Xanthine oxidase inhibitors from an endophytic fungus *Lasiodiplodia pseudotheobromae*

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ABSTRACT

Two new compounds, lasdiplactone (**1**) and lasdiploic acid (**2**) and one known compound **3** were isolated from the chloroform extract of cell free filtrate of the endophytic fungus *Lasiosdiplodia pseudotheobromae*. The structures of new compounds were determined by interplay of spectral techniques (IR, mass, ^{1}H NMR, ^{13}C NMR, DEPT, and 2D NMR). The absolute configuration at C-4 position of **1** was established as *S* using a process similar to modified Mosher's method. The absolute configuration of **2** was established by comparing its ECD spectrum with the calculated ECD spectra of all possible isomers. In the *in vitro* XO inhibition assay, the highest inhibition was exhibited by **3** with an IC$_{50}$ of $0.38 \pm 0.13\,\mu g/ml$, followed by **2** with an IC$_{50}$ of $0.41 \pm 0.1\,\mu g/ml$ and the least in **1**. The oxidized form of **1** also showed high XO inhibition with IC$_{50}$ of $0.35 \pm 0.13\,\mu g/ml$.

1. Introduction

The enzyme xanthine oxidase (XO) plays a critical role in human purine metabolism apart from its role in oxidative stress related diseases [1]. Over–activity of XO results in higher serum urate levels resulting in the development of hyperurecemia, which is a pre–disposing factor for gout, atherosclerosis, chronic heart failure [2], tissue injury and ischemia [3–6]. Therefore, XO serves as a prime drug target for exploration of non–purine based selective XO inhibitors (NP–SIXO's) as anti–hyperuricemia agents for their possible development into uricostatic drugs. Till date, only two drugs, allopurinol and febuxostat are the cornerstone of anti–gout therapeutics. Allopurinol is a purine based competitive inhibitor of XO, while febuxostat is the only NP–SIXO which is recommended for those suffering from Allopurinol Hypersensitivity Syndrome (AHS) [7]. Hence, the emphasis world over is on exploring new NP–SIXO's which can be used for the management of hyperuricemia and related oxidative stress related diseases.

Endophytic fungi have been widely recognized as an important resource of structurally unique and bioactive natural products, which can be used as templates for development of new medicinal agents [8–11]. During an *in vitro* screening program of endophytic fungi as possible sources of XO inhibitors, cell free culture filtrate of an endophytic fungus *Lasiodiplodia pseudotheobromae* (#1048 AMSTITYEL) exhibited

potent XO inhibition. Further partial purification of cell free filtrate of *Lasiodiplodia pseudotheobromae* (#1048 AMSTITYEL) indicated that the XO inhibitory activity resided in the chloroform extract with an IC$_{50}$ value of $0.61\,\mu g/ml$ which was better than allopurinol (IC$_{50}$ of $0.94\,\mu g/ml$) [12].

Thus, the present work is oriented towards isolation and identification of secondary metabolites present in the chloroform extract of the culture filtrate of *Lasiodiplodia pseudotheobromae* (#1048 AMSTITYEL), followed by evaluation of their *in vitro* XO inhibitory potential. Here, we report the isolation and structure elucidation of two new compounds, lasdiplactone (**1**) and lasdiploic acid (**2**) together with one known compound (**3**) and their evaluation as XO inhibitors.

2. Results and discussion

In earlier study, ethyl acetate and chloroform extracts from culture filtrates from 42 endophytic fungi (*Lasiodiplodia, Fusarium, Muscodor*) isolated from different parts of various medicinal plants were investigated for xanthine oxidase inhibitory activity. The chloroform extract from the culture filtrate of *Lasiodiplodia pseudotheobromae* exhibited strong XO inhibitory activity [12]. The cell free filtrate of the fungal culture was obtained by growing *Lasiodiplodia pseudotheobromae* on potato dextrose broth (PDB) for three weeks. The filtrate was

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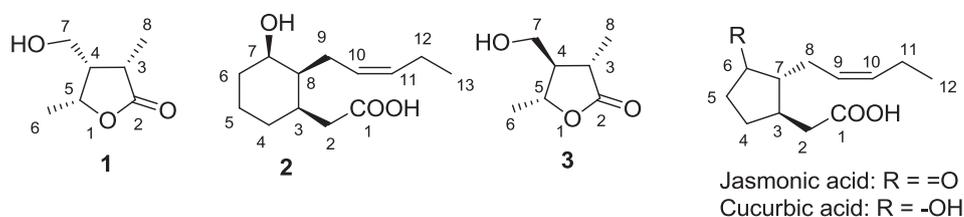
Fig. 1. Structures of isolated compounds and reported compounds.

subsequently extracted with distilled chloroform. Three secondary metabolites (**1**, **2** and **3**) were isolated from the chloroform extract using silica gel column chromatography as detailed in the experimental section. The structures of all compounds were established by analyses of $^1H/^{13}C$ NMR, 2D NMR, IR and ESI–MS spectral data as well as chemical correlations (Fig. 1).

The $^1H/^{13}C$ NMR, IR and ESI–MS spectra of **1** and **3** were very similar indicating structural similarity between both the compounds. However, **1** and **3** showed opposite optical rotation, the specific rotations being $+12°$ (c = 0.1, CHCl$_3$) and $-13°$ (c = 0.1, CHCl$_3$), respectively. The spectral data of **3** was identical to a reported compound (3S,4R,5R)–4–hydroxymethyl–3,5–dimethyldihydro–2–furanone (specific rotation $-18°$, c = 0.3, CHCl$_3$) [13,14]. These data suggested that the **1** and **3** may have identical planar structure but a different stereochemistry.

The ESI–MS of **1** gave [M + 1]$^+$ peak at m/z 145 and a fragment peak at m/z 126 indicating the loss of H$_2$O molecule. The IR spectrum indicated the presence of primary alcohol (3405 cm^{-1}) and a lactone or an ester (1747 cm^{-1}). The ^{13}C NMR, ^1H NMR and mass spectra together indicated that molecular formula of **1** is C$_7$H$_{12}$O$_3$. The two degrees of unsaturation included one carbonyl group (δ_C 179.5) and one ring.

The ^{13}C NMR spectrum of **1** showed seven signals whereas DEPT–45 spectrum showed the presence of six protonated carbons and one quaternary carbon (δ_C 179.5). The DEPT–135 spectrum showed one negative signal at δ 60.2 indicating the presence of –CH$_2$OH group, also confirmed by a pair of double doublets (δ 3.81 and 3.75) in the ^1H NMR spectrum. The ^1H NMR also showed two doublets (δ 1.45 and 1.26) for two –CH$_3$ groups adjacent to –CH group.

The structure of lasdiplactone (**1**) was established by using ^1H–^1H COSY (Fig. 2) and HMBC experiment as shown in Table 1. The methyl protons at δ 1.26 showed HMBC correlation with carbons at δ 179.5 (C-2), 37.6 (C-3) and 53.3 (C-4), whereas methyl at δ 1.45 showed HMBC correlation with carbons at δ 77.4 (C-5) and 53.3 (C-4). The hydroxymethylene protons at δ 3.81 and 3.75 showed HMBC correlation with carbons at δ 37.6 (C-3), 53.3 (C-4) and 77.4 (C-5). The ^1H–^1H COSY spectrum showed correlation of hydroxymethylene protons with H-4; methyl protons at δ 1.26 and 1.45 with H-3 and H-5, respectively. These data established the presence of five member lactone ring with methyl substitutions at C-3 and C-5 and hydroxymethylene substitution at C-4 confirming its planar structure.

A number of chiral derivatizing agents have been reported for determination of absolute configuration of compounds containing secondary alcohol, secondary amine or carboxylic acid functionalities by using modified Mosher's method [16,17]. The presence of primary alcohol functionality at C-4 indicated that a process similar to the

modified Mosher's method reported by Nagai and Kusumi [18] for determining the absolute stereochemistry of chiral carboxylic acids could be applied for **1** after oxidizing its primary alcohol functionality into an acid. Chiral anisotropic reagents, R– and S–phenylglycine methyl ester (PGME) have been used for the determination of absolute configuration of chiral carboxylic acids via their amide derivatives [19,20]. The primary alcohol at C-4 position of **1** was oxidized to yield carboxylic acid **4** using pyridinium dichromate. (R)– and (S)–amides of **4** were synthesized using (R)– and (S)–phenylglycinyl methyl ester hydrochloride (PGME) (Scheme 1) [21,22] and purified using silica gel column chromatography. All the protons were assigned by detailed analyses of 1D and 2D NMR spectral data and $\Delta\delta$ (δ_S–δ_R) calculated. Based on the chiral anisotropic effects of the aromatic ring, a conformation was drawn as shown in Fig. 3A, in which H–C4–CO–NH–C–CO–OMe are in the plane of the paper, C-5 and C-6 with negative $\Delta\delta$ values are above the plane (toward the viewer) and C-3 and C-8 with positive $\Delta\delta$ values are below the plane of paper (away from viewer). Model 3B in which substituents having positive $\Delta\delta$ values were placed on the right–hand side and those with negative $\Delta\delta$ values were placed on the left–hand side represents the correct absolute configuration of carboxylic acid **4** as shown in 3C. The absolute configuration at C-4 of the acid **4** was therefore determined to be R. The absolute configuration at C-4 of **1** replacing the carboxylic acid with alcohol functionality was determined to be S as shown in 3D. The configuration at position C-3 and C-5 relative to C-4 was established using ^1H–^1H NOESY experiment (Table 2). Cross peaks between hydroxymethylene protons and both the methyl groups indicated their *cis* relationship. Also, correlation observed between protons at C-3 and C-4 indicated their *cis* relationship. It suggested that the configuration at C-3 and C-5 are S and R, respectively. Compound **1** was therefore characterized as (3S,4S,5R)–4–hydroxymethyl–3,5–dimethyldihydro–2–furanone (Fig. 1).

The ESI–MS spectrum of lasdiploic acid (**2**) showed [M + Na]$^+$ peak at m/z 248.93. The IR spectrum indicated the presence of secondary alcohol (3385, 1081, 1277 cm^{-1}) and a carbonyl group (1706 cm^{-1}). The ^{13}C NMR showed signals for thirteen carbons and the DEPT spectra showed the presence of one methyl, six methylenes, five methines (two in the olefinic region) and one quaternary carbon (a carboxyl group δ_C 178.7). The combined analysis of $^1H/^{13}C$ NMR, IR and mass spectrum indicated a molecular formula of C$_{13}$H$_{22}$O$_3$ for **2** showing three degrees of unsaturation which included a double bond, a carboxyl group and one ring. The above data showed similarity with cucurbic acid (C$_{12}$H$_{20}$O$_3$), however, with an additional methylene group [23].

The ^{13}C NMR spectrum of **2** showed signals at δ 133.3 and 127.0 and the corresponding ^1H NMR multiplets centered at δ 5.48 and δ 5.38 for one proton each were indicative of the presence of a double bond. ^1H NMR showed a triplet (δ 0.98, 3H) indicating the presence of –CH$_3$ group adjacent to –CH$_2$ group, one proton signal at δ 4.04 (corresponding carbon at δ 77.6) indicative of a secondary hydroxyl group and two other methine protons centered at δ 2.69 and 1.59. It also showed a pair of distinct double doublets (δ 2.47 and 2.26) besides several overlapping signals at δ 2.1–1.9 (7H) and 1.4–1.3 (3H).

The structure of **2** was established by detailed analyses of 2D NMR spectral data including ^1H–^1H COSY, HMBC and NOESY experiments as shown in Fig. 2 and Table 1. The methyl protons at δ 0.98 showed HMBC correlation with carbons at δ 20.7 (C–12) and 133.3 (C–11),
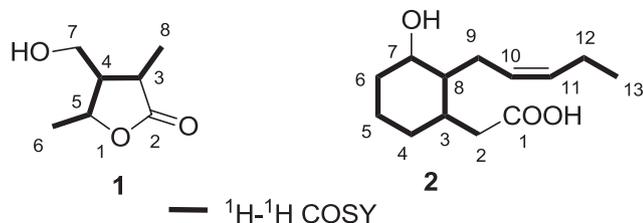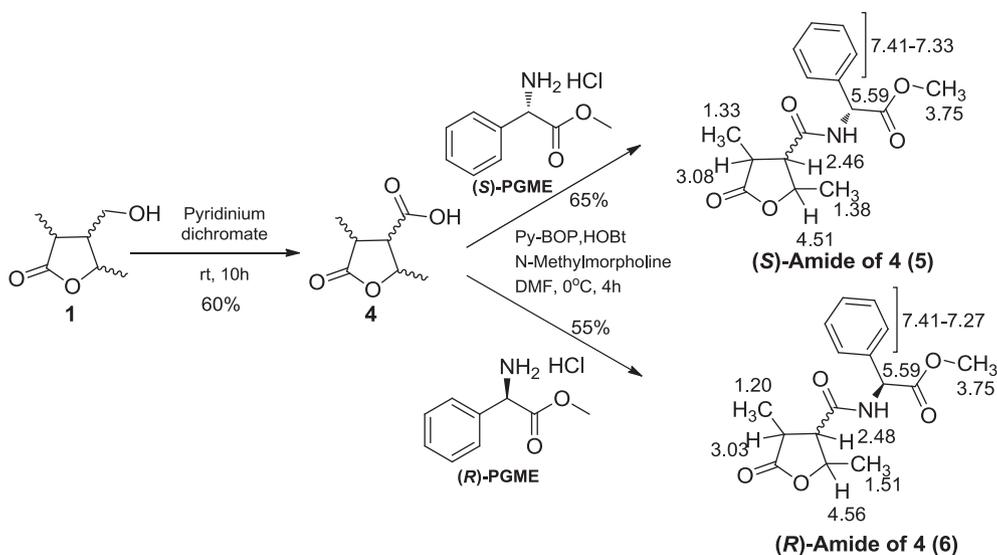

Fig. 2. ^1H–^1H COSY correlations of **1** and **2**.

Table 1
^{1}H (400 MHz, CDCl$_3$), ^{13}C NMR (100 MHz, CDCl$_3$) and HMBC data of **1** and **2**.

Position	1			2		
	δ_C	δ_H (*J* in Hz)*	HMBC	δ_C	δ_H (*J* in Hz)	HMBC
1				178.7		H-2
2	179.5		H–3, H–8	35.3	2.47 (dd, 15.3, 9.4, 1H)	
					2.26 (dd, 15.5, 9.2, 1H)	
3	37.6	2.64 (dq, 10.7, 7.1, 1H)	H–4, H–7, H–8	36.6	2.69 (m, 1H)	H–2
4	53.3	1.90–1.83 (m, 1H)	H–3, H–6, H–7, H–8	28.3	2.15–1.91 (m, 1H)	H–2
					1.40–1.27 (m, 1H)	
5	77.4	4.42 (dq, 9.3, 6.2 Hz, 1H)	H–4, H–6, H–7	29.7	1.40–1.27 (m, 2H)	H–4
6	20.0	1.45 (d, 6.2 Hz, 3H)	H–4, H–5	32.5	2.15–1.91 (m, 1H)	H–5
					1.62–1.56 (m, 1H)	
7	60.2	3.81 (dd, 11.0, 4.5 Hz, 1H)	H–3, H–5	77.6	4.04 (m, 1H)	H–6, H–9
		3.75 (dd, 11.0, 4.8 Hz, 1H)				
8	14.0	1.26 (d, 7.1 Hz, 3H)	H–3, H–4	50.5	2.15–1.91 (m, 1H)	H–2, H–3, H–9
9				25.3	2.15–1.91 (m, 2H)	H–10
10				127.0	5.38 (m, 1H)	H–9
11				133.3	5.48 (m, 1H)	H–9, H–13
12				20.7	2.15–1.91 (m, 2H)	H–11, H–13
13				14.2	0.98 (t, 7.5, 3H)	H–12

* The coupling constants for complex first-order multiplets were calculated as described in literature [15].

Scheme 1. Synthesis and ^{1}H NMR data of (*S*)–amide and (*R*)–amide of compound **4**.

whereas H$_2$–9 showed correlation with carbons at δ 50.5 (C-8), 77.6 (C-7), 127.0 (C–10) and 133.3 (C–11). The above HMBC correlations with C-8 and C-7 together with ^{1}H–^{1}H COSY correlation of H-8 with H$_2$–9 and H-7 confirmed the location of attachment of pentenyl chain with

the ring and the substitution of –OH group at C-7. The proton at C-8 also showed HMBC correlation with C-7 (δ 77.6). The HMBC correlation of H$_2$–2 with C-3 (δ 36.6), C-4 (δ 28.3), C-8 (δ 50.5), C-1 (δ 178.7) as well as ^{1}H–^{1}H COSY correlation between H-2 and H-3 confirmed the
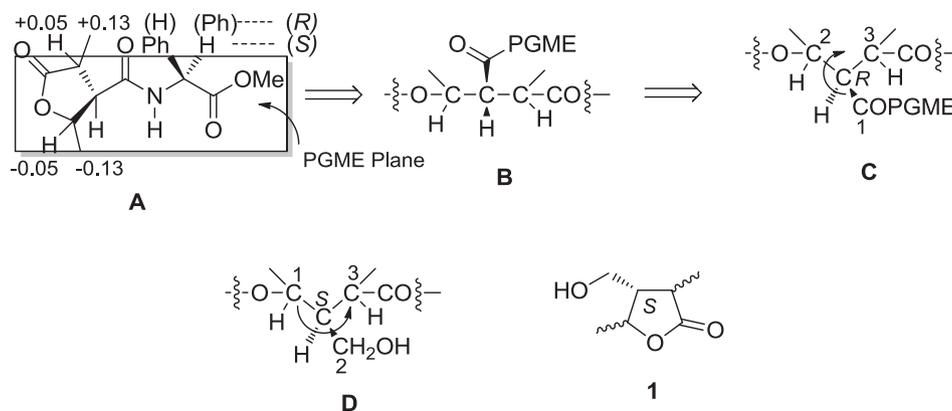

Fig. 3. Absolute configuration of compound **1**.

Table 2
^{1}H–^{1}H NOESY (500 MHz, CDCl$_3$) data of **1** and **2**.

	1			**2**	
Position	δ_H	NOESY	Position	δ_H	NOESY
3	2.64	H–8, H–4	3	2.69	H–4, H–8
4	1.90–1.83	H–3	7	4.04	H–6, H–8
5	4.42	H–6	10	5.38	H–9
7	3.81	H–4, H–6, H–8	11	5.48	H–12
			12	2.15–1.91	H–13
			13	0.98	H–12

attachment of carboxyl group to C-3 through the methylene carbon C-2. The above correlations together with other HMBC as well as ^{1}H–^{1}H COSY experiments (Fig. 2 and Table 1) confirmed the structure containing a trisubstituted cyclohexane ring.

Compound **2** contained one additional methylene group as compared to cucurbic acid, which has a five member ring and is structurally related to jasmonic acid [14]. This additional methylene could either be a part of the ring or may be present in one of the side chains. In order to further confirm that this additional methylene is in fact present in the ring, 1 mg of **2** was oxidized with Jones reagent. The product showed an *m/z* at 125 suggesting the conversion of alcohol to ketone and an IR spectrum showed a band at 1716 cm^{-1} thereby confirming that **2** contained a six member ring.

The relative stereochemistry at chiral centers of **2** was suggested by 2D NOESY experiments (Table 2). Correlations between H-3 and H-8, as well as H-8 and H-7 indicated that all substituents were *cis* with respect to each other. The absolute configuration of **2** was determined by comparing experimental electronic circular dichroism (ECD) spectrum with calculated ECD spectra of all possible isomers. TDDFT calculations were carried out using the CAM-B3LYP method and aug-cc-pVDZbasis set. The compound **2** has three chiral centers and hence eight stereoisomers are, in principle, possible. All the stereoisomers were initially optimized at B3LYP/6-31 + G(d) level. The absolute energies of all eight stereoisomers were calculated and are given in supplementary data (Table S1). On a relative scale the stereoisomers 3A-8E-7A (3axial-8equatorial-7axial) and 3E-8E-7A are preferred structures. 3A-8E-7A isomer being only marginally less stable by 0.29 kcal/mol than the global minimum 3E-8E-7A stereoisomer. In TDDFT calculations, twenty excited states were included. UV and CD spectra of all stereoisomers were generated using GuassView 5.0.9 [24] by applying the band shape width 0.33 eV exponential half-width. The experimental CD spectrum of **2** (in acetonitrile) is comparable with the calculated CD spectra of 3A-8E-7A and 3E-8E-7A steroisomers (Fig. 4). Based on the relative all *cis* stereochemistry noticed from 2D-NOESY correlations (Table 2) and the comparative CD spectra (Fig. 4 and supplementary data), we propose that absolute stereochemistry of compound **2** is 3*R*, 8*S*, 7*R* with 3A-8E-7A structure.

2.1. Biological studies

In the *in vitro* XO inhibition assay the best inhibition was exhibited by **3** with IC$_{50}$ of 0.38 ± 0.13 μg/mL, followed by **2** with IC$_{50}$ of 0.41 ± 0.1 μg/mL and the least in **1**. Compounds **1** and **3** are isomers differing in absolute configuration at C-4. Both compounds **1** and **3** have lactone nucleus. The isomer **3** was seven times more potent than isomer **1** in XO inhibitory activity. It is well known that stereochemistry plays an important role in drug action, for example, stereoisomers of methyl jasmonate (JA-Me) show different biological activities. (l*R*,2*S*)– and (l*R*,2*R*)–JA–Me strongly inhibited growth of soybean callus, whereas (l*S*,2*R*)– and (l*S*,2*S*)–JA–Me showed very low inhibitory effect [25]. The oxidized form of **1** *i.e.* compound **4** gave the best XO inhibition with IC$_{50}$ of 0.35 ± 0.13 μg/ml. However, by Tukey's post hoc analysis, the *in vitro* XO inhibition did not differ in **2**, **3** and **4**

significantly. The amides of **4** *i.e.* compounds **5** and **6** did not exhibit a good XO inhibitory profile as evident from their IC$_{50}$ values (Table 3). None of the isolated compounds showed XO inhibitory activity either equal to or better than positive control allopurinol. However, chloroform extract of the *L. pseudotheobromae* culture filtrate showed better XO inhibitory activity than allopurinol [12]. This result may be due to synergistic effect of two or more compounds on enzyme XO.

3. Experimental

3.1. General

The chiral reagents and other chemicals were purchased from Sigma–Aldrich, Hyderabad, India. The compounds were isolated and purified using silica gel (Merck, Germany) column chromatography. Silica gel coated aluminum sheets (TLC Silica gel 60 F$_{254}$, 0.25 mm thickness, Merck, Germany) were used for TLC. ^{1}H NMR and ^{13}C NMR spectra were recorded on 400/500 and 100/125 MHz (Bruker FT–NMR Avance II, USA/JEOL, Japan) spectrometer, respectively, using tetramethylsilane as an internal standard. The chemical shifts are reported in δ units. Infra–red (IR) spectra were recorded by using Perkin Elmer–Spectrum II instrument. Mass spectra were recorded on Thermo LTQ–XL mass spectrometer (Thermo, USA). Optical rotation was recorded on Autopol® IV, Automatic polarimetry, Rudolph Research Analytical (Hackettstown, USA). Circular Dichroism spectrum was recorded on JASCO J-815CD spectrometer, Japan.

3.2. Re–culturing and maintenance of the endophytic fungi

Lasiodiplodia pseudotheobromae #1048AMSTYEL was previously isolated from *Aegle marmelos*, Yelandur, Karnataka and ITS sequence deposited at Gen Bank with an accession number of KF540145 [12]. For further studies, the strain was sub–cultured on PDA (potato dextrose agar) plates and incubated at 26 ± 2 °C for 7 days. Subsequently, 5 mm mycelial plugs were prepared from 7 days old culture and aseptically transferred to PDA slants and vials and incubated at 26 ± 2 °C till fungal mycelia were observed.

3.3. Production of culture filtrate

For secondary metabolite production, 200 mL of pre–sterilized Potato Dextrose Broth (PDB, Hi–Media, India) in 1 L Erlenmeyer flask (Borosil, India, 4980021) was aseptically inoculated with 10 mm mycelial disk of a week old fungal culture. The flask was then incubated at 26 ± 2 °C, 120 rpm for 2 weeks. After the culmination of the incubation period, the mycelium was separated from the spent broth by filtration through Whatman paper 4 (Sigma Aldrich, USA, Z240567) followed by centrifugation at 12,000 rpm for 10 min and subsequently passing it through a 0.2 μm nitrocellulose membrane (Merck Millipore, USA, PR04066) for making it cell–free. Cell free culture filtrate (13 L), obtained by filtration was lyophilized and stored at −20 °C for further use.

3.4. Extraction and isolation

The mycelia were filtered out from fungal culture (13 L) and the filtrate was extracted with chloroform. The chloroform part was concentrated under vacuum to yield an extract (1.26 g). A portion of extract (1.1 g) was washed with petroleum ether and subjected to silica gel column chromatography. Thirty four fractions were collected by increasing the polarity of mobile phase from 50% to 100% CHCl$_3$ in hexane followed by 1–20 % MeOH in CHCl$_3$. Compounds **1** (70 mg) and **3** (4 mg) were isolated from fraction 9 by repeated silica gel column chromatography. Compound **2** (10 mg) was isolated from fraction 22.

(3*S*,4*S*,5*R*) - Lasdiplactone (**1**): light yellow liquid; [α]$_D^{25}$ + 12° (*c* = 0.1, CHCl$_3$), +17° (*c* = 0.3, CHCl$_3$); IR (CHCl$_3$) v cm^{-1} 3405,
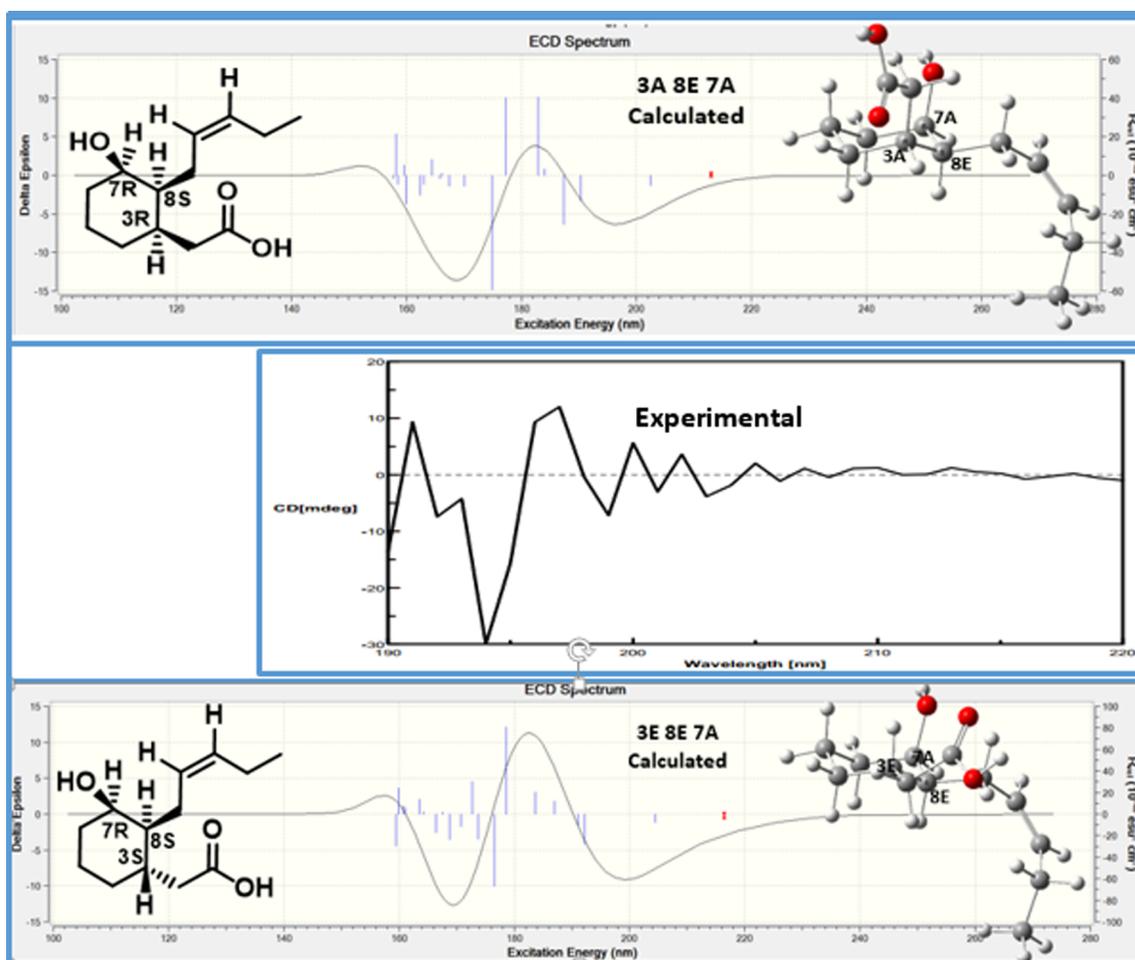
Fig. 4. Calculated (top and bottom) and experimental (middle) ECD spectra.

Table 3

Quantitative assessment of XO inhibition by compounds isolated from chloroform fraction of culture filtrate of *Lasiodiplodia pseudotheobromae*.

S. No.	Sample	XO Inhibitory activity IC$_{50}$ (µg/ml)[**]
1	**1**	2.81^a ± 0.71
2	**2**	0.41^{bc} ± 0.10
3	**3**	0.38^{bc} ± 0.13
4	**4**	0.35^{bc} ± 0.13
5	**5**	1.83^{ab} ± 0.4
6	**6**	2.50^a ± 0.5
7	**Allopurinol (Standard)**	0.18^c ± 0.02

Means with different superscript letters are different by Tukey's post hoc test (p < 0.05).

[**] Data presented are mean ± standard deviation of three replicates.

2920, 2851, 1747, 1458, 1381, 1191; ^{1}H and ^{13}C NMR (Table 1); ESI–MS: $[M + 1]^+$ 144.81, $[M – H_2O]^+$ 126.84 for $C_7H_{12}O_3$.

(3R, 7R, 8S) - Lasdiploic acid (**2**): light yellow liquid; $[\alpha]_D^{25} + 8°$ (c = 0.4, MeOH); IR (CHCl$_3$) v cm^{-1} 3385, 2927, 1706, 1462, 1408, 1277, 1190, 772; ^{1}H and ^{13}C NMR data (Table 1); ESI–MS: $[M + 1]^+$ 227.12, $[M + Na]^+$ 249.18 for $C_{13}H_{22}O_3$.

(3S, 4R, 5R)–4–(Hydroxymethyl)–3,5–dimethyldihydrofuran–2(3H) –one (**3**): light yellow liquid; $[\alpha]_D^{25}$ –13° (c = 0.1, CHCl$_3$); IR (CHCl$_3$) v cm^{-1} 3414, 2922, 2852, 1749, 1458, 1378, 1191; ^{1}H NMR (400 MHz, CDCl$_3$): δ 4.40 (dq, J = 9.2, 6.2 Hz, 1H), 3.84 (dd, J = 10.9, 4.1 Hz, 1H), 3.77 (dd, J = 10.9, 5.3 Hz, 1H), 2.61 (dq, J = 11.3, 7.1 Hz, 1H), 1.92–1.84 (m, 1H) 1.47 (d, J = 6.2 Hz, 3H), 1.28 (d, J = 7.0 Hz, 3H);

^{13}C NMR (100 MHz, CDCl$_3$): δ178.8, 77.1, 60.8, 53.2, 37.7, 20.1, 14.1; ESI–MS: $[M + 1]^+$ 144.84, $[M – H_2O]^+$ 126.92 for $C_7H_{12}O_3$.

3.5. Oxidation of compound 1

Compound **1** was oxidized using reported method [17,18]. In brief, compound **1** (0.1 mmol, 14.4 mg) was dissolved in DMF (1 mL). Pyridinium dichromate (4.5 equiv., 170 mg in 1 mL DMF) was added and the solution was stirred at room temperature for 10 h. The completion of reaction was monitored with TLC. After completion of the reaction, water (5 mL) was added and extracted with diethyl ether (10 mL × 3). The organic solvent extracts were combined and passed through anhydrous sodium sulphate. The solvent was evaporated under vacuum to yield pure acid (**4**) (60%, 9.5 mg). The reaction was repeated two times to generate sufficient amount for further reactions. Compound **4**: White solid; $[\alpha]_D^{25} + 12°$ (c = 0.3, CHCl$_3$); IR (CHCl$_3$) v cm^{-1} 3385, 2922, 2852, 1748, 1458, 1378, 1192; ^{1}H NMR (400 MHz, CDCl$_3$): δ 4.61–4.55 (m, 1H), 3.04–2.96 (m, 1H), 2.67 (t, J = 10.6 Hz, 1H), 1.54 (d, J = 6.1 Hz, 3H), 1.38 (d, J = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 176.6, 174.8, 75.7, 55.5, 39.9, 20.2, 14.5; ESI–MS: $[M]^+$158.0 for $C_7H_{10}O_4$.

3.6. Condensation of 4 with (S)–PGME

The mixture of **4** (0.038 mmol, 6 mg) and (S)–PGME was dissolved in dimethylformamide (DMF) (1 mL) under nitrogen. A solution of PyBOP (0.038 mmol, 19.8 mg) and HOBt (0.038 mmol, 5.8 mg) in DMF (1 mL), followed by N–methylmorpholine (3 equiv., 15 µl) was added. The reaction mixture was stirred at 0 °C for 4 h. Completion of the

reaction mixture was monitored by TLC. After the completion of the reaction, chloroform (10 mL) was added and washed with water. The organic layer was passed through anhydrous sodium sulphate, followed by evaporation under vacuum. Compound (**5**) was purified using silica gel column chromatography (yield 55%). Compound **5**: Colorless liquid; IR (CHCl$_3$) υ cm^{-1} 3351, 2954, 2919, 2851, 1740, 1647, 1457, 1188; ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.41–7.33 (m, 6H), 5.59 (d, $J = 6.9$ Hz, 1H), 4.51(dq, $J = 9.3$, 6.2, 1H), 3.75 (s, 3H), 3.08 (dq, $J = 11.7$, 7.5, 1H), 2.46 (dd, $J = 11.0$, 9.9 Hz, 1H), 1.38 (d, $J = 6.2$ Hz, 3H), 1.33 (d, $J = 7.1$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 177.0, 171.0, 168.5, 135.9, 129.2, 128.9, 127.2, 76.2, 58.6, 56.8, 53.1, 40.7, 19.7, 13.9; ESI–MS: [M + 1]$^+$ 306.18, [M + Na]$^+$ 328.11 for C$_{16}$H$_{19}$NO$_5$.

3.7. Condensation of 4 with (R)–PGME

Compound **4** was condensed with (*R*)–PGME under same conditions as above to give **6** (yield 65%). Compound **6**: Colorless liquid; IR (CHCl$_3$) υ cm^{-1} 3322, 2955, 2924, 2852, 1744, 1651, 1456, 1175; ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.41–7.31 (m, 6H), 5.59 (d, $J = 7.1$ Hz, 1H), 4.56 (dq, $J = 9.5$, 6.2, 1H), 3.75 (s, 3H), 3.03 (dq, $J = 11.7$, 7.4, 1H), 2.48 (dd, $J = 11.3$, 9.7 Hz, 1H), 1.51 (d, $J = 6.2$ Hz, 3H), 1.20 (d, $J = 7.1$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl$_3$): δ 177.1, 171.1, 168.6, 135.7, 129.2, 128.9, 127.2, 76.5, 58.5, 56.8, 53.1, 40.5, 19.7, 13.8; ESI–MS: [M + 1]$^+$ 306.20, [M + Na]$^+$ 328.26 for C$_{16}$H$_{19}$NO$_5$.

3.8. In vitro assay for xanthine oxidase inhibition

All the six compounds (3 isolated and 3 synthesized) were subjected for determination of the xanthine oxidase (XO) inhibitory activity using microtiter plate based NBT assay as described with minor modifications [12]. The method was based upon the amount of formazan formed due to the interaction of NBT with superoxide radicals which is produced during the oxidation of hypoxanthine to xanthine and then to uric acid with the help of xanthine oxidase. The reduction in the intensity of the formazan product indicates xanthine oxidase inhibition. Briefly, 50 μl of test compound was pre–incubated with 30 μl (220 mU/ml) of xanthine oxidase at 37 °C for 1 h before assaying the activity. The reaction was initiated by adding 90 μl of xanthine (10 mM) followed by 30 μl of 2 mM NBT to the pre–incubated master–mix. After 1 h incubation at 37 °C, the amount of formazan produced was estimated by measuring the absorbance at 575 nm using a Biotek throughput reader, Powerwave 340. The control well comprised all reagents except test compound. Allopurinol was used as a positive control. All the test and control experiments were performed in triplicates, and the data was represented as Mean ± SD values.

4. Conclusion

The present study revealed that the XO inhibitory activity of chloroform fraction of *Lasiodiplodia pseudotheobromae* # 1048AMSTYEL was attributed largely to one new compound **2** and one known compound **3**. The oxidized form of **1** (*i.e.* compound **4**) statistically gave a similar XO inhibition when compared to **2** and **3**.

Acknowledgements

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.12.008.

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