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Crepidatumines C and D, Two New Indolizidine Alkaloids from *Dendrobium crepidatum* Lindl. ex Paxt.

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Abstract: Two new indolizidine alkaloids, crepidatumines C (**1**) and D (**2**), together with crepidine (**3**), isocrepidamine (**4**), and crepidamine (**5**) were isolated from the *Dendrobium crepidatum* Lindl. ex Paxt. X-ray diffraction experiments established the absolute configurations of known compounds **3** and **4**. The planar structures and relative configurations of new compounds **1** and **2** were elucidated by extensive spectra analysis including HR-ESI-MS, NMR (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HSQC, HMBC, and NOESY spectra), and the absolute configurations of **1** and **2** were suggested on the basis of possible biosynthetic pathways. The biological results confirmed that isocrepidamine (**4**) displayed a potent hypoglycemic effect in vitro without cytotoxicity.

Keywords: *Dendrobium crepidatum* Lindl. ex Paxt.; indolizidine alkaloids; crepidatumines; structure elucidation; hypoglycemic effect

1. Introduction

Dendrobium, a genus of Orchidaceae, is distributed in south of China [1,2]. The stems of several *Dendrobium* species are used as precious traditional Chinese medicines with the effect of maintaining gastric tonicity, enhancing the production of body fluids, and relieving and curing symptoms of dryness and body heat [3]. *Dendrobium crepidatum* Lindl. ex Paxt. Is considered as one of the sources of "Shi-Hu". It produces indolizine-type alkaloids, and so far, only several indolizine-type alkaloids and two stilbene derivatives have been isolated from this medicinal plant [4–9]. Two indolizidine alkaloids, crepidatumines a and B, with novel skeletons, together with the stereoisomer of dendrocrepidine B and dendrocrepine, were isolated during our previous chemical investigation of this medicinal plant [10]. Actually, the biosynthesis of indolizidine alkaloids from the *Dendrobium crepidatum* Lindl. ex Paxt. is still not clear. In order to obtain the potential intermediates or shunt products of the biosynthesis, five indolizidine alkaloids including the two new analogs crepidatumines C (**1**) and D (**2**) together with crepidine (**3**), isocrepidamine (**4**), and crepidamine (**5**) were isolated from the total alkaloid extract [5]. (Figure 1) In this paper, the structure elucidation, biological evaluation, and possible biogenetic origin of compounds **1**–**5** arereported.

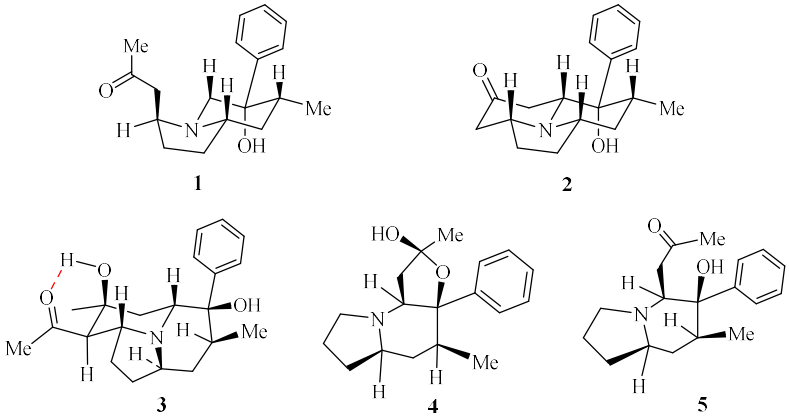


Figure 1. Structures of **1**–**5**.

2. Results and Discussion

The structures of compounds **3** and **4** were determined to be crepidine and isocrepidamine, which were supported by X-ray diffraction experiments (Figure 2), and compound **5** was characterized to be crepidamine based on the NMR data [5].

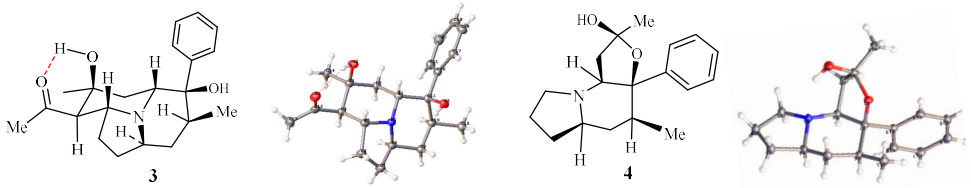


Figure 2. X-ray crystal structure of **3** and **4**.

The molecular formula of **1** was determined to be $C_{18}H_{25}NO_2$ based on the HRESIMS (*m/z* 288.1968 $[M + H]^+$, calcd 288.1964). The ^1H, ^{13}C and HMQC spectra of **1** (Table 1) revealed the presence of two methyls, five methylenes, three methines, an oxygenated carbon with chemical shift value δ_C = 76.5, a keto group with chemical shift value δ_C = 206.8, and a mono-substituted phenyl ring. In addition, a singlet proton formed as a free hydroxyl or amino group.The ^1H-^1H COSY correlations established three isolated spin-systems including a mono-substituted phenyl unit, and a fragment: –C-12–C-7–C-8–C-9–C-10–C-11–C-1–C-2–. Analysis of HMBC correlations elucidated the structure of **1** (Figure 3). The HMBC cross peaks from 6-OH to C-5, C-6, C-7, and C-1′ determined the direct connectivities of C-6 with C-5, C-7, and C-1′ with a hydroxyl group anchored at C-6; the correlations of CH_3-4 with C-2 and C-3 established the linkage of CH_3-4 with C-2 and C-3; and correlations of CH_2-5 with C-1 and C-9, and H-9 with C-1, established an indolizidine ring system. Thus, the planar structure of **1** was characterized. The relative configuration of **1** was determined on the basis of analysis of NOESY correlations. The NOESY correlations from H-5b, H-7 to H-2′ (H-6′), and from H-5b to H-9 confirmed that these protons or groups were on the same side of the corresponding piperidine ring; the correlations of –CH_2-2 with H-5b, and of H-1 and 6-OH with H-5a demonstrated that these protons were close to each other in space. Thus, the relative configuration of **1** was determined (Figure 3).

The HRESIMS (*m/z* 286.1809 $[M + H]^+$, calcd 286.1807) assigned the molecular formula of **2** as $C_{18}H_{23}NO_2$. The ^1H, ^{13}C and HMQC spectra of **2** (Table 1) revealed the presence of one methyl, five methylenes, four methines, an oxygenated carbon with chemical shift value δ_C = 76.5, a keto group with chemical shift value δ_C = 208.5, and a mono-substituted phenyl ring. In addition, there are two singlet protons as free hydroxyl or amino groups. These data accounted for all the ^1H and ^{13}C-NMR resonances together considering the degrees of unsaturation, implying that **2** possessed a tricyclic system. The ^1H-^1H COSY correlations established three isolated proton spin-systems including a mono-substituted phenyl unit, and two fragments: –C-12–C-7–C-8–C-9–C-10–C-11–C-1–C-2– and

–C-4–C-5–. Analysis of HMBC correlations elucidated the structure of **2** (Figure 4). Correlations of H-5 with C-1 and C-9, and H-9 with C-1 established an indolizidine ring system. The HMBC cross peaks from 6-OH to C-5, C-6, C-7, and C-1′ determined the direct connectivities of C-6 with C-5, C-7, and C-1′ with a hydroxyl group anchored at C-6.The correlations of H-4 with C-2 and C-3 established the linkage of C-4 with C-2 and C-3. Thus, the planar structure of **2** was characterized. The relative configuration of **2** was determined on the basis of analysis of NOESY correlations. The correlations from H-5 with H-1, H-7, H-9 and 7-OH implied that these groups possessed β-configurations. The NOESY correlations from 12-Me to H-2′ (H-6′) confirmed that 12-Me and the mono-substituted phenyl group were on the same side of the corresponding furan ring. Thus, the relative configuration of **2** was determined (Figure 4).

Table 1. NMR spectroscopic data of **1** and **2** in (DMSO-d_6) (δ in ppm and J in Hz) [a,b].

Pos	1		2	
	δ_C [b], Type	δ_H [a], mult. (*J* in Hz)	δ_C [b], Type	δ_H [a], mult. (*J* in Hz)
1	74.8, CH	4.10, m	59.9, CH	3.37, m
2	48.6, CH_2	2.46, m	44.1, CH_2	2.33, dd (10.8, 13.8) 1.92, ddd (1.8, 4.2, 13.8)
3	206.8, qC		209.6, qC	
4	30.9, CH_3	2.07, s	38.6, CH_2	1.13, ddd (1.8, 3.0, 13.2) 2.63, t (13.2)
5a 5b	67.1, CH_2	2.95, d (10.8) 2.69, d (10.8)	66.7, CH	3.00, dd (3.0, 13.2)
6	76.5, qC		76.2, qC	
7	38.4, CH	1.96, m	31.7, CH	2.46, m
8	36.3, CH_2	1.49, m	35.5, CH_2	1.46, m 1.74, dt (3.0, 11.4)
9	63.9, CH	2.43, m	51.4, CH	3.15, m
10	30.2, CH_2	1.65, m 1.46, m	29.7, CH_2	1.40, m 1.97, m
11	30.8, CH_2	1.67, m 1.25, m	29.1, CH_2	1.39, m 2.04, m
12	14.4, CH_3	0.48, d (6.6)	16.1, CH_3	0.71, d (6.6)
1′	146.1, qC		143.9, qC	
2′/6′	128.2, CH	7.46, dd (1.2, 8.4)	126.9, CH	7.41, br d (7.2)
3′/5′	125.6, CH	7.31, br d (8.4)	128.2, CH	7.34, dd (7.2)
4′	126.8, CH	7.21, br t (8.4)	127.0, CH	7.24, dd (7.2)
6-OH		4.76, s		4.53, s

[a] Assignments were based on HSQC, HMBC, and ^{1}H-^{1}H COSY experiments. [b] NMR spectroscopic data were recorded at 600 MHz (^{1}H NMR), 150 MHz (^{13}C NMR).

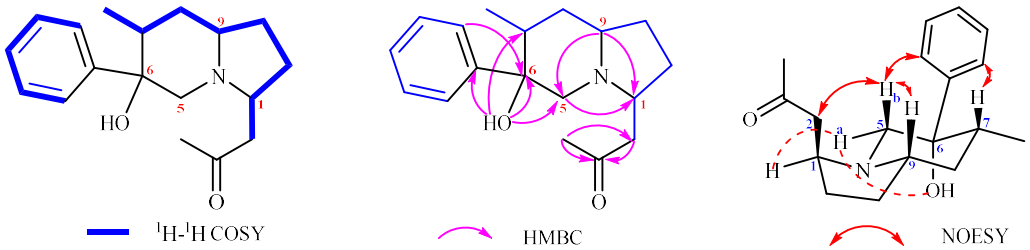


Figure 3. ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of **1**.

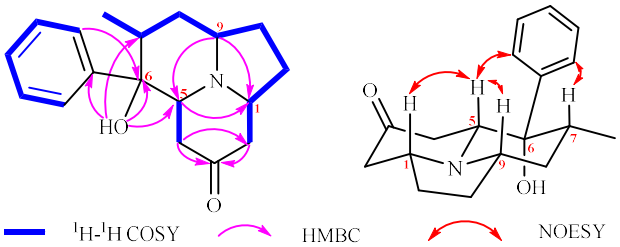


Figure 4. ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of **2**.

The absolute configurations of **1** and **2** were suggested to be same as those of **3**–**5** on the basis of similar biosynthesis pathway (Figure 5, Supplementary Materials). Structures of **3** and **4** were determined by the X-ray diffraction experiments, and showed that **4** is a racemate.

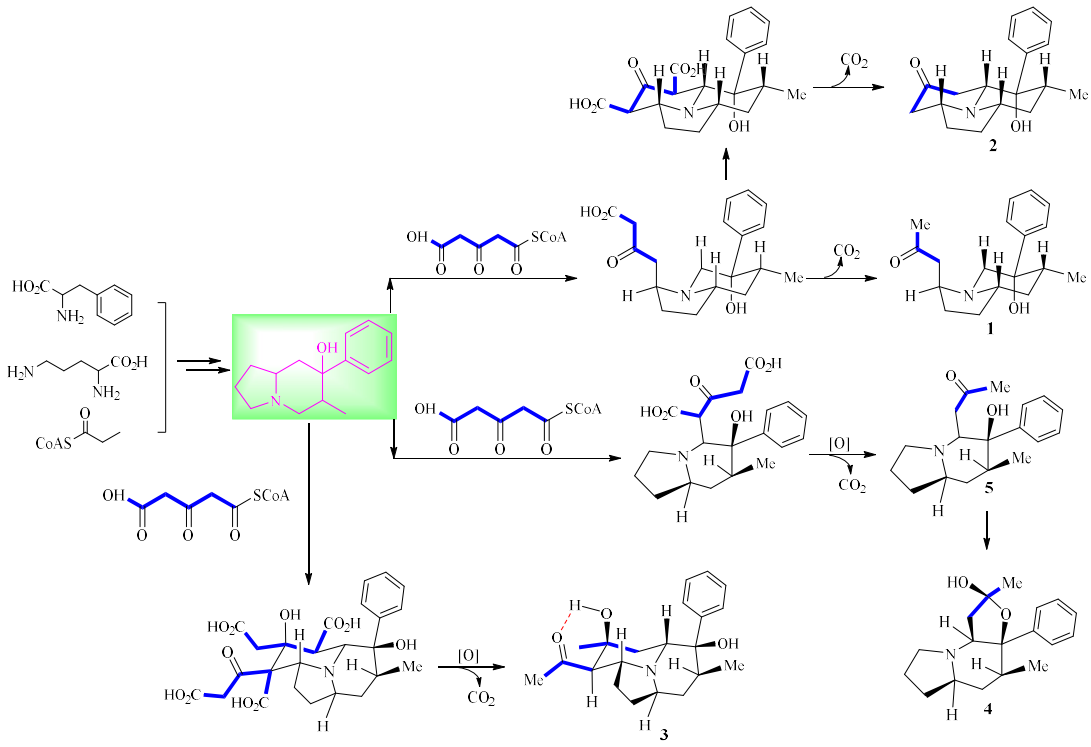
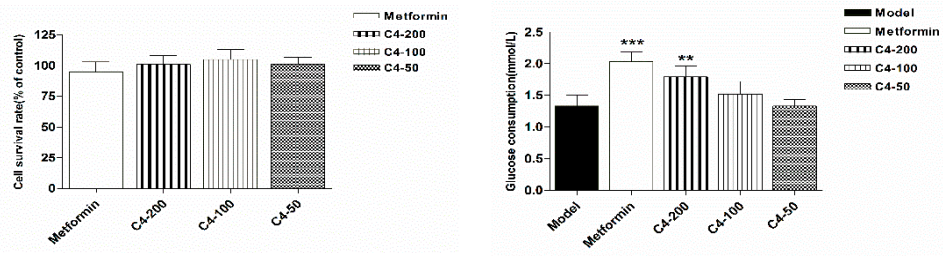


Figure 5. The possible biosynthetic pathway of compounds **1**–**5**.

The hypoglycemic effect of compound **4** (isocrepidamine) was evaluated using the high glucose model of HepG2 cells. As a result, at the concentrations of 200 μmol/L, this compound significantly increased the glucose consumption by 34% compared with the model group, which hadnon-cytotoxicity as per the cell counting kit-8 (CCK-8) assay (Figure 6).



(**A**) (**B**)

Figure 6. Effect of compound 4 (C4) on cell viability (**A**) and glucose consumption (**B**) in the HepG2 cell. Data are shown as the mean ± SD (n = 6). ** $p < 0.01$, *** $p < 0.001$ versus model.

3. Experimental Section

3.1. General Experimental Procedures

Optical rotations were measured on a PerkinElmer 241 polarimeter (Perkin Elmer, Inc., Waltham, MA, USA), and UV data were determined on a ThermoGenesys-10S UV-vis spectrometer (Fisher Scientific, Illkirch, France). IR data were recorded using a Nicolet IS5FT-IR spectrophotometer (Shimadzu, Kyoto, Japan). CD spectra were obtained on a JASCO J-810 spectrometer (JASCO, Tokyo, Japan). ^{1}H and ^{13}C-NMR data were acquired with a Bruker 600 spectrometer (Bruker, Rheinstetten, Germany) using solvent signals (DMSO-d_6; δ_H 2.50/δ_C 39.5) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. HRESIMS were obtained using a TOF-ESI-MS (Waters Synapt G2, Milford, MA, USA). Semipreparative HPLC separation was carried out using a Lumtech instrument packed with a YMC-Pack ODS-A column (YMC Co., Ltd., Kyoto, Japan, 5 μm, 250 × 10 mm). Sephadex LH-20(Pharmacia Biotech AB, Uppsala, Sweden) and silica gel (200–300 mesh) (Qingdao Marine Chemical Plant, Qingdao, China) were used.

3.2. Plant Materials

The stems of *Dendrobium crepidatum* Lindl. ex Paxt. were collected from Ruili Resource Nursery of Dendrobium Germ Plasm and Resources, the Ministry of Agriculture and Rural Affairs of the People's Republic of China (Yunnan, China) in August 2017. The sample was identified by one of the co-authors Ze-Sheng Li from Yunnan Dehong Institute of Tropical Agricultural Science (Yunnan, China). a voucher specimen was deposited in the herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences (Beijing, China).

3.3. Extraction and Isolation

The dried stems of *Dendrobium crepidatum* Lindl. ex Paxt. (9.0 kg) were extracted under reflux with 95% ethanol (50 L × 3 h, three times). The combined extract was suspended with water, and extracted with petroleum ether and CH_2Cl_2 three times separately. The fraction of CH_2Cl_2 was concentrated into extracts, and dissolved in 5% hydrochloric acid filtered, then adjusted to pH 10 with ammonia water. Finally, it was extracted by CH_2Cl_2 three times at room temperature. The CH_2Cl_2 extract was obtained the total alkaloids 90 g of crude extract. The original extract was fractionated on a silica gel CC eluted with petroleum ether- acetone (50:1, 40:1, 30:1, 20:1, 15:1, 10:1, 5:1, 2:1 and 0:1, *v*/*v*, each 6.6 L) to give five fractions (Fr.1 to Fr.5). Fr.2 (10 g) was fractionated on a silica gel column chromatography (CC) using petroleum ether-acetone isocratic elution (30:1) to afford six fractions (Fr.2.1–Fr.2.6). Fr.2.1 (10.0 g) was purified by semi-preparative HPLC (60–100% MeOH-H_2O for 30.0 min, *v*/*v*, 2 mL/min) to obtain crepidine (**3**; 105 mg, t_R 29.0 min), and isocrepidamine (**4**; 2.0 g, t_R 32.7 min). Separation of Fr.2.4 (2.0 g) was performed over Sephadex LH-20 (CH_2Cl_2: MeOH/1:1) to give four fractions (Fr.2.4.1–Fr.2.4.4). Fr.2.4.2 (500 mg) was further purified by semi-preparative HPLC (60–100% MeOH-H_2O for 30 min, *v*/*v*, 2 mL/min) to obtain crepidamine (**5**; 30.0 mg, t_R 21.0 min). Fr.2.4.3 (1.0 g) was purified by

semi-preparative HPLC (60–100% MeOH-H_2O for 30.0 min, *v*/*v*, 2 mL/min) to obtain crepidatumine C (**1**; 4.0 mg, t_R 32.8 min), and crepidatumine D (**2**; 25.0 mg, t_R 24.7 min).

Compound **1**: white powder; $[\alpha]_D^{25}$ −3.00 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 206 (3.68); IR (neat) υ_{max} 2930, 1714, 1036, 766, 704 cm^{-1}; for ^{1}H-NMR and ^{13}C-NMR data see Table 1; Positive HR-ESI-MS: *m*/*z* 288.1964 (calcd. for $C_{18}H_{26}NO_2$ $[M + H]^+$, 288.1968).

Compound **2**: white powder; $[\alpha]_D^{25}$ −4.00 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 209 (3.81); IR (neat) υ_{max} 3482, 2964, 1708, 999, 776, 709 cm^{-1}; for ^{1}H-NMR and ^{13}C-NMR data see Table 1; Positive HR-ESI-MS: *m*/*z* 286.1807 (calcd. for $C_{18}H_{24}NO_2$ $[M + H]^+$, 286.1809).

3.4. X-Ray Crystallographic Analysis of ***3*** *and* ***4****.*

Upon crystallization from *n*-Hexane–CH_2Cl_2 (10:1) using the vapor diffusion method, colorless crystals were obtained for **3**. $C_{21}H_{29}NO_3$, M = 343.45, orthorhombic, a = 5.7476(3) Å, b = 17.5786(5) Å, c = 17.7942(6) Å, U = 1797.84(11) Å3, T = 109.1(3), space group P212121 (No. 19), Z = 4, μ(Cu Kα) = 0.666, 9652 reflections measured, 3383 unique (Rint = 0.0609), which were used in all calculations. The final wR (F2) was 0.1367 (all data).

Crystallographic data for the structure of **3** has been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 1936544) (Table 2).

Table 2. Crystal data and structure refinement for **3.**

Identification Code	**3**
Empirical formula	$C_{21}H_{29}NO_3$
Formula weight	343.45
Temperature/K	109.1(3)
Crystal system	orthorhombic
Space group	$P2_12_12_1$
a/Å, b/Å, c/Å	5.7476(3), 17.5786(5), 17.7942(6)
α/°, β/°, γ/°	90, 90, 90
Volume/Å^3	1797.84(11)
Z	4
ϱ_{calc}/mg mm^{-3}	1.269
μ/mm^{-1}	0.666
F (000)	744
Crystal size/mm^3	0.35 × 0.12 × 0.02
2θ range for data collection	7.06 to 142.74°
Index ranges	−6 ≤ h ≤ 7, −18 ≤ k ≤ 21, −21 ≤ l ≤ 19
Reflections collected	9652
Independent reflections	3383[R(int) = 0.0609 (inf-0.9Å)]
Data/restraints/parameters	3383/0/231
Goodness-of-fit on F^2	1.030
Final R indexes [I > 2σ (I) i.e., F_o > 4σ (F_o)]	R_1 = 0.0510, wR_2 = 0.1302
Final R indexes [all data]	R_1 = 0.0551, wR_2 = 0.1367
Largest diff. peak/hole/e Å^{-3}	0.275/−0.288
Flack Parameters	0.2(2)
Completeness	0.993

Upon crystallization from *n*-Hexane–CH_2Cl_2 (10:1) using the vapor diffusion method, colorless crystals were obtained for **4**. $C_{20}H_{27}NO_4$, M = 345.42, orthorhombic, a = 6.6679(4) Å, b = 10.7681(4) Å, c = 24.2111(9) Å, U = 1738.38(13) Å3, *T* = 107.75(10), space group $P2_12_12_1$ (no. 19), Z = 4, μ (Cu Kα) = 0.737, 5698 reflections measured, 3256 unique (Rint = 0.0271), which were used in all calculations. The final wR (F2) was 0.1106 (all data).Crystallographic data for the structure of **4** has been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 1908235) (Table 3).

Table 3. Crystal data and structure refinement for compound **4**.

Identification Code	**4**
Empirical formula	$C_{20}H_{27}NO_4$
Formula weight	345.42
Temperature/K	107.75(10)
Crystal system	orthorhombic
Space group	$P2_12_12_1$
a/Å, b/Å, c/Å	6.6679(4), 10.7681(4), 24.2111(9)
α/°, β/°, γ/°	90, 90, 90
Volume/Å^3	1738.38(13)
Z	4
ϱ_{calc}/mg mm^{-3}	1.320
μ/mm^{-1}	0.737
F (000)	744
Crystal size/mm^3	0.350 × 0.340 × 0.100
2θ range for data collection	8.988 to 142.446°
Index ranges	$-4 \le h \le 7$, $-11 \le k \le 13$, $-29 \le l \le 29$
Reflections collected	5698
Independent reflections	3256[R(int) = 0.0271 (inf-0.9Å)]
Data/restraints/parameters	3256/0/230
Goodness-of-fit on F^2	1.054
Final R indexes [I > 2σ (I) i.e., $F_o > 4\sigma(F_o)$]	R_1 = 0.0397, wR_2 = 0.1072
Final R indexes [all data]	R_1 = 0.0421, wR_2 = 0.1106
Largest diff. peak/hole/e Å^{-3}	0.312/−0.240
Flack Parameters	−0.13(14)
Completeness	0.9984

3.5. In Vitro Evaluation of Compound **4**

Cell culture: Human hepatoma cells HepG2 were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone). The medium was supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (HyClone) in a humidified atmosphere of 5% CO_2 and 37 °C.

Assay for cell viability: The assay for cell viability was determined with the cell counting kit-8 (CCK-8). HepG2 cells were seeded in 96-well plates as 2.5×10^3 cells each well. After culturing for 24 h, the control group was added with serum-free medium, while the experimental groups were with the medium containing different concentrations (50, 100, and 200 μmol/L) of compound **4** or 200 μmol/L of metformin for another 24 h. Then the cells were treated with CCK-8 for 4 h. Finally, the absorbance was measured at 450 nm. The cell survival rate was calculated as the absorbance of each treated well divided by the control.

Assay for hypoglycemic activity: For the experiment, the cells were seeded in 96-well plates as 1×10^4 cells each well. After culturing for 24 h, the medium containing different concentrations (50, 100 and 200 μmol/L) of compound **4** was added for 24 h. The cells with 200 μmol/L metformin treatment were taken as positive control and the cells with phenol red-free DMEM as control. After the drug treatment, the glucose concentrations of the medium were determined with the glucose oxidase method. The glucose consumption of each well was obtained by subtracting the glucose concentrations of the experimental medium from the control group.

4. Conclusions

Two new indolizidine alkaloids crepidatumines C (**1**) and D (**2**) together with crepidine (**3**), isocrepidamine (**4**), and crepidamine (**5**) were isolated from the *Dendrobium crepidatum* Lindl. ex Paxt., and their structures were determined by HR-ESI-MS, NMR (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HSQC, HMBC, and NOESY spectra), and X-ray diffraction experiments. The results enrich the chemical diversity and further provide the key intermediates in the biosynthetic pathway of indolizidine alkaloids from *Dendrobium crepidatum* Lindl. ex Paxt., implying that more minor intermediates or shunt products might exist in the medicinal plants. In addition, the biological study showed a potent hypoglycemic effect of isocrepidamine (**4**) in vitro without cytotoxicity.

Supplementary Materials: The following including NMR, IR, UV and HR-ESI-MS spectra of compounds **1** and **2** are available online.

Author Contributions: X.X. performed the isolation; Z.L., H.Z. and Y.B. provided and identified the medicinal plants; Y.Y. performed the biological experiments; M.Y. provided the mass data; G.D. analyzed the NMR spectra; G.D. and B.L. wrote the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds (**1** and **2**) are available from the authors.