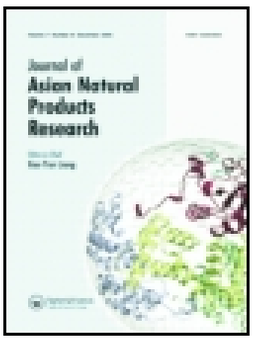

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Three new amino acid derivatives from edible mushroom *Pleurotus ostreatus*

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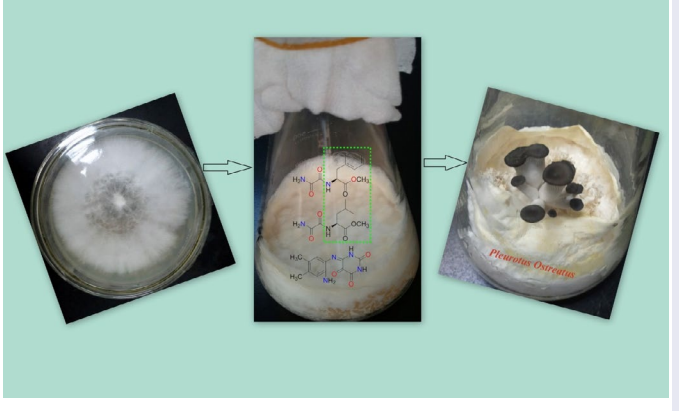
ABSTRACT

Three new amino acid derivatives, oxalamido-*L*-phenylalanine methyl ester (**1**), oxalamido-*L*-leucine methyl ester (**2**), and lumichrome hydrolyzate (**3**), together with nine known compounds (**4–12**), were isolated from the solid culture of edible mushroom *Pleurotus ostreatus*. Their structures were elucidated on the basis of extensive spectroscopic analysis. The absolute configurations of **1** and **2** were established by the chiral synthesis and confirmed by circular dichroism (CD) analysis of their total synthesis products and natural isolates. All new compounds were evaluated for their antioxidant effects, antimicrobial activities, and cytotoxic activity. Compounds **1–3** showed weak antifungal activities against *Candida albicans* with minimum inhibitory concentration (MIC) value of 500 μg/ml.



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1. Introduction

Mushrooms have become increasingly attractive as a functional food due to their bioactive metabolites in the development of drugs and nutraceuticals [1]. Mushroom mycelia have been considered as alternatives and replacement of dried fruiting bodies for their rich bioactive metabolites. The technique of solid-stated fermentation refers to micro-organism growing on moist solid substrates without free flowing water. Recently, fermented foods produced by inoculating the edible and medicinal mushrooms on the cooked rice medium have attracted wide attention due to enhancement of diversity of pharmacological bioactivities and surprising metabolites [2,3]. Examples include sesquiterpenoids from *Pleurotus cystidiosus* with inhibitory activity against α-glucosidase and protein tyrosine phosphatase–1B (PTP1B) [4], terpenoids from *Astraeus odoratus* with antibacterial and cytotoxic activities [5], lectin from *Agaricus bisporus* with immunomodulatory effect [6], β-D-glucans from *Cookeina tricholoma* with antinociceptive property [7]. In this work, we tried to grow the edible mushroom on the cooked rice medium for their bioactive secondary metabolites.

Pleurotus ostreatus, also known as the oyster mushroom, is a fresh edible mushroom frequently consumed in Southeast Asia and belongs to the phyla Basidiomycete, order Agaricales, family Pleurotaceae, genus *Plearotus*. Compounds with medicinal properties have been isolated from the fruiting body and mycelia culture of this mushroom, including benzofuranone monoterpenoids with nitric oxide production (NO) inhibitory activity [8], bisablolene sesquiterpenes with PTP1B inhibitory activity [9], pleurospiroketal sesquiterpenes with NO inhibitory activity [10], cyclododecane skeleton diterpenoid and tetrahydrobenzofuran sesquiterpenoid with cytotoxicity against Hela and HepG2 cells [8], protein hydrolysates with antioxidant activity [11], protein with antitumor activity [12], polysaccharides with antitumor activity [13], mine elements with antioxidant and immunomodulatory activity [14], and sterol with osteomalacia- and rickets-prevented activity [15]. In the present research, we reported the isolation and structure elucidation of three new amino acid derivatives (**1–3**), along with nine known compounds (**4–12**) from the EtOAc extract of the solid culture of *P. ostreatus* (Figure 1). The new structures were elucidated by extensive spectroscopic methods. Finally, the absolute configurations of **1** and **2** were established by the chiral synthesis. All new compounds were evaluated for their antioxidant effects by 2,2-diphenyl-1-picryldrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazolin-6-sulfonic acid) (ABTS) free radical scavenging activities, antimicrobial activities against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*, and cytotoxicity against HL-60 cell line. Unfortunately, these compounds were found to be inactive.

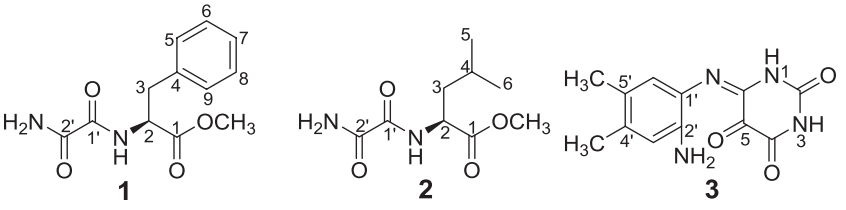


Figure 1. The structures of compounds **1–3**.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. Its HRESIMS exhibited a $[M + Na]^+$ at *m/z* 273.0858, corresponding to a molecular formula of $C_{12}H_{14}N_2O_4$. The infared absorption (IR) spectrum indicated the presence of an amino group at 3385, 3304 cm^{-1}, an ester carbonyl at 1744 cm^{-1}, and amide carbonyl at 1662 cm^{-1}. The ^1H NMR (nuclear magnetic resonance) and ^{13}C NMR spectra of **1** exhibited the presence of a mono-substituted benzene ring with signals at δ_H 7.18–7.29 (5H, m) and δ_C 137.3, 129.0 (×2), 128.3 (×2), 126.6, which were unambiguously confirmed by the 2D-NMR. And a methyl ester group with the signals at δ_H 3.65 (3H, s) and δ_C 52.1, 171.1 was observed. The ^{13}C NMR (100 MHz, DMSO-d_6) and heteronuclear single quantum coherence (HSQC) (Table 1) spectra showed 12 carbon signals, and the multiplicity of carbon signals was classified into nine sp2 carbons (δ 171.1, 161.4, 160.2, 137.3, 129.0, 129.0, 128.3, 128.3, 126.6, including three carbonyls, five methines, one quaternary), three sp3 carbons (53.6, 52.1, 35.7, including one methylene, one methine, and one methoxy) by analysis of HSQC data.

The HMBC (heteronuclear multiple bond correlation) correlations of structure **1** between H-3 (δ_H 3.10, 3.14) and C-1 (δ_C 171.1), C-2 (δ_C 53.6), C-4 (δ_C 137.3), C-5 (δ_C 129.0), between H-2 (δ_H 4.56, td) and C-1 (δ_C 171.1), C-3 (δ_C 35.7), C-4 (δ_C 137.3), C-1′ (δ_C 160.2), between OCH_3 (δ_H 3.65, s) and C-1 (δ_C 171.1), between NH (δ_H 8.86, d) and C-1′ (δ_C 160.2), as well as between NH_2 (δ_H 7.79, s) and C-1′ (δ_C 160.2) (Figure 2) permitted the identification of the phenylalanine structure unit. Meanwhile, these data indicated that the compound was an amino acid derivative with carboxylate group and 2-amino-2-oxacetyl group. Thus, the planar structure of **1** was deduced as shown in Figure 1. Despite its relative structural simplicity, difficulties were encountered during the determination of its absolute configuration using amide hydrolysis and comparing optical data method due to the limited yield. A straightforward and efficient method was developed to assign the stereochemical configuration. Optical pure *L*-amino acid derivatives were synthesized using the specific *L*-amino acid as initial materials. The final assignment of oxalamido-*L*-phenylalanine methyl

Table 1 ^1H and ^{13}C NMR spectral data for **1** and **2** in DMSO-d_6[a]

	1		**2**	
Position	δ_H, muti (*J* in Hz)		δ_H, muti (*J* in Hz)	
Phe				
1	171.1, C	–	172.0, C	–
2	53.6, CH	4.56, td (8.7, 5.7)	50.5, CH	4.33, td (8.4, 3.6)
3	35.7, CH_2	3.10, dd (13.9, 9.5); 3.14, dd (14.5, 5.9)	38.7, CH_2	1.51–1.53, m 1.76–1.86, m
4	137.3, C	–	24.3, CH	1.53–1.55, m
5	129.0, CH	7.19–7.21, m	21.0, CH_3	0.84, d (6.0)
6	128.3, CH	7.25–7.27, m	22.8, CH_3	0.88, d (6.0)
7	126.6, CH	7.19–7.21, m		
8	128.3, CH	7.25–7.27, m		
9	129.0, CH	7.19–7.21, m		
1′	160.2, C	–	160.5, C	–
2′	161.4, C	–	161.6, C	–
1-OCH_3	52.1, CH_3	3.65, s	52.0, CH_3	3.63, s
2-NH		8.86, d (8.4)		8.92, d (8.0)
2′-NH_2		8.03, s 7.79, s		8.10, s 7.85, s

[a]400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. Data were assigned based on the HSQC, HMBC, ^1H-^1H COSY, and NOESY experiments.

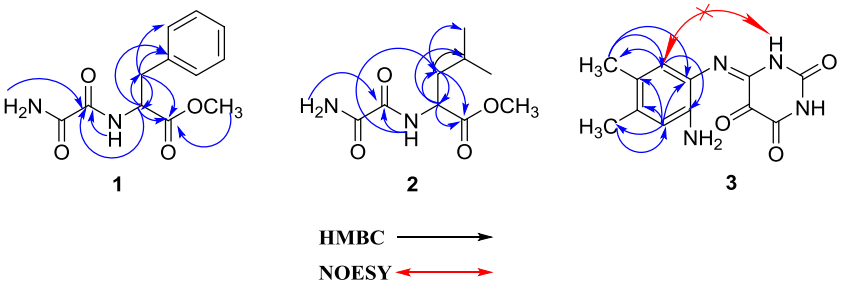


Figure 2. Key HMBC (blue) and NOESY (red) correlations of **1–3**.

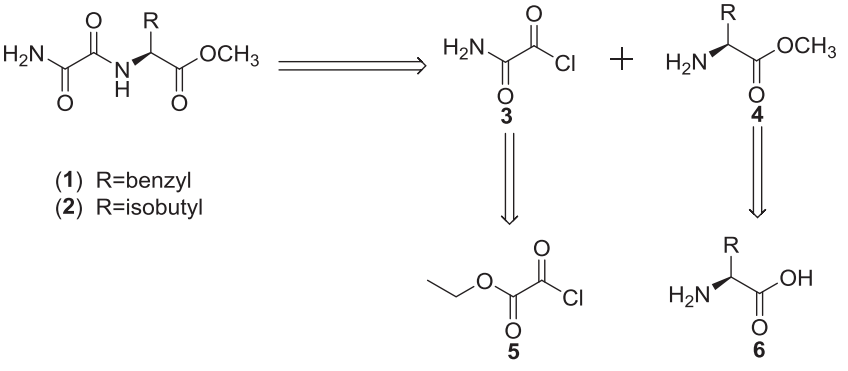
ester (**1**) was made by comparison of the circular dichroism (CD) spectrum of **1** with that of *L*-chiral synthetic product.

Compound **2** was obtained as a white amorphous powder. Its HRESIMS exhibited a $[M + H]^+$ at *m/z* 217.1150, $[M + Na]^+$ at *m/z* 239.1059, corresponding to a molecular formula of $C_9H_{16}N_2O_4$. The IR spectrum indicated the presence of an amino group at 3381, 3313 cm^{-1}, ester carbonyl at 1743 cm^{-1}, and amide carbonyl at 1667 cm^{-1}. Comparison of NMR data of **2** with that of **1** suggested both of them shared the same basic structure except for the amino acid residue (Table 1). The structure of **2** was elucidated by the analysis of 2D NMR data including the HSQC, HMBC and COSY spectra (Figure 2). In the HMBC spectrum, correlations from H-3 (δ_H 1.52, m and δ_H 1.81, m) to C-1 (δ_C 172.0), C-2 (δ_C 50.5), C-4 (δ_C 24.3), C-5 (δ_C 21.0), from H-2 (δ_H 4.33, td) to C-1 (δ_C 172.0), C-3 (δ_C 38.7) and C-4 (δ_C 24.3), from H-5 (δ_H 0.84, 3H, d) to C-2 (δ_C 50.5), C-3 (δ_C 38.7), C-4 (δ_C 24.3) and C-6 (δ_C 22.8), from $-OCH_3$ (δ_H 3.63, s) to C-1 (δ_C 172.0), from NH (δ_H 8.92, d) to C-1′ (δ_C 160.5), and from NH_2 (δ_H 7.85, s) to C-1′ (δ_C 160.5) afforded the leucine structure unit. Furthermore, the tert-butyl methine was confirmed by ^1H-^1H COSY correlation. The residues of compound **2** were the same as that of **1**. Consequently, the planar structure of **2** was identified as shown in Figure 1. Using the same synthetic strategy in **1**, the absolute configuration of **2** was identified as oxalamido-*L*-leucine methyl ester.

Compound **3** was obtained as yellow amorphous powder. Its HRESIMS exhibited $[M-H_2O + H]^+$ at *m/z* 243.0875 and $[M-H_2O + Na]^+$ at *m/z* 265.0684, corresponding to a molecular formula of $C_{12}H_{12}N_4O_3$, with 9° of unsaturation. The IR spectrum indicated the presence of an amino group at 3388, 3180 cm^{-1}, and the presence of ester carbonyl at 1707 cm^{-1} and amide carbonyl at 1580 cm^{-1}. Analysis of ^1H and ^{13}C NMR data of compound **3** showed the existence of two methyl singlets at δ_H 2.47, 2.49 and δ_C 19.6, 20.2, which were unambiguously confirmed by the HSQC spectrum. The HSQC spectrum of compound **3** showed two aromatic proton signals at δ_H 7.71, 7.92 and its carbon signals at δ_C 125.8, 128.7. Meanwhile, four amino group protons were deduced according to the HSQC spectrum (Table 2). Careful comparison of the ^1H and ^{13}C NMR data of **3** (Table 2) with those of known lumichrome [16] revealed that compound **3** is the hydrolyzate of lumichrome (Figure 1), which was confirmed by its two primary amine acute IR peaks at 3388 and 3180 cm^{-1}. Nitrogen double-bond configuration was determined as *E* relationship based on the NOESY (nuclear overhauser effect spectroscopy) crosspeaks. On the basis of the above evidence, the structure of **3** was established as (*E*)-6-((2-amino-4,5-dimethylphenyl)imino) dihydropyrimidine-2,4,5(3*H*)-trione.

Table 2. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data of **3** in DMSO-d_6.

No.	δ_H	δ_C	HMBC (^{1}H→^{13}C)
2	–	150.1, C	–
4	–	160.7, C	–
5	–	162.5, C	–
6	–	146.5, C	–
1′	–	141.6, C	–
2′	–	138.3, C	–
3′	7.92, s	128.7, CH	1′, 4′, 5′, 4′-$\underline{C}H_3$
4′	–	144.7, C	–
5′	–	138.9, C	–
6′	7.71, s	125.8, CH	2′
1-NH	11.7, s	–	–
3-NH	11.8, s	–	–
2′-NH	7.98, s	–	-
	7.69, s		–
4′-CH_3	2.47, s	19.6, CH_3	3′
5′-CH_3	2.49, s	20.2, CH_3	1′, 6′

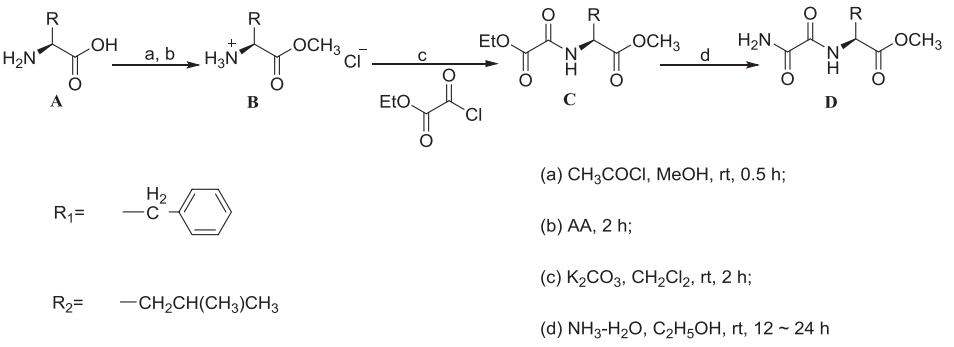


Scheme 1. Retrosynthetic analysis.

To verify absolute configurations of **1** and **2**, the total synthesis of **1** and **2** was carried out. Our retrosynthetic analysis of **1** and **2** was shown in Scheme 1. Compounds **1** and **2** were composed of 2-amino-2-oxacetyl chloride (**3**) and corresponding amino acid methyl ester (**4**). Amino acid methyl ester (**4**) could be prepared by the corresponding amino acid (**6**). Ethyl 2-chloro-2-oxoacetate (**5**), instead of 2-amino-2-oxacetyl chloride (**3**), was afforded to form a peptide bond because 2-amino-2-oxacetyl chloride (**3**), itself is very unstable.

To synthesize compound **1**, we firstly performed the condensation of *L*-phenylalamine methyl ester with ethyl 2-chloro-2-oxoacetate. And the target compound **1** was afforded by aminolysis of ethyl ester of **C** [17]. Similarly, we also have synthesized compound **2** by the same procedures (Scheme 2). The spectroscopic data of synthetic **1** and **2**, including ^{1}H NMR, ^{13}C NMR, circular dichroism, and some ESI mass spectra, almost matched the data originated for natural products **1** and **2**, respectively. Therefore, the absolute configurations of compounds **1** and **2** were established as oxalamido-*L*-phenylalanine methyl ester (**D1**), oxalamido-*L*-leucine methyl ester (**D2**), respectively (Figure 3).

The known compounds (**4–12**) were identified as uracil (**4**) [18], nicotinamide (**5**) [19], 1*H*-indole-2,3-dione (**6**) [20], 1*H*-indole-3-carboxaldehyde (**7**) [21], 1*H*-indole-3-carboxylic



Scheme 2. Synthesis of amino acid derivatives.

acid (**8**) [18], cyclo (Leu-Pro) (**9**) [22], uridine (**10**) [23], 2′-O-methyluridine (**11**) [18], and adenosine (**12**) [24] by comparing its NMR spectral data with those reported in the literatures.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter (Perkin-Elmer Co., Jena, Germany). Ultraviolet Visible (UV) spectra were measured on Shimadzu UV-1601 (Shimadzu, Tokyo, Japan). IR spectra were measured on a Bruker IFS-55 infrared spectrophotometer (Bruker Co., Zurich, Switzerland). The NMR spectral data were recorded on Bruker AV-400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) with tetramethylsilane (TMS) as the internal standard (Bruker Co.). The HR-ESI-MS data were obtained on the micro TOF-Q mass instrument (Bruker Co.). CD spectra were measured on Bio-logic MOS 450 (Bio-Logic, Paris, France). Chromatography was carried out on silica gel (200–300 mesh; Qingdao Haiyang Chemical Factory, Qingdao, China), Sephadex LH-20 (Pharmacia, Piscataway, NJ, USA), HPLC (high-performance liquid chromatography) (Shimadzu LC-8A vp, Kyoto, Japan), and Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA).

2,2-Diphenyl-1-picrylhudrazyl (DPPH·), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid, and *L*-amino acid were purchased from Sigma-Alddrich (St. Louis, MO, USA). All chemicals used for synthesis were analytical reagent grade with no further purification.

3.2. Fungal material

The strain of *Pleurotus ostreatus* used in this work was purchased from Institute of Edible Fungi, Shenyang Agricultural University, Liaoning, China, and deposited in School of Traditional Chinese Material Medica, Shenyang Pharmaceutical University, China. *P. ostreatus* strain was cultured on the Petri dish of potato dextrose agar at 25 °C for 10 days. Agar plugs were inoculated in 500-ml Erlenmeyer flasks containing 120 ml of medium (0.4% glucose, 1% malt extract, and 0.4% yeast extract), and the final pH of the medium

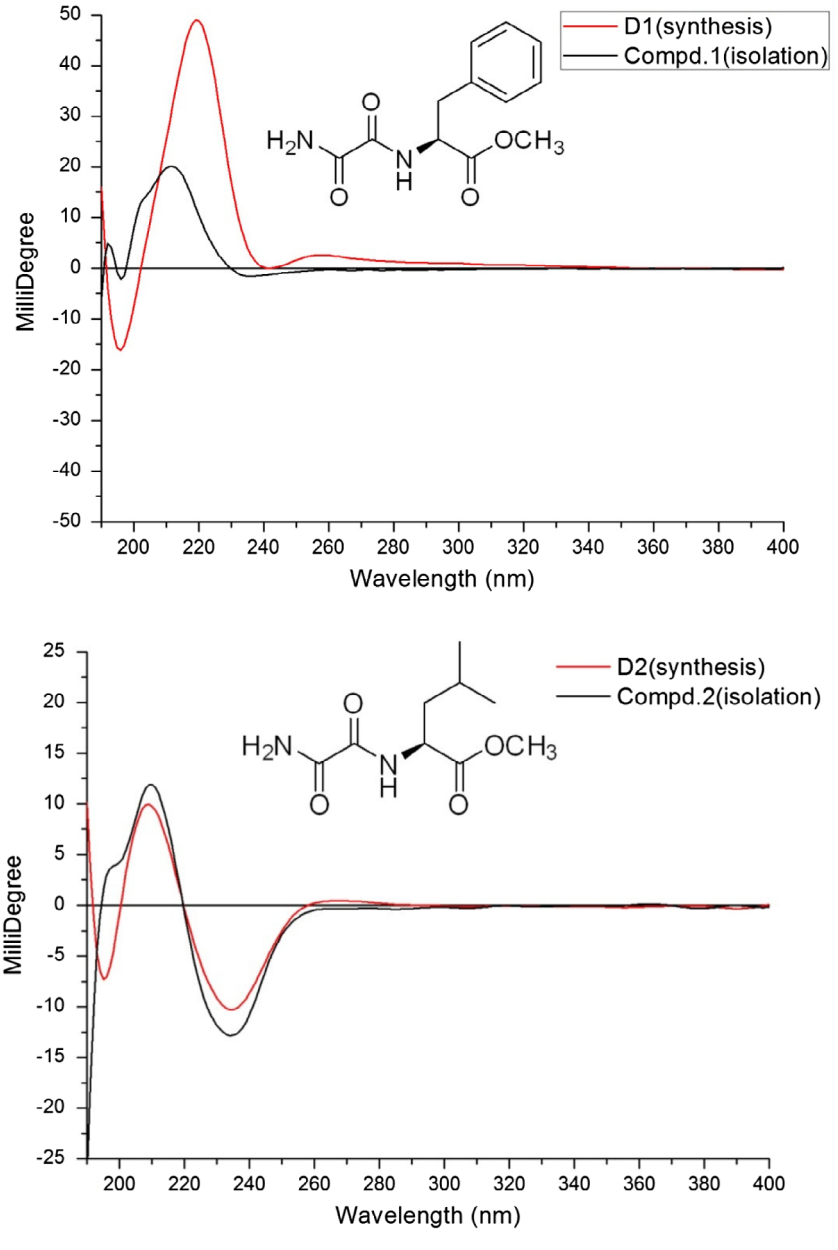


Figure 3. Isolated and synthetic ECD spectra of oxalamido-*L*-phenylalanine methyl ester and oxalamido-*L*-leucine methyl ester.

was adjusted to 6.5 before sterilization, and incubated at 25 °C on a rotary shaker at 170 rpm for 7 days. Large-scale fermentation was carried out in 263 of 500 ml Fernbach flasks, each containing 80 g of rice and 120 ml of distilled H_2O. Each flask was inoculated with 15.0–20.0 ml culture medium and incubated at 25 °C for 25 days.

3.3. Extraction and isolation

The fermented rice substrate in 263 flasks was soaked with exhaustive ethyl acetate (EtOAc) and extracted five times under ultrasonic. The combined organic solvent was evaporated to dryness under vacuum to afford the crude extracts (70.7 g). Similarly, *n*-BuOH extracts (137.7 g) were obtained using the same method. EtOAc extracts were subjected to sillica gel column chromatography using a stepwise solvent gradient method with CH_2Cl_2/MeOH (100:0→0:100, v/v) to give 12 fractions (Fr.1–Fr.12). Fr.1 (5 g) was subjected to CC on silica gel column (6 × 55 cm) using a stepwise solvent gradient method with petroleum ether (PE)/EtOAc (100:0–0:100) to give six subfractions (Fr.1.1–Fr.1.6). Fr.1.4 (400 mg) was chromatographed over Sephadex LH-20 (2.5 × 40 cm) eluted with MeOH, followed by semi-preparative HPLC (45% CH_3OH/H_2O, a flow rate at 3 ml/min) to yield compound **6** (50 mg, t_R 7.5 min). Fr.2 (15 g) was separated over silica gel CC (7.5 × 120 cm) eluting with CH_2Cl_2/MeOH (100:0–0:100) to yield eight subfractions (Fr.2.1–Fr.2.2). Fr.2.2 (1.7 g) was chromatographed over Sephadex LH-20 (4.5 × 45 cm) eluting with MeOH, followed by semi-preparative HPLC (30% CH_3OH/H_2O, a flow rate at 3 ml/min) to yield compound **7** (15 mg, t_R 31 min). Fr.2.3 (1.6 g) was also chromatographed over Sephadex LH-20 (4.5 × 45 cm) eluting with MeOH to yield six subfractions (Fr.2.3.1–Fr.2.3.6). Fr.2.3.4 (300 mg) was purified by HPLC (27% CH_3OH/H_2O, a flow rate at 3 ml/min) to give compound **8**. Fr.2.3.5 (200 mg) was separated over semi-preparative HPLC (33% CH_3OH/H_2O, a flow rate at 3 ml/min) to yield compound **1** (2.0 mg, t_R 63.4 min). Fr.3 (2.5 g) was also purified by Sephadex LH-20 (MeOH), followed by semi-preparative HPLC (36% CH_3OH/H_2O, a flow rate 3 ml/min) to yield compound **2** (4.5 mg, t_R 29.5 min). Compound **10** (6.4 mg) was obtained by recrystallization after Sephadex LH-20 (MeOH). The *n*-BuOH extracts were also subjected to silica gel column chromatography eluted with a solvent gradient of CH_2Cl_2/MeOH (100:0→100:100, v/v) to give six fractions (Fr.1–Fr.6). Fr.1 was subjected to silica gel column eluting with PE/EtOAc (100:0–0:100) to yield six subfractions (Fr.1–Fr.6). Fr.6 was chromatographed over Sephadex LH-20 (CH_2Cl_2/MeOH, 1:1), followed by semi-preparative HPLC (24% MeOH/H_2O, a flow rate 3 ml/min) to give compound **9** (5.1 mg, t_R 63 min). Fr.2 (4.9 g) was separated on silica gel CC, eluted with petroleum ether/acetone (100:2–100:50) to give four subfractions (Fr.2.1–Fr.2.4). Subfraction Fr.2.3 was subjected to Sephadex LH-20 eluting with MeOH to give four subfractions (Fr.2.3.1–Fr.2.3.4). Compound **3** (3.0 mg) was obtained from subfraction Fr.2.3.4 by recrystallization. Fr.3 (10 g) was subjected to CC on silica gel column (6 × 60 cm) eluting with CH_2Cl_2/MeOH (100:0–0:100) to yield seven subfractions (Fr.3.1–Fr.3.7). Fr.3.4 (1.6 g) was chromatographed over Sephadex LH-20 (4.5 × 45 cm) eluting with MeOH to give four subfractions (Fr.3.4.1–Fr.3.4.4). Fr.3.4.2 (247 mg) was purified by semi-preparative HPLC (10% CH_3OH/H_2O, a flow rate at 3 ml/min) to yield compounds **5** (53 mg, t_R 20 min) and **11** (5.7 mg, t_R 26.5 min). Compound **4** (6.0 mg) was obtained by recrystallization from Fr.3.4.4. Fr.4 (30 g) was applied to silica gel CC eluting with CH_2Cl_2/MeOH (100:0–0:100) to yield six subfractions (Fr.3.1–Fr.3.6). Fr.3.5 (5 g) was subjected to Sephadex LH-20 eluting with MeOH to yield six subfractions (Fr.3.5.1–Fr.3.5.6). Compound **12** (100 mg) was obtained by recrystallization after gel permeation chromatography on Sephadex LH-20 eluted with MeOH.

3.3.1. Oxalamido-L-Phenylalamine methyl ester (1)

White amorphous powder; $[\alpha]_D^{25}$ +50 (*c* 0.02, MeOH). UV (MeOH) λ_{max} (log ε) 205 (3.53) nm; IR (KBr) ν_{max} (cm^{-1}): 3385, 3304, 2956, 2925, 1744, 1662, 1605, 1541; ^1H and ^{13}C NMR spectral data are shown in Table 1; HR-ESI-MS (positive) *m/z*: 273.0858 $[M + Na]^+$ (calcd for $C_{12}H_{14}N_2O_4Na$, 273.0846).

3.3.2. Oxalamido-L-leucine methyl ester (2)

White amorphous powder; $[\alpha]_D^{25}$ +22.2 (*c* 0.045, MeOH); UV (methanol) λ_{max} (log ε) 209 (3.44) nm; IR (KBr) ν_{max}(cm^{-1}): 3381, 3313, 2956, 2927, 2855, 1743, 1667, 1208, 1025, 802; ^1H and ^{13}C NMR and 2D NMR spectral data were shown in Table 1; HR-ESI-MS (positive) *m/z*: 217.1150 $[M + H]^+$ (calcd for $C_9H_{17}N_2O_4$, 217.1183), 239.1059 $[M + Na]^+$ (calcd for $C_9H_{16}N_2O_4Na$, 239.1002).

3.3.3. (E)-6-((2-Amino-4,5-dimethylphenyl)imino)dihydropyrimidine-2,4,5(3H)-trione (3)

Yellow amorphous powder; UV (MeOH) λ_{max} (log ε) 206 (2.97), 246 (2.57), 258 (2.56) nm; IR (KBr) ν_{max}(cm^{-1}): 3388, 3180, 3000, 2923, 2854, 2805, 1707, 1580, 1362, 1345, 1005; ^1H and ^{13}C NMR and 2D NMR data were shown in Table 2, HR-ESI-MS (positive) m/z: 243.0875 $[M–H_2O + H]^+$ (calcd for $C_{12}H_{13}N_2O_2$, 243.0877), 265.0684 $[M–H_2O + Na]^+$ (calcd for $C_{12}H_{12}N_4O_3Na$, 265.0696).

3.4. Synthetic experiment

3.4.1. General procedure for the synthesis of intermediates B1–B2

At the room temperature, 10-ml acetyl chloride was slowly added dropwise to 40 ml of methanol. After the mixture was cooled to r.t., *L*-amino acid hydrochloride was added. The reaction was stirred for 30 min, and then solutions were concentrated under reduced pressure to give desired *L*-amino acid methyl ester hydrochloride as white solid in almost quantitative yields. No further purification was required unless indicated as below.

3.4.2. General procedure for the synthesis of intermediates C1–C2

To a slurry of *L*-amino acid methyl ester hydrochloride substrate in 20–30 volumes of dry dichloromethane at room temperature, three equivalents of anhydrous potassium carbonate was added followed by a solution of 1.1 equivalents of commercially available ethyl 2-chloro-2-oxoacetate by dropwise. Reaction mixture was slowly stirred at room temperature for 2 h. After completion of the reaction monitored by TLC, the residue was quenched with water and extracted twice with CH_2Cl_2. The merged organic layer was washed three times with saturated brine and dried over anhydrous $MgSO_4$ for 30 min, then filtered and concentrated under reduced pressure to afford intermediate **C** as yellow oil with good yields. No further purification was required unless indicated as below.

Methyl (2-ethoxy-2-oxoacetyl) -L-phenylalaninate (**C1**): light yellow oil; ^1H-NMR (400 MHz, DMSO-d_6): δ 9.26 (1H, d, *J* = 8.0 Hz, 2-NH), 7.29–7.19 (5H, m, H-5–H-9), 4.56 (1H, ddd, *J* = 9.8, 8.0, 5.1 Hz, H-2), 4.21 (2H, qd, *J* = 7.1, 1.6 Hz, 2′-OC<u>H</u>$_2$CH$_3$), 3.64 (3H, s, 1-OCH$_3$), 3.14 (1H, dd, *J* = 13.9, 5.1 Hz, H-3a), 3.06 (1H, dd, *J* = 13.9, 9.8 Hz, H-3b), 1.24 (3H, t, *J* = 7.1 Hz, 2′-OCH$_2$C<u>H</u>$_3$).

Methyl (2-ethoxy-2-oxoacetyl) -L-leucinate (**C2**): light yellow oil; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.24 (1H, d, J = 8.0 Hz, 2-NH), 4.35 (2H, ddd, J = 10.7, 8.0, 4.0 Hz, H-2), 4.25 (2H, q, J = 7.1 Hz, 2′-OC$\underline{H}_2$CH$_3$), 3.64 (3H, s, 1-OCH$_3$), 1.76 (1H, td, J = 11.0, 4.0 Hz, H-3a), 1.61–1.52 (2H, m, H-3b, H-4), 1.28 (3H, t, J = 7.1 Hz, 2′-OCH$_2$C$\underline{H}_3$), 0.89 (3H, d, J = 6.4 Hz, H-5 or 4-CH$_3$), 0.85 (3H, d, J = 6.2 Hz, 4-CH$_3$ or H-5).

To a solution of intermediate **C** in five volumes of ethanol cooled at 0 °C, 2–10 equivalents of aq. ammonia (14.8 N) were added. Reaction mixture was slowly warmed up to room temperature overlight. Concentration under reduced pressure gave target product as white solid in good yields. Furthermore, the residue was purified by column chromatography on silica gel (3:1 PE/EA) and HPLC (35% Methol-H$_2$O) to give desired product as white powder.

Oxalamido-L-phenylalanine methyl ester (**D1**): white powder; CD (c 2.00 mM, MeOH) $\Delta\varepsilon_{196\,nm}$ − 2.45, $\Delta\varepsilon_{219\,nm}$ + 7.43, $\Delta\varepsilon_{258\,nm}$ + 0.394; The ^{1}H-NMR spectral data were same as compound **1**: δ 8.87 (1H, d, J = 8.5 Hz, 2-NH), 8.03 (1H, s, 2′-NH$_2$), 7.80 (1H, s, 2′-NH$_2$), 7.29–7.18 (5H, H-5–H-9), 4.56 (1H, dt, J = 8.5, 5.4 Hz, H-2). 3.65 (3H, s, 1-OC$\underline{H}_3$), 3.15 (1H, dd, J = 13.7, 5.4 Hz, H-3a), 3.11 (1H, dd, J = 13.7, 5.4 Hz, H-3b).

Oxalamido-L-leucine methyl ester (**D2**): white powder; CD (c 2.30 mM, MeOH) $\Delta\varepsilon_{196\,nm}$ − 0.95, $\Delta\varepsilon_{209\,nm}$ + 1.31, $\Delta\varepsilon_{234\,nm}$ − 1.35; The ^{1}H-NMR spectral data were same as natural isolates: δ 8.91 (1H, d, J = 8.4 Hz, 2-NH), 8.10 (1H, s, 2′-NH$_2$), 7.85 (1H, s, 2′-NH$_2$), 4.33 (1H, ddd, J = 8.4, 3.9, 3.0 Hz, H-2), 3.63 (3H, s, 1-OC$\underline{H}_3$), 1.84–1.78 (1H, m, H-3a), 1.59–1.51 (2H, m, H-3a, H-4), 0.88 (3H, d, J = 6.2 Hz, H-5 or 4-C$\underline{H}_3$), 0.84 (3H, d, J = 6.0 Hz, 4-C$\underline{H}_3$ or H-5).

3.5. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH·) scavenging assay

The DPPH· scavenging activity was assessed according to the described method with minor modifications [25], using lower DPPH concentration (0.2 mM) and different sample to DPPH ratio (1:1). To a 100 µl aliquot of the sample with different concentrations, 100 µl of DPPH solution (0.2 mM) was added in a 96-well microplate. The mixture was shaken vigorously and incubated in darkness for 30 min. The absorbance of the reaction solution at 517 nm was recorded using a varioskan flash multimode reader. Ascorbic acid was used as a positive control. The percentage of scavenging DPPH versus concentration was plotted using the following equation: DPPH scavenging activity (%) = $[1 - (S - S_B)/(C - C_B)] \times 100\%$, where S, S_B, C, and C_B are the absorption of the sample, the blank sample, the control and the blank control, respectively. All experiments were tested in triplicate.

3.6. 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) free radical ($ABTS^+$) scavenging assay

The determination of $ABTS^+$ scavenging was carried out on the basis of the previous method with minor modifications [25]. ABTS radical cations ($ABTS^+$) were obtained by reacting 7 mM stock solution of $ABTS^+$ with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 14 h before use. The $ABTS^+$ solution was diluted with ethanol to the absorbance of 0.70 ± 0.05 at 734 nm. To a 100 µl aliquot of the sample with different concentrations, 150 µl $ABTS^+$ solution in a 96-well microplate was added. After reacting at room temperature for 30 min, the absorbance was recorded using varioskan flash multimode reader at 734 nm. The percentage of $ABTS^+$ scavenging activity

was calculated using the same equation in DPPH assay. Similarly, all experiments were carried out in triplicate, and ascorbic acid was used as positive control.

3.7. Antimicrobial bioassay

The minimum inhibitory concentration (MIC) values for all compounds were determined by the dilution method. For sample preparation, each of the test compounds was dissolved in DMSO and then diluted with sterile broth to a concentration of 500 μg/ml. Further dilutions of the compounds in the test medium were prepared at the required quantities of 250, 125, etc. down to 3.9 μg/ml. Chloramphenicol and fluconazole were used as positive controls for bacteria and fungus, respectively. The *in vitro* antimicrobial activity of the compounds was tested by tube-dilution technique using individually packaged, flat bottomed, 96-well microtitre plates (NCCLS. 2000). Bacterial strains were maintained on LB medium for 48 h at 37 °C and fungal strains were on PDA medium for 48 h at 28 °C. The cell density for bacteria was 2–4 × 10^7 CFU/ml and 2–4 × 10^5 CFU/ml for fungus. A serial dilution of compounds were performed in the microplates and incubated for 12 h. The last tube with no growth of micro-organism was recorded to represent the MIC value expressed in μg/ml.

3.8. Cytotoxicity assay

Antitumor activity of **1–3** was evaluated by the MTT assay against HL-60 cell lines. HL-60 cell (American Type Culture Collection) was maintained in RPMI-1640 medium (Gibco) with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1 mmol/L L-glutamine, and 10% (v/v) heat-inactivated FBS in a humidified atmosphere of 5% CO_2 at 37.0 °C. Logarithmic phase cells were used for experiments. Appropriate dilutions of the test samples were added to the cultures. The growth inhibition was calculated comparing with control cells. 5-Fluorouracil was used as a positive control.

3.9. Antioxidation of compounds 1–3

Antioxidant activities of compounds **1–3** were evaluated by their ability to scavenge DPPH and ABTS radicals. All compounds with IC_{50} (50% inhibitory concentration) values of above 200 μM were inactive in the DPPH radical scavenging assay and ABTS assay, comparable with that of the standard compound ascorbic acid with an IC_{50} value of 10.2 μM.

3.10. Antibacterial and antifungal activity of compounds 1–3

The antimicrobial activities of compounds **1–3** were assessed for *B. subtilis*, *S. aureus*, *E. coli* and *Candida albicans*. None of these compounds exhibited antibacterial activities against *B. subtilis*, *S. aureus* and *E. coli*. Compounds **1–3** showed weak antifungal activities against *C. albicans* with MIC value of 500 μg/ml. Fluconazole was used as positive control against *C. albicans* (SC5314) with MIC value of 500 μg/ml.

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Disclosure statement

The authors declare no competing financial interest.

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