Two New Alkaloids from *Brachystemma calycinum* and Their Inhibitory Effects on Lymphocyte Proliferation

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Two new alkaloids, brachystemidines F (**1**) and G (**2**), were isolated from the roots of *Brachystemma calycinum*. Their structures were established on the basis of detailed spectroscopic analyses, including extensive NMR and HR-MS techniques. Compound **2**, which exhibits an unusual *N*-hydroxydiazenyl (HO−N=N) moiety, is a potent immunosuppressive agent, as demonstrated by inhibition of mouse T- and B-lymphocyte proliferation, with IC_{50} values of 6.33 and 5.60 μg/ml, resp.

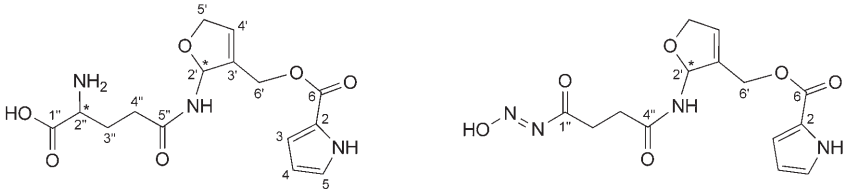
Introduction. – Immunosuppressants are widely used for controlling autoimmune diseases and preventing rejection of transplanted organs. Ideal immunosuppressive drugs should be highly effective, and exhibit low toxicity and high selectivity. They should be able to desensitize the immune response of the patients to the allograft, and meanwhile maintain normal immunological functions of the host to avoid opportunistic infections and malignance [1]. However, most of the current therapeutic agents suffer from low selectivity and significant adverse effects. Thus, it is highly desirable to find new types of immunosuppressants that can overcome the range of side effects and enhance selectivity.

Rheumatism is likely to be related with the immune system. Therefore, it was hypothesized that the investigation of traditional medicines with effects on rheumatic diseases may speed up the discovery of new immunosuppressants. *Brachystemma calycinum* D. Don (Caryophyllaceae) is a traditional folk medicine distributed in Southeast Asia and Southwest China [2]. It is being used for treating rheumatism, limb numbness, impotence, and edema of the feet [3]. Previous chemical investigations on this species have led to the isolation of several new cyclic peptides and alkaloids [4].

Herein, we report on the isolation, characterization, and biological properties of two new alkaloids, brachystemidines F (**1**) and G (**2**), which were obtained from the BuOH-soluble extract of the roots of *B. calycinum*.

Results and Discussion. – 1. *Chemistry.* Compound **1**, isolated as a colorless, oily solid from the BuOH-soluble extract of *B. calycinum*, had the molecular formula $C_{15}H_{19}N_3O_6$, as deduced by HR-FAB-MS (*m/z* 338.1351 ($[M+H]^+$; calc. 338.1352)). The NMR data of **1** (*Table 1*)[1]) were very similar to those of brachystemidines A–E,

[1]) Arbitrary atom numbering.



which had been previously isolated from this plant [4c]. Comparison of their ^{13}C-NMR data, along with the observation of a key HMBC correlation between H–C(6′) and C(2′), implied the presence of a 2′-substituted 2,5-dihydrofuran-3-yl 1*H*-pyrrole-2-carboxylate moiety [4c]. The remaining two C=O signals at δ(C) 172.0 and 170.2, two CH_2 groups at δ(C) 31.4 and 26.3, and one CH group at δ(C) 52.7 were indicative of a glutamic acid residue. This was confirmed by *i*) ^{1}H,^{1}H-COSY correlations of H–C(3″) with both H–C(2″) and H–C(4″); *ii*) HMBC interactions of H–C(2″)/C(1″), H–C(3″)/C(5″), and H–C(4″)/C(5″); and *iii*) positive ninhydrin color reaction on a TLC plate, indicating an NH_2 group. The connection of these two units through an amide bond was established on the basis of additional HMBC correlations between H–C(2′) and C(5″), the amide H-atom and C(5″), and the ^{1}H,^{1}H-COSY interaction of H–C(2′) with the amide H-atom. The observation of a 'zigzag' coupling between H–C(2′) and one H-atom of CH_2(5′) indicated that these two H-atoms were in one plane and that the five-membered ring most likely adopts an envelope conformation in solution. However, we were unable to determine the relative or absolute configurations at C(2′) and C(2″) due to the scarcity of material.

From the above data, the structure of compound **1** was assigned as *N*-(2,5-dihydro-3-{[(1*H*-pyrrol-2-ylcarbonyl)oxy]methyl}furan-2-yl)glutamine (=2-amino-5-oxo-5-[(2,5-dihydro-3-{[(1*H*-pyrrol-2-ylcarbonyl)oxy]methyl}furan-2-yl)amino]pentanoic acid), and named *brachystemidine F*.

Compound **2**, obtained as a colorless gum from the BuOH-soluble extract of *B. calycinum*, showed the $[M-H]^-$ quasi-molecular ion peak at *m*/*z* 335.0966 in the high-resolution FAB mass spectrum, corresponding to the molecular formula $C_{14}H_{16}N_4O_6$. The structure of **2** resembled that of **1** in sharing a 2′-substituted 2,5-dihydrofuran-3-yl 1*H*-pyrrole-2-carboxylate moiety, as inferred from the NMR spectroscopic data (*Table 1*). This was confirmed by a key HMBC correlation between H–C(6′) and C(2′). ^{1}H,^{1}H-COSY Cross-peaks between H–C(2″) and H–C(3″), and HMBC correlations of H–C(2″) and H–C(3″) with the C=O C-atoms C(1″) and C(4″) established the partial structure from C(1″) to C(4″). Considering the structural similarity with brachystemidines A–F, this unit should connect to C(2′) *via* an amide bond, as supported by HMBC interactions of the amide H-atom at δ(H) 6.70 (*d*, J = 8.0 Hz) to both C(2′) and C(4″). Taking the molecular formula and chemical shift of C(1″) into consideration, the remaining 48 mass units, corresponding to HN_2O, should be located at C(1″) *via* a C–N bond. Development of a brown color upon exposure to ferric chloride on a TLC plate together with a very strong IR absorption at 3442 cm^{-1} indicated the presence of an OH group [5]. Thus, the HN_2O moiety, corresponding to one degree of unsaturation, should be an *N*-hydroxydiazenyl (HO–N=N) group.

Table 1. *^{1}H- and ^{13}C-NMR Data of* **1** *and* **2**. At 400 and 100 MHz, resp., δ in ppm, J in Hz. Arbitrary atom numbering.

Position	**1**[a])		**2**[b])	
	δ(H)	δ(C)	δ(H)	δ(C)
H–N(1)	11.96 (br. *s*)		9.91 (br. *s*)	
2	–	121.4	–	121.9
3	6.84 (br. *s*)	115.6	6.96 (*d*, *J* = 1.2)	116.2
4	6.18 (br. *s*)	109.7	6.25–6.26 (*m*)	110.6
5	7.05 (br. *s*)	124.4	6.98 (*dd*, *J* = 2.4, 1.2)	123.8
6	–	159.9	–	160.4
2′	6.30 (br. *s*)	84.8	6.52–6.58 (*m*)	86.1
3′	–	134.1	–	134.4
4′	6.30 (br. *s*)	127.8	6.11–6.12 (*m*)	127.9
5′	4.41 (br. *d*, *J* = 13.5), 4.53 (br. *d*, *J* = 13.5)	73.0	4.56–4.61 (*m*), 4.62–4.67 (*m*)	73.9
6′	4.73–4.81 (*m*)	58.4	4.69 (br. *d*, *J* = 10.0), 4.91 (*t*, *J* = 10.0)	58.5
1″	–	170.2	–	162.6
2″	3.40–3.44 (*m*)	52.7	2.90–2.96 (*m*)[c])	23.2[c])
3″	1.91–1.94 (*m*)	26.3	2.69–2.73 (*m*)[c])	31.4 [c])
4″	2.26–2.29 (*m*)	31.4	–	171.0
5″	–	172.0	–	–
CON*H*	8.68 (*d*, *J* = 9.2), 8.79 (*d*, *J* = 9.2)[d])		6.70 (*d*, *J* = 8.0), 6.60 (*d*, *J* = 8.0)[d])	

[a]) Recorded in (D_6)DMSO. [b]) Recorded in $CDCl_3$. [c]) Assignments within column may be interchanged. [d]) Split signals due to rotational isomers; the less-intensive signals are the latter ones each.

Notably, such a functionality is very rare in Nature, but a few natural products with similar groups have been isolated from microbial sources [5].

From the above data, the structure of **2** was, thus, assigned as (2,5-dihydro-2-{[4-(hydroxydiazenyl)-4-oxobutanoyl]amino}furan-3-yl)methyl 1*H*-pyrrole-2-carboxylate, and named *brachystemidine G*.

It is interesting to note that the amide H-atoms of **1** and **2** both displayed distinct pairs of peaks, with very close chemical shifts, in a ratio of *ca.* 2:1 in the ^{1}H-NMR spectra. This was attributed to the presence of rotamers arising from the isomerization of the amide function [6].

2. *Biology.* The antiproliferative action of brachystemidines F (**1**) and G (**2**) was tested against T- and B-lymphocytes of Balb/c mice, with cyclosporin A being used as positive control (*Table 2*) [7]. Alkaloid **2** inhibited the proliferation of these two types of cells, with IC_{50} values of 6.33 and 5.60 μg/ml, respectively. In contrast, alkaloid **1** was inactive towards either cells, at concentrations of up to 10.0 μg/ml. Given the structural similarities of **1** and **2**, but the significant difference in bioactivity, it is hypothesized that the *N*-hydroxydiazenyl (HO–N=N) moiety is crucial for the inhibitory activity of **2**.

Table 2. *Inhibitory Effects of* **1** *and* **2** *on Lymphocyte Proliferation*

Compound	IC_{50} [μg ml^{-1}]	
	T-Cells	B-Cells
1	n.a.[a])	n.a.
2	6.33	5.60
Cyclosporin A[b])	0.30	0.41

[a]) Not active. [b]) Positive control.

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Experimental Part

General. All reagents were distilled before use. Column chromatography (CC): silica gel (200–300 mesh, 10–40 μm; *Qingdao Marine Chemical Factory*, China); C_{18} reverse-phase silica gel (40–63 μm; *Daiso Co.*, Japan); *Sephadex LH-20* gel (*Amersham Pharmacia Biotech*, Sweden). TLC: silica gel GF_{254} (10–40 μm; *Qingdao*). UV Spectra: *Shimadzu UV-2401PC* spectrophotometer; λ_{max} in nm. Optical rotations: *Jasco-20C* digital polarimeter. IR Spectra: *Bruker Tensor-27* FT-IR spectrophotometer, with KBr discs; in cm^{-1}. ^{1}H- and ^{13}C-NMR Spectra: *Bruker AM-400* spectrometer; chemical shifts δ in ppm rel. to Me_4Si, J in Hz. ^{1}H,^{1}H-COSY, HSQC, HMQC, and HMBC Spectra: *Bruker DRX-500* spectrometer. FAB-MS (neg. or pos. ion mode): *VG Auto Spec-3000* mass spectrometer. ESI- and HR-ESI-MS: *API QSTAR Pulsar-1* mass spectrometer; in *m*/z.

Plant Material. The roots of *Brachystemma calycinum* D. Don were collected in Xishuangbanna (Yunnan Province, China) at the end of March 1999, and identified by Prof. *H. Wang*, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences. A voucher specimen (No. CHYX0001) was stored in the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. The dried and milled roots of *B. calycinum* (13.0 kg) were extracted with 95% aq. EtOH at reflux (3×). The combined extracts were concentrated *in vacuo*, and then partitioned sequentially between H_2O and petroleum ether (PE), AcOEt, and BuOH, resp. An aliquot of the BuOH-soluble extract (70.0 g) was subjected to CC (SiO_2; $CHCl_3$/MeOH/H_2O 70:30:2) to afford four fractions: *Fr. 1–Fr. 4. Fr. 3* (503 mg) was further separated by CC (1. SiO_2, $CHCl_3$/MeOH/H_2O 80:18:2; 2. C_{18}, MeOH/H_2O 20:80→55:45; 3. SiO_2, $CHCl_3$/i-PrOH 3:1) to afford **2** (4.8 mg). Further fractionation of *Fr. 4* (150 mg) by CC (1. SiO_2, $CHCl_3$/MeOH/H_2O 80:18:0.5, 70:30:5; 2. *Sephadex LH-20*, MeOH; 3. SiO_2, $CHCl_3$/MeOH/H_2O 80:18:2) finally afforded **1** (6.0 mg).

Antiproliferative Assay. Lymphocytes were isolated from the spleen of Balb/c mice. Proliferation of lymphocytes was analyzed *in vitro* using the MTT (= [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay [7]. The lymphocytes were seeded into 96-well microculture plates at a cell density of 1×10^5 cells per well. Activation of T- and B-lymphocytes was induced by Con A and lipopolysaccharides (LPS) at final concentrations of 5 and 15 μg/ml, resp. A dose–response curve was established using different concentrations of **1** and **2**. The final concentrations of test compounds in the assay were 0.625, 1.25, 2.5, 5, and10 μg/ml. Wells containing various concentrations of cyclosporine A and *RPMI-1640* medium were used as pos. and neg. controls, resp. All assays were carried out in duplicate (n=2). After 72 h of incubation, MTT reagent (final concentration 4 mg/ml) was added, and the cells were cultured for 4 h, and then dissolved by addition of DMSO. Spectrophotometric measurement was carried out at 540 nm using a microplate reader.

Brachystemidine F (=*2-Amino-5-oxo-5-[(2,5-dihydro-3-{[(1*H*-pyrrol-2-ylcarbonyl)oxy]methyl}furan-2-yl)amino]pentanoic Acid*; **1**). Colorless, oily solid. $[\alpha]_D^{24} = +9.3$ (c=0.24, MeOH). UV (MeOH):

266, 202. IR (KBr): 3429, 2923, 1637, 1412, 1310, 1126, 1079, 1053. ^{1}H- and ^{13}C-NMR: see *Table 1*. ESI-MS (neg.): 336 ($[M-H]^-$). FAB-MS (pos.): 338 ($[M+H]^+$). HR-ESI-MS (pos.): 338.1351 ($[M+H]^+$, $C_{15}H_{20}N_3O_6^+$; calc. 338.1352).

Brachystemidine G (*=(2,5-Dihydro-2-{[4-(hydroxydiazenyl)-4-oxobutanoyl]amino}furan-3-yl)-methyl 1*H*-Pyrrole-2-carboxylate*; **2**). Colorless gum. UV (MeOH): 269, 203. $[\alpha]_D^{24} = +71.4$ ($c = 0.13$, MeOH). IR (KBr): 3442, 2927, 1702, 1640, 1402, 1318, 1163, 1021. ^{1}H- and ^{13}C-NMR: see *Table 1*. FAB-MS (pos.): 337 ($[M+H]^+$). FAB-MS (neg.): 672 ($[2M]^-$), 336 (M^-). HR-ESI-MS: 335.0966 ($[M-H]^-$, $C_{14}H_{15}N_4O_6^-$; calc. 335.0992).

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