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Acortatarins A and B, Two Novel Antioxidative Spiroalkaloids with a Naturally Unusual Morpholine Motif from *Acorus tatarinowii*

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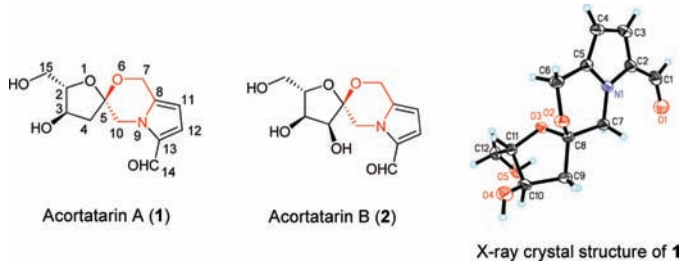
ABSTRACT



Acortatarins A (1) and B (2), two novel spiroalkaloids with a naturally unusual morpholine motif, were isolated from the rhizome of *Acorus tatarinowii*. Their structures with absolute configuration were determined by spectroscopic methods, X-ray diffraction analysis, and Mosher's method. Importantly, compound 1 could significantly inhibit reactive oxygen species production in high-glucose-stimulated mesangial cells in a dose- and time-dependent manner.

Diabetic nephropathy (DN) is a major complication of diabetes and a leading cause of end-stage renal disease (ESRD) worldwide.[1] Although hyperglycemia is the underlying metabolic abnormality, many other mechanisms have also been proposed for the development of DN.[2] Increasing evidence shows that the overproduction of reactive oxygen species (ROS) in renal cells plays an important role in the development and progression of DN. Therefore, a search for molecules that potentially inhibit renal oxidative stress could have an important impact on the intervention of DN.

The rhizome of *Acorus tatarinowii* Schott (Araceae) has been widely used as a traditional Chinese medicine for

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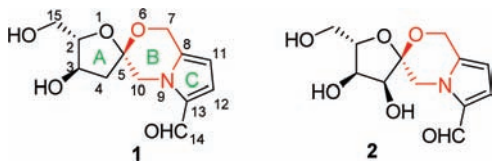
treating central nervous system disorders.[3] In the course of our search for bioactive compounds from this plant, two novel spiroalkaloids, acortatarins A (**1**) and B (**2**), were isolated and identified to bear a naturally unusual morpholine ring. The two compounds were also assessed for their effects on inhibition of high-glucose-induced ROS production in renal mesangial cells. Herein, we describe the isolation, structural determination, and biological evaluation of these two novel spiroalkaloids.



The air-dried powders of *A. tatarinowii* rhizome (50 kg) were extracted with boiling water (2 × 100 L) and then EtOH (1 × 100 L) under reflux to give an extract (4.7 kg), which was suspended in water (6 L) followed by an exhaustive partition with EtOAc and *n*-BuOH. The *n*-BuOH soluble extract (315 g) was separated by silica gel column chromatography eluted with gradient $CHCl_3$−MeOH−H_2O to supply eight fractions (I−VIII). Fraction IV (9.3 g) was passed through a MCI gel CHP 20P column with gradient aqueous MeOH to yield four subfractions (IV-A−IV-D). Subfraction IV-B (1.6 g) was purified by C_{18} silica gel, Sephadex LH-20 and semipreparative HPLC (Agilent 1100 HPLC system, Zorbax SB-C-18, Agilent, 9.4 mm ×25 cm, MeOH−H_2O, 20:80) to afford compounds **1** (7.3 mg) and **2** (3.4 mg).

Acortatarin A (**1**),[4] a colorless needle crystal, presented a molecular formula of $C_{12}H_{15}NO_5$ deduced from its HRESIMS at *m/z* 276.0843 $[M + Na]^+$ (calcd 276.0847), requiring 6 degrees of unsaturation. UV absorption at 296 nm revealed the presence of a pyrrole-2-aldehyde moiety,[5] consistent with NMR observations of δ_H 9.32 (s, H-14) and δ_C 180.2 (C-14) for an aldehyde group, and two vicinal olefinic protons at δ_H 6.98 (d, J = 4.3 Hz, H-12) and 6.08 (d, J = 4.3 Hz, H-11) characteristic of a 2,5 disubstituted pyrrole ring (Table 1).[6] This partial structure (ring C) was further supported by $^1H-^1H$ COSY correlations of H-11/H-12 and HMBC correlations of H-14/C-13, H-12/C-8, C-11, C-13, C-14, and H-11/C-7, C-8, C-12, C-13.

The ^{13}C NMR and DEPT spectra showed 12 carbons due to four methylenes, five methines (one aldehydic carbon and two olefinic ones), and three quaternary carbons (two olefinic ones). These data occupied only 4 degrees of unsaturation, and thus there should be two additional rings in the structure of **1**. Analysis of ^{13}C NMR spectrum disclosed the presence

Table 1. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data of **1** and **2** in CD_3OD

	1		**2**	
position	δ_H	δ_C	δ_H	δ_C
2	4.03 (ddd, 4.9, 4.9, 3.7)	89.3 (d)	3.87 (ddd, 7.7, 7.1, 3.3)	84.0 (d)
3	4.24 (ddd, 8.5, 4.9, 2.4)	72.3 (d)	4.06 (t, 7.7)	75.9 (d)
4a	2.10 (dd, 14.0, 2.4)	45.8 (t)	3.91 (d, 7.7)	80.9 (d)
4b	2.29 (dd, 14.0, 8.5)			
5		104.5 (s)		100.5 (s)
7a	4.80 (d, 15.9)	58.7 (t)	4.88 (d, 15.4)	58.5 (t)
7b	4.96 (d, 15.9)		5.13 (d, 15.4)	
8		137.6 (s)		137.3 (s)
10a	4.17 (d, 14.0)	52.0 (t)	4.30 (d, 13.7)	50.7 (t)
10b	4.55 (d, 14.0)		4.50 (d, 13.7)	
11	6.08 (d, 4.3)	106.1 (d)	6.08 (d, 4.4)	106.2 (d)
12	6.98 (d, 4.3)	125.9 (d)	7.03 (d, 4.4)	126.1 (d)
13		132.4 (s)		132.6 (s)
14	9.32 (s)	180.2 (d)	9.37 (s)	180.3 (d)
15a	3.57 (dd, 12.2, 4.9)	63.0 (t)	3.61 (dd, 12.1, 7.1)	64.7 (t)
15b	3.65 (dd, 12.2, 3.7)		3.72 (dd, 12.1, 3.3)	

of a deoxysugar moiety,[7] corresponding to six carbon resonances at δ_C 52.0 (C-10), 104.5 (C-5), 45.8 (C-4), 71.3 (C-3), 89.3 (C-2), and 63.0 (C-15). This fragment (ring A) was further proved by $^1H-^1H$ COSY pairs of H-15/H-2/H-3/H-4 and HMBC correlations of H-10/C-4, C-5, H-4/C-2, C-3, C-5, C-10, H-3/C-5, C-15, and H-15/C-2, C-3 (Figure 1). The remaining structure (ring B) was concluded to be a

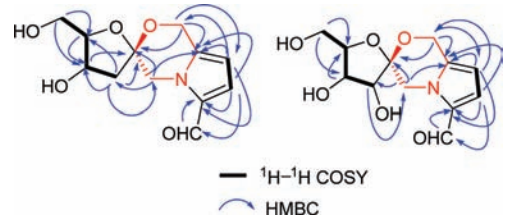


Figure 1. Key COSY and HMBC correlations of **1** and **2**.

naturally rare morpholine moiety, consisting of partial atoms of rings A and C. This was supported by HMBC couplings of H-7/C-5, C-8, C-11, and H-10/C-4, C-5, C-8. Accordingly, the planar structure of **1** was elucidated as shown. The X-ray crystallographic diffraction analysis revealed the presence of a naturally unusual morpholine ring in **1** and the relative configurations at C-2, C-3, and C-5 (Figure 2).

The absolute configuration at C-3 of compound **1** was determined, using the Mosher method,[8] by comparing the ^1H NMR chemical shifts of (*S*)- and (*R*)-MTPA esters. To prepare the Mosher esters, the hydroxyl at C-15 was first selectively protected with TBS to produce **1a**.[9] Then, the secondary hydroxyl at C-3 was treated with Mosher chloride

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(4) **Acortatarin A (1):** colorless needles, mp 164−166 °C; $[\alpha]^{27}_D$ +178.4 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε) 296 (4.25), 254 (3.75) nm; IR (KBr) ν_{max} 3426, 2928, 2855, 1734, 11713, 1641, 1462, 1185 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; FABMS: *m/z* 254 $[M + H]^+$ (100), 206 (9); HRESIMS *m/z* 276.0843 (calcd for $[M + Na]^+$ 276.0847).

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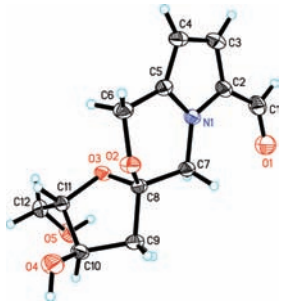

Figure 2. X-ray crystal structure of **1** showing relative configuration.

to give Mosher ester derivatives of **1a**, and their ^{1}H NMR data were assigned according to ^{1}H−^{1}H COSY data (Table 2). According to the Mosher arguments, C-3 was assigned

Table 2. ^{1}H NMR Data of the (*S*)- and (*R*)-MTPA Esters of **1a** in $CDCl_3$ at 500 MHz

proton	δ_H(*S*)-MTPA of **1a**	δ_H (*R*)-MTPA of **1a**	$\Delta\delta_H$ ($\delta_S - \delta_R$)
2	4.28 (m)	4.32 (m)	−0.04
3	5.46 (m)	5.44 (m)	*R*
4a	2.40 (m)	2.37 (m)	+0.03
7a	4.71 (d, 15.6)	4.74 (d, 15.6)	
7b	4.89 (d, 15.6)	4.93 (d, 15.6)	
10a	4.28 (d, 13.7)	4.28 (d, 13.7)	
10b	4.65 (d, 13.7)	4.65 (d, 13.7)	
11	5.99 (d, 4.1)	5.99 (d, 4.1)	
12	6.91 (d, 4.1)	6.91 (d, 4.1)	
14	9.46 (s)	9.46 (d)	
15a	3.68 (dd, 11.0, 3.2)	3.71 (dd, 11.0, 3.2)	−0.03
15b	3.83 (dd, 11.0, 2.7)	3.90 (dd, 11.0, 2.7)	−0.07

as *R*-configuration, since the signs of $\Delta\delta_H$ ($\delta_S - \delta_R$) were positive for H-4 (+0.03) and negative for H-2 (−0.04) and H-15 (−0.03, −0.07). As the relative stereochemistry at C-2, C-3, and C-5 of **1** has been confirmed by the X-ray crystallographic diffraction analysis, the absolute configuration of C-2 (*R*) and C-5 (*S*) were readily determined. Thus, the absolute configuration of compound **1** was elucidated as illustrated in Figure 1, and named as acortatarin A.

Acortatarin B (**2**)[10] was obtained as a white powder. The molecular formula of **2** was established as $C_{12}H_{15}NO_6$ by HRESIMS at *m*/*z* 292.0803 $[M + Na]^+$ (calcd 292.0797). Its UV spectrum indicated the presence of a pyrrole-2-aldehyde moiety (295 nm). The NMR spectral data of **2** were very similar to those of **1**, differing in that C-4 of **1** was oxygenated in **2**, which resulted in the downfield shift of C-4 from δ_C 45.8 in **1** to 80.9 in **2**. This conclusion was also supported by ^{1}H−^{1}H COSY correlations of H-15/H-2/H-3/H-4 (δ_H 3.91, d, J = 7.7 Hz) and HMBC interactions of H-4/C-3, C-10, and H-10/C-4 (Figure 1). The planar structure of **2** was thus established as shown (Figure 1). The relative configuration of **2** was assigned via ROESY experiments (Figure 3), which showed cross peaks of H-15/H-3,

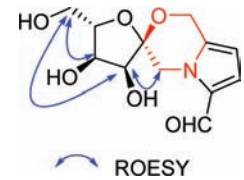

Figure 3. Key ROESY correlations of **2**.

H-4/H-15, H-10, suggesting these protons to be spacially vicinal. From a biogenetic point of view, **2** was proposed to have the same absolute configuration as that of **1**. As a result, the structure of **2** was determined as shown.

ROS are produced by various stressors as well as endogenous metabolic activities, including glucose metabolism. High-glucose-induced ROS overproduction has been directly implicated in DN. Compounds **1** and **2** were examined for their antioxidative effects in high-glucose-induced mesangial cells. Established rat mesangial cells were pretreated with indicated concentration of **1**, **2**, or cytosolic Cu/Zn superoxide dismutase (c-SOD, positive control) at 37 °C for 1 h and then exposed to either 5.6 mM (normal glucose, NG) or 25 mM (high glucose, HG) D-glucose for 3 h as described in previous reports.[11] The intracellular production of ROS was detected by 2′,7′-dichlorofluorescein (DCF) fluorescence. As shown in Figure 4A and B, high-glucose-induced ROS generation was significantly attenuated by pretreating mesangial cells with **1** and to lesser extent with **2**. The antioxidative activity of **1** and **2** was dose-dependent and reached the maximum at 10 μM (**1**) and 50 μM (**2**), respectively.

To further confirm the antioxidative effect of **1**, mesangial cells were pretreated with 10 μM **1** or 100 u/mL of c-SOD for 1 h and then incubated with 5.6 or 25 mM D-glucose for 1−24 h. As shown in Figure 4C, the pretreatment of **1** decreased ROS production by almost 50% at various time points, suggesting that **1** inhibited high-glucose-induced ROS generation in mesangial cells in a time-dependent fashion.

The trypan blue exclusion test was performed in every experiment to exclude the possibility that the effects of **1** and **2** were related to their cellular toxicity. The results showed that there was no statistically significant difference among the groups (data not shown).

The morpholine motif is a common pharmacophore present in many inhibitors, such as phosphoinositide 3-kinase related

(10) **Acortatarin B (2):** white amorphous powders; $[\alpha]^{27}_D$ 92.7 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 295 (4.05), 255 (3.63) nm; IR (KBr) ν_{max} 3427, 2923, 2852, 1640, 1033 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; FABMS *m*/*z* 270 $[M + H]^+$ (81), 255 (23), 225 (100), 207 (31), 193 (10), 179 (6), 163 (33), 115 (35), 74 (22); HRESIMS *m*/*z* 292.0803 (calcd for $[M + Na]^+$ 292.0797).

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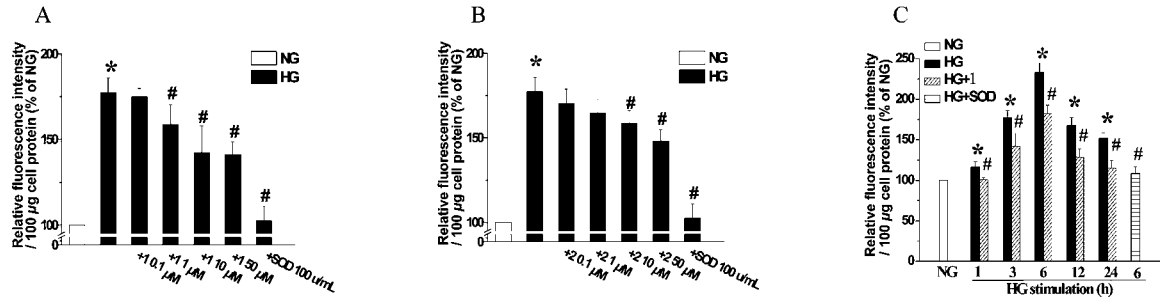

Figure 4. Compounds **1** and **2** inhibited high-glucose-induced ROS production in mesangial cells. Data are expressed as mean ± SD of three independent experiments. ANOVA, $p < 0.001$ in A, B, and C; *$p < 0.05$ vs NG; #$p < 0.05$ vs HG.

kinases,[12] TNF-α converting enzymes,[13] matrix metalloproteinases, and tumor necrosis factor inhibitors.[14] The oxygen in morpholine has been shown to participate directly in a key hydrogen bonding interaction in the ATP-binding site of PI3Kc.[15] Therefore, the importance of the morpholine motif has attracted great interest in oriented synthesis by medicinal chemists.[16] Our findings of bioactive natural morpholine-containing alkaloids will provide a new template for further exploration in drug discovery of DN.

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Supporting Information Available: Experimental procedures, NMR spectra, MS spectra, IR spectra, and X-ray crystallographic data in CIF format of acortatarin A (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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