spectroscopic and spectrometric studies (1D, 2D NMR, ESI-MS, and MS/MS).



Basic limonoids, a major class of tetranortriterpenoids, possess a 4,4,8-trimethyl-17-furanylsteroidal skeleton and are well-known for insecticidal and diverse pharmacological activity (Figure 1).[1−3] They are also named as ring-intact limonoids due to the presence of intact A, B, C, and D rings which upon further rearrangements lead to the formation of highly oxygenated and structurally complex *seco*-limonoids.[1,4] The abundance of basic limonoids is mainly restricted to the plants of families Meliaceae and Rutaceae.[1,5] Epoxyazadiradione (**1**), azadiradione (**2**), and gedunin (**3**), three representative members of the basic limonoid family, have been reported to possess numerous bioactivities such as anti-feedant,[2] anti-carcinogenic,[6,7] anti-inflammatory,[8,9] antimalarial,[10,11] and anti-ulcerogenic.[12] All of them share common structural features in A, B, and C-rings and the variability lies only within the D-ring (Figure 1). The structures of basic limonoids are featured by conformational rigidity of the tetracyclic framework, steric hindrance created by skeletal substitutions (Me-18, 19, 28, 29, 30, C-17 furan, and C-7 acetate) and the presence of inactive methylene carbons. As a result, conventional synthetic methods are sometimes ineffective or time-consuming to achieve the semisyntheses of targeted derivatives. In addition, the existence of oxygenated functional groups such as epoxide, furan and ester make their structures sensitive toward external chemical reagents. Despite having the choice of numerous chemical reagents for the modification of functional groups, it is indeed synthetically challenging to functionalize the inactive methylene carbons (C-6, 11, or 12) of basic limonoid skeletons. In this endeavor, biocatalysts offer a "green" approach for the regio- and stereoselective hydroxylation on the inactive carbon centers.[3,13−16] Whole-cell microorganism is a preferred choice over purified enzymes for the cofactor dependent biocatalytic reactions. Although cofactors are available commercially, it is

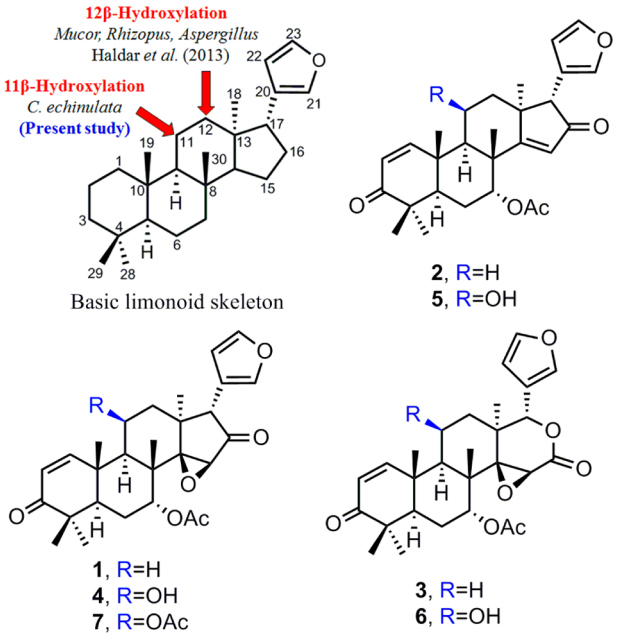


Figure 1. Structures of basic limonoid skeleton, substrates (**1**−**3**), and metabolites (**4**−**6**).

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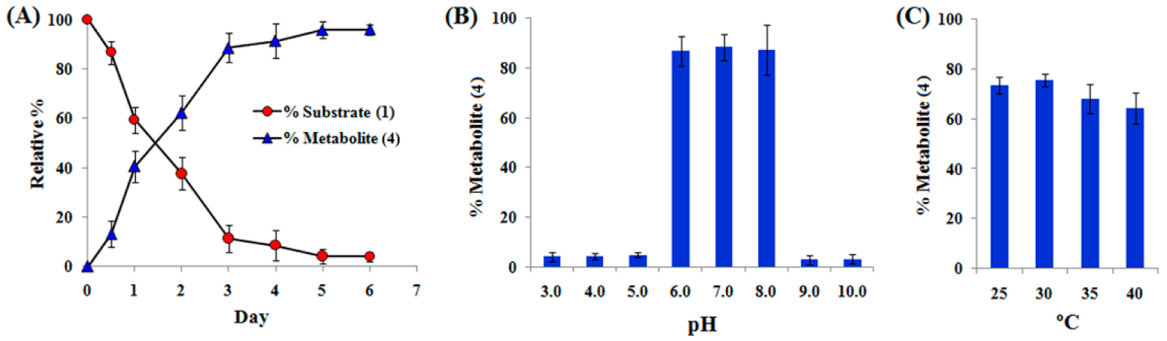

Figure 2. Kinetic study and optimization of the fermentation conditions: (A) graphical representation of the time-course experiment (substrate concentration 0.2 g/L), (B) initial pH (3.0−10.0) optimization study, and (C) temperature (25, 30, 35, and 40 °C) optimization study.

easier and less expensive to use metabolically active cells as the source of cofactors and machinery for their regeneration in situ.

The use of whole cells is easy and inexpensive and the enzymes are well-protected and stable in the cellular environment.[15−20] In the previous report, we have investigated fungi (*Mucor*, *Rhizopus*, *Aspergillus* sp.) mediated 12β-hydroxylation on a range of basic limonoids.[3] The present study was aimed at 11β-hydroxylation on basic limonoids (**1**, **2**, and **3**) and it was achieved by the fungal whole cell of *Cunninghamella echinulata*. 11β-Hydroxylation is well-known on steroidal skeletons.[21−24] Limonoid skeletons, however, are richer in chemically sensitive oxygenated functionalities and possess more skeletal complexity due to the presence of methyls (C-4, 4, and 8) and furan ring (C-17). Therefore, high-efficiency regio- and stereoselective oxygenation reactions, irrespective of the reagents that cause this transformation, are highly challenging.

Screening results revealed *C. echinulata* (National Collection of Industrial Microorganisms; catalogue no. 691) efficiently carried out biotransformation of epoxyazadiradione (**1**), an isolated limonoid from Neem (*Azadirachta indica*) fruits.[25] Highest conversion (91.5%) of the substrate was achieved by *C. echinulata* with formation of a single metabolite with 0.2 g/L substrate concentration (20 mg substrate in 100 mL fermentation medium) and 4 days incubation period (Supporting Information; Table S1). The metabolite was found to be absent in substrate control (i.e., substrate without organism) and organism control (i.e., organism without substrate) experiments, thus indicating the transformation to be whole-cell catalyzed. A resting cell experiment also showed the formation of the same metabolite which further authenticated the whole-cell mediated transformation of epoxyazadiradione (**1**) (Supporting Information; Figure S1). Further, the same transformation observed by spore suspension of *C. echinulata* confirmed the responsible enzyme to be constitutive to the fungus.

The fermentation conditions affecting the catalytic activity of the whole cell such as pH and temperature were optimized, and kinetics of the bioconversion was studied (Figure 2). Evaluation of the resting cell mediated bioconversion with varying initial pH (3.0 to 10.0) revealed highest transformation (%) in the pH range 6.0−8.0. Beyond this pH range, drastic reduction in the biocatalytic transformation was observed (Figure 2B). Varying temperature in the range 25−40 °C did not show significant effect on the level of formation of the metabolite (Figure 2C). Time-course experiments clearly indicated that *C. echinulata* could efficiently carry out the transformation with substrate concentration 0.2 g/L (20 mg in 100 mL culture medium) and 6 days incubation period at 30 °C and 180 rpm. In the early period of fermentation (2 days), around 62% epoxyazadiradione (**1**) was converted into the metabolite, whereas after 3 days around 88% conversion was achieved. With prolonged incubation up to 6 days, conversion of the substrate (**1**) was slowly increased to around 96% (Figure 2A). However, higher substrate concentration resulted in unmetabolized substrate in the fermentation broth even after 6 days of incubation. Therefore, the optimized substrate concentration 0.2 g/L with incubation period of 6 days at 30 °C and pH (initial) 7.0 was established as the most favorable condition for the biocatalytic transformation and further used for the preparative scale fermentation.

The biocatalytic process was further performed in preparative scale under identical conditions with an aim of isolation and structural characterization of the metabolite. The crude biotransformation products (594 mg) of epoxyazadiradione (**1**, 700 mg) obtained after extracting the fermentation broth with ethyl acetate was purified over silica gel flash column chromatography to furnish a novel metabolite which was identified as 11β-hydroxyepoxyazadiradione (**4**, 41 mg) on the basis of IR, 1D (^{1}H, ^{13}C, DEPT-135) (Table 1) and 2D NMR (COSY, NOESY, HSQC, HMBC) (Figure 3), and high resolution ESI(+)-MS and MS/MS studies (Supporting Information; Figure S2−S10). The molecular formula of the metabolite (**4**) was established as $C_{28}H_{34}O_7$ on the basis of major ion peaks at *m*/*z* 505.2202 ($C_{28}H_{34}O_7Na$, $[M + Na]^+$) and 483.2381 ($C_{28}H_{35}O_7$, $[M + H]^+$) in the ESI(+)-MS spectrum, demonstrating the insertion of an oxygen atom to the epoxyazadiradione ($C_{28}H_{34}O_6$) skeleton. In addition, the appearance of a broad absorption band at 3500 cm^{-1} in the IR spectrum and ion peaks at *m*/*z* 465.2277 $[(M + H - H_2O)^+]$ and 405.2060 $[(M + H - AcOH - H_2O)^+]$ in the ESI(+)-MS/MS spectrum indicated the presence of hydroxyl functionality. In comparison to the substrate (**1**), none of the skeletal functional groups showed significant alternation in the chemical shift values as evident from ^{1}H and ^{13}C NMR spectra. Also, the comparative analysis of ^{13}C and DEPT-135 spectra with the substrate revealed loss of one secondary (methylene) carbon along with the increment of a tertiary (methine) carbon in the metabolite implying hydroxylation at one of the methylenes (6, 11, or 12). No significant change was observed for the chemical shift value of C-6 in ^{13}C NMR spectrum, while C-11 and C-12 underwent downfield shift from 16.1 to 65.4 ppm and 29.1 to 43.0 ppm, respectively. The position of hydroxylation was established to be C-11 on the basis of its drastic downfield shift. The appearance of multiplet at δ_H 4.92 ppm (1H, m, H-11) in ^{1}H NMR spectrum also substantiated the hydroxylation at C-11. The connectivity and stereo-

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Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data of the novel metabolites (4 and 5) in $CDCl_3$

	4[a]		5[a]	
position	δ_C type	δ_H mult. (J)	δ_C type	δ_H mult. (J)
1	156.8 CH	7.40[b]	157.1 CH	7.48[b]
2	125.6 CH	5.92 d (10.3)	125.8 CH	5.93 d (10.3)
3	203.9 C	-	204.0 C	-
4	44.1 C	-	44.4 C	-
5	47.4 CH	2.15 m	47.0 CH	2.17 m
6	24.6 CH_2	2.05 m (α), 1.86 m (β)	23.8 CH_2	2.10 m (α), 1.93 m (β)
7	74.9 CH	4.66 t (2.7)	75.1 CH	5.27 m
8	42.4 C	-	44.0 C	-
9	43.9 CH	2.48 d (3.2)	42.9 CH	2.38 d (5.9)
10	40.9 C	-	41.0 C	-
11	65.4 CH	4.92 m	65.2 CH	4.83 m
12	43.0 CH_2	2.43 m (α), 2.11 m (β)	43.8 CH_2	2.63 dd (13.4, 8.8) (α), 1.86 m (β)
13	41.7 C	-	46.8 C	-
14	72.0 C	-	192.2 C	-
15	57.1 CH	3.49 s	124.1 CH	5.96 s
16	208.1 C	-	204.5 C	-
17	51.1 CH	3.95 s	60.4 CH	3.46 s
18	23.9 CH_3	0.97 s	25.9 CH_3	0.97 s
19	22.1 CH_3	1.61 s	22.0 CH_3	1.64 s
20	116.3 C	-	118.2 C	-
21	141.6 CH	7.54 m	141.7 CH	7.47[b]
22	110.8 CH	6.23 m	111.0 CH	6.27 m
23	142.6 CH	7.41[b]	142.9 CH	7.44 m
28	26.8 CH_3	1.07 s	26.8 CH_3	1.09 s
29	21.1 CH_3	1.09 s	21.4 CH_3	1.10 s
30	22.3 CH_3	1.48 s	28.3 CH_3	1.60 s
COMe	169.6 C	-	169.3 C	-
COMe	21.3 CH_3	2.00 s	20.9 CH_3	1.91 s

[a]The assignments are based on ^{1}H–^{1}H COSY, NOESY, HSQC, and HMBC correlations. J values are reported in Hz. [b]Overlapped.

chemistry of hydroxyl functionality and assignment of chemical shift values were further evaluated through homo- and heteronuclear 2D NMR studies. The presence of a spin system locating H-9–11–12 in the ^{1}H–^{1}H COSY spectrum and key correlation of C-11 with H-12 in the HMBC spectrum further confirmed the hydroxylation at C-11 (Figure 3A). On the basis of strong NOESY cross-peaks H-11/Me-18, H-11/H-9, and H-11/H-1, the orientation of H-11 was found to be equatorial (α-face) which in turn confirmed the axial hydroxylation (β-face) (Figure 3E). The low coupling constant (J = 3.18 Hz) between H-9 (axial) and H-11 was also reminiscent of the equatorial orientation of H-11. NMR analyses of the acetylated metabolite

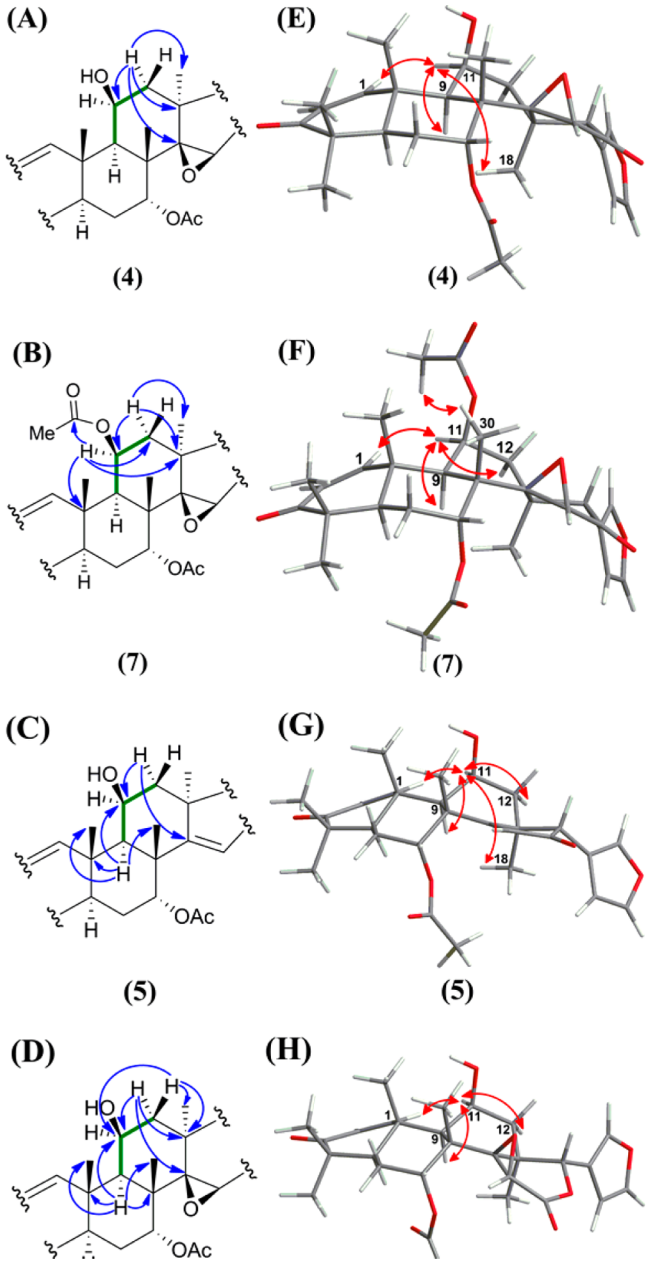

Figure 3. Key correlations in 2D NMR of the limonoids: (**A**–**D**) ^{1}H–^{1}H COSY (green —) and HMBC (blue H→C), (**E**–**H**) NOESY (red H↔H). The Arabic numerals in parentheses represent the numbering of limonoids.

(7) further supported the occurrence of 11β-hydroxylation on epoxyazadiradione (Supporting Information; Figures S11–S18). The hydroxylation at C-11 was confirmed by HMBC correlations (H-11 with C-10; 11-COMe, 12, 13, and C-11 with H-9, 12) and the existence of H-9–11–12 spin-system in ^{1}H–^{1}H COSY spectrum of 7 (Figure 3B). The β-orientation of the acetyl group at C-11 was authenticated through strong NOESY cross-correlations such as H-11/12α, H-11/H-9, H-11/H-1, and H-11-COMe/Me-30 (Figure 3F).

To further evaluate the substrate scope of *C. echinulata* to carry out 11β-hydroxylation, another two ring-intact limonoids (**2** and **3**) were subjected to same biocatalytic reaction. Fermentation was carried out in preparative scale for azadiradione (**2**) and gedunin (**3**) with subsequent isolation

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and characterization of the metabolites (Supporting Information; Figures S19−S36). Major ion peak at m/z 489.2254 ($C_{28}H_{34}O_6Na$, $[M + Na]^+$) in the ESI(+)-MS spectrum of azadiradione metabolite (**5**) corresponded to the molecular formula $C_{28}H_{34}O_6$. Broad and strong absorption band at 3489 cm^{-1} in the IR spectrum, multiplet at δ_H 4.83 ppm in the ^1H NMR spectrum and a highly abundant ion peak at m/z 389.2112 ($[M + H - AcOH - H_2O]^+$) in ESI(+)-MS/MS spectrum inferred the appearance of a hydroxyl moiety on the azadiradione ($C_{28}H_{34}O_5$) skeleton. Increment in the number of methine carbon at the cost of methylene carbon along with extensive downfield shift of C-11 (δ_C 15.8 to 65.2 ppm) in the ^{13}C NMR spectrum evidenced the 11-hydroxylation. Key HMBC correlations (C-11 with H-9 and 12) and spin-system consisting C-9−11−12 in COSY spectrum further corroborated the existence of hydroxyl moiety at C-11 (Figure 3C). Strong NOESY cross peaks H-11/H-1, H-11/H-9, H-11/H-12α, and H-11/Me-18 affirmed the 11β-hydroxylation (Figure 3G). Therefore, by analyzing all the spectroscopic data the metabolite was unequivocally identified as novel 11β-hydroxyazadiradione (**5**). Similarly, the molecular formula of gedunin metabolite (**6**) was established as $C_{28}H_{34}O_8$ on the basis of major ion peak at m/z 521.2156 ($C_{28}H_{34}O_8Na$, $[M + Na]^+$) in ESI(+)-MS spectrum. Downfield shift of C-11 (δ_C 15.0 to 64.8 ppm), H-9−11−12 spin-system in $^1H-^1H$ COSY spectrum and strong HMBC correlations of C-11 with H-9,12 elucidated the position of hydroxylation certainly to be C-11 (Figure 3D). By analyzing the strong NOESY cross peaks (H-11/H-1, H-11/H-9, and H-11/H-12α), orientation of the hydroxyl moiety was determined to be on the β-face (Figure 3H). Thus, the structure of metabolite was unambiguously characterized as 11β-hydroxygedunin (**6**) which has previously been isolated from the cortex of *Cedrela sinensis*.[26] 11β-Hydroxy ring-intact limonoids and its esterified metabolites have been reported previously from various species of *Meliaceae*. More than a dozen of 11β-hydroxy/acetoxy derivatives of walsuranolide have been isolated from *Walsura yunnanensis* and several hirtin analogues bearing 11β-hydroxy/acetoxy moiety have been identified from *Trichilia pallida*.[1,27−29] Although the isolated yields of 11β-hydroxy metabolites were low (<10%),[30] the whole-cell fungal system *C. echinulata* was able to mimic the plant monooxygenase system to introduce hydroxyl functionality at 11β-position of the basic limonoid skeletons. This study clearly indicated that *C. echinulata* can be used for the isolation of gene which encodes 11β-hydroxylase for large scale production of 11β-hydroxy derivatives of basic limonoids (**1**, **2**, and **3**) using recombinant DNA technology.

In conclusion, *C. echinulata* could efficiently carry out C−H activation of chemically inert methylene carbon through regio- and stereoselective 11β-hydroxylation on the basic limonoid skeleton. Using the whole cell of *C. echinulata*, novel 11β-hydroxy derivatives of epoxyazadiradione and azadiradione were generated under mild fermentation conditions, which sustained sensitive skeletal functionalities. The developed biocatalytic process once again exerted the potential of fungal system, especially *Cunninghamella* species, to generate structurally novel analogues of natural products.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation of the purified metabolites was measured in polarimeter at 589 nm in $CHCl_3$ (50 mm cell, c in g/100 mL). Thin film IR spectra were recorded in FT-IR spectrometer in $CHCl_3$. Recording of 1D and 2D NMR spectra was performed by 500 MHz NMR spectrometer in $CDCl_3$. Residual solvent signals were labeled as the reference in the NMR spectra. UPLC-ESI-MS runs were carried out in ESI positive ion mode through quadrupole/orbitrap mass spectrometer. Components of the samples were resolved through UPLC C18 column (2.1 × 100 mm, particle size 1.7 μm, injection volume 5.0 μL, flow rate 0.3 mL/min) with a gradient solvent program of 35.0 min (0.0 min, 40.0%; 5.0 min, 50.0%; 10.0 min, 60.0%; 25.0 min, 65.0%; 30.0 min, 90.0%; 32.0 min, 90.0%; 34.0 min, 40.0%; 35.0 min, 40.0% methanol in water). ESI(+)-MS/MS experiments were performed in data dependent acquisition mode (t-ms^2) utilizing $[M + H]^+$ as the precursor ion with normalized collision energy (NCE) 35%.[25,31] TLC experiments were performed on silica-gel G coated plates (analytical, 0.25 mm). Plates were run in 5.0% methanol in dichloromethane twice; spots were visualized under UV light and developed by dipping in the charring solution (3.2% anisaldehyde, 2.8% H_2SO_4, 2.0% acetic acid, and 92.0% ethanol) followed by heating through hot air-gun. Extracts were purified through silica-gel (230−400 mesh) flash column chromatography to obtain purified metabolites.

Chemicals and Microorganisms. All the chemicals and reagents were obtained from commercial suppliers and used without further purification. Milli-Q water was used for the preparation of media and performing UPLC-ESI-MS runs. Technical grade solvent for the extraction and chromatography (silica-gel) purpose was double distilled prior to use. The fungal strains were obtained from the National Collection of Industrial Microorganisms (NCIM), CSIR-NCL, Pune, India. Limonoids (**1** and **2**) were isolated from Neem (*Azadirachta indica*) fruits and gedunin (**3**) was semisynthesized from epoxyazadiradione (**1**).[25]

Maintenance and Fermentation. The cultures were stored on Potato−Dextrose Agar (PDA) slant at 4 °C and subcultured at 30 °C. Fresh (48 h) well-grown culture on PDA slant was used for the preparation of inoculum. Sterile Czapek Dox medium (100 mL, pH 5.8) in 250 mL Erlenmeyer flask was inoculated under aseptic condition with 3% (v/v) inoculum. The flasks were subjected to orbital shaking on rotary shaker at 180 rpm.

Screening Experiment. To the well-grown culture (48 h) of various fungal species, 20 mg limonoid was added by dissolving in 0.2 mL acetone. The flask was placed on an orbital shaker for 4 days at 30 °C and 180 rpm. Both the broth and the biomass were extracted with ethyl acetate (100 mL × 2), and a concentrated ethyl acetate layer was subjected to analysis through TLC and UPLC-ESI(+)-MS. In addition, two individual control experiments were set up: one in the absence of epoxyazadiradione (organism control) and another in the absence of the organism (substrate control). They were extracted and further analyzed in similar manner.

Resting Cell Experiment, pH, and Temperature Optimization. From the well-grown culture of *C. echinulata*, mycelia was separated by filtration through muslin cloth and repeatedly washed by distilled water to completely remove the fermentation medium. Further, mycelia (wet weight ∼5 g) were added to phosphate buffer (75 mL) of pH 7.0 along with 250 mg dextrose and 3.0 mg epoxyazadiradione (**1**) (in 0.2 mL acetone). After incubation at 30 °C and 180 rpm for 36 h, both mycelia and buffer was extracted with ethyl acetate (75 mL × 2), and concentrated organic layer was analyzed by UPLC-ESI(+)-MS. Substrate control and organism control experiments were also processed through similar procedures. For pH optimization study, eight individual sets of resting cell experiments were carried out by varying the buffer pH from 3.0 to 10.0 keeping other parameters identical. Also, for temperature optimization study resting cell experiments were kept in four independent sets with different temperatures of 25, 30, 35, and 40 °C maintaining other conditions identical. Each set of experiments were carried out in triplicate.

Spore Suspension Experiment. The spore suspension was prepared from sporulated surface culture of *C. echinulata* by gently rubbing the surface of the slant by wire loop in the presence of sterile water. Epoxyazadiradione (**1**, 2.0 mg in 20 μL acetone) was added to the spore suspension (25 mL) and incubated at 30 °C and 180 rpm for

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36 h. Further, the reaction mixture was extracted with ethyl acetate (25 mL × 2) and the organic layer was analyzed by UPLC-ESI(+)-MS.

Time-Course Experiment. For studying the time dependent progress of the bioconversion, 10 mL aliquots of broth were aseptically taken out at regular intervals from the reaction flask (20 mg **1** in 100 mL medium), extracted with ethyl acetate (10 mL × 2), and monitored through UPLC-ESI(+)-MS. The abundance (%) of individual components (**1** and **4**) in the extract was analyzed through area under the respective peaks in extracted ion ($[M + H]^+$) chromatograms.

Preparative Scale Fermentation. The pH of well-grown culture (*C. echinulata*) in 250 mL Erlenmeyer flask containing 100 mL medium was adjusted to ∼6.0−7.0 under aseptic condition by the addition of sterile aqueous solution of 1 M K_2HPO_4. Epoxyazadiradione (0.7 g in 7.0 mL acetone) was equally distributed in 35 such flasks. The batch of flasks was incubated in orbital shaker at 30 °C and 180 rpm for 6 days. Subsequently, the broth and mycelia were separately extracted with ethyl acetate (3.5 L × 3 and 300 mL × 3, respectively) and concentrated organic layers were individually analyzed by TLC and UPLC-ESI(+)-MS. The mycelial extract (35 mg) did not show the presence of metabolites. The broth extract (594 mg) was subjected to silica-gel flash column (40 × 2 cm) chromatography using gradient mixture of methanol in dichloromethane as the eluent with increasing polarity. Unmetabolized substrate (**1**) and the metabolite (**4**, 41 mg) were eluted at 0.2% and 0.8% methanol in dichloromethane, respectively. Similar procedures were followed for the preparative scale fermentation, extraction and purification of the metabolites for the other two substrates (**2** and **3**). For the preparative scale fermentation 600 mg substrates (**2** and **3**) were used. Azadiradione (**2**) and its metabolite (**5**, 38 mg) were eluted at 0.4% and 1.2%, whereas gedunin (**3**) and its 11β-hydroxy metabolite (**6**, 51 mg) were eluted at 0.3% and 1.0% methanol in dichloromethane, respectively.

Characterization of the Metabolites. The isolated metabolites were structurally characterized by exhaustive IR, NMR, and MS analyses.

*11β-Hydroxyepoxyazadiradione (**4**).* A 41 mg (0.08 mmol) amount of **4** was isolated from 700 mg (1.50 mmol) of **1** in 5.6% yield. White solid; $[\alpha]^{25}_D$ +26.8 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) ν_{max} (cm^{-1}): 3500, 1746, 1666, 1377, 1242, 1034; ^1H NMR ($CDCl_3$, 500 MHz) δ: see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) δ: see Table 1; HRMS (ESI+) *m*/*z*: $[M + Na]^+$ Calcd for $C_{28}H_{34}O_7Na$, 505.2197; found 505.2202.

*11β-Hydroxyazadiradione (**5**).* A 38 mg (0.08 mmol) amount of **5** was isolated from 600 mg (1.29 mmol) of **2** in 6.3% yield. White solid; $[\alpha]^{25}_D$ +18.1 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) ν_{max} (cm^{-1}): 3489, 1733, 1702, 1670, 1380, 1243, 1029; ^1H NMR ($CDCl_3$, 500 MHz) δ: see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) δ: see Table 1; HRMS (ESI+) *m*/*z*: $[M + Na]^+$ Calcd for $C_{28}H_{34}O_6Na$, 489.2248; found 489.2254.

*11β-Hydroxygedunin (**6**).* A 51 mg (0.10 mmol) amount of **6** was isolated from 600 mg (1.24 mmol) of **3** in 8.2% yield. White solid; $[\alpha]^{25}_D$ +19.1 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) ν_{max} (cm^{-1}): 3495, 1739, 1667, 1382, 1239, 1029; ^1H NMR ($CDCl_3$, 500 MHz) δ: 7.42 (2H, m, H-21, 23), 7.39 (1H, d, *J* = 10.4 Hz, H-1), 6.36 (1H, m, H-22), 5.91 (1H, d, *J* = 10.4 Hz, H-2), 5.66 (1H, s, H-17), 4.81 (1H, m, H-11), 4.54 (1H, m, H-7), 3.57 (1H, s, H-15), 2.35 (1H, d, *J* = 5.2 Hz, H-9), 2.17 (1H, m, H-12α), 2.13 (1H, m, H-5), 2.08 (3H, s, H−CO<u>Me</u>), 1.71 (1H, m, H-12β), 1.63 (3H, s, H-19), 1.39 (3H, s, H-30), 1.19 (3H, s, H-18), 1.07 (6H, s, H-28, 29); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 203.8 (C-3), 169.7 (C-<u>C</u>OMe), 167.3 (C-16), 156.7 (C-1), 143.2 (C-23), 141.3 (C-21), 125.8 (C-2), 120.2 (C-20), 109.9 (C-22), 78.0 (C-17), 74.5 (C-7), 69.3 (C-14), 64.8 (C-11), 56.0 (C-15), 47.1 (C-5), 44.2 (C-9), 43.9 (C-4), 42.5 (C-8), 41.2 (C-10), 39.5 (C-12), 38.1 (C-13), 26.8 (C-28), 23.7 (C-6), 22.5 (C-19), 21.3 (C-29), 21.0 (C−CO<u>Me</u>), 20.5 (C-30), 16.6 (C-18); HRMS (ESI+) *m*/*z*: $[M + Na]^+$ Calcd for $C_{28}H_{34}O_8Na$, 521.2146; found 521.2156.

Semisynthesis of 11β-Acetoxyepoxyazadiradione (7). 11β-Hydroxyepoxyazadiradione (**4**, 18.0 mg, 0.037 mmol) taken in 2.0 mL anhydrous dichloromethane was added by acetic anhydride (10.5 μL, 0.111 mmol), triethylamine (15.6 μL, 0.111 mmol), and 4-(dimethylamino)pyridine (catalytic) sequentially in stirring condition. After addition, the reaction mixture was left for stirring (3 h) at room temperature. Further, it was partitioned between dicholoromethane/brine solution and the concentrated organic layer was purified through silica-gel flash column chromatography. Elution of the column with 20% ethyl acetate in hexane afforded 12.0 mg (0.023 mmol, ∼62% yield) pure acetylated product (**7**). White solid; $[\alpha]^{25}_D$ = +28.1 (*c* 0.5, $CHCl_3$); IR ($CHCl_3$) ν_{max} (cm^{-1}): 1739, 1673, 1375, 1235, 1034; ^1H NMR ($CDCl_3$, 500 MHz) δ: 7.55 (1H, m, H-21), 7.41 (1H, t, *J* = 1.53 Hz, H-23), 7.23 (1H, d, *J* = 10.4 Hz, H-1), 6.22 (1H, m, H-22), 5.90 (1H, d, *J* = 10.4 Hz, H-2), 5.89 (1H, m, H-11), 4.71 (1H, m, H-7), 3.90 (1H, s, H-17), 3.50 (1H, s, H-15), 2.70 (1H, d, *J* = 3.7 Hz, H-9), 2.58 (1H, dd, *J* = 14.3, 9.5 Hz, H-12α), 2.18 (3H, s, H-11-CO<u>Me</u>), 2.02 (3H, s, H-7-CO<u>Me</u>), 1.46 (3H, s, H-30), 1.44 (3H, s, H-19), 1.08 (6H, s, H-28, 29), 0.99 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 207.8 (C-16), 203.5 (C-3), 170.2 (11-<u>C</u>OMe), 169.5 (7-<u>C</u>OMe), 155.4 (C-1), 142.7 (C-23), 141.7 (C-21), 126.2 (C-2), 116.0 (C-20), 110.8 (C-22), 74.6 (C-7), 71.7 (C-14), 66.9 (C-11), 56.9 (C-15), 51.0 (C-17), 47.6 (C-5), 44.1 (C-4), 42.9 (C-9), 42.3 (C-8), 41.5 (C-13), 40.5 (C-10), 39.1 (C-12), 26.8 (C-28), 24.5 (C-6), 23.8 (C-18), 21.9 (C-30), 21.6 (C-19 and 11-CO<u>Me</u>), 21.3 (C-7-CO<u>Me</u>), 21.2 (C-29); HRMS (ESI+) *m*/*z*: $[M + Na]^+$ Calcd for $C_{30}H_{36}O_8Na$, 547.2302; found 547.2303.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

UPLC-ESI(+)-MS chromatograms, screening results, copies of 1D, 2D NMR, ESI(+)-MS, and MS/MS spectra of the metabolites. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b00417.

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Notes

The authors declare no competing financial interest.

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