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Three New Diketopiperazines from the Previously Uncultivable Marine Bacterium *Gallaecimonas mangrovi* HK-28 Cultivated by iChip

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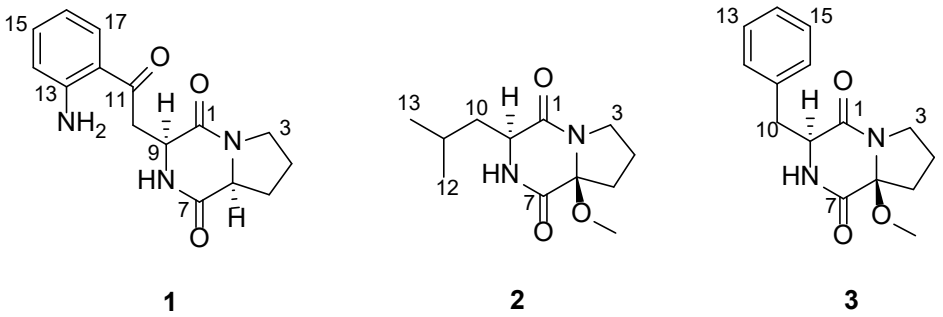
Keywords: iChip, *Gallaecimonas mangrovi,* diketopiperazines, antibacterial activity

Introduction

Mangrove-associated microorganisms have proven to be a new resource of structurally novel and biologically active natural products that could be used to develop new medicinal agents.[1−3] However, the bulk of microorganisms from the mangrove environment will not grow under laboratory conditions.[4−7] Therefore, The vast majority of biological and chemical resources related to uncultivable microorganisms have been limitedly studied so far. In order to deeply mine the bioactive agents from mangrove-derived microorganisms, a novel iChip device (isolation chip) was developed to simulate the natural environment to *in situ* cultivate uncultivable microbial organisms. The cultivation approach reportedly has been proven to increase microbial recovery by 5 to 300 times.[6]

In this study, the extracts produced from 360 mangrove microbial isolates obtained using iChip method were screened against the marine pathogen *Vibrio harveyi*. A novel bacterium *Gallaecimonas mangrovi* HK-28 which was reported by our lab previously,[8] exhibited antibacterial activity and was selected for in-depth chemical investigation. Subsequently, fermentation of the strain HK-28 in large scale and bioassay-guided analysis of the EtOAc extract of the fermentation broth were carried out. Three new diketopiperazines were discovered in the course of this research, named gallaecimonamides A−C (**1**−**3**) (Figure 1). Herein, the isolation, structure elucidation and biological activity of these new diketopiperazines (DKPs) were reported.



1 **2** **3**

Figure 1. Chemical structures of compounds **1**−**3**.

Results

Structure elucidation of compounds 1−3

Gallaecimonamide A (**1**) was isolated as a white amorphous powder. Analysis of HRESIMS and NMR (Table 1) data allowed for the determination of the molecular formula of **1** as being $C_{15}H_{17}N_3O_3$ (*m/z* 288.1341 [M + H]+). The IR spectrum of **1** showed a strong absorption at 1648 cm^{-1}, indicating that there may be carbonyl groups. The ^1H NMR spectrum of **1** recorded in DMSO-d_6 displayed signals indicative of an amide NH (δ_H 8.07), an aromatic amine (δ_H 7.13), four aromatic protons [δ_H 6.76 (1H, d, J = 8.4 Hz, H-14), 7.25 (1H, dd, J = 8.4, 6.9 Hz , H-15), 6.55 (1H, dd, J = 8.1, 6.9 Hz, H-16), 7.74 (1H, d, J = 8.1 Hz, H-17)] and four sets of methylene multiplets [δ_H 3.41−3.43 (1H, m, H-3), 3.32−3.34 (1H, m, H-3), 1.85−1.89 (1H, m, H-4), 1.79−1.84 (1H, m, H-4), 2.13−2.18 (1H, m, H-5), 1.90−1.94 (1H, m, H-5), 3.59 (1H, dd, J = 12.5, 5.2 Hz, H-10), 3.22 (1H, dd, J = 12.5, 5.4 Hz, H-10)]. In addition, analysis of the ^{13}C NMR spectrum of **1** indicated the presence of a characteristic diketopiperazine ring, including two typical amide carbonyl groups [δ_C 165.8 (C-1) and 169.5 (C-7)]. The cross peaks between H-14/H-15, H-15/H-16 and H-16/H-17 in the ^1H-^1H COSY (Figure 2) and the correlations from 8-NH to C-1 and C-9, from H-9 to C-1, C-10 and C-11, from H-17 to C-11, C-15, C-16 and C-13, from H-16 to C-14 and C-17, from H-15 to C-13 and C-17 and from NH_2-13 to C-12 in the HMBC spectrum (Figure 2) demonstrated the presence of a kynurenine (Kyn) residue in **1**. Analysis of the ^1H-^1H COSY spectrum data, a liner correlations at H_2-3/H_2-4, H_2-4/H_2-5 and H_2-5/H-6, as well as the HMBC correlations from H_2-4 to C-5 and from H-6 to C-5, indicating that compound **1** contains a proline (Pro) residue. Finally, the HMBC cross peaks from H-9 to C-1 and from 8-NH to C-6 indicated the cyclic dipeptide nature of **1**, allowing the planar diketopiperazine structure of **1** to be assigned (Figure 1).

Gallaecimonamide B (**2**) and C (**3**) were also obtained as white amorphous powders, and their molecular formulas were respectively found to be $C_{12}H_{20}N_2O_3$ (*m/z* 241.1545 $[M + H]^+$) and $C_{15}H_{18}N_2O_3$ (*m/z* 275.1389 $[M + H]^+$) based on HRESIMS data. Compound **2** displayed the presence of characteristic ^{13}C NMR chemical shift values observed for a diketopiperazine ring system, which included the typical amide carbonyl groups at C-1 (δ_C 167.8) and C-8 (δ_C 165.0), a heteroatom deshielded methine residue at C-10 (δ_C 55.3) and a hemi-aminal quaternary carbon [δ_C 90.7 (C-6)]. In addition, a methoxy group (δ_H/ 3.18, δ_C 51.4) was furthermore observed in the HSQC spectrum of **2**, which suggested the existence of a methoxy group attached to C-6. This position assignment was further confirmed by the observation of an HMBC correlation from 6-*O*-CH_3 to C-6. The cross peaks between H_2-3/H_2-4 and H_2-4/H_2-5 in the ^1H-^1H COSY spectrum (Figure 3) indicated that **2** possessed three consecutive methylene groups characteristic of a proline residue. Similarly, the unaccounted for observation of two methyl groups, a methylene group and a methine group in **2**, suggested the presence of a leucine (Leu) residue. The HMBC correlations (Figure 3) observed from H-9 to C-1, C-10 and C-11 along with

those from H_2-10 to C-1, C-9, C-12 and C-13 allowed the construction of a *cyclo*-leucine-methoxyproline diketopiperazine structure for **2** (Figure 1). Compound **3** also displayed the typical NMR data for a diketopiperazine ring (Table 1), which encompassed the characteristic amide carbonyl groups [δ_C 166.7 (C-1) and 164.8 (C-7)], a heteroatom deshielded methine residue at [δ_C 58.2 (C-9)] and hemi-aminal quaternary carbon [δ_C 90.8 (C-6)]. In addition, the presence of a methoxy group ($\delta_{H/}$ 3.00, δ_C 50.9) was observed in the HSQC spectrum of **3**, and it could be assigned based on an HMBC correlation (Figure S28) observed from 6-*O*-CH_3 to C-6. The COSY spectrum (Figure 3) displayed the correlations for geminal couplings in H_2-3, H_2-4 and H_2-5 and correlations between H_2-3/H_2-4 and H_2-4/H_2-5, indicating the presence of three consecutive methylene groups' characteristic of a proline residue in **3**. Similarly, the presence of five aromatic protons and a benzylic group in **3** suggested the existence of a phenylalanine residue that would account for all remaining atoms in the molecular formula. The cross peaks between H-9/H_2-10 in the ^{1}H-^{1}H COSY spectrum, together with the HMBC correlations from H-9 to C-1 and C-11, from H_2-10 to C-11 and C-12 revealed a cyclic-phenylalanine-methoxyproline diketopiperazine structure for **3** as shown in Figure 1.

Table 1. ^{1}H and ^{13}C NMR data of compounds **1**–**3**.

	1[a]		**2**[a]		**3**[a]	
position	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	165.8		167.8		166.7	
3	45.0	3.41−3.43, m 3.32−3.34, m	45.1	3.45−3.48, m	44.90	3.53−3.57, m 3.40−3.44, m
4	22.4	1.85−1.89, m 1.79−1.84, m	19.3	1.83−1.85, m	19.2	1.82−1.85, m
5	27.7	2.13−2.18, m 1.90−1.94, m	31.0	2.29−2.31, m 1.86−1.89, m	31.5	2.26−2.28, m 1.85−1.87, m
6	58.7	4.26 (t, *J* = 8.1)	90.7		90.8	
7	169.5		165.0		164.8	
8-NH		8.07, s		8.56 (d, *J* = 4.2)		8.34 (d, *J* = 4.2)
9	50.9	4.56 (t, *J* = 5.3)	55.3	3.72 (ddd, *J* = 9.6, 4.8, 4.3)	58.2	4.01 (ddd, *J* = 8.0, 5.6, 4.2)
10	38.3	3.59 (dd, *J* = 12.5, 5.2) 3.22 (dd, *J* = 12.5, 5.4)	44.3	1.64−1.69, m 1.52−1.55, m	40.4	3.06 (dd, *J* = 8.0, 5.6)
11	197.7		24.0	1.73−1.76, m	136.9	
12	116.5		21.6	0.87 (d, *J* = 6.5)	129.7	7.19 (d, *J* = 7.1)
13	151.1		23.1	0.90 (d, *J* = 6.6)	128.30	7.30 (t, *J* = 7.1)
14	116.9	6.76 (d, *J* = 8.4)			126.60	7.23 (t, *J* = 7.1)
15	134.1	7.25 (dd, *J* = 8.4, 6.9)			128.30	7.30 (t, *J* = 7.1)
16	114.4	6.55 (dd, *J* = 8.1, 6.9)			129.7	7.19 (d, *J* = 7.1)
17	130.9	7.74 (d, *J* = 8.1)				
13-NH_2		7.1, s				
6-*O*CH_3			51.4	3.18, s	50.9	3.00, s

[a]measured at 600 MHz for ^{1}H NMR data and 150 Hz for ^{13}C NMR data in DMSO-d_6.

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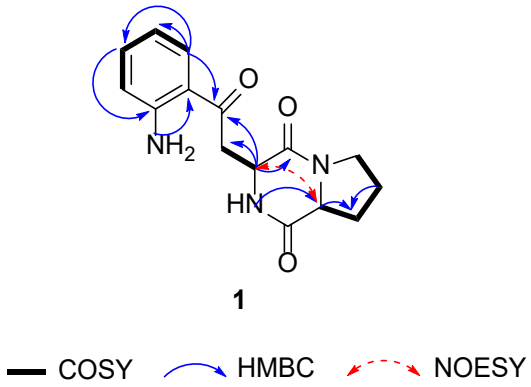

Figure 2. Key COSY, HMBC, and NOESY correlations of **1**.

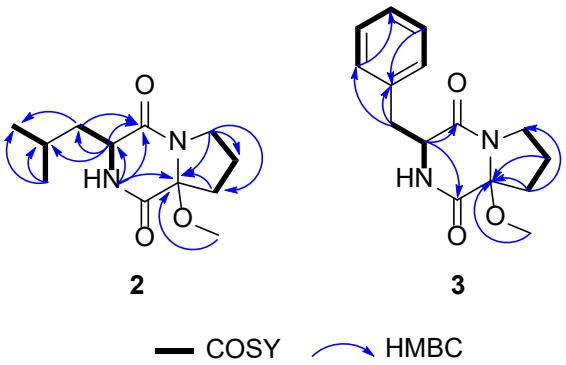

Figure 3. Key COSY and HMBC correlations of **2** and **3**.

The absolute configuration of the proline moiety for **1** was determined by optical rotation measurement, following literature reports that the sign of $[\alpha]^D$ for proline containing diketopiperazines were negative or positive depending only on the proline chiral α carbon being *S* or *R*, respectively, even when the remaining residues were other amino acids.[9, 10] The $[\alpha]^D$ value of **1** in methanol at 20 °C were -86.2°, so the absolute configuration of Pro were determined as *S* in **1**. Furthermore, the NOSEY correlation (Figure 2) between H-6 and H-9 in **1** indicated that they were on the same face of the diketopiperazine ring. Therefore, the absolute configuration of Kyn was determined as *S*, and the absolute configuration of **1** should be *cyclo* (*S*-Pro-*S*-Kyn) (Figure 1). No correlation was observed between H-6 and 9-*O*Me for **2** and **3** in the NOESY experiments, suggesting that these signals could be placed on the opposite side of the diketopiperazine ring and also on the basis of biogenetic considerations. The relative configurations at C-6 and C-9 of **2** and **3** were tentatively proposed as 6*S*,9*S* or 6*R*,9*R*, respectively. The absolute configurations of **2** and **3** were confirmed by time-dependent density functional theory (TDDFT) electronic circular dichroism (ECD) calculations.[11] Conformational analyses for the molecules 6*S*,9*S*-**2**/**3** and 6*R*,9*R*-**2**/**3** were carried out using the MMFF94S force field in the Sybyl-X 2.0. Combined with experimental and calculated ECD spectral data for analysis, the absolute configurations of both **2** and **3** were assigned to be 6*S*,9*S*, because the calculated curves of (6*S*,9*S*)-**2** and 6*R*,9*R*-**2**/**3** were a good match with the experimental curves of **2** and **3** (Figure 4).

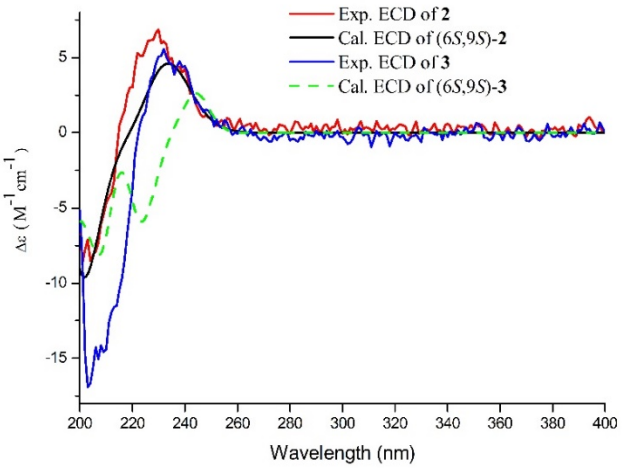

Figure 4. Experimental and calculated ECD spectra of compounds **2** and **3** in MeOH.

Biological Assays

The crude EtOAc extract of *G. mangrovi* HK-28 showed antibacterial activity against *V. harveyi* with a MIC value of 300 μg/mL when tested *in vitro*. Compounds **1**−**3** were evaluated for the biological inhibition of *V. harveyi* and *V. parahaemolyticus* growth using a previously reported protocol.[12, 13] Chloramphenicol was used as a positive control. Compound **1** exhibited modest antibacterial activity against *V. harveyi* with a MIC value of 50 μM. However, compounds **2** and **3** showed no antibacterial activity against *V. harveyi,* and none of these compounds inhibited *V. parahaemolyticus* at the tested concentrations up to 300 μM. The isolated compounds **1**−**3** were also tested for antimalarial activity against *P. falciparum* W2. None of them exhibited antimalarial activity toward the parasite (EC_{50} > 100 μg/mL).

Table 2. Antibacterial activities of compounds **1**−**3**.

	MIC (μM)	
Compounds	***V. harveyi***	***V. parahaemolyticus***
1	50	>300
2	>300	>300
3	>300	>300
Chloramphenicol[a]	6.25	12.5

[a]Chloramphenicol as positive control.

Conclusion

The chemical investigation was performed on an EtOAc extract of a novel mangrove-derived bacterial species *G. mangrovi* HK-28 isolated from the iChip platform, and three new compounds were successfully isolated. Notably, *in vitro* biological assays of **1**−**3**, only **1** displayed selectively antibacterial activity against *V. harveyi* with a MIC value of 50 μM, while the others were inactive against *V. harveyi* (MIC > 300 μM), *V. parahaemolyticus* (MIC > 300 μM) and *P. falciparum* W2 (EC_{50} > 100 μg/mL). Collectively, it is expected that continued applications of the new device to cultivate microorganisms will improve the efficiency of discovering new microbial source for the production of chemically diverse and pharmaceutically useful compounds

Experimental Section

General experimental procedures

Optical rotations were obtained using a JASCO P-2000 digital polarimeter (Hachioji, Japan). UV-vis absorption spectra were acquired with an Evolution 201 UV-vis spectrophotometer (Thermo Fisher, Waltham, MA, USA). FT-IR spectra were recorded using a Nicolet is5 spectrometer (Thermo Fisher). NMR spectra were collected on a Varian 600 MHz (Palo Alto, CA, USA) spectrometer. The signals of the solvent peaks were used as internal chemical shift references (δ_H 2.49 and δ_C 39.5 for DMSO-d_6). High-resolution ESI(±)MS data were acquired on a prototype Bruker TIMS-QTOF mass spectrometer. Semipreparative HPLC was performed using a Waters 2695 series HPLC instrument (Alliance 2695, Milford, MA, USA) equipped with a Waters 2996 detector and an ODS column (250 × 10 mm, 5 μm, YMC Co. Ltd., Tokyo, Japan). Medium-pressure liquid chromatography was carried out using a FLEXA Purification System (Angela Technologies, Tianjin, China). Column chromatography was carried out on normal phase silica gel (200−300 mesh, Qingdao Marine Chemical Inc. Qingdao, PR China) and Sephadex LH-20 (Amersham Biosciences, Piscataway, NJ, USA).

Bacterial material

The bacterium investigated in this study was previously isolated from a mangrove sediment sample collected from Haikou, Hannan Province, People′s Republic of China (110°34′E, 19°58′N), in May 2017,[8] and identified as a novel species of the genus *Gallaecimonas* based on 16S rRNA gene sequence analysis.[14] The bacterium strain HK-28 was then received for deposit in the China Center For Type Culture Collection with accession number CCTCC M2019824. Furthermore, a voucher specimen was maintained in culture at the Department of Marine Pharmacy, College of Food and Pharmaceutical Sciences, Ningbo University, using marine broth 2216 medium (MB, peptone 5 g, yeast extract 1 g, NaCl 19.45 g, $MgCl_2$ 12.6 g, $MgSO_4$ 6.64 g, $CaCl_2$ 1.8 g, KCl 0.55 g, $NaHCO_3$ 0.16 g, $FeC_6H_5O_7$ 0.1 g, $SrCl_2$ 57 mg, KBr 80 mg, H_3BO_3 22 mg, $NaSiO_3 \cdot 9H_2O$ 9.3 mg, NaF 2.4 mg, NH_4NO_3 2.4 mg, dissolved in 1 L of distilled H_2O, pH 6.0-7.0) at -80 ℃ with 25% (*v/v*) glycerol.

Fermentation, extraction, and isolation

The bacterium *G. mangrovi* HK-28 was incubated in replicates in 1 L Erlenmeyer flasks containing 400 mL of the seed medium (MB, Difco). Flask cultures were inoculated on a rotatory shaker at 150 rpm at 28 ℃ for 24 h. 20 L of the seed cultures were transferred to 500 × 1 L Erlenmeyer flasks containing 400 mL of MB medium. The fermentation broth was inoculated on a rotatory shaker at 150 rpm at 28 ℃ for 6 days. At harvest, the culture broth was filtered, and 200 L of filtrate was extracted with an equal volume of EtOAc three times. The EtOAc extract was evaporated to yield 22 g of a brown crude extract. The crude extract (22 g) was first fractionated on a silica gel VLC column using a step gradient elution with a mixture of petroleum ether/EtOAc (20:1, 10:1, 5:1, 2:1, 1:1, 2:5, 0:1, v/v) and then with EtOAc/MeOH (8:1, 0:1, v/v), producing four fractions. Fraction 2 (3.6 g) was further isolated by Sephadex LH-20, with MeOH/CH_2Cl_2 (1:1) as eluent, producing three subfractions (Fr.2-1−Fr.2-3). Fr.2-2 (2.0 g) was further subjected to MPLC separation on an ODS column eluting with MeOH/H_2O (25 to 60% MeOH, 150 min, 20.0 mL/min), and additionally separated by semipreparative reversed-phase HPLC to yield **1** (1.6 mg) (20% MeCN in water; 2.0 mL/min; t_R 25 min), **2** (2.2 mg) (15% MeCN in water; 4.0 mL/min; t_R 51 min) and **3** (1.0 mg) (18% MeCN in water; 4.0 mL/min; t_R 43 min).

Gallaecimonamide A (**1**): White amorphous powder; $C_{15}H_{17}N_3O_3$; $[\alpha]^{20}_D$ -86.2 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 227 (3.81), 256 (3.26), 366 (3.15); IR (KBr) ν_{max} 3438, 2917, 1648, 1450, 752 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* $[M + H]^+$ 288.1341 (calcd for $C_{15}H_{18}N_3O_3$, 288.1344).

Gallaecimonamide B (**2**): White amorphous powder; $C_{12}H_{20}N_2O_3$; $[\alpha]^{20}_D$ +18.0 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.14); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 226 (6.1), 202 (-8.7) nm; IR (KBr) ν_{max} 2956, 1670, 1559, 1540, 1436, 1053, 483 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* $[M + H]^+$ 241.1545 (calcd for $C_{12}H_{21}N_2O_3$, 241.1548).

Gallaecimonamide C (**3**): White amorphous powder; $C_{15}H_{18}N_2O_3$; $[\alpha]^{20}_D$ +43.6 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.45); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 232 (5.6), 205 (-15.6) nm; IR (KBr) ν_{max} 3248, 2918, 1676, 1431, 1052, 701 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* $[M + H]^+$ 275.1389 (calcd for $C_{15}H_{19}N_2O_3$, 275.1398).

Antibacterial Assay

Antibacterial activities of compounds **1**−**3** were evaluated using 96-well microplates containing the bacterial strains *V. harveyi* KP635244 and *V. parahaemolyticus* ATCC 17802. In each sterile 96-well microtiter plate, *V. harveyi* and *V. parahaemolyticus* were cultured in MB medium. Test compounds were dissolved in DMSO and added to a 96-well microtiter plate and inoculated with a bacterial suspension (10^4–10^5 CFU/mL) and incubated at 28°C for 24 h. Each well was comprised of 20 µL different concentrations of the test compounds and 180 µL freshly prepared bacterial suspension. The final concentration of the test compound in the 96-well microtiter plate is 300 µM to 0.1 µM. Chloramphenicol and dimethyl sulfoxide (DMSO) were used as positive and negative controls, respectively. Tests were carried out in triplicate. The minimum inhibitory concentration (MIC) for *V. harveyi* and *V. parahaemolyticus* was determined based on visible growth inhibition of each pathogen.

Antimalarial Assay

Compounds **1**−**3** were evaluated for the *in vitro* antimalarial activity against the parasite (*P. falciparum* W2) using a previously described method.[15] Chloroquine, atovaquone, and artemisinin were selected as positive controls against *P. falciparum* W2 with EC_{50} values of 112, 2.5, and 160 nM, respectively.

Supplementary Material

Supplementary data to this article can be found online at

Acknowledgments

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Author Contribution Statement

S.H. and L.D. designed experiments. P.X., W.Z., Y.Y., X.H., D.S., Y.S., and J.E.H.L. performed the experiments. P.X., L.D., C.B.N., H.J., X.Y., B.W. and S.L. analyzed the data. L.D., P.X., and S.H. wrote the manuscript.

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