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New protein tyrosine phosphatase inhibitors from fungus *Aspergillus gorakhpurensis* F07ZB1707†

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Twelve new compounds, aspergorakhins A–L (1–12) coupled with one known xanthone leptosphaerin D (13), were isolated from the extract of soil-derived fungus *Aspergillus gorakhpurensis* F07ZB1707. Their structures were elucidated by spectroscopic data analysis including UV, IR, NMR, and HRESIMS. The absolute configurations of 5 and 8–11 were identified using ECD and OR calculations. All compounds were tested by enzyme inhibitory activity assay *in vitro*. Aspergorakhin A (1) showed selective activities against PTP1B and SHP1 over TCPTP with IC₅₀ values 0.57, 1.19, and 22.97 μM, respectively. Compounds 1 and 2 exhibited modest cytotoxicity against tumor cell lines A549, HeLa, Bel-7402, and SMMC-7721 with IC₅₀ values in the range of 6.75–83.4 μM.

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Introduction

Protein tyrosine phosphatases (PTPs) are signaling enzymes that regulate tyrosine phosphorylation. They have shown an important role in cellular processes including proliferation, metabolism, motility, and survival. The disorder of PTPs could induce human diseases such as tumor, diabetes, autoimmune diabetes, and infectious diseases. A number of PTPs such as PTP1B and SHP1 have been explored as targets for drug discovery.^{1–3} However the highly conserved active site of PTPs' family members increases the difficulty of achieving selective inhibitors and drug candidates.⁴

Natural products have played an important role in the history of drug discovery. Over 50% of all current clinically approved drugs are derived from natural products.⁵ Since Alexander

Fleming discovered penicillin in 1928, fungi have become a considerable resource for novel compounds and new drugs.^{6,7} In our continuous search for active compounds from fungi, twelve new compounds were obtained from the extract of soil-derived fungus *Aspergillus gorakhpurensis* F07ZB1707, which was fermented by a salt-containing medium. These compounds included one 5α,8α-epidioxysterol aspergorakhin A (1), three nortriterpenoids aspergorakhins B–D (2–4) with a rare carboxyl or carbonyl groups in C-19, eight aromatic polycyclic polyketides aspergorakhins E–J, L, and leptosphaerin D (5–10, 12, 13), and one cyclohexanone derivative aspergorakhin K (11) (Fig. 1). Compounds 1, 2, and 11 showed inhibitory effect against PTPs including PTP1B, SHP1, and TCPTP *in vitro*. Among them, compound 1 might exhibit selective activities against PTP1B and SHP1 over TCPTP with IC₅₀ values 0.57, 1.19, and 22.97 μM, respectively. Their inhibitory effects against cathepsin B and cytotoxicity in the human tumor cell lines were

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Fig. 1 The chemical structures of 1–13.

also screened. Herein, we report the isolation, structural elucidation, and biological evaluation of these compounds.

Results and discussion

Compound **1** was obtained as a white amorphous powder. The molecular formula was determined as $C_{27}H_{40}O_3$ on the basis of the HRESIMS peak at m/z 413.3047 $[M + H]^+$ (calcd for 413.3056), indicates at the eight degrees of unsaturation. The NMR data (Table 1) of **1** showed 27 carbon resonances including five methyl carbons, seven methylene carbons, five methine carbons, five protonated olefinic carbons, and five quaternary carbons with one sp^2 quaternary carbon. The IR spectrum showed a strong absorption at ν_{max} 3386 cm^{-1} , implying the existence of a hydroxyl group. The five sp^2 olefinic and one sp^2

quaternary carbons account for three degrees of unsaturation; then, the remaining five degrees of unsaturation suggest that **1** had five rings. Furthermore, the characteristic signals of four singlet methyl groups at δ_H 0.83 (s, H-18), 0.88 (s, H-19), 1.73 (s, H-26), and 1.75 (s, H-27) and one doublet methyl at δ_H 1.03 (d, J = 6.7 Hz, H-21) (Table 1), indicated the steroid skeleton of **1**.^{8,9} Two contiguous coupled olefinic protons at δ_H 6.24 (d, J = 8.5 Hz, H-6) and 6.50 (d, J = 8.5 Hz, H-7), and an oxygenated methine at δ_H 3.97 (m, H-3) were the characteristics of a 3 β -hydroxy-6-en-5 α ,8 α -epidioxy steroid skeleton,¹⁰ which was also corresponding to the ^{13}C NMR signals at δ_C 66.5 (CH, C-3), 82.1 (C, C-5), 135.4 (CH, C-6), 130.7 (CH, C-7), and 79.4 (C, C-8). All these data showed great similarity with the known compound yalongsterol A¹¹ except for different side chains at C-17. The COSY correlations H-20/H-21, 22, H-23/H-22, 24, and the HMBC

Table 1 1H and ^{13}C NMR data of **1–4** (J in Hz, δ in ppm)^a

No.	1 ^b		2 ^b		3 ^c		4 ^c	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1 α	34.7	1.96, m	29.0	2.23, m	34.8	2.69, m	34.3	2.49, m
1 β		1.69, m		1.76, m		1.20, m		1.02, m
2 α	30.1	1.86, m	23.0	2.12, m	29.8	1.95, m	29.8	1.96, m
2 β		1.55, m		2.00, m		1.72, m		1.67, m
3	66.5	3.97, m	84.4	4.13, dd (3.7, 1.6)	78.9	3.21, dd (12.0, 4.6)	79.3	3.19, dd (12.0, 4.6)
4 α	36.9	2.12, m	36.9		40.3		40.4	
4 β		1.91, dd (13.7, 11.5)						
5	82.1		47.8	1.61, m	50.1	1.29, dd (11.9, 4.3)	52.3	1.25, m
6 α	135.4	6.24, d (8.5)	25.2	2.13, m	24.6	2.15, m	18.6	2.42, m
6 β				1.87, m		2.71, m		1.71, m
7 α	130.7	6.50, d (8.5)	119.0	5.48, d (7.2)	123.7	5.64, d (6.6)	27.3	2.19, m
7 β								2.15, m
8	79.4		143.3		142.3		140.9 ^d	
9	51.1	1.50, m	131.5		139.2		128.2 ^d	
10	37.0		46.9		48.0		49.5	
11 α	20.6	1.60, m	124.8	5.58, t (4.5)	122.7	5.60, d (6.2)	23.3	2.18, m
11 β								1.90, m
12 α	39.3	1.95, m	38.0	2.28, d (4.5)	39.2	2.26, d (17.9)	32.3	1.79, m
12 β		1.24, m				2.12, dd (17.9, 6.2)		1.71, m
13	44.7		44.3		45.4		46.2	
14	51.6	1.56, m	49.7		51.2		51.3	
15 α	23.4	1.53, m	31.3	1.73, m	32.6	1.74, m	32.0	1.74, m
15 β		1.23, m		1.35, m		1.40, m		1.27, m
16 α	28.6	1.78, m	27.3	1.98, m	28.3	2.01, m	28.7	1.96, m
16 β		1.36, m		1.42, m		1.40, m		1.40, m
17	56.2	1.25, m	47.1	1.70, m	48.5	1.69, m	48.2	1.58, m
18	12.9	0.83, s	15.7	0.77, s	16.3	0.59, s	16.2	0.74, s
19	18.2	0.88, s	175.7		179.1		179.9 ^d	
20	39.7	2.13, m	39.2	1.59, m	40.5	1.51, m	40.8	1.52, m
21	20.5	1.03, d (6.7)	16.5	1.03, d (6.5)	17.2	1.01, d (6.5)	17.4	1.00, d (6.5)
22 α	137.7	5.38, dd (15.0, 8.7)	68.1	3.67, dd (10.6, 2.9)	68.0	3.58, dd (10.7, 3.0)	68.1	3.57, dd (10.7, 3.0)
22 β				3.40, dd (10.6, 7.0)		3.27, dd (10.7, 7.1)		3.25, dd (10.7, 7.1)
23	124.5	6.14, dd (15.0, 10.8)	24.2	0.89, s	25.4	0.91, s	25.0	0.94, s
24	125.1	5.75, br d (10.8)	28.8	1.12, s	27.2	1.00, s	27.2	0.98, s
25	133.1		23.2	1.07, s	14.8	0.79, s	14.7	0.77, s
26	18.2	1.73, s						
27	25.9	1.75, s						

^a 700 MHz for 1H , 175 MHz for ^{13}C . Tetramethylsilane (TMS) as an internal standard. ^b Recorded in $CDCl_3$. ^c Recorded in CD_3OD . ^d Extracted from HMBC.

correlations H-26, 27/C-24, 25 disclosed the presence of the 6-substituted 2-methylhepta-2,4-diene side chain, which was linked with C-17. The configuration of the C-22/C-23 double bond was assigned as *E* on the basis of the coupling constant ($J = 15.0$ Hz) of olefinic protons H-22 and H-23.

The relative configuration of **1** was determined according to the NOESY correlations. The NOESY correlations of H-19/H-4 β and H-2 β imply that this methyl was axial and H-3 (3.97, m) was also axial from the J values. The strong NOESY correlation of H-18/H-7 indicated that the second ring was in the boat geometry with a double bond and CH₃-18 was axial and in *cis*-configuration to CH₃-19. Then, the peroxide bridge was in *trans*-orientation with respect to CH₃-19. The NOESY correlation of H-17/H-14 together with no correlation between H-18 and H-14 indicated that H-18 was in *trans*-configuration with respect to H-14 and H-17. Given the NOESY correlation of H-18/H-20 and no NOESY correlation between H-18 and H-21, it suggested that H-18 and H-20 were β -oriented. A comparison of the ¹³C NMR data of C-20 (δ_C 39.7) with the literature value¹¹ also supported the β -orientation of H-20 in **1**. Furthermore, the β -orientation of H-20 was consistent with several 5 α ,8 α -epidioxysterols in many studies from different origins, whose structures confirmed were by X-ray diffraction analysis. Thus, the structure of compound **1** was consequently identified to be (22*E*)-5 α ,8 α -epidioxy-6,22,24-trien-3 β -ol, named aspergorakhin A.

Compound **2** was obtained as a white amorphous powder. The molecular formula was established as C₂₅H₃₆O₃ through the pseudo molecular ion [M + Na]⁺ peak at m/z 407.2556 (calcd for 407.2562), accounting for seven degrees of unsaturation. The presence of the hydroxy group was indicated by the obvious absorption at $\nu_{\max}$ 3390 cm⁻¹ in the IR spectrum. The ¹H NMR data exhibited five methyls (δ_H 0.77, 0.89, 1.03, 1.07, and 1.12), two olefinic protons (δ_H 5.48 and 5.58), one oxygenated methine (δ_H 4.13), and one oxygenated methylene (δ_H 3.67 and 3.40). The ¹³C NMR data revealed 25 carbons containing four olefinic carbons (δ_C 143.3, 131.5, 124.8, and 119.0) and two oxygenated carbons (δ_C 84.4 and 68.1). The NMR data of **2** was analogous to those of 23,24,25,26,27-pentanorlanost-7,9(11)-dien-3 β ,22-diol closely,¹² except for the absence of one methyl group (δ_C 22.7, CH₃-19) and the presence of one carbonyl group (δ_C 175.7), which was oxidized to carbonyl group and then formed an ester group (δ_C 175.7) with the 3 β -hydroxyl group in **2**. The key COSY correlations, as shown in Fig. 2, demonstrated clearly that **2** was a triterpenoid nucleus. The key HMBC correlations of H-23/C-8, H-11/C-9, C-10, and C-13, H-12/C-9 and C-11 implied that two double bonds were at C-7(8) and C-9(11). There were five methyls in the structure, of which two singlet methyls were germinal; they were correlated in the HMBC with C-3 (δ_C 84.4), a quaternary carbon (δ_C 36.9), and C-5 (δ_C 47.8). Therefore, the singlet methyls (CH₃-24, CH₃-25) were located at C-4 (δ_C 36.9). There were another two singlet protons of methyl at δ_H 0.89 and 0.77, which were both coupled to the same quaternary carbons (δ_C 44.3/49.7) (C-13/C-14). The protons of methyl at δ_H 0.89 were further coupled to a CH₂ (C-15) and to an olefin carbon C-8 (δ_C 143.3); thus, the methyl should be attributed to CH₃-23. The other protons of methyl at δ_H 0.77 was correlated to a CH₂ (C-12) with the COSY correlated to the olefinic proton (H-11) at δ_H 5.58,

Fig. 2 Key COSY (—), HMBC (—→), and NOESY(---→) correlations of compounds **1**–**12**.

while the CH carbon (C-17) was correlated in HMBC to a doublet protons of methyl at δ_H 0.77. Therefore, the protons of methyl (δ_H 0.77) were attributed to CH₃-18. The remaining doublet protons of methyl (δ_H 1.03) was correlated to C-17, C-20, and C-22 in the HMBC spectrum, which should account for CH₃-21.

The relative configuration of **2** was determined by the key NOESY correlations of H-18/H-15 β ; H-23/H-15 α , H-17, implying that CH₃-18 was axial and in *trans*-configuration with respect to CH₃-23 and H-17. Obviously, H-3 was equatorial and 3-OH was β -oriented, forming an up bridge with C-19 (δ_C 175.7). The key NOESY correlations of H-3/H-25 and H-5/H-25 indicated that H-3 and H-5 were *cis*-orientated with respect to each other. Finally, CH₃-21 was assigned the β -orientation compared with the NMR data of cladosporide A, whose absolute configuration was confirmed by X-ray diffraction analysis.¹³ On the basis of the evidences mentioned above, **2** was consequently identified as aspergorakhin B.

Compound **3** was isolated as a white amorphous powder, whose molecular formula was C₂₅H₃₈O₄, as determined by the HRESIMS peak at m/z 403.2850 [M + H]⁺ (calcd for 403.2848). The NMR data of **3** exhibited great similarity with compound 23,24,25,26,27-pentanorlanost-7,9(11)-dien-3 β ,22-diol except for the absence of a methyl group (δ_C 22.7, CH₃-19), the presence of a carboxyl group (δ_C 179.1, C-19), the upfield shift of an olefinic carbon (δ_C 139.2 in **3**, C-9), and the downfield shift of a quaternary carbon (δ_C 48.0 in **3**, C-10),¹² which indicated that the methyl group was oxidized to the carbonyl group. The relative configuration of **3** was assigned by the NOESY correlations of H-1 α /H-3, 5, H-24/H-3, 5, H-18/H-15 β , H-23/H-15 α , 17, together with the NMR data comparing with those of the known compounds,¹² implying that **3** had the same absolute configuration as that of 23,24,25,26,27-pentanorlanost-7,9(11)-dien-3 β ,22-diol. On the basis of the evidences mentioned above, the structure of **3** was consequently identified as aspergorakhin C.

Compound **4** was isolated as a white amorphous powder. Its molecular formula was confirmed to be C₂₅H₄₀O₄ by the HRESIMS peak at m/z 405.3014 [M + H]⁺ (calcd for 405.3005). The NMR data of **4** was closely similar to that of **3**, except for the absence of two olefinic signals (δ_H 5.64, δ_C 123.7) and (δ_H 5.60,

δ_{C} 122.7) in **3**, which indicated that there was only one couple of double bonds in **4**. The key HMBC correlation of H-23/C-8 verified that the double bond was located between C-8 and C-9. The key NOESY correlations of H-3/H-5, 24, H-18/H-15 β , H-23/H-15 α , H-17 (Fig. 2) together with the same J values of H-3 (dd, $J = 12.0, 4.6$ Hz) and almost the same chemical shifts for C-20 (δ_{C} 40.5 in **3**, δ_{C} 40.8 in **4**) indicated that the absolute configuration of **4** was the same as that of **3**. Consequently, **4** was named as aspergorakhin D.

Compound **5** was isolated as a pale brown powder. The molecular formula was resolved as $\text{C}_{15}\text{H}_{20}\text{O}_5$ from the $[\text{M} + \text{Na}]^+$ peak at m/z 303.1207 (calcd for 303.1208). The strong absorption bands at 3408 and 1731 cm^{-1} in the IR spectrum revealed the presence of a hydroxyl group and a lactone carbonyl group. Comprehensive analysis of the NMR data (Table 2) suggested that **5** was a phthalide derivative, similar to isochracein and (S)-3-ethyl-6-hydroxyphthalide.¹⁴ The NMR data of **5** showed two adjacent aromatic protons at δ_{H} 7.52 (d, $J = 7.4$ Hz, H-5) and δ_{H} 6.92 (d, $J = 7.4$ Hz, H-6), indicating a typical 1,2,3,4-tetra-substituted benzene moiety with a hydroxyl substituted at C-3, which was determined by the HMBC correlations of H-5/C-3 and H-11/C-3 (Fig. 2). The side chain and the lactone ring were assigned by the key HMBC data (Fig. 2). The absolute configuration of **5** was determined by the calculated ECD together with OR using the time-dependent density functional theory (TDDFT) (ESI S114† for details). Notably, the calculated

ECD spectrum for (8*R*,12*R*)-**5** and (8*R*,12*S*)-**5** were both in accordance with the experimental curve of **5** (Fig. 3) and the quantum calculation of optical rotations (OR) for (8*R*,12*R*)-**5** and (8*R*,12*S*)-**5** were +22.4 and +118.6, respectively. The experimental OR of **5** was +107.7 (c 0.13, MeOH). Thus, the absolute configuration of **5** was identified to be (8*R*,12*S*).

Compound **6** was isolated as a brown oil with the molecular formula $\text{C}_{21}\text{H}_{22}\text{O}_5$ and eleven degrees of unsaturation, as determined by the $[\text{M} + \text{Na}]^+$ peak at m/z 377.1374 (calcd for 377.1365). The proton spectrum (Table 2) of **6** showed signals of five olefinic or aromatic protons (δ_{H} 6.20, 6.63, 6.42, and 7.11), an oxygenated proton of methyl group (δ_{H} 3.64), and four protons of methyl groups (δ_{H} 0.93, 0.93, 2.00, and 2.28). The ^{13}C NMR data exhibited 21 carbons in **6**, including one ketone carbonyl carbon (δ_{C} 204.1), one oxygenated non-protonated carbon (δ_{C} 78.6), and fourteen olefinic or aromatic carbons. These fragments accounted for eight degrees of unsaturation, requiring three additional rings in **6**. The detailed analysis of its NMR data (Table 2) indicated that **6** contained fragments or signals of pentasubstituted and 1,2,3,4-tetrasubstituted benzene rings, a 1,2-disubstituted olefin (δ_{C} 122.5, 128.0), two aryl methyl groups (δ_{C} 17.1, 13.3), coincident or identical methyl groups (δ_{C} 27.5, 27.5) on an oxygenated quaternary carbon (δ_{C} 78.6), a methoxy group (δ_{C} 56.5), and a ketone carbon (δ_{C} 204.1), suggesting that **6** was very similar to massarinins A,¹⁵ except for the absence of an formaldehyde moiety and the difference in

Table 2 ^1H and ^{13}C NMR data of **5**–**9** (J in Hz, δ in ppm)^a

No.	5		6		7		8		9	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1	172.9		204.1		203.7		75.1	6.94, s	150.6	
2	112.8		133.4		132.7		136.2		129.7	
3	156.3		150.4		150.2		148.0		120.9	
4	129.1		112.0	6.63, s	112.0	6.66, s	122.2	7.03, s	65.3	4.88, d (3.2)
5	139.7	7.52, d (7.4)	127.0		127.6		127.6		38.0	2.37, ddd (10.7, 3.9, 3.2)
6	113.6	6.92, d (7.4)	148.0		148.2		148.9		44.2	2.37, dd (16.9, 3.9) 2.60, dd (16.9, 10.7)
7	150.7		123.2		123.3		115.0		196.2	
8	83.9	5.45, dd (6.9, 4.2)	164.7		171.4		165.0		165.6	
9	28.6	2.10, m 1.78, m	109.9	6.42, d (8.4)	112.3	6.62, d (8.9)	110.5	6.55, d (8.8)	110.5	6.55, d (8.8)
10	8.8	0.95, t (7.3)	135.4	7.11, d (8.4)	135.3	7.94, d (8.9)	129.6	7.66, d (8.8)	130.5	7.78, d (8.8)
11	33.2	3.07, dd (14.2, 1.9) 2.64, dd (14.2, 10.5)	114.4		113.8		114.6		114.1	
12	79.8	3.60, dd (10.5, 1.9)	156.9		164.2		162.6		162.3	
13	73.8		113.3		113.1		111.0		107.9	
14	25.0	1.25, s	122.5	6.20, d (9.8)	192.3		193.1		193.4	
15	25.7	1.25, s	128.0	5.38, d (9.8)	48.4	2.55, s	48.8	2.58, d (16.7) 2.38, d (16.7)	48.9	2.66, s
16			78.6		81.9		80.9		81.1	
17			27.5	0.93, s	26.0	0.99, s	24.9	0.77, s	26.7	1.32, s
18			27.5	0.93, s	26.0	0.99, s	27.2	1.14, s	26.8	1.32, s
19			56.5	3.64, s	56.6	3.63, s	57.0	3.60, s	140.7	7.66, s
20			17.1	2.28, s	17.2	2.29, s	15.4	2.30, s	16.1	1.13, d (6.9)
21			13.3	2.00, s	13.3	2.03, s	174.2			

^a 600 MHz for ^1H , 150 MHz for ^{13}C , CD_3OD . TMS as an internal standard.

Fig. 3 The chemical structures of the isomers and the comparison of the experimental and calculated ECD spectra of **5**.

the substitution of the aromatic ring. Comprehensive analysis of the HSQC and HMBC data of **6** (Fig. 2) confirmed a 2-methoxyl-4,6-dimethyl-5-hydroxyl benzol unit by key HMBC correlations of H-4/C-2, C-3, and C-6, H-19/C-3, H-20/C-4, C-5, and C-6, H-21/C-2, C-6, and C-7. The ketone carbon (C-1) was as a bridge carbon connecting to C-2 and C-13 on the basis of a weak four-bond HMBC correlation of H-4/C-1.

Compound **7**, a pale yellow powder, was assigned a molecular formula of $C_{21}H_{22}O_6$ with eleven degrees of unsaturation by the $[M + H]^+$ peak at m/z 371.1492 (calcd for 371.1495) in the HRESIMS spectrum. A detailed comparison of the NMR data with that of **6** suggested that **7** was similar to **6** except for a carbonyl group (δ_C 192.3, C-14) and a methylene (δ_C 48.4, C-15) in **7**, replacing the 1,2-disubstituted olefin (δ_C 122.5, 128.0) in **6**. Therefore, the substituent groups on the pentasubstituted aromatic ring in **7** were revealed. Homogeneously, C-1 was linked to C-2 and C-13 on the basis of weak 4J HMBC correlations of H-4/C-1.

Compound **8** was obtained as a pale yellow powder. The HRESIMS spectrum yielded an $[M + Na]^+$ ion peak at m/z 407.1102 (calcd for 407.1107), which suggested its molecular formula to be $C_{21}H_{20}O_7$ with twelve degrees of unsaturation. The NMR data (Table 2) indicated that **8** and **7** had the same 2,2-dimethyl-benzopyrone unit, except for the presence of a lactone ring and the difference of the pentasubstituted aromatic ring in **8**. The ^{13}C NMR data of δ_C 75.1 (C-1), 136.2 (C-2), 148.0 (C-3), 122.2 (C-4), 127.6 (C-5), 148.9 (C-6), 115.0 (H-7), and 174.2 (C-21) exposed the presence of an isobenzofuranone group. In

addition, the key HMBC correlations of H-1/C-13, C-2, and C-21 confirmed that C-1 (δ_C 75.1) was connected to C-13 (δ_C 112.0), accomplishing the assignment of **8**.

The absolute configuration of **8** was elucidated by the ECD calculation. The calculated ECD spectrum of enantiomer (1R)-**8** was consistent with that of the experimental ECD spectrum of **8**, which exhibited one strong negative Cotton effect at 213 nm together with two weak positive Cotton effects at 246 and 274 nm (Fig. 4). Thus, the absolute configuration of **8** was identified as (1R).

Compound **9** was a pale yellow solid and its molecular formula $C_{20}H_{20}O_6$ was elucidated by the $[M + H]^+$ peak at m/z 357.1333 (calcd for 357.1338), indicating the presence of eleven degrees of unsaturation. The 1H and ^{13}C NMR data of **9** (Table 2) revealed a hydroxyl-substituted 2,2-dimethylbenzopyrone fragment, which was consistent with **7** and **8**. Coincidentally, the comparison of the 2D NMR spectra between **9** and massarinins B indicated that the planar structure of **9** contained the upper part of **7** and the lower part of massarinins B.¹⁵ The description mentioned above was proved by the key 2D NMR correlations shown in Fig. 2. In addition, C-1 (δ_C 150.6) was connected to C-13 (δ_C 107.9) by a weak four-bond HMBC correlation of H-9 at δ_H 6.55 (d, J = 8.8 Hz) with C-1. The J -value for H-5 with H-6 β was 10.7 Hz, which revealed that they were both axial orientations. H-4 was equatorially oriented and *cis*- to H-5, according to the $J_{4,5}$ -value (3.2 Hz) and the half chair form of the cyclohexanone group. The absolute configuration of **9** was further determined to be (4S,5S) by the comparison of the calculated ECD of (4S,5S)-**9** and (4R,5R)-**9** with the experimental CD curve (Fig. 5).

Compound **10** was isolated as a light yellow solid. Its molecular formula $C_{18}H_{16}O_5$ was confirmed by the $[M + Na]^+$ peak at m/z 335.0890 (calcd for 335.0895) with eleven degrees of unsaturation. The 1D NMR (Table 3) and HSQC spectra revealed signals for twelve olefinic carbons, one methine, two methylenes, one methyl, and one carbonyl group. Moreover, the COSY correlations shown in Fig. 2, together with the HMBC correlations of H-11/C-10, H-9/C-10, H-9/C-9a, H-9/C-4a, H-8/C-4a, H-8/

Fig. 4 The chemical structures of isomers of **8** and the comparison of the experimental and calculated ECD spectra of **8**.

Fig. 5 The structures of the isomers and the comparison of the experimental and calculated ECD spectra of **9**.

C-1, and H-8/C-5, implied the presence of an isobenzofuranone group and an isobenzofuran group in **10**. The HMBC correlations of H-9 to C-6, H-7 to C-5, and H-9 to C-5, indicated that the isobenzofuranone group and the isobenzofuran group were linked by C5–C9 and an extra ethyl side chain was attached to C-5. The absolute configuration of **10** was determined by ECD and

OR calculations. All four isomers were considered in the ECD calculations. The calculated curves of the (5*S*,9*S*)-**10** and (5*R*,9*S*)-**10** isomers both showed a good consistency with the experimental spectrum (Fig. 6). Furthermore, the calculated ORs for (5*S*,9*S*)-**10** and (5*R*,9*S*)-**10** were +31.1 and –14.7, respectively. The experimental OR of **10** was –10.4 (c 0.10, MeOH). Thus, compound **10** was identified as (5*R*,9*S*)-**10**, aspergorakhin J.

Compound **11** was isolated as a white amorphous powder with the molecular formula of $C_{10}H_{16}O_3$, as determined from the $[M + Na]^+$ peak at m/z 207.0989 (calcd for 207.0997). Its 1D-NMR revealed a total of 10 carbons including one carbonyl, two olefinic quaternary carbons, one oxygenated methine, one methyl, and five methylene carbons, including an oxygenated methylene (δ_C 56.9). The COSY spectrum revealed two sets of contiguous protons of H-6/H-5/H-4 and H-8/H-9/H-10. Then, the key HMBC correlations of H-6/C-1, H-4/C-2, H-4/C-8, H-10/C-8, H-7/C-1, H-7/C-2 (Fig. 2) indicated that **11** was an α,β -unsaturated cyclohexanone derivative. Combining the molecular formula and the degree of unsaturation, we confirmed two oxygenated alkyl groups C-5 (δ_C 66.1) and C-7 (δ_C 56.9) connected to the hydroxyl groups. The absolute configuration of **11** was established by the ECD calculations. The calculated ECD spectrum of (5*R*)-**11** was consistent with the experimental ECD, which showed one strong positive Cotton effect at 207 nm and one strong negative Cotton effect at 239 nm with one weak

Table 3 1H and ^{13}C NMR data of **10**–**13** (J in Hz, δ in ppm)^a

10 ^b			11 ^c		12 ^d		13 ^d	
No.	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	152.9		199.1		163.5		166.6	
2	115.9	6.75, d (7.8)	134.4		110.4	6.56, d (8.2, 1.2)	112.4	6.64, dd (8.2, 1.2)
3	130.3	7.13, t (7.8)	158.7		137.2	7.38, t (8.2)	138.7	7.48, t (8.2)
4	115.2	6.63, d (7.8)	39.6	2.71, dd (17.9, 4.3) 2.49 ^e	110.0	6.47, d (8.2, 1.2)	110.5	6.54, dd (8.2, 1.2)
4a	141.8				162.6		163.5	
5	94.9		66.1	4.26, tt (8.6, 4.3)	145.5		144.9	
6	27.6	1.58, dq (14.5, 7.4) 1.85, dq (14.5, 7.4)	46.7	2.76, dd (16.4, 4.3) 2.51 ^e	132.3		137.9	
7	7.4	0.65, t (7.4)	56.9	4.37, d (12.4) 4.35, d (12.4)	117.0	6.99, s	120.7	6.85, s
8	73.6	5.30, d (11.8) 5.20, d (11.8)	36.5	2.35, m	152.5		156.0	
8a	126.7				128.7		121.8	
9	85.5	5.63, s	21.5	1.55 ^e	196.7		198.6	
9a	133.0				113.8		113.8	
10	172.2		14.1	0.97, t (7.4)				
11	117.5	7.35, d (7.7)			66.6	5.32, s	67.5	5.35, s
11a	129.7				125.1		124.8	
12	132.5	7.46, t (7.7)			17.3	2.36, s	17.5	2.36, s
13	122.6	7.18, d (7.7)			57.1	3.76, s		
14	154.0							
1-OH						12.55, s		13.25, s
5-OH						7.75, s		7.71, s
8-OH								10.49, s

^a TMS as an internal standard. ^b 600 MHz for 1H , 150 MHz for ^{13}C , CD_3OD . ^c 700 MHz for 1H , 175 MHz for ^{13}C , $CDCl_3$. ^d 600 MHz for 1H , 150 MHz for ^{13}C , $(CD_3)_2CO$. ^e Overlapped.

Fig. 6 The structures of the isomers and the comparison of the experimental and calculated ECD spectra of **10**.

positive Cotton effect at 283 nm (Fig. 7). Thus, **11** was affirmed to be (5*R*)-**11**, aspergorakhin K.

Compound **12** was obtained as a light-yellow amorphous powder and its molecular formula $C_{16}H_{14}O_5$ was identified by the $[M + H]^+$ peak at m/z 287.0924 (calcd for 287.0919). The characteristics of **12** in NMR were extremely similar to those of leptosphaerin D (**13**),¹⁶ except for an extra methoxyl group at C-8, which was confirmed by the HMBC correlations from H-13 to C-8.

The potential inhibitory activity of **1–13** was screened against PTP1B, SHP1, TCPTP, and cathepsin B *in vitro*. Cathepsin B, as an important tumor-promoting factor, acts as a target of new chemotherapeutics.^{17–19} Compound **1** showed significant

Fig. 7 The structures of the isomers and the comparison of the experimental and calculated ECD spectra of **11**.

inhibition of four enzymes with IC_{50} values of 0.57, 1.19, 22.97, and 8.18 μM , respectively (Table 4). Aspergorakhin B (**2**) inhibited SHP1 and TCPTP with IC_{50} values of 6.18 and 7.50 μM . Also, the IC_{50} value of aspergorakhin K (**11**) against PTP1B was 12.06 μM . However, the other compounds did not exhibit inhibitory activity with the concentration of 13.33 $\mu g mL^{-1}$ (34.69 μM for **2** and 72.41 μM for **11**). In addition, **1–4** were assayed for their cytotoxicity toward human cancer cells A549, HeLa, Bel-7402, and SMMC-7721. The results (Table 5) included that **1** and **2** exhibited modest cytotoxicity against the four cancer cells with IC_{50} values in the range of 6.75–83.40 μM .

Experimental section

General information

Optical rotations were recorded on a SGW-533 digital polarimeter (INESA Co., Shanghai, China). The NMR spectra were recorded on Bruker Avance III 600 or 700 spectrometers (Bruker, USA) with tetramethylsilane (TMS) or deuterated solvent as the internal standard. The ECD data were collected using a MOS-450/SFM300 spectropolarimeter (Bio-Logic, Seyssinet-Pariset, France). The ESIMS and HR-ESIMS data were recorded with an Agilent 6120 (Agilent, Santa Clara, CA, USA) or AB SCIEX TripleTOF 5600+ (AB SCIEX, Boston, MA, USA; Shimadzu LC-20A, Tokyo, Japan) LC/MS/MS system. Preparative HPLC was performed with BRIX 1860 (Orienda Ltd., Beijing, China) equipped with an UV dual wavelength detector. Preparative HPLC was carried out using GRACE Allsphere ODS-2 column (22 mm $\times$ 250 mm, 5 μm ; Alltech, Chicago, IL, USA).

Strain materials

The fungus *Aspergillus gorakhpurensis* F07ZB1707 was isolated from the mountainous region of Shennongjia Forestry District, Hubei Province of China in 2007. It was identified according to the morphological characteristics and ITS gene sequences (GenBank accession No. MT994887, ESI S117†). The fungus was preserved in New Drug Research & Development Center of North China, Pharmaceutical Group Corporation (NCPG).

Fermentation, extraction, and isolation of the compounds

The strain was cultivated on PDA slant media at 26 $^{\circ}C$ for 7 days. The fungal colony scraped from the slant media was transferred to 250 mL flasks, each containing 60 mL seed media (2.0% starch, 0.2% soybean cake starch, 1.0% glucose, 0.3% yeast

Table 4 Inhibitory activity of **1**, **2**, and **11** with IC_{50} (μM) against four enzymes *in vitro*

Compound	PTP1B	SHP1	TCPTP	Cathepsin B
1	0.57	1.19	22.97	8.18
2	>34.69	6.18	7.50	>34.69
11	12.06	>72.41	>72.41	>72.41
$Na_3VO_4^a$	1.10	4.40	2.40	—
Leupeptin ^a	—	—	—	7.50

^a Positive control.

Table 5 Cytotoxic activity of **1** and **2** with IC₅₀ (μM) against four human cancer cell lines *in vitro*

Compound	A549	HeLa	Bel-7402	SMMC-7721
1	6.75	27.61	53.30	41.21
2	83.40	61.90	—	—
Taxol ^a	0.45	4.80	28.20	4.27

^a Positive control.

extract, 0.6% malt extract, 0.2% NaCl, 0.2% CaCO₃, and 0.1% MgSO₄, pH 7.0). Flasks with the media were cultured at 26 °C for 72 h on a rotary shaker at 200 rpm. A total of 225 Erlenmeyer flasks (1000 mL), each of which contained 200 mL liquid media (20.0 g maltose, 10.0 g monosodium glutamate, 20.0 g sorbitol, 33.0 g sea salt, 3.0 g yeast extract, 0.5 g KH₂PO₄, 0.3 g MgSO₄, 0.5 g L-tryptophan per liter of purified water, pH 6.5) were individually inoculated with 10 mL seed broth and incubated at 26 °C on a rotary shaker at 200 rpm for 7 days. All the media were sterilized at 121 °C for 30 min before use.

The culture of large-scale liquid fermentation (45.0 L) of the fungus was filtered under reduced pressure to obtain the filtrate and a mycelia cake. The filtrate and mycelia cake were extracted with EtOAc three times. The EtOAc solution was concentrated under reduced pressure at 40 °C to produce the crude extract (35.0 g). The extract was separated by silica gel column chromatography (CH₂Cl₂/MeOH, 100 : 0–0 : 100) and yielded twelve subfractions (Fr. 1–Fr. 12). Fr. 5 (2.1 g) was separated by CC over Sephadex LH-20 (CH₂Cl₂ : MeOH = 1 : 1) to obtain 10 subfractions (Fr. 5.1–Fr. 5.10). Fr. 5.3 (0.9 g) was subjected to silica gel column chromatography (PE/EtOAc, 40 : 1–1 : 1) to yield 8 fractions (Fr. 5.3.1–5.3.8). Fr. 5.3.7 (80.0 mg) was purified by ODS HPLC (GRACE Allsphere ODS-2, 22 mm × 250 mm, 5 μm) and eluted with CH₃CN/H₂O/FA (45 : 55 : 0.1 to 100 : 0 : 0.1, 20 mL min^{−1}) to afford **1** (1.5 mg) at *t*_R 34.0 min and **2** (4.8 mg) at *t*_R 19.0 min. Fr. 5.5 (1.15 g) was subjected to Sephadex LH-20 CC (MeOH) again to obtain subfractions (Fr. 5.5.1–Fr. 5.5.8). Fr. 5.5.6 (127.0 mg) was purified by preparative HPLC with an ODS column and eluted with CH₃CN/H₂O/FA (45 : 55 : 0.1 to 60 : 40 : 0.1, 20 mL min^{−1}) to afford **6** (5.9 mg) at *t*_R 21.0 min, **7** (8.5 mg) at *t*_R 13.5 min, **8** (2.3 mg) at *t*_R 8.2 min, and **10** (5.6 mg) at *t*_R 11.1 min. Fr. 9 (0.4 g) was directly purified by ODS HPLC and eluted with CH₃CN/H₂O/FA (10 : 90 : 0.1 to 65 : 35 : 0.1, 20 mL min^{−1}) to afford **3** (15.9 mg) at *t*_R 17.8 min, **4** (3.3 mg) at *t*_R 19.0 min, and **11** (3.8 mg) at *t*_R 6.9 min. Fr. 7 (2.3 g) was separated by CC over Sephadex LH-20 (MeOH) to obtain 13 subfractions (Fr. 7.1–Fr. 7.13). Fr. 7.4 (467.0 mg) was purified by ODS HPLC and eluted with CH₃CN/H₂O/FA (15 : 85 : 0.1 to 40 : 60 : 0.1, 20 mL min^{−1}) to afford **5** (9.9 mg) at *t*_R 18.2 min. Fr. 7.5 (311.0 mg) subjected to ODS MPLC (CH₃CN/H₂O/FA, 15 : 85 : 0.1–100 : 0 : 0.1) to obtain Fr. 7.5.B3, and then Fr. 7.5.B3 (95.0 mg) was purified by ODS HPLC and eluted with CH₃CN/H₂O/FA (15 : 85 : 0.1 to 45 : 55 : 0.1, 20 mL min^{−1}) to afford **9** (3.9 mg) at *t*_R 19.0 min. Fr. 12 (2.6 g) was separated by CC over Sephadex LH-20 (MeOH) to obtain 10 subfractions (Fr. 12.1–Fr. 12.10). Fr. 12.9 (100.0 mg) was purified by ODS HPLC

and eluted with CH₃CN/H₂O/FA (20 : 80 : 0.1 to 45 : 55 : 0.1, 20 mL min^{−1}) to afford **12** (50.3 mg) at *t*_R 14.8 min and **13** (30.5 mg) at *t*_R 15.2 min.

Spectral data

Aspergorakhin A (1). White amorphous powder; [α]_D²⁰ −72.0 (c 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.37), 230 (4.10), and 273 (3.34); IR ν_{max} 3386, 2924, 2871, 2855, 1732, 1651, 1557, 1456, 1377, 1260, 1061, 1026, 798 cm^{−1}; ¹H and ¹³C NMR data, Table 1; HR-ESIMS: *m/z* 413.3047 ([M + H]⁺, calcd for C₂₇H₄₁O₃, 413.3056).

Aspergorakhin B (2). White amorphous powder; [α]_D²⁰ +46.5 (c 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.45) and 242 (4.55); IR ν_{max} 3390, 2924, 1739, 1673, 1607, 1464, 1355, 1257, 1131, 1060, 969 cm^{−1}; ¹H and ¹³C NMR data, Table 1; HR-ESIMS: *m/z* 407.2556 ([M + Na]⁺, calcd for C₂₅H₃₆O₃Na, 407.2562).

Aspergorakhin C (3). White amorphous powder; [α]_D²⁰ +296.5 (c 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.18) and 244 (4.00); IR ν_{max} 3353, 2924, 2873, 1687, 1557, 1373, 1238, 1032, 983, 802, 578 cm^{−1}; ¹H and ¹³C NMR data, Table 1; HR-ESIMS: *m/z* 403.2850 ([M + H]⁺, calcd for C₂₅H₃₉O₄, 403.2848).

Aspergorakhin D (4). White amorphous powder; [α]_D²⁰ +132.2 (c 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 203 (3.74); IR ν_{max} 3390, 2920, 2874, 1683, 1583, 1450, 1374, 1237, 1030, 593 cm^{−1}; ¹H and ¹³C NMR data, Table 1; HR-ESIMS: *m/z* 405.3014 ([M + H]⁺, calcd for C₂₅H₄₁O₄, 405.3005).

Aspergorakhin E (5). Pale brown powder; [α]_D²⁰ +107.7 (c 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 206 (5.34) and 303 (4.39); IR ν_{max} 3408, 2971, 2932, 1731, 1631, 1598, 1443, 1277, 1106, 1077, 967, 798 cm^{−1}; ¹H and ¹³C NMR data, Table 2; HR-ESIMS: *m/z* 303.1207 ([M + Na]⁺, calcd for C₁₅H₂₀O₅Na, 303.1208).

Aspergorakhin F (6). Pale brown oil; UV (MeOH) λ_{max} (log ε) 204 (4.55) and 278 (4.07); IR ν_{max} 3412, 2921, 2850, 1682, 1609, 1470, 1429, 1344, 1204, 1123, 804, 724 cm^{−1}; ¹H and ¹³C NMR data, Table 2; HR-ESIMS: *m/z* 377.1374 ([M + Na]⁺, calcd for C₂₁H₂₂O₅Na, 377.1365).

Aspergorakhin G (7). Pale yellow powder; UV (MeOH) λ_{max} (log ε) 206 (4.51), 247 (4.48), 276 (4.44), and 343 (3.88); IR ν_{max} 3400, 2916, 2849, 1668, 1585, 1424, 1294, 1236, 1092, 1054, 829 cm^{−1}; ¹H and ¹³C NMR data, Table 2; HR-ESIMS: *m/z* 371.1492 ([M + H]⁺, calcd for C₂₁H₂₃O₆, 371.1495).

Aspergorakhin H (8). Pale yellow powder; [α]_D²⁵ −29.3 (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 218 (4.24), 275 (3.68), and 320 (3.75); IR ν_{max} 3300, 2916, 2849, 1730, 1581, 1539, 1466, 1417, 1047, 944, 873, 814, 720 cm^{−1}; ¹H and ¹³C NMR data, Table 2; HR-ESIMS: *m/z* 407.1102 ([M + Na]⁺, calcd for C₂₁H₂₀O₇Na, 407.1107).

Aspergorakhin I (9). Pale yellow powder; [α]_D²⁵ +103.2 (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.00) and 278 (3.30); IR ν_{max} 3359, 2919, 2851, 1644, 1465, 1377, 1097, 1028 cm^{−1}; ¹H and ¹³C NMR data, Table 2; HR-ESIMS: *m/z* 357.1333 ([M + H]⁺, calcd for C₂₀H₂₁O₆, 357.1338).

Aspergorakhin J (10). Pale yellow powder; [α]_D²⁵ −10.4 (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 214 (3.96) and 301 (3.11); IR ν_{max} 3234, 2957, 2921, 2850, 1759, 1741, 1599, 1472, 1290, 1023, 789,

736 cm^{-1} ; ^1H and ^{13}C NMR data, Table 3; HRESIMS m/z 335.0890 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{18}\text{H}_{16}\text{O}_5\text{Na}$, 335.0895).

Aspergorakhin K (11). White amorphous powder; $[\alpha]_{\text{D}}^{20} -16.0$ (c 0.10, MeOH); UV (MeOH) λ_{max} ($\log \epsilon$) 204 (4.16) and 276 (3.18); IR ν_{max} 3366, 2921, 2850, 1650, 1465, 1378, 1273, 1016, 600 cm^{-1} ; ^1H and ^{13}C NMR data, Table 3; HR-ESIMS: m/z 207.0989 $[\text{M} + \text{H}]^+$, calcd for $\text{C}_{10}\text{H}_{16}\text{O}_3\text{Na}$, 207.0997).

Aspergorakhin L (12). Pale yellow powder; UV (MeOH) λ_{max} ($\log \epsilon$) 205 (4.11), 280 (3.63), and 365 (3.54); IR ν_{max} 3359, 2919, 2851, 1644, 1465, 1377, 1097, 1028 cm^{-1} ; ^1H and ^{13}C NMR data, Table 3; HR-ESIMS: m/z 287.0924 $[\text{M} + \text{H}]^+$, calcd for $\text{C}_{16}\text{H}_{15}\text{O}_5$, 287.0919).

Leptosphaerin D (13). Pale yellow powder; ^1H and ^{13}C NMR data, Table 3; HR-ESIMS: m/z 273.0761 $[\text{M} + \text{H}]^+$, calcd for $\text{C}_{15}\text{H}_{13}\text{O}_5$, 273.0763).

Electronic circular dichroism (ECD) and optical rotation (OR) calculation

Conformational searches were performed by means of the BARISTA software (CONFLEX Corporation) using the MMFF94S force field. The conformers with energy from 0–5 kcal mol^{-1} were used for optimization at the B3LYP/6-31G(d) level in the gas phase using the Gaussian 09 package. These conformations with 0–2.5 kcal mol^{-1} were further optimized at the B3LYP/6-311+G(d) level in the gas phase. The calculation of ECD and OR was performed at the B3LYP/6-311++G(2d,p) level. Compounds 5 and 8–11 (ESI S114^+) were selected for the calculations.^{20–25} The Boltzmann sums were performed to simulate the ECD curves and the OR values for the selected chiral compounds.

Inhibitory activity of the enzymes assay

Human phosphatase PTP1B, SHP1, and TCPTP were used for inhibitory activity assays. Na_3VO_4 was used as the positive control. The detailed procedure is placed in ESI S116,† the same as that described in the literature.²⁶ The cathepsin B assays were carried out in triplicate according to a published method²⁷ with modification in ESI S116.† Leupeptin was used as the positive control.

Cytotoxicity assays

Human cancer cells A549, Bel-7402, SMMC-7721, and HeLa were selected for cytotoxicity assays.²⁸ A549, Bel-7402, SMMC-7721, and HeLa cells were cultured and then dealt with various concentrations of 1–4 for 48 h and detected by the MTT assay. Taxol was used as the positive control. All the human cell lines were provided by the Hebei Key Laboratory of Forensic Medicine, College of Forensic Medicine, Hebei Medical University.

Conclusions

In summary, 12 new metabolites aspergorakhin A–L (1–12) and leptosphaerin D (13) were isolated from the cultivation of the fungus *Aspergillus gorakhpurensis* F07ZB1707 cultured in a salt-containing medium. The structures of these compounds included: sterol (1), nortriterpenes (2–4), aromatic polycyclic

polyketides (5–10, 12–13), and cyclohexanone derivative (11). All the compounds were obtained from *Aspergillus gorakhpurensis* without precedent and this study was helpful to fill the research gap in the field of novel compounds' discovery of this strain. Aspergorakhin A (1) showed selective activities against PTP1B and SHP1 over TCPTP with IC_{50} values of 0.57, 1.19, and 22.97 μM , respectively.

Author contributions

Yannan Ji: conceptualization, data curation, formal analysis, investigation, validation, writing – original draft. Qiqi Zhou: data curation. Guosheng Liu: data curation. Tianhui Zhu: data curation, formal analysis. Yufang Wang: data curation, formal analysis. Yan Fu: data curation. Yeying Li: data curation, methodology. Ruolan Li: data curation, methodology, formal analysis. Xuexia Zhang: data curation, methodology, formal analysis. Mei Dong: resources, methodology, formal analysis. Françoise Sauriol: data curation, methodology, formal analysis. Yucheng Gu: funding acquisition, writing – review & editing. Qingwen Shi: conceptualization, supervision, project administration, writing – review & editing. Xinhua Lu: resources, methodology, writing – review & editing. Zhiyu Ni: conceptualization, methodology, writing – review & editing.

Conflicts of interest

The authors declare no conflicts of interest.

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