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ARTICLE TYPE

Biocatalyst mediated functionalization of salannin, an insecticidal limonoid

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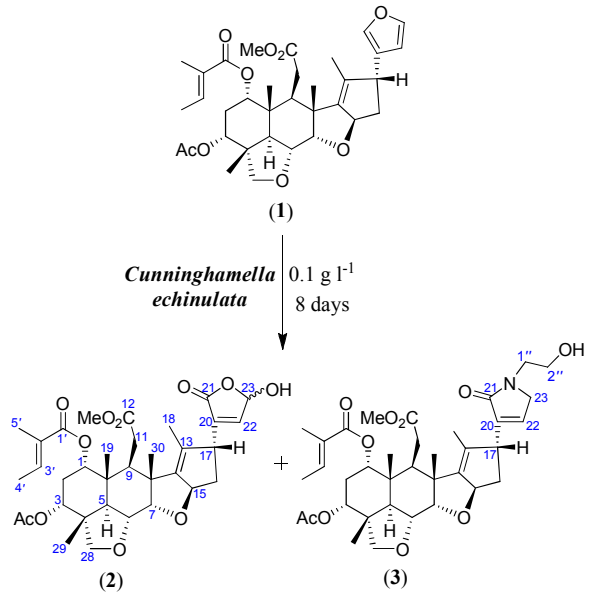
Transformation of salannin, an insecticidal C-seco limonoid was investigated using a fungal system, *Cunninghamella echinulata*. Salannin was efficiently converted into two metabolites, where C-17 furan moiety was transformed into γ-hydroxybutenolide (salanninolide) and N-(2-hydroxyethyl)-α,β-unsaturated-γ-lactam (salanninactam) analogues. Present studies have indicated salanninolide to be a metabolite in the C-seco limonoid biosynthetic pathway.

Limonoids, structurally characterized as tetranortriterpenoids have been shown to possess wide spectrum of biological properties.[1-3] Salannin (**1**), a C-seco limonoid is well-known due to its strong anti-feedant and growth-inhibiting properties towards insects.[2, 4-6] Abundance of salannin among plant resources is restricted to the family of *Maliaceae* and specifically in the genera of *Azadirachta* and *Melia* (*e.g. Azadirachta indica*, *Melia dubia*, *Melia volkensii*).[2] Modifications in various functional groups flanked around its skeleton can lead to the variability in its potency as an insecticidal molecule. For example, hydrogenation of furan moiety at C-17 resulted in an enhanced activity against Colorado potato beetle.[6] However, the complex skeletal architecture and presence of sensitive functional groups make the use of conventional synthesis for structural and functional modifications tedious and time consuming. On the other hand, biocatalysts offer synthetically challenging highly regio- and stereo-selective structural and functional modifications on these complex natural products at mild conditions. Biocatalysts are known to modify natural products to generate bioactive lead derivatives.[7-10] The pronounced insecticidal activity and our interest on triterpenoids and their biocatalytic functionalization,[11, 12] have prompted to study biocatalyst mediated transformation of salannin. In this report, we describe a novel bioconversion of salannin (**1**) by using the fungal strain *Cunninghamella echinulata* to produce two metabolites salanninolide (**2**) and salanninactam (**3**) of which metabolite **3** is hitherto not known (Scheme 1).

Various fungal systems belonging to the genera of *Mucor*, *Aspergillus*, *Rhizopus*, *Neurospora*, *Penicillium* and *Cunninghamella* were screened for the novel and efficient biotransformation of salannin. Of these, *Cunninghamella echinulata* (National Collection of Industrial Microorganisms, catalogue no. 691) was found to carry out efficient transformation of salannin into two metabolites, which were absent in substrate control (*i.e.* substrate without organism) as well as organism control (*i.e.* organism without substrate) experiments as monitored by TLC and LC-ESI-HRMS (ESI, Fig. S1†). Stability of salannin in the substrate control experiment excluded the possibility of degradation in the experimental condition. Fermentation, maintenance and propagation of the microorganisms were carried out as reported earlier (ESI†).[12, 13]



Scheme 1 Biotransformation of salannin (**1**) by *Cunninghamella echinulata*.

The substrate concentration of 0.1 g l^{-1} with an incubation period of 8 days was optimized from the substrate concentration and time course experiments (ESI, Fig. S1†). Large-scale fermentation of salannin (400 mg) with *Cunninghamella echinulata* yielded a crude extract (433 mg) which upon TLC and LC-ESI-HRMS analyses indicated the presence of three limonoids including un-reacted **1**. The extract was subjected to column chromatography and the metabolites were eluted with methanol-dichloromethane gradient mixture. The purified metabolites **2** (78 mg, R_f: 0.23, R_t: 13.8 min) and **3** (11 mg, R_f: 0.21, R_t: 13.3 min) were structurally characterized on the basis of HRMS, IR, one- and two-dimensional NMR spectrometric and

crystallographic studies (Table 1, Fig. 1).

Table 1 ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) assignments of metabolites **2** and **3** in $CDCl_3$.

No.	Salanninolide (**2**)		Salanninactam (**3**)	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	70.5 CH	4.89, t (2.4)	71.6 CH	4.73, t (2.4)
2	28.2 CH_2	[a]	27.4 CH_2	[b]
3	71.2 CH	4.98, t (2.4)	71.3 CH	4.95, t (2.4)
4	42.6 C	-	42.7 C	-
5	40.2 CH	2.72, d (12.8)	39.9 CH	2.79, d (12.5)
6	72.5 CH	3.98, dd (12.5, 3.4)	72.5 CH	3.99, dd (12.8, 3.4)
7	86.0 CH	4.25, d (3.0)	85.6 CH	4.20, d (3.0)
8	48.3 C	-	49.4 C	-
9	39.2 CH	2.53, m	39.4 CH	2.71, t (6.1)
10	40.5 C	-	40.6 C	-
11	29.9 CH_2	[a]	30.8 CH_2	[b]
12	174.6 C	-	173.2 C	-
13	132.7 C	-	133.4 C	-
14	147.8 C	-	148.4 C	-
15	87.4 CH	5.42, m	87.6 CH	5.30, m
16	40.2 CH_2	[a]	40.0 CH_2	[b]
17	48.7 CH	3.49, d (6.7)	49.1 CH	[c]
18	13.3 CH_3	1.83, s	13.2 CH_3	1.72, s
19	15.3 CH_3	0.94, s	15.0 CH_3	1.01 , s
20	137.4 C	-	141.2 C	-
21	171.3 C	-	172.7 C	-
22	141.6 CH	6.76, s	135.3 CH	6.80, m
23	96.8 CH	5.97, s	52.6 CH_2	3.97, s
23-OH	-	5.30, br s	-	-
28	77.6 CH_2	3.72, d (7.6) 3.61, d (7.6)	77.6 CH_2	[c]
29	19.4 CH_3	1.21, s	19.7 CH_3	1.22, s
30	16.3 CH_3	1.30, s	16.9 CH_3	1.30, s
12-OMe	52.5 CH_3	3.44, s	51.5 CH_3	3.43, s
3-OAc	21.0 CH_3 170.3 C	2.00, s	20.8 CH_3 170.3 C	1.92, s
1′	166.5 C	-	166.7 C	-
2′	128.9 C	-	129.1 C	-
3′	137.3 CH	6.96, m	137.1 CH	6.92, m
4′	14.4 CH_3	1.85, d (7.0)	14.4 CH_3	1.79, d (7.0)
5′	11.9 CH_3	1.90, s	11.9 CH_3	1.90, s
1″	-	-	46.6 CH_2	[c]
2″	-	-	62.0 CH_2	3.83, t (4.9)

Overlapped in the region [a] δ_H 2.10-2.40, [b] δ_H 2.04-2.32 and [c] δ_H 3.57-3.68.

LC-ESI-HRMS analysis of metabolite **2** showed an ion peak at *m/z* 651.2762 ($C_{34}H_{44}O_{11}Na$; $[M+Na]^+$) indicating insertion of two oxygen atoms to the salannin ($C_{34}H_{44}O_9$) skeleton maintaining same degree of unsaturation. Exhibition of a broad and strong absorption band at 3397 cm^{-1} (broad and strong) in IR spectrum and $[M+H-H_2O]^+$ ion peak at *m/z* 611.2848 ($C_{34}H_{43}O_{10}$) in LC-ESI-HRMS implied the presence of hydroxyl functionality in the metabolite **2**. In comparison to salannin (**1**), NMR spectra (^{1}H and ^{13}C) of metabolite **2** revealed significant alternation only in the chemical shifts of furan moiety. Therefore, basic skeleton of salannin (**1**) consisting of A, B, C and D rings was concluded to be intact in the metabolite **2**. The conversion of furan moiety to γ-hydroxybutenolide attached to C-17 in metabolite **2** was confirmed on the basis of NMR signals observed for hemiacetal (δ_C 98.6, CH) and α,β-unsaturated lactone (δ_C 137.4, C and 141.6, CH for double bond; δ_C 171.3, C for lactone carbonyl). The presence of α,β-unsaturated lactone and hemiacetal was further substantiated by the peaks at δ_H 6.76 (1H, s) and 5.97 (1H, s) respectively in ^{1}H NMR. The structural connectivity and assignment of NMR chemical shift values were further evaluated through homo- and hetero-nuclear 2D NMR studies (COSY, NOESY, HSQC and HMBC) (Fig. 1A). Presence of a spin-system constituting H-17-22-23 in COSY spectrum and correlations (H-22 with C-20, 21, 23, 17 and H-17 with C-20, 22) in HMBC spectrum further affirmed the existence of γ-hydroxybutenolide ring (α,β-unsaturated-γ-hydroxy-γ-lactone) at

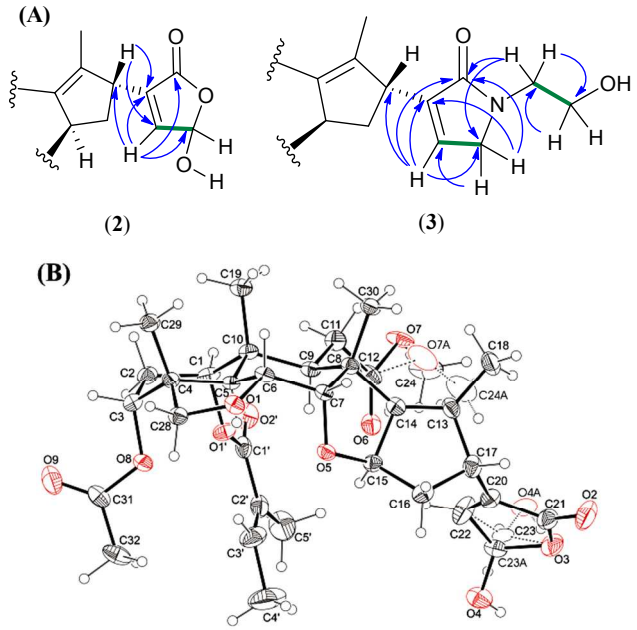


Fig. 1 (A) Key COSY (—) and HMBC (H→C) correlations of metabolite **2** and **3**, **(B)** ORTEP of salanninolide (**2**) (CCDC no. 964596). The hydroxyl group at C-23 displayed statistical disorder over two configurational positions with occupancies 0.7 (R) and 0.3 (S).

C-17. Analyzing all the spectroscopic data, metabolite **2** was identified as salanninolide (γ-hydroxybutenolide analogue of salannin) which was previously isolated from the seeds of *Azadirachta indica* (Neem).[14, 15] However, metabolite **2** was isolated as an inseparable diastereomeric mixture (86:14 from ^{1}H NMR spectrum) at C-23 as γ-hydroxybutenolides are highly susceptible to isomerisation at the hydroxy-bearing centre as observed with scalarane sesterterpene skeletons.[16] The structure

of metabolite **2** and its existence as diastereomeric mixture was further unambiguously confirmed on the basis of single crystal X-ray diffraction data (Fig. 1B).[17]

LC-ESI-HRMS spectrum of metabolite **3** showed major ion peak at *m/z* 678.3251 ($C_{36}H_{49}O_{10}NNa$; $[M+Na]^+$) corresponding to the insertion of two carbons, one oxygen and one nitrogen atoms to the salannin ($C_{34}H_{44}O_9$) skeleton. Absorption bands at 3422 (broad) and 1671 (sharp) cm^{-1} in IR spectrum indicated the presence of hydroxyl and amide/lactam functionality. As observed with metabolite **2**, chemical shift values for the rings A, B, C and D were similar to salannin for metabolite **3** except the furan moiety at C-17. Presence of α,β-unsaturated lactam was predicted on the basis of peaks at δ_C 141.2, 135.3 (C and CH respectively, double bond) and 172.7 (C, lactam carbonyl) in ^{13}C NMR spectrum. The peak at δ_H 6.80 (1H, m) in ^1H NMR spectrum further supported the presence of α,β-unsaturated lactam containing tri-substituted double bond. ^{13}C and DEPT-135 NMR data indicated the presence of two additional methylene carbons in metabolite **3** compared to salannin. Considering the degree of unsaturation (*i.e.* 13, same as that of salannin) and chemical shift values of methylene carbons (^{13}C NMR δ_C: 62.2, 52.6 and 46.6) for the side chain of metabolite **3**, a N-(2-hydroxyethyl) substituted α,β-unsaturated-γ-lactam structure was constructed. Existence of methylene protons at δ_H 3.97 (2H, s), 3.83 (2H, t) and 3.57-3.68 (2H, overlapped with H-17 and 28) in ^1H NMR and their correlation with δ_C 52.6, 62.2 and 46.6 respectively in HSQC spectrum further supported the structural framework. COSY spectrum showed the presence of only two spin systems (H-22-23 and H-1″-2″) in the side chain at C-17. Key correlations such as H-22↔C-20, C-21, C-23, C-17 and H-23↔C-20, C-21, C-22 in HMBC spectrum further upheld the presence of α,β-unsaturated lactam moiety. The presence of N-(2-hydroxyethyl) was confirmed on the basis of correlations H-2″↔C-1″ and H-1″↔C-2″, C-23, C-21 as observed by HMBC analysis. The observations obtained from COSY and HMBC spectra (Fig. 1A) were in full agreement with the construction and arrangement of various functionalities in the metabolite **3**. Thus, metabolite **3** was structurally characterized as novel N-substituted α,β-unsaturated-γ-lactam analogue of salannin on the basis of spectral data and named as salanninactam.

Time course experiments with salannin (**1**) revealed that during early stages of incubation (48 h) nearly 48% of **1** was transformed into metabolites **2** and **3**. However, prolonging the incubation period to seven days, the transformation increased to 95% (ESI, Fig. S1†). In resting cell experiment, about 80% of the substrate (**1**) was transformed into two metabolites (**2** and **3**) after 36 h of incubation period with substrate concentration 2mg/3g of mycelia (ESI, Fig. S1†). Further, formation of same level of metabolites was observed when the resting cell experiments were carried out in dark indicating that these metabolites were formed through enzymatic transformation and ruling out the possibility of photo-oxidation of the substrate.

Salanninolide (**2**), a minor constituent of the diverse limonoid pool from Neem was reported to be photo-oxidized product of salannin (**1**) in organic solvent.[18, 19] Consequently, salanninolide and other γ-hydroxybutenolides from Neem were proposed either to be photo-degraded products of the corresponding limonoids or alternatively an intermediate in the formation of C-17 furan ring.[20] In this study, fungi mediated transformation indicated an enzymatic pathway leading to the biosynthesis of salanninolide from salannin. Therefore, the abundance of salanninolide (**2**) in Neem might not be due to the photo-oxidation of salannin and indicated it as a metabolic product of limonoid biosynthetic pathway.

Photo-mediated oxidation of salannin (**1**) is reported to produce salanninolide (**2**) along with two other isomeric analogues, namely isosalanninolide and Δ^{17}-isosalanninolide.[18] In comparison, microbial system selectively transformed furan to γ-hydroxybutenolide as a major metabolite which is truly rare.[21] Bioconversion reaction leading to the transformation of furan to α,β-unsaturated-γ-lactam ring (**3**) is one of the rare biotransformation reactions.[22] In fact, the natural existence of α,β-unsaturated five-membered lactam ring at the side chain of limonoids has been reported rarely.[23-26]

In the present study, rare and novel bioconversion of furan moiety of salannin to γ-hydroxybutenolide and N-(2-hydroxyethyl) substituted α,β-unsaturated-γ-lactam analogue demonstrated the potential of microbial system, especially *Cunninghamella echinulata* to transform complex natural products into novel metabolites which might be useful further for the large scale production of these metabolites for various applications.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures, LC-ESI-HRMS chromatograms, time-course experiment and copies of NMR, ESI-HRMS and IR spectra. See DOI: 10.1039/b000000x/

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Highlight:

Novel and efficient modification was achieved on furan moiety of salannin by fungi mediated biocatalysis.

Graphical Abstract: