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•Original article•

Steroids and dihydroisocoumarin glycosides from *Xylaria* sp. by the one strain many compounds strategy and their bioactivities

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[ABSTRACT] The fungus *Xylaria* sp. KYJ-15 was isolated from *Illigera celebica*. Based on the one strain many compounds (OSMAC) strategy, the strain was fermented on potato and rice solid media, respectively. As a result, two novel steroids, xylarsteroids A (**1**) and B (**2**), which are the first examples of C_{28}-steroid with an unusual *β*- and *γ*-lactone ring, respectively, along with two new dihydroisocoumarin glycosides, xylarglycosides A (**3**) and B (**4**), were identified. Their structures were elucidated by spectroscopic methods, X-ray diffraction and electronic circular dichroism (ECD) experiments. All isolated compounds were evaluated for cytotoxicity, DPPH radical scavenging activity, acetylcholinesterase inhibitory and antimicrobial effect. Compound **1** exhibited potent AChE inhibitory activity with an IC_{50} value of 2.61 ± 0.05 μmol·L^{-1}. The *β*-lactone ring unit of **1** is critical for its AChE inhibitory activity. The finding was further confirmed through exploring the interaction of **1** with AChE by molecular docking. In addition, both compounds **1** and **2** exhibited obvious antibacterial activity against *Bacillus subtilis* with a minimum inhibitory concentration (MIC) of 2 μg·mL^{-1}. Compounds **3** and **4** exhibited antibacterial activities against *Staphylococcus aureus* with MICs of 4 and 2 μg·mL^{-1}, respectively, which also exhibited DPPH radical scavenging activity comparable to the positive control with IC_{50} values of 9.2 ± 0.03 and 13.3 ± 0.01 μmol·L^{-1}, respectively.

[KEY WORDS] *Xylaria* sp.; OSMAC strategy; Secondary metabolite; Bioactivity
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Introduction

Microbial secondary metabolites are widely considered to be a major source of lead compounds for drug development [1]. However, many of the biogenetic gene clusters that encoding these secondary metabolites are silent under standard laboratory conditions [2-4]. Recently, new approaches have been developed to improve the diversity of metablites by activating the silenced genes. The one strain many compounds (OSMAC) strategy is a simple and effective approach, which aims to diversify medium and culture conditions, induce the expression of silent biological gene clusters, and enrich metabolites [5]. For instance, a series of azaphilones were successfully isolated from fungi by the OSMAC strategy, cocultivation, and epigenetic modification [6-8].

Steroids are the old class of natural products and ubiquitously exist in the kingdoms of animals, plants, and fungi, which exhibit diverse biological functions and have many potential biomedical applications [9-11]. So far, more than 100 steroidal drugs have been approved by the FDA for clinical treatment of various diseases, such as inflammatory diseases, cancer, and heart failure [12]. Therefore, further discovery of natural steroids with novel frameworks and strong biological activity has aroused great interest among chemists and pharmacologists [13-21].

Our group has been working on the discovery of structurally interesting and biologically significant natural products from the genus *Illigera* and its endophytes [22-24]. For example, a chemical investigation of secondary metabolites of *Xylaria* sp. KYJ-15 isolated from *Illigera celebica* was conducted. Inspired by the OSMAC strategy, this strain was fermented with two media (potato and rice solid media). As a result, fermentation of the fungus on potato and rice solid media yielded two novel steroids [xylarsteroids A (**1**) and B (**2**)] and

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two new dihydroisocoumarin glycosides [xylarglycoside A (**3**) and B (**4**)], respectively (Fig. 1). Both xylarsteroids A (**1**) and B (**2**) were the first examples of C_{28}-steroid with an unusual *β*- and *γ*-lactone ring, respectively. All isolated compounds were evaluated for cytotoxicity, DPPH radical scavenging activity, acetylcholinesterase (AChE) inhibitory and antimicrobial effect. Interestingly, the AChE inhibitory activity of compound **1** was significantly better than that of compound **2**, which indicated that the *β*-lactone ring unit of **1** was the main factor for enhancing its activity. This result was further supported by molecular docking. Herein, the details of isolation, structural characterization and bioactivities for all compounds and molecular docking study of **1** were described.

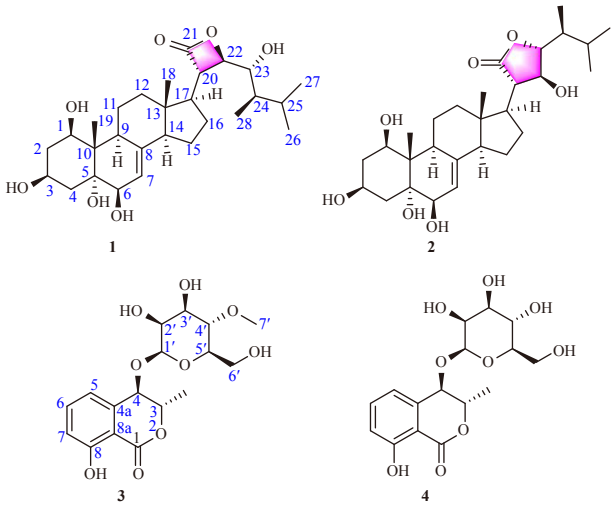

Fig. 1 Structures of compounds 1–4

Results and Discussion

Compound **1** was isolated as colorless crystals. Its molecular formula was determined to be $C_{28}H_{44}O_7$ by positive HR-ESI-MS (*m/z* 515.2976 $[M + Na]^+$, Calcd. for 515.2979), indicating 7 degrees of unsaturation. The ^1H and ^{13}C NMR spectra of **1** showed five methyls [C-18 (δ_C 12.3, δ_H 0.73), C-19 (δ_C 10.6, δ_H 1.05), C-26 (δ_C 20.2, δ_H 0.97), C-27 (δ_C 17.0, δ_H 0.92), C-28 (δ_C 8.6, δ_H 0.95)], six methylenes [C-2 (δ_C 40.5, δ_H 1.56, 1.98), C-4 (δ_C 39.1, δ_H 1.68, 2.21), C-11 (δ_C 24.5, δ_H 1.58, 2.34), C-12 (δ_C 38.0, δ_H 1.27, 2.25), C-15 (δ_C 23.1, δ_H 1.69), C-16 (δ_C 26.6, δ_H 1.47, 1.98)], twelve methines [C-1 (δ_C 73.8, δ_H 3.96), C-3 (δ_C 64.8, δ_H 4.04), C-6 (δ_C 73.2, δ_H 3.57), C-7 (δ_C 117.3, δ_H 5.33), C-9 (δ_C 44.5, δ_H 2.24), C-14 (δ_C 54.2, δ_H 2.04), C-17 (δ_C 54.6, δ_H 3.52), C-20 (δ_C 49.7, δ_H 2.02), C-22 (δ_C 76.2, δ_H 4.31), C-23 (δ_C 72.3, δ_H 3.75), C-24 (δ_C 40.6, δ_H 1.46) and C-25 (δ_C 29.1, δ_H 1.76)], and four quaternary carbons [C-5 (δ_C 75.8), C-8 (δ_C 42.8), C-10 (δ_C 42.2), C-13 (δ_C 43.5)], which were similar to those of stigmasta-7,22-dien-3*β*,5*α*,6*α*-triol [25], indicating that **1** was also a steroid (Table 1).

The correlations observed in the ^1H-^1H COSY spectrum indicated the presence of four independent spin systems (C1-C4, C6-C7, C9-C12, C14-C28) in **1** (Fig. 2). The HMBC correlations from H-4 to C-6, from H-6 to C-8 and C-10, from H-10 to C-3, from H-7 to C-9 and C-14, from H-11 to C-8 and C-13, from H-15 to C-8 and C-13, from H-18 to C-12 and C-17 and from H-19 to C-1, C-5 and C-9 revealed the same carbon skeleton as that of stigmasta-7,22-dien-3*β*,5*α*,6*α*-triol [25]. Furthermore, the HMBC correlations from H-17 and H-22 to C-21 (δ_C 171.4) and the remaining one degree unsaturation of **1** indicated that a *β*-lactone ring was connected between C-20 and C-22, and the chemical shifts of C-1 (δ_C 73.8), C-3 (δ_C 64.9), C-5 (δ_C 75.9), C-6 (δ_C 73.2) and C-23 (δ_C 72.3) indicated that these corresponding positions were all substituted by hydroxyl groups. Thus, the planar structure of **1** was elucid-

Table 1 ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectral data in CD_3OD of compounds 1–2

No.	1 (in CD_3OD)		2 (in CD_3OD)	
	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type
1	3.96, m	73.8, CH	3.93, m	73.8, CH
2*α*	1.98, m	40.5, CH_2	1.95, m	40.4, CH_2
2*β*	1.56, m		1.53, m	
3	4.04, m	64.8, CH	4.01, m	64.8, CH
4*α*	2.12, m	39.1, CH_2	2.10, m	39.0, CH_2
4*β*	1.68, m		1.64, m	
5		75.8, C		75.8, C
6	3.57, d (5.4)	73.2, CH	3.55, d (5.6)	73.2, CH
7	5.33, d (5.4)	117.3, CH	5.30, d (5.4)	117.1, CH
8		142.8, C		143.0, C
9	2.24, m	44.5, CH	2.20, m	44.6, CH
10		42.2, C		42.1, C
11*α*	1.58, m	24.5, CH_2	1.55, m	24.5, CH_2
11*β*	2.34, m		2.31, m	
12*α*	2.25, m	38.0, CH_2	2.31, m	37.8, CH_2
12*β*	1.27, m		1.55, m	
13		43.5, C		43.5, C
14	2.04, m	54.2, CH	2.00, m	54.3, CH
15	1.69, m	23.1, CH_2	1.65, m	22.6, CH_2
16*α*	1.47, m	26.6, CH_2	1.67, m	24.7, CH_2
16*β*	1.98, m		1.87, m	
17	3.52, dd (3.5, 10.7)	54.6, CH	2.60, dd (5.5, 7.5)	49.8, CH
18	0.73, s	12.3, CH_3	0.74, s	11.9, CH_3
19	1.05, s	10.6, CH_3	1.03, s	10.7, CH_3
20	2.02, m	49.7, CH	1.91, m	50.4, CH
21		171.3, C		177.8, C
22	4.31, dd (3.6, 5.9)	76.2, CH	4.11, t (5.0)	73.6, CH
23	3.75, t (5.8)	72.3, CH	4.01, m	87.6, CH
24	1.46, m	40.6, CH	1.63, m	42.2, CH
25	1.76, m	29.1, CH	1.99, m	27.5, CH
26	0.97, d (6.8)	20.2, CH_3	0.96, d (7.0)	19.7, CH_3
27	0.92, d (6.9)	17.0, CH_3	0.87, d (6.8)	15.1, CH_3
28	0.95, d (6.9)	8.6, CH_3	0.91, d (7.1)	8.2, CH_3

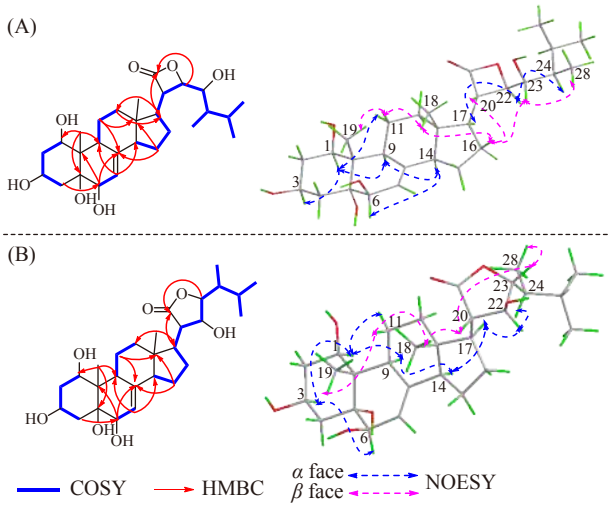

Fig. 2 Key 2D NMR correlations of 1 (A) and 2 (B)

ated (Fig. 1).

In the NOESY spectrum of **1**, the correlations of H-3/H-1/H-9/H-11α, H-9/H-14/H-6, H-17/H-22/H-24, H-19/H-11β/H-18/H-16β/H-20 and H-16β/H-23/H-28 suggested that H-1, H-3, H-6, H-9, H-14, H-17, H-22 and H-24 were on the same side, while H-18, H-19, H-20 and H-23 were on the other side. However, the configuration of OH-5 was not assigned. Fortunately, high-quality crystals of **1** were obtained and characterized by X-ray diffraction with Cu Kα radiation. The X-ray diffraction result (Fig. 3) not only confirmed the planar structure and relative configuration of **1**, but also unambiguously determined its absolute configuration as 1*R*, 3*S*, 5*R*, 6*R*, 9*S*, 10*S*, 13*R*, 14*R*, 17*R*, 20*S*, 22*R*, 23*R*, 24*R* with a small Flack parameter of 0.00(11) (CCDC 2160472). Thus, the structure of compound **1** was revealed and named xylarsteroid A.

Compound **2** was isolated as a white solid. The HR-ESI-MS data indicated a molecular formula of $C_{28}H_{44}O_7$ (*m/z* 515.2979 $[M + Na]^+$, Calcd. for 515.2979). The ^1H and ^{13}C NMR data (Table 1) of **2** closely resembled the data of **1**, which indicated that they shared an identical carbon skeleton. A detailed comparison of their 2D NMR data showed that the only difference was the presence of a γ-lactone ring between C-20 and C-23 in **2**, which was proved by the key HMBC interaction from H-23 to C-21 (δ_C 177.8) (Fig. 2). The NOESY correlations (Fig. 2) of H-1/H-3/H-6, H-11α/H-1/H-9/H-14/H-17/H-22/H-24, H-11β/H-19, and H-11β/H-18/H-20/H-23/H-28 indicated that H-1, H-3, H-6, H-9, H-14, H-17, H-22 and H-24 are α-oriented, while H-18, H-19, H-20, H-22, H-23 and H-24 are β-oriented. Therefore, the relative configuration of **2** was assigned as 1R^*, 3S^*, 6R^*, 9S^*, 10S^*, 13R^*, 14R^*, 17R^*, 20S^*, 22S^*, 23R^*, 24S^*. The absolute configuration of the remaining OH-5 and other chiral centers except C-22 and C-24 should be the same as those of **1** by biosynthetic consideration and comparison of NMR data of **1** and **2**. The configurations of C-22 and C-24 in compounds **1** and **2** are different only because their α-lactone and β-lactone lead to a change in the priority of the groups around the chiral centers C-22 and C-24, while the spatial orientations of their chiral centers are unchanged. This assignment was further supported by the very similar experimental ECD curves of **1** and **2** (Fig. 3). Finally, the absolute configuration of **2** was determined to be 1*R*, 3*S*, 5*R*, 6*R*, 9*S*, 10*S*, 13*R*, 14*R*, 17*R*, 20*S*, 22*S*, 23*R*, 24*S*. Thus, the structure of compound **2** was revealed and named xylarsteroid B.

Compound **3** was isolated as colorless needles. Its molecular formula, $C_{17}H_{22}O_9$ (*m/z* 393.1175 $[M + Na]^+$, Calcd. for 393.1156), with 7 degrees of unsaturation, was determined by HR-ESI-MS data. The ^1H and ^{13}C NMR spectra of **3** revealed the presence of 17 carbons, which were assigned as

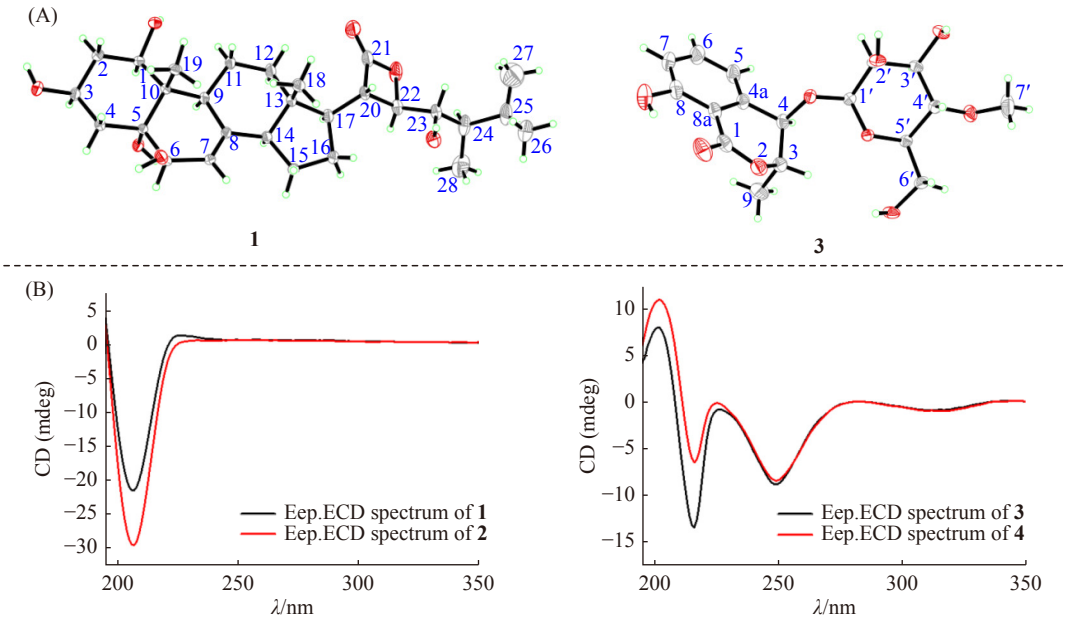

Fig. 3 X-ray crystal structures of 1 and 3 (A), and experimental ECD spectrum of 1–4 (B)

an ester carbonyl (δ_C 167.9), one methyl group (δ_C 16.5; δ_H 1.32), five methines including three aromatic (δ_C 120.4, 136.2, 118.3; δ_H 7.05, 7.61, 7.07) and two oxygenated (δ_C 79.6, 71.6; δ_H 5.12, 4.98), and three aromatic quaternary carbons (δ_C 135.4, 161.9, and 107.2), in addition to a glycosyl group (δ_C 96.6, 71.1, 73.8, 77.1, 76.2, 61.3 and 59.6; δ_H 4.34, 3.69, 3.45, 3.28, 3.20, 3.92, 3.76 and 3.55). The 1D NMR data of dihydroisocoumarin moiety of **3** closely resembled those of 7-hydroxymellein [26]. The 1D and 2D NMR data of **3** (Fig. 4) further confirmed that the planar structure of the dihydroisocoumarin moiety was the same as that of 7-hydroxymellein [26]. In addition, the structure of the sugar residue as well as its location at C-4 were determined according to the 1D and 2D NMR data. The relative configuration of **3** was identified by analysis of its NOESY data (Fig. 4). Finally, the single-crystal X-ray diffraction analysis using Cu Kα radiation was performed for **3**, and the absolute configuration of **3** possessing a 4-methoxy-β-D-mannopyranose was unequivocally determined to be 3*S*, 4*R* (Fig. 3). Thus, the structure of compound **3** was revealed and named xylarglycoside A.

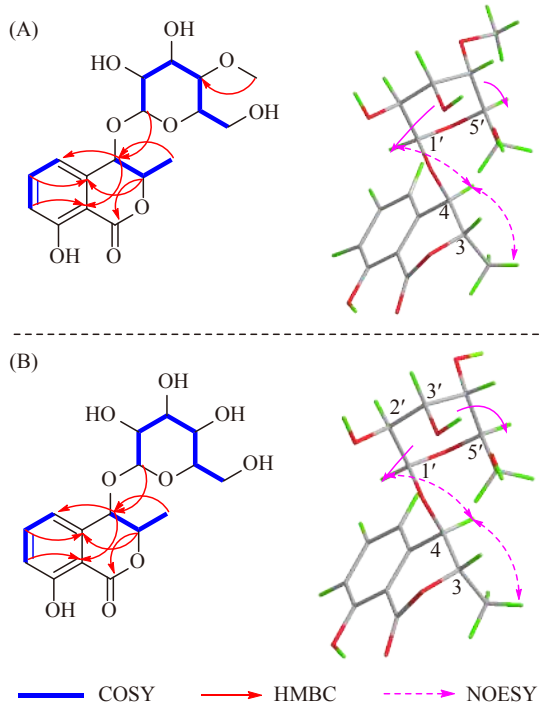

Fig. 4 Key 2D NMR correlations of 3 (A) and 4 (B)

Compound **4** has a molecular formula of $C_{16}H_{20}O_9$, which was confirmed by the HR-ESI-MS ion at *m/z* $[M + Na]^+$ 379.1000 (Calcd. for $C_{16}H_{20}O_9$ $[M + Na]^+$, 379.1000), with 7 degrees of unsaturation. A detailed comparison of the 1D NMR data for **4** (Table 2) with those of **3**, and their difference was that the C-4' position of the sugar moiety of **4** was substituted with a hydroxy group rather than an oxymethyl group, which was further supported by the NMR data of **4** (Table 2 and Fig. 4). The carbon signals at δ_C 98.4, 71.9, 73.3, 72.9, 70.0, and 60.8 were assigned to the hexose moiety. A detailed comparison of the 1D NMR data of the sugar moieties of **3** and **4** suggested that the glucose residue of **4** was also in β-configuration. This assignment was further supported by a NOESY correlation (Fig. 4) between H-1′ and H-5′. Additionally, both **3** and **4** showed positive optical rotations [compound **3**: $[a]_D^{20}$: +5.1 (*c* = 0.11, MeOH) and **4**: $[a]_D^{20}$: +5.3 (*c* = 0.22, MeOH)]. The experimental ECD curves (Fig. 3) of **3** and **4** were almost the same, which suggested that the configurations of their corresponding chiral centers were identical. Through the above analysis and biosynthetic considerations, compound **4** was accompanied by an β-D-mannopyranose, and the absolute configuration of the aglycone moiety was determined to be 3*S*, 4*R*. Thus, the structure of compound **3** was revealed and named xylarglycoside B.

Table 2 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data in CD_3OD of compounds 3–4

No.	3 (in CD_3OD)		4 (in CD_3OD)	
	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type
1		167.9, C		168.1, C
3	5.12, m	79.6, CH	5.04, m	78.4, CH
4	4.98, d (2.1)	71.6, CH	4.65, d (1.9)	73.6, CH
4a		135.4, C		137.0, C
5	7.05, d (7.3)	120.4, CH	7.01, d (7.2)	120.7, CH
6	7.61, t (7.5)	136.2, CH	7.49, t (7.4)	136.7, CH
7	7.07, d (8.4)	118.3, CH	6.92, d (8.4)	117.9, CH
8		161.9, C		162.3, C
8a		107.2, C		107.4, C
9	1.32, d (6.8)	16.5, CH_3	1.22, d (6.8)	16.8, CH_3
1′	4.34, br s	96.6, CH	5.11, d (3.6)	98.4, CH
2′	3.69, d (3.3)	71.1, CH	3.36, m	71.9, CH
3′	3.45, m	73.8, CH	3.44, m	73.3, CH
4′	3.28, m	77.1, CH	3.22, m	72.9, CH
5′	3.20, m	76.2, CH	3.23, m	70.0, CH
6′	3.92, dd (2.2, 11.9)	61.3, CH_2	3.51, brs	60.8, CH_2
	3.76, dd (6.0, 11.9)			
7′	3.55, s	59.6, OCH_3		

In the bioassay, all isolated compounds were evaluated for cytotoxicity, DPPH radical scavenging activity, AChE inhibitory and antimicrobial effect. None of these compounds exhibited obvious cytotoxicity at 40 μmol·L^{-1}. Both compounds **1** and **2** showed obvious antibacterial activity against *Bacillus subtilis* with a minimum inhibitory concentration (MIC) of 2 μg·mL^{-1}, and they also showed weak DPPH free radical scavenging activities (Table 3). Compounds **3** and **4** showed antibacterial activities against *Staphylococcus aureus* with MICs of 4 and 2 μg·mL^{-1}, respectively, and they also exhibited DPPH radical scavenging activity comparable to the positive control with IC_{50} values of 9.2 ± 0.03 and 13.3 ± 0.01 μmol·L^{-1}, respectively (Table 3). Remarkably, at a concentration of 50 μmol·L^{-1}, compound **1** had potential AChE inhibitory activity with an IC_{50} value of 2.61 ± 0.05 μmol·L^{-1}

Table 3 Antimicrobial effect, AChE inhibitory effect and DPPH radical scavenging activity of compounds 1–4 (mean ± SD, *n* = 3)

Compound	MIC/(μg·mL^{-1})				IC_{50}/(μmol·L^{-1})	
	C. albicans	*B. subtilis*	*E. coli*	*S. aureus*	AChE	DPPH
1	64	2	> 256	> 256	2.61 ± 0.05	32.7 ± 0.06
2	64	2	> 256	> 256	NT[d]	27.1 ± 0.05
3	32	64	64	4	NT[d]	9.2 ± 0.03
4	32	64	32	2	NT[d]	13.3 ± 0.01
Nystatin[a]	1	–	–	–	–	–
Kanamycin[b]	–	1	1	1	–	–
Tacrine[c]	–	–	–	–	0.45 ± 0.03	–
Vitamin C[e]	–	–	–	–	–	12.1 ± 0.02

[a] Nystatin is a positive control for antifungal activity against *C. albicans*; [b] Kanamycin is a positive control for antibacterial activity against *S. aureus*, *B.subtilis* and *E. coli*; [c] Tacrine is a positive control forAChE inhibitory activity; [d] The compound has no significant AChE inhibitory activity; [e] Vitamin C is a positive control for DPPH radical scavenging activity

(Table 3), while compound **2** showed weak inhibitory activity with an inhibition rate of 12.98%. This result indicated that the *β*-lactone ring of the structure of **1** exerts significant effect on the AChE inhibitory activity. Therefore, the mechanism of action of compound **1** on AChE (PDB ID: 1acj) was further investigated by molecular docking [27, 28]. The molecular docking result for compound **1** showed that the ligand was well accommodated in the active site with a binding energy of −8.59 kcal·mol^{-1}, where Ser-235, Leu-305, His-398, Trp-524 and Asn-525 are some of the amino acid residues involved in the binding with the active site of AChE (Fig. 5). Compound **1** interacted with the above amino acid residues to form six hydrogen bonds, where the *β*-lactone ring accounted for three hydrogen bonds (the *β*-lactone ring formed three hydrogen bonds with amino acid residues His-398, Trp-524 and Asn-525), which further confirmed that the *β*-lactone ring unit of **1** exert positive effect on its AChE inhibitory activity. These findings will not only increase the chemical diversity of steroids, but also provide new insights into the structural modification of anti-neurodegenerative lead compounds.

Experimental

General experimental procedures

Melting points were determined on an XRC-1 melting point apparatus and uncorrected (Sichuan University Science Instrument Co., Chengdu, China). NMR spectra were recorded on a Bruker DRX-400 (Bruker, Karlsruhe, Germany). Chemical shifts are expressed in ppm and CD_3OD (δ_H 3.31/δ_C 49.0) signals. Optical rotation values were determined by a Jasco P-1020 digital polarimeter (Jasco, Tokyo, Japan). Electronic circular dichroism (ECD) spectra were obtained using an Applied Photophysics spectropolarimeter (Bio-Logic Science Instruments, Seyssinet-Pariset, France). UV spectra were measured on a Shimadzu UV-2401PC spectrometer (Shimadzu, Tokyo, Japan). HR-ESI-MS data were acquired on an Agilent 1290 UPLC/6545Q-TOF-MS spectrometer (Agilent, Santa Clara, CA, USA). HPLC was performed on an Agilent 1260 instrument equipped with a Zorbax SB-C_{18} column (5 μm, 9.4 mm × 250 mm) (Agilent, Santa Clara, CA, USA). The crystallographic data were acquired on a Bruker Apex DUO diffractometer equipped with a graphite monochromator using Cu K*α* radiation (*λ* = 1.541 78 Å) (Agilent Technologies Inc., Waldbronn, Germany). Column chromatography (CC) was performed using RP-18 (50 μm, Merck, Germany), Si gel (200–300 mesh, Qingdao Marine Chemical Co., Ltd.), and Sephadex LH-20 (GE Chemical Corporation). Thin-layer chromatography (TLC) (GF_{254}, Qingdao Ocean Chemical Co., Ltd.) was used to monitor fractions.

Fungal material and fermentation

The fungus *Xylaria* sp. KYJ-15 was isolated from the stems of *Illigera celebica* collected from Yunnan Province, China. The species was identified as *Xylaria* based on their morphological and genetic (ITS) characteristics (GenBank accession No. JQ862705.1). The strain was preserved in School of Chemical Science and Technology, Yunnan University, China. The strain was maintained on modified PDA medium (1 L of water, 15 g of agar, 20 g of glucose, 200 g of potato, and natural pH), and cultured in a constant temperature incubator at 28 °C for 3 days. Then, aliquots (5 kg) of potatoes were placed in 50 glass inoculum flasks as a medium, followed by sterilization at 121 °C for 30 min. Finally, the fungus was added to the medium and incubated at room temperature for 30 dats. Fermentation of this strain with rice medium was also performed based on the similar method described above.

Extraction and isolation

After fermentation, the potato medium was ultrasonically extracted three times for 30 min in each. The mass of the extract after concentration on the rotary evaporator was 20 g. The extract was first fractionated by column chromatography using silica gel with a gradient of $CHCl_3$−MeOH (100 : 0, 30 : 1, 3 : 1, *V*/*V*) to obtain three fractions (Fr.1−Fr.3). Fr. 2 (2.0 g) was separated by repeated silica gel column chromatography using Sephadex LH-20 (MeOH), column chromatography using silica gel and further purified by preparative

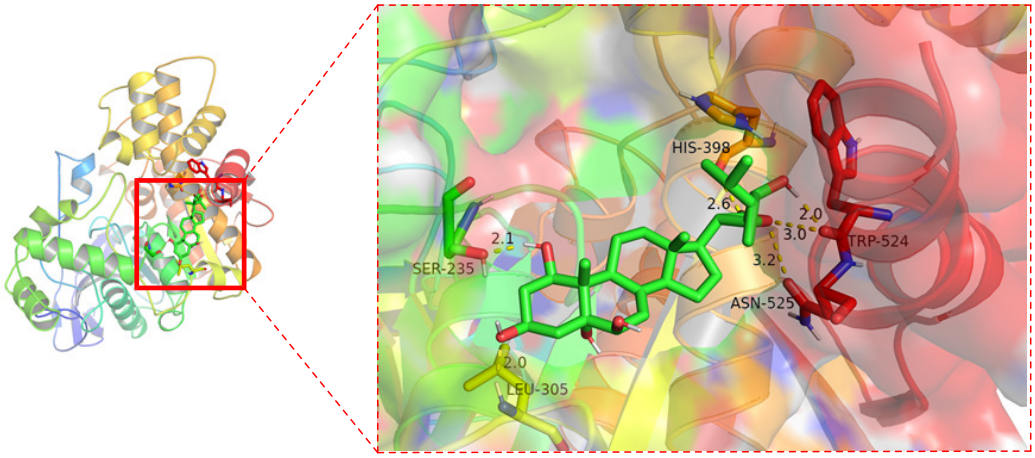

Fig. 5 Docking model for compound 1 with the crystallographic structure of AChE (PDB ID: 1acj). Hydrogen bonds are indicated by yellow dashed lines between the atoms involved

HPLC with the eluent MeOH−H_2O (3 : 7, *V*/*V*) to obtain **1** (8.5 mg, t_R 12 min, *v* 2.0 mL·min^{-1}) and **2** (6.0 mg, t_R 12.5 min, *v* 2.0 mL·min^{-1}). After treatment of the fermented rice medium with the same method as above, 30 g of the extract was obtained. Three fractions (Fr.1−Fr.3) were obtained after repeating the above column chromatography of the obtained extract. Fr. 3 (5.0 g) was fractionated by repeated column chromatography on a Sephadex LH-20 column eluting with MeOH and further purified by preparative HPLC with the eluent MeOH−H_2O (1 : 4, *V*/*V*) to obtain **3** (10.0 mg, t_R 8.5 min, *v* 2.0 mL·min^{-1}) and **4** (12.0 mg, t_R 8 min, *v* 2.0 mL·min^{-1}).

Xylarsteroid A (**1**): colorless crystals; m.p. 184−186 °C; $[a]_D^{20}$: −54.2 (*c* = 0.15, MeOH); UV (MeOH) λ_{max} (log ε): 198 (3.02) nm; ^{1}H and ^{13}C NMR data see Table 1; HR-ESI-MS *m/z* 515.2976 $[M + Na]^+$ (Calcd. for $C_{28}H_{44}O_7Na^+$, 515.2979).

Xylarsteroid B (**2**): a white solid; m.p. 209−211°C; $[a]_D^{20}$: −34.7 (*c* = 0.30, MeOH); UV (MeOH) λ_{max} (log ε): 198 (2.93) nm; ^{1}H and ^{13}C NMR data see Table 1; HR-ESI-MS *m/z* 515.2979 $[M + Na]^+$ (Calcd. for $C_{28}H_{44}O_7Na^+$, 515.2979).

Xylarglycoside A (**3**): colorless needles; m.p. 188−190 °C; $[a]_D^{20}$: +5.1 (*c* = 0.11, MeOH); UV (MeOH) λ_{max} (log ε): 210 (2.39) nm; ^{1}H and ^{13}C NMR data see Table 2; HR-ESI-MS *m/z* 393.1175 $[M + Na]^+$ (Calcd. for $C_{17}H_{22}O_9Na^+$, 393.1156).

Xylarglycoside B (**4**): a white solid; m.p. 201−203 °C $[a]_D^{20}$: +5.3 (*c* = 0.22, MeOH); UV (MeOH) λ_{max} (log ε): 210 (3.28) nm; ^{1}H and ^{13}C NMR data see Table 2; HR-ESI-MS *m/z* $[M + Na]^+$ 379.1000 (Calcd. for $C_{16}H_{20}O_9Na^+$, 379.1000).

Crystallographic data of xylarsteroid A (1)

The suitable crystals of **1** were obtained from chloroform. Crystal data for **1**: $C_{28}H_{44}O_7$, *M* = 524.67, *a* = 6.8675 (3) Å, *b* = 9.9530 (10) Å, *c* = 20.493 (2) Å, α = 90°, β = 90°, γ = 90°, *V* = 1390.3 (2) Å^3, *T* = 293 (2) K, space group P2(1), *Z* = 2, μ(Cu Kα) = 0.729 mm^{-1}, 20 155 reflections measured, 5425 independent reflections (R_{int} = 0.0626). The final R_1 value was 0.0526 [$I > 2\sigma(I)$]. The final $wR(F^2)$ value was 0.1346 [$I > 2\sigma(I)$]. The final R_1 value was 0.0598 (all data). The final $wR(F^2)$ value was 0.1423 (all data). The goodness of fit on F^2 was 1.071. Flack parameter = 0.00 (11) (CCDC 2160472).

Crystallographic data of xylarglycoside A (3)

The suitable crystals of **3** were obtained from methanol. Crystal data for **3**: $C_{17}H_{22}O_9$, *M* = 370.34, *a* = 8.2028 (14) Å, *b* = 7.8879 (13) Å, *c* = 13.788 (2) Å, α = 90°, β = 90°, γ = 90°, *V* = 881.6 (3) Å^3, *T* = 298 (2) K, space group P2(1), *Z* = 2, μ(Cu Kα) = 0.971 mm^{-1}, 22 331 reflections measured, 3183 independent reflections (R_{int} = 0.0201). The final R_1 values were 0.0247 [$I > 2\sigma(I)$]. The final $wR(F^2)$ values were 0.0676 [$I > 2\sigma(I)$]. The final R_1 values were 0.0247 (all data). The final $wR(F^2)$ values were 0.0676 (all data). The goodness of fit on F^2 was 1.048. Flack parameter = 0.055 (19) (CCDC 2160452).

Antimicrobial activity assay

One strain of pathogenic fungi and three strains of pathogenic bacteria (*Candida albicans*, *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*) were chosen for antimicrobial assay, and the antimicrobial activity of compounds **1**−**4** was evaluated by the previously reported method [29]. Nystatin was used as the positive control for antifungal activity against *C. albicans*, while kanamycin was used as the positive control for antibacterial activity against *S. aureus*, *B.subtilis* and *E. coli*. All experiments were repeated three times.

AChE inhibitory assay

The AChE inhibitory activity of compounds **1**−**4** was evaluated by the spectrophotometric method developed by Ellman *et al*. with slight modification [30]. Tacrine was used as the positive control, and all experiments were repeated three times.

Cytotoxicity assay

Five human cancer cell lines (breast cancer MCF-7, colon cancer SW480, hepatocellular carcinoma SMMC-7721, lung cancer A549, and myeloid leukemia HL-60) were chosen for cytotoxicity assay, and the cytotoxicity of compounds **1**−**4** was evaluated *in vitro* by the MTS method [31]. Cisplatin (DDP) was used as the positive control.

Molecular docking

A molecular docking study of **1** was carried out to further investigate the mechanism of the inhibitory effects of **1**

on AChE. The crystallographic structure of AChE (PDB ID: 1acj) was downloaded from the RCSB Protein Data Bank. Molecular docking was conducted using Autodocktools-1.5.6 and PyMOLol-2.3.4. Autodocktools-1.5.6 was used to study the interactions. Pymol-2.3.4 was applied to analyze the docking results.

Supplementary Materials

Supplementray information can be acquired by e-mail to the corresponding authors.

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