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Alkaloids from the Leaves of *Uncaria rhynchophylla* and Their Inhibitory Activity on NO Production in Lipopolysaccharide-Activated Microglia

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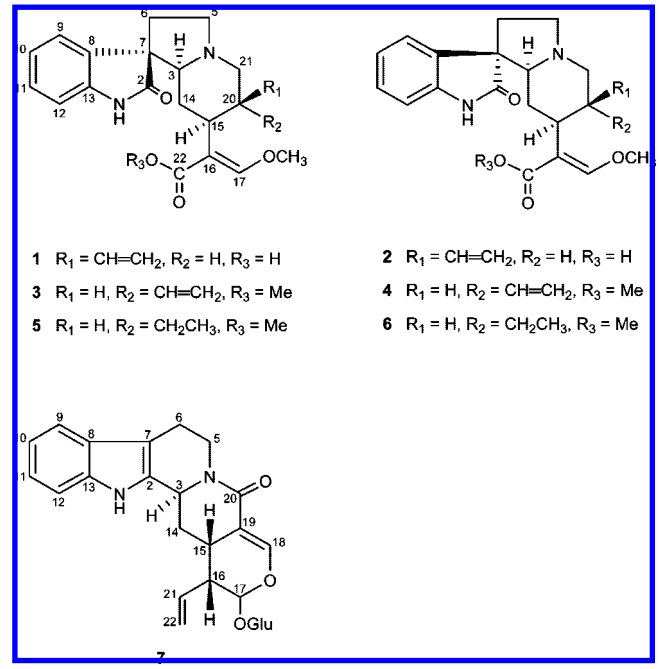
Two new isomeric alkaloids, 18,19-dehydrocorynoxinic acid B (**1**) and 18,19-dehydrocorynoxinic acid (**2**), were isolated from the $CHCl_3$ extract of the leaves of *Uncaria rhynchophylla*, together with four known rhynchophylline-type alkaloids, corynoxeine (**3**), isocorynoxeine (**4**), rhynchophylline (**5**), and isorhynchophylline (**6**), and an indole alkaloid glucoside, vincoside lactam (**7**). The structures of compounds **1** and **2** were elucidated by spectroscopic methods including UV, IR, HREIMS, 1D and 2D NMR, and CD experiments. The activity assay showed that compounds **3**−**6**, with a C-16 carboxylic ester group, and **7** exhibited inhibitory activity on lipopolysaccharide (LPS)-induced NO release in primary cultured rat cortical microglia (IC_{50}: 13.7−19.0 μM). However, only weak inhibitory activity was observed for compounds **1** and **2**, with a C-16 carboxylic acid group (IC_{50}: >100 μM).

The leaves of *Uncaria rhynchophylla* (Miq.) Jacks., a folk herb, are used to treat hypertension, headache, and stroke,[1] similar to the hooks of the plant that is a well-known herbal medicine mentioned in Chinese and Japanese Pharmacopoeias.[2,3] Previous phytochemical studies on parts of the hooks and stems reported the isolation of many unique oxindole or indole alkaloids, such as rhynchophylline, isorhynchophylline, corynoxeine, isocorynoxeine, and hirsutine.[4,5] These alkaloids exhibit a number of pharmacological effects such as lowering blood pressure, vasodilatation, sedation, and protection against ischemia-induced neuronal damage.[6–9] In addition, detailed quantitative analysis of *U. rhynchophylla* showed the similarity of the chemical profile of oxindole alkaloids from the leaves to those of the hooks and stems and also the existence of some minor alkaloids.[10] Although six oxindole and indole alkaloids as well as phenolic constituents were isolated from the leaves,[11,12] the minor alkaloids have not yet been studied. Further investigation is important with regard to resource utilization because of the richer botanical nature of the leaves compared with that of the hooks and stems of the plant. The present study describes the isolation and structural elucidation of two new diastereoisomeric corynoxinic acids (**1**, **2**) and five known compounds (**3**−**7**) and their inhibitory activity on NO release in LPS-activated microglial cells.

The EtOH extract of the leaves of *U. rhynchophylla* was prepared by percolation. It exhibited little inhibitory activity on NO production in LPS-activated primary rat cortical microglial cells at a concentration of 15 μg/mL, and cytotoxic effects appeared at higher concentrations. The *n*-hexane-, $CHCl_3$-, and *n*-BuOH-soluble fractions obtained by partitioning the crude EtOH extract were also examined for inhibitory activity. Only the $CHCl_3$-soluble fraction was found to be significantly active. The isolation of the active fraction led to two new compounds, 18,19-dehydrocorynoxinic acid B (**1**) and 18,19-dehydrocorynoxinic acid (**2**), together with four known oxindole alkaloids, corynoxeine (**3**), isocorynoxeine (**4**), rhynchophylline (**5**), and isorhynchophylline (**6**), and an indole alkaloid glucoside, vincoside lactam (**7**). The structures of compounds **1** and **2** were elucidated using 1D and 2D NMR, UV, FT-IR, MS, and CD techniques. Compounds **3**−**7** were identified by comparing their NMR and MS data with reported values.[11,13–15]

The activity of these alkaloids on the activation of LPS-induced rat primary microglial cells was assayed for the first time. The inhibitory activities of five known alkaloids were confirmed, whereas both new compounds showed little activity at the maximum concentration (100 μM).



Compound **1** was isolated as a colorless, amorphous solid. The molecular formula was determined to be $C_{21}H_{24}N_2O_4$ from the molecular ion peak at *m/z* 368.1760 (calcd 368.1736) in the HREIMS. This implies that it has 11 degrees of unsaturation. The EI mass spectrum displayed a molecular ion at *m/z* 368, indicating the loss of a methylene group (14 mass units) from compounds **3** and **4** (*m/z* 382). The UV absorptions at 212.4, 241.8, and 280.9 nm revealed an oxindole nucleus. The IR spectrum showed absorption bands due to a hydroxy and amino group (3420 cm^{-1}), a carboxylic carbonyl (1703 cm^{-1}), an amide carbonyl (1647 cm^{-1}), and a vinyl group (984.1 and 918.7 cm^{-1}). The resonances for the carbons and protons located on the tetracyclic framework had a close resemblance to those of the known corynoxine B, and they were assigned

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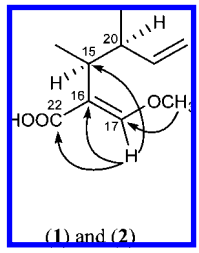

Figure 1. HMBC correlations of C-15 substituents of compounds **1** and **2**.

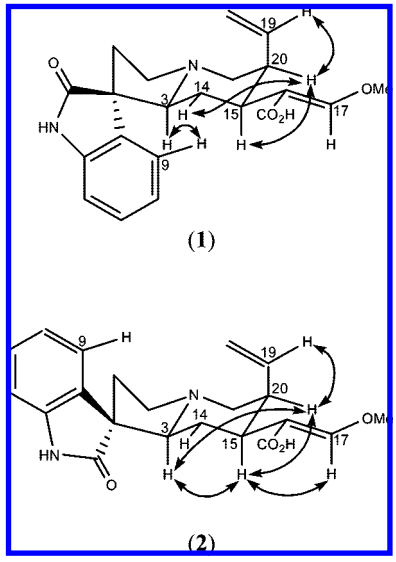

Figure 2. Key NOESY correlations of compounds **1** and **2**.

according to the literature values of the ^{1}H and ^{13}C NMR data for corynoxine B[12] as well as its own HSQC spectrum. The tetracyclic moiety accounted for a partial molecular formula of $C_{15}H_{16}N_2O$ with eight degrees of unsaturation; thus, the remaining molecular formula $C_6H_8O_3$ consisted structurally of two chain substituents, accounting for three degrees of unsaturation and being attached to the cyclic framework separately at C-15 and C-20. The resonances for the carbons and protons of both substituents in the ^{1}H and ^{13}C NMR as well as HSQC spectra included a vinyl group (δ_H 5.03, d, J = 17.0 Hz; δ_H 4.93, d, J = 10.3 Hz and δ_H 5.50, m, δ_C 115.3 and 139.0), as evidenced by the IR absorption bands at 984.1 and 918.7 cm^{-1}, a carboxylic carbonyl (δ_C 172.2), as evidenced by the IR absorption band at 1703 cm^{-1}, a methoxy (δ_H 3.74, 3H, s, δ_C 60.9), and an sp^2 methine (δ_H 7.32, s, δ_C 159.8) that should be directly attached to the oxygen. The constitution of one of the substituents, $C_4H_5O_3$, and its location at C-15 were deduced from the HMBC spectrum (Figure 1). The HMBC correlations of the substituents were determined between the methoxy protons and C-17 and between H-17 and C-15, C-16, and C-22, respectively. Subsequently, the remaining substituent, a vinyl group (−CH=CH$_2$), could be attached only to C-20.

The absolute configuration of compound **1** was determined from the CD spectrum and 2D NOESY correlation (Figure 2). As a tetracyclic oxindole alkaloid, its 7*R* configuration was established according to a positive Cotton effect (CE) at 285 nm and a negative CE at 220 nm in the CD spectrum.[16,17] Meanwhile, a negative CE at 265 nm indicated that H-3 had an α-orientation,[17] as evidenced by the substantial NOE correlation between H-3 and aromatic H-9. The strong NOE correlations between α-H-14 at δ_H 2.22 in the downfield region and H-20 and between H-20 and H-15 indicate that both H-20 and H-15 are cofacial with α-H-14. Thus, compound **1** possesses 7*R*, 3*S*, 15*S*, 20*S* absolute configurations, which are consistent with the *allo* B configuration for corynoxine B.[17] The geometry of the *trans* C-16−C-17 double bond was confirmed on the basis of the olefinic proton in a downfield shift relative to the corresponding signals of the *cis* compounds.[18]

Compound **2** was isolated as a colorless, amorphous solid. The molecular ion peak at *m*/*z* 368.1747 (calcd 368.1736) in the HREIMS data demonstrated the same molecular formula of $C_{21}H_{24}N_2O_4$ as that of compound **1**. Its NMR data (Table 1) suggested that its tetracyclic framework was similar to that of corynoxine.[12] The constitution and location of both substituents $C_4H_5O_3$ and C_2H_3 were also deduced from the HMBC spectrum (Figure 1). For compound **2**, the 7*S* configuration and α-orientation of H-3 were deduced on the basis of the presence of a negative CE at 285 nm, a positive CE at 220 nm, and a negative CE at 265 nm in the CD data, respectively.[16,17] The α-orientations of H-20 and H-15 were indicated by the strong NOESY correlations of H-3/H-20 and H-3/H-15 (Figure 2). Thus, compound **2** possesses 7*S*, 3*S*, 15*S*, 20*S* absolute configurations, which are consistent with the *allo* A configuration for corynoxine.[17] The 16-carboxy derivatives possessing an α-orientation of H-20 have not been reported, although several tetracyclic oxindole alkaloids possessing β-H-20 have been isolated from the hooks and stems of *U. sinensis*.[19]

Microglial activation plays an important role in the progression of several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and HIV-associated dementia.[20,21] The inhibitory activity of seven isolates on microglial activation was investigated. The cytotoxic activity of the tested fractions and compounds on primary cultured rat cortical microglial cells in the presence of LPS was assessed by the MTT assay. Cotreatment of both unstimulated and stimulated microglial cells with all samples did not affect the cell viability at the tested concentrations (data not shown). As shown in Table 2, compounds **3**−**7** as well as the $CHCl_3$-soluble fraction potently inhibited NO release by LPS-activated microglia, with the IC_{50} value of the $CHCl_3$-soluble fraction being 6.3 μg/mL and that of the pure compounds ranging from 13.7 to 19.0 μM. In a parallel experiment, resveratrol, a naturally occurring polyphenol present in red wine and various dietary products, significantly suppressed NO production induced by LPS, which was consistent with our previous report.[22] The C-7 configuration of compounds **3**−**7** seemed not to affect their microglial activation activity, although it has been reported that the antagonistic activity of these compounds on 5-HT2A receptors in the brain is closely related to the 7*S* configuration of the oxindole moiety.[23] These results suggest that the five rhynchophyllinoid alkaloids may be of therapeutic potential in neurodegenerative diseases related to microglial activation. Nevertheless, compounds **1** and **2** were not active at the maximum concentration (100 μM). The inactivity may be due to the C-20 configuration or C-22 carboxylation. Further isolation of corynoxeinic acid and isocorynoxeinic acid might be helpful for elucidation of the structure−activity relationships of rhynchophyllinoid alkaloids on microglial activation.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241MC automatic polarimeter. UV spectra were obtained using a Shimadzu UV-2201 spectrophotometer. CD spectra were recorded on a JASCO CD-2095 plus spectrophotometer. IR spectra were obtained on a Bruker IFS-55 infrared spectrometer. 1D and 2D NMR spectra were recorded separately on a Bruker ARX-600 or ARX-300 spectrometer. HREIMS were acquired on a JEOL JMS-GCmate mass spectrometer with a resolution of 5000. Preparative HPLC was carried out using a Shimadzu LC-8A solvent delivery pump and Shimadzu SPD-10AVP detector.

Plant Materials. Leaves of *U. rhynchophylla* were collected in October 2005 in Fujian Province, China. A voucher specimen is maintained in the Department of Traditional Chinese Medicines, Shenyang Pharmaceutical University. Species identification was confirmed by Professor Zerong Jiang, Shenyang Pharmaceutical University.

Biological Materials. Fetal bovine serum (FBS) and Iscove's modified Dulbecco's medium (IMDM) were purchased from Gibco

Table 1. NMR Data of Compounds **1** and **2** in $CDCl_3$

	compound **1**				compound **2**			
position	δ_C[a] mult.	δ_H[b] (J in Hz)	HMBC[c]	NOESY	δ_C[b]	δ_H[b] (J in Hz)	HMBC[c]	NOESY
2	183.3, qC				182.6			
3	74.6, CH	2.36 br, s		9,14β	72.4	2.89 br, s		5α,14β,15,20
5	54.4, CH_2	2.47 br, t α (7.8)	7	20	53.4	2.54 br, s α	21	3,21β
		3.41 br, s β	7	21α,21β		3.63 br, s β		6β,21β
6	34.0, CH_2	2.03, dd α (11.8, 7.3)	7,8	9	36.6	2.28, m α		
		2.53, dd β (19.9, 7.7)	5			2.35 br, s β		5β
7	56.1, qC				56.2			
8	133.5, qC				132.2			
9	122.7, CH	7.23, d (7.0)	13	3,6α	125.3	7.27, s		1,10,14β
10	122.2, CH	7.05, dd (7.3, 7.2)	8,9,11,12,13		123.5	6.44 br, s		9,11,12
11	127.5, CH	7.18, dd (7.6, 7.6)	8,9,12		127.9	7.02 br, s		10
12	109.5, CH	6.88, d (7.6)	8,10,13		109.4	6.86 br, s	8,10	1,10
13	140.8, qC				140.0			
14	28.4, CH_2	2.22, m α		20	29.4	2.20, m α		
		1.15 br, d β (9.7)		3,15		1.18 br, d β (9.9)		3,15,17
15	37.0, CH	2.42, dd (13.3, 10.2)	14	14β,19,20, 21α,21β	37.7	2.64 br, d (11.9)		3,14β,17,19, 20,21β,$-OCH_3$
16	112.0, qC				112.6			
17	159.8, CH	7.32, s	15,16,22,$-OCH_3$	$-OCH_3$	159.5	7.27 s	15,16,22,$-OCH_3$	15,$-OCH_3$
18	115.3, CH_2	5.03, d (17.0)		20	116.3	5.00 d (18.0)		20,21β
		4.93, d (10.3)		19		4.99 d (10.4)		19
19	139.0, CH	5.50, m		15,18,20,21α	138.8	5.51 m		15,18,20,21β
20	41.7, CH	3.12, m		5α,14α,15, 18,19,21α	40.8	3.49 br, s	16	3,15,18,19
21	58.2, CH_2	1.93 br, t α (10.2)	15	5β,15,19,20	58.5	2.25 m α	18	
		3.29 br, d β (7.8)		5β,15		3.31 br, s β		5α,5β,18,19
22	172.2, qC				171.0			
-OCH_3	60.9, CH_3	3.74, s	17	17	61.1	3.72 s	16,17	15,17
NH (1)						8.55 br, s		9,12

[a], [b] Measured separately at 300 and 600 MHz. [c] HMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon. All chemical shift assignments were carried out on the basis of HSQC and HMBC NMR techniques.

Table 2. Effect of the $CHCl_3$ Fraction and Compounds **1**−**7** on NO Release by LPS-Activated Microglia[c]

	NO (% of LPS control)					
sample	cont.[a]	conc. 0[b]	3	5	15 (μg/mL)	IC_{50} (μM)
fr. $CHCl_3$	31.4 ± 0.8	100.0 ± 5.2$^{###}$	93.8 ± 5.5	69.5 ± 4.2	46.2 ± 2.1*	6.3 (μg/mL)
		conc. 0	1	10	100 (μM)	
1	24.9 ± 0.9	100.0 ± 3.9$^{###}$	81.9 ± 1.2	84.2 ± 2.1	88.9 ± 2.7	>100.0
2	30.5 ± 1.4	100.0 ± 3.3$^{###}$	98.8 ± 2.0	106.6 ± 0.0	92.7 ± 1.8	>100.0
3	38.7 ± 0.7	100.0 ± 0.0$^{###}$	86.9 ± 1.4*	74.5 ± 1.3***	37.9 ± 3.2***	15.7
4	34.6 ± 1.7	100.0 ± 4.1$^{###}$	92.2 ± 3.0	73.2 ± 1.3***	40.5 ± 0.6***	13.7
5	32.0 ± 0.7	100.0 ± 4.1$^{###}$	106.5 ± 4.7	75.8 ± 1.7***	35.9 ± 1.3***	18.5
6	33.8 ± 1.8	100.0 ± 4.8$^{###}$	95.2 ± 3.2	76.9 ± 0.9**	42.1 ± 1.8***	19.0
7	24.3 ± 0.9	100.0 ± 1.6$^{###}$	71.8 ± 2.2***	65.7 ± 0.7***	38.6 ± 1.6***	16.4
		conc. 0	3	10	30 (μM)	
resveratrol	32.8 ± 1.5	100.0 ± 0.6$^{###}$	92.0 ± 0.9*	72.4 ± 3.0*	43.4 ± 1.1***	11.5

[a] In unstimulated microglial cells, only small amounts of NO_2^- (5.7 ± 1.2 μM) could be detected in the medium. [b] Stimulation of microglial cells with LPS resulted in a marked increase in NO_2^- production (18.1 ± 0.4 μM). Data are represented as mean ± SEM of triplicate cultures. [c] Primary microglial cells were treated with serial dilutions of the fraction or compounds in the presence of LPS (1 μg/mL) and then incubated for 48 h. Absorbance of 540 nm was determined after mixing the culture supernatants with Griess reagent as described in the Experimental Section. $^{###}p < 0.001$ vs control group (cultured in medium alone). $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$ vs the groups treated with LPS alone. Resveratrol was used as a positive control.

BRL (Grand Island, NY). LPS (E5:055) was purchased from Sigma (St. Louis, MO). Resveratrol was purchased from Xian Guanyu Biotech Co. Ltd. (China), and its purity was shown to be 98% by HPLC. Compounds **1**−**7**, resveratrol, the extract of *U. rhynchophylla*, and three partitioned fractions were dissolved initially in DMSO and then diluted with PBS for experiments. DMSO at the highest concentration possibly present under the experimental conditions used (0.1%) was not toxic to cells.

Microglial Cell Cultures. Primary rat microglia were prepared from the cortex of newborn Wistar rats (1 day).[24] Briefly, meninges and blood vessels were removed from the rat cortex. Tissues were dissociated with 0.25% trypsin at 37 °C for 15 min, and then the cell suspension was filtered through a 50 μm diameter nylon mesh. The cells were collected by centrifugation at 1200 rev/min for 10 min, resuspended in IMDM supplemented with 5% FBS, and then plated in culture flasks. After 11−14 days, the flasks were placed in a rotary shaker at 250 rev/min for 1 h. The resulting cell suspension rich in microglia was placed on culture dishes, to which the cells adhered after 30 min at 37 °C. The purity of the cells obtained was more than 95%. The cells were plated at a density of 5×10^5 cells/cm^2 onto 96-well microtiter plates for MTT and nitrite assay.

Cell Viability. Cell viability was determined in microglial cells by the MTT reduction assay.[25] In brief, cells were seeded in 96-well microtiter plates and treated with various test sample solutions with LPS (1 μg/mL) for 48 h. The treated cells were incubated with MTT (0.25 mg/mL) at 37 °C for 3 h. The formazan crystals in the cells were solubilized with a solution containing 50% DMF and 20% sodium dodecyl sulfate (pH 4.7). The level of MTT formazan was assayed by measuring the absorbance at a wavelength of 490 nm with a Spectra (Shell) Reader (TECAN, Austria).

Nitrite Assays. Accumulated nitrite in the culture supernatants, an indicator of NO synthase activity, was measured by the Griess reaction.[24] Briefly, cells were seeded in 96-well microtiter plates and treated with various test sample solutions with LPS (1 μg/mL) for 48 h. Then, 50 μL culture supernatants were mixed with 50 μL Griess reagent (part I: 1% sulfanilamide; part II: 0.1% naphthylethylene diamide

dihydrochlride and 2% phosphoric acid) at room temperature. Fifteen minutes later, the absorbance was determined at 540 nm using the Spectra (Shell) Reader. The nitrite concentration was calculated with reference to a standard curve of sodium nitrite obtained using known concentrations. The results were expressed as a percentage of the response of the related LPS-treated groups that were designated as 100%. IC_{50} values for the inhibition of NO release were determined on the basis of linear or nonlinear regression analysis of the concentration response data curves ($n = 3$). Dunnet's *t*-test was used for statistical analysis.

Extraction and Isolation. The air-dried leaves (2.5 kg) of *U. rhynchophylla* were percolated with 75% aqueous EtOH (25 L). The EtOH extract (670 g) was partitioned between *n*-hexane (3 × 4 L) and 5% aqueous EtOH (1 L), and the aqueous phase was further extracted with $CHCl_3$ (3 × 4 L) to give a $CHCl_3$ fraction (55 g; $IC_{50} = 6.3$ μg/mL). The dried $CHCl_3$-soluble fraction was subjected to Si gel CC (10 × 150 cm, 200−300 mesh, Qindao Ocean Chemical Co., China) eluting with a $CHCl_3$−MeOH gradient.

One gram of the fraction eluted with $CHCl_3$−MeOH (20:1) (total 2.3 g) was passed through a Sephadex LH-20 column (3.5 × 60 cm, GE Healthcare, Sweden) and eluted with $CHCl_3$−MeOH (50:50), and the eluates were grouped on the basis of TLC analysis into four major fractions (F1−F4). Fraction F3 (150 mg) was further separated by open column chromatography using an MDS-5 reversed-phase packing (3.5 × 30 cm, 200−300 mesh, Bejing Medicine Technology Center, China) and eluting with a gradient of MeOH−H_2O (50:50−100:0) to yield four major fractions (F3-1, F3-2, F3-3, and F3-4). Fraction F3-3 was further subjected to ODS open column chromatography (3.0 × 30 cm, 50 μm, YMC Co., Ltd., Japan.) eluting with MeOH−H_2O (50:50), and finally, preparative HPLC (Shim-pack PRC-ODS, 2.0 × 30 cm, Shimadzu Co. Ltd., Japan) was carried out using MeOH−H_2O (40:60) plus 0.04% Et_2NH as an eluent at a flow rate of 12 mL/min to afford compounds **1** (8 mg) and **2** (28 mg). Fraction F2 (310 mg) was subjected to ODS open column chromatography (3.0 × 30 cm) eluting with MeOH−H_2O (60:40) and, finally, to preparative HPLC using MeOH−H_2O (65:35) plus 0.04% Et_2NH as an eluent at a flow rate of 12 mL/min to give compounds **3** (16 mg), **4** (50 mg), **5** (7 mg), and **6** (36 mg).

One gram of the fraction eluted with $CHCl_3$−MeOH (10:1) (total 7.9 g) was subjected to MDS-5 open column chromatography (3.5 × 40 cm) eluting with a gradient of MeOH−H_2O (50:50−100:0). The fraction eluted with MeOH−H_2O (70:30) was purified a further two times using MDS-5 open column chromatography to give compound **7** (89 mg).

18,19-Dehydrocorynoxinic acid B (1): colorless, amorphous solid; $[\alpha]^{24}_D$ −97.8 (*c* 0.45, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 212 (5.16), 242 (4.85), 281 (3.92); IR (KBr) ν_{max} 3420, 2928, 1703, 1647, 1621, 984, 919 cm^{-1}; ^1H NMR and ^{13}C NMR ($CDCl_3$) data are shown in Table 1; CD (0.72 mM, MeOH, 25 °C); LREIMS *m*/*z* 368 $[M]^+$ (43), 324 (63), 310 (11), 222 (12), 178 (18), 164 (29), 144 (32), 130 (49), 117 (32), 108 (100), 91 (39), 82 (44), 69 (70), 55 (58); HREIMS (positive) *m*/*z* 368.1760 (calcd for $C_{21}H_{24}N_2O_4$, 368.1736).

18,19-Dehydrocorynoxinic acid (2): colorless, amorphous solid; $[\alpha]^{24}_D$ +90.5 (*c* 1.0 $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 210 (5.14), 242 (4.79), 281 (3.88); IR (KBr) ν_{max} 3423, 2930, 1710, 1645, 1620, 986, 914 cm^{-1}; ^1H NMR and ^{13}C NMR ($CDCl_3$) data are shown in Table 1; CD (0.52 mM, MeOH, 25 °C); LREIMS *m*/*z* 368 $[M]^+$ (89), 324 (38), 223 (19), 192 (10), 178 (14), 164 (16), 159 (13), 144 (30), 130 (43), 121 (13), 117 (29), 108 (100), 95 (19), 91 (20), 82 (41), 77 (29), 69 (45), 61 (11), 54 (34); HREIMS (positive) *m*/*z* 368.1747 (calcd for $C_{21}H_{24}N_2O_4$, 368.1736).

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Supporting Information Available: ^1H NMR, ^{13}C NMR, HSQC, HMBC, NOESY, and CD spectra of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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