Selective Inhibition of Collagen-Induced Platelet Aggregation by a Cyclic Peptide from *Drymaria diandra*

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Four cyclic peptides, diandrine A – D (**1** – **4**), were isolated from the MeOH extract of Formosan *Drymaria diandra*. Their structures were elucidated by chemical and spectroscopic analyses as cyclo(-Gly[1]-Pro[2]-Trp[3]-Pro[4]-Tyr[5]-Phe[6]-), cyclo(-Gly[1]-Pro[2]-Leu[3]-Pro[4]-Leu[5]-Trp[6]-Ser[7]-Ser[8]-), cyclo(Gly[1]-Gly[2]-Pro[3]-Tyr[4]-Trp[5]-Pro[6]-), and cyclo(Gly[1]-Gly[2]-Pro[3]-Tyr[4]-Trp[5]-Pro[6]-), respectively. Compounds **3** and **4** were stable conformational isomers. Cyclopeptide **1** showed a selective inhibitory effect on collagen-induced platelet aggregation with an IC_{50} value of 44.2 μM.

1. Introduction. – Recently, much attention has been devoted to higher-plant cyclopeptides and their bioactivities. Most of them have been isolated from the family Caryophyllaceae, Rutaceae, Annonaceae, and Rhamnaceae [1]. The genus *Drymaria* WILLD. (Caryophyllaceae), including 40 species, is mainly distributed in tropical America. However, only one species, *Drymaria diandra* BL. (*Drymaria cordata* (L.) WILLD. subsp. *diandra* (BL.) I. DUKE ex HATUSIMA), grows in Taiwan [2]. It is used as a folk medicine for the treatment of fever, malaria, and cancer. In previous studies, norditerpenes, alkaloids, and flavonoids were isolated from the genus *Drymaria* [3 – 7]. In our recent paper, we reported the isolation of the sesquiterpenes 3-oxo-α-ionol and megastigma-4,7-diene-3,9-dione and anemonin from this plant [8]. The last-mentioned metabolite was the major one and showed anticancer activity *in vitro*. Herein, we report the isolation and structure elucidation of four new cyclic peptides, diandrines A – D (**1** – **4**) *(Fig. 1)*. The isolates were tested for antiplatelet activity. One of them, **1**, exhibited selective inhibition against collagen-induced platelet aggregation. Additionally, compounds **1** – **4** did not induce any cytotoxicity in MCF-7 and HepG2 cancer cell lines in a screening assay.

2. Results and Discussion. – Compound **1** was obtained as pale yellow powder. The molecular formula, $C_{41}H_{45}N_7O_7$, was determined by HR-FAB-MS ($[M+H]^+$ at *m/z* 748.3456, calc. 748.3380). The NMR spectra of **1** (*Table 1*) showed four amide signals (δ 8.18 (*d*), 9.28 (*d*), 9.49 (*d*), 10.52 (*d*)) and six carbonyl resonances (δ 171.6, 172.2 (2×), 171.2, 172.5, and 168.5), indicating that **1** might belong to a peptide class of compounds. A negative ninhydrin test indicated its cyclic nature. IR Absorptions at 3300, 1630, and 1516 cm^{-1} were characteristic for amide, carbonyl, and aromatic moieties. The presence

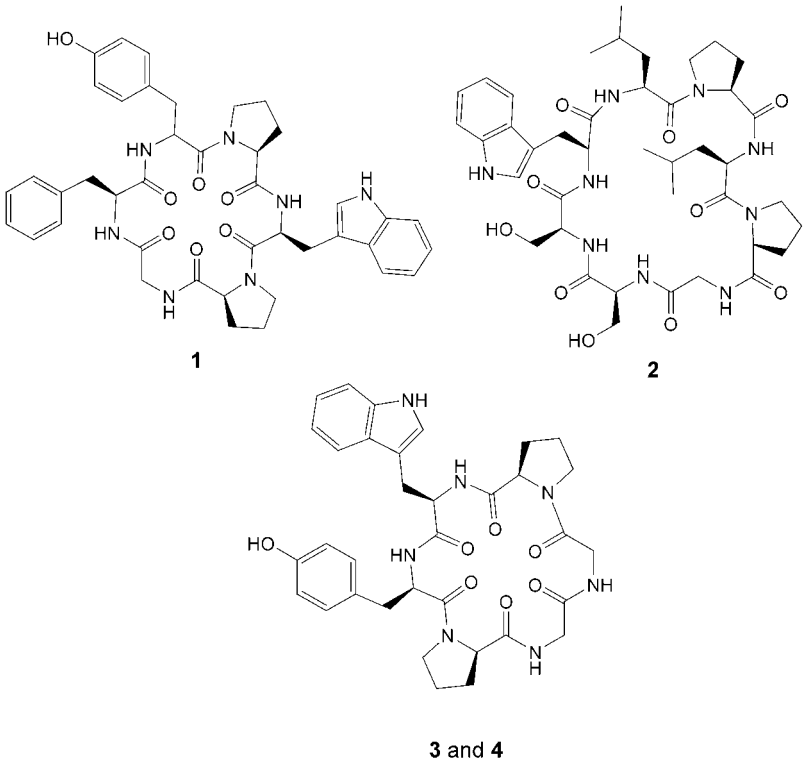

Fig. 1. *Structures of compounds* **1**–**4**

of the amino acid residues Gly, Pro (2×), Trp, Try, and Phe was established by the analysis of 2D NMR data (HMBC, TOCSY, and COSY). The sequence was determined by HMBC and ROESY cross-peaks. Finally, the configuration of each amino acid residue was assigned to be L, which was deduced by acid hydrolysis in a *MicroVessel*™ in a domestic microwave oven and *Marfey*'s analysis [9][10]. The ROESY cross-peaks, $Trp^3H-C(\alpha)/Pro^2CH_2(\delta)$, $Trp^3H-C(\alpha)/Pro^4CH_2(\delta)$, and Gly^1-$CH_2(\alpha)/Pro^4CH_2(\delta)$, provided evidence of these connectivities, indicating that the amide bonds of both Pro residues adopted *cis* geometry. This was further supported by the difference of the ^{13}C-NMR chemical shifts of Pro^2 ($\Delta\delta(C(\beta)-C(\gamma))=8.3$) and Pro^4 ($\Delta\delta(C(\beta)-C(\gamma))=9.4$) [11][12]. The CD spectrum exhibited negative *Cotton* effects at *ca.* 195 and 208 (sh) nm and positive ones at *ca.* 230 and 270 nm. Additionally, a dihedral angle (ϕ) $Phe^6H-N-C(\alpha)-H$ of 148° was determined by the coupling constants [13], and the distance between $Trp^3C(\alpha)$ and $Phe^6C(\alpha)$ was *ca.* 7.18 Å as calculated with the molecular-modeling software MM2 (*Fig. 2*). The information implied a type-IV β-turn between Phe^6 and Trp^3, and the ROESY correlations between $Tyr^5H-C(\alpha)$ and Trp^3NH, and $Tyr^5H-C(\alpha)$ and $Phe^6H-C(\alpha)$ confirmed this assumption [14][15].

Compound **2** was isolated as pale yellow powder. The molecular formula, $C_{41}H_{59}N_9O_{10}$, was determined by HR-FAB-MS ($[M+H]^+$ at *m/z* 838.4469, calc.

Table 1. *^{1}H-NMR* (400 MHz) *and ^{13}C-NMR* (100 MHz) *Data of* **1** *in C_5D_5N.* δ in ppm, J in Hz.

		δ(H)	δ(C)[a]	ROESY (δ(H))
Gly[1]	C=O	–	168.5 (*s*)	
	NH	9.49 (*d*, J = 9.6)	–	5.15
	$CH_2(\alpha)$	4.08 (*d*, J = 15.4)	42.9 (*t*)	9.28, 5.15
		5.15 (*dd*, J = 15.4, 9.6)		9.49
Pro[2]	C=O	–	171.6 (*s*)	
	H–C(α)	3.82 (*d*, J = 9.6)	61.0 (*d*)	5.32, 0.61
	$CH_2(\beta)$	2.12 (*dd*, J = 12.0, 6.0)	30.3 (*t*)	0.61
		0.61 (*ddd*, J = 12.4, 12.0, 9.6)		2.12
	$CH_2(\gamma)$	1.35 (*m*)	22.0 (*t*)	
	$CH_2(\delta)$	3.39, 3.68 (2*m*)	46.7 (*t*)	
Trp[3]	C=O	–	172.2 (*s*)	
	NH	10.32 (*d*, J = 2.4)	–	5.61
	H–C(α)	5.33 (*m*)	55.5 (*d*)	3.82, 3.67
	$CH_2(\beta)$	3.48 (*dd*, J = 13.6, 5.2)	28.3 (*t*)	
		3.70 (*d*, J = 13.6)		
	Ar	–	109.9 (*s*)	
		7.37 (*d*, J = 1.6)	125.0 (*d*)	
		7.60 (*d*, J = 8.0)	111.9 (*d*)	
		7.16 (*t*, J = 8.0)	119.2 (*d*)	
		7.20 (*t*, J = 8.0)	122.0 (*d*)	
		7.89 (*d*, J = 8.0)	119.5 (*d*)	
		–	137.3 (*s*)	
		–	128.2 (*s*)	
		12.10 (*d*, J = 1.6)		
Pro[4]	C=O	–	171.2 (*s*)	
	H–C(α)	3.67 (*m*)	61.5 (*d*)	5.32
	$CH_2(\beta)$	1.81, 1.30 (2*m*)	31.6 (*t*)	
	$CH_2(\gamma)$	1.23 (*m*)	22.2 (*t*)	
	$CH_2(\delta)$	3.54 (*m*)	47.2 (*t*)	
Tyr[5]	C=O	–	172.2 (*s*)	
	NH	8.18 (*d*, J = 8.8)		
	H–C(α)	5.61 (*dt*, J = 10.3, 4.4)	53.9 (*d*)	5.32, 10.32, 7.16, 3.43
	$CH_2(\beta)$	3.22 (*dd*, J = 14.2, 10.3)	36.9 (*t*)	
		3.43 (*dd*, J = 14.2, 4.4)		5.61
	Ar	–	128.0 (*s*)	
		7.16 (*d*, J = 7.6)	130.6 (*d*)	5.61
		7.06 (*d*, J = 7.6)	115.9 (*d*)	
		–	157.4 (*s*)	
Phe[6]	C=O	–	172.5 (*s*)	
	NH	9.28 (*d*, J = 8.4)	–	
	H–C(α)	5.32 (*m*)	53.2 (*d*)	
	$CH_2(\beta)$	3.04 (*dd*, J = 12.0, 4.8)	40.7 (*t*)	5.32
		3.13 (*t*, J = 12.0)		
	Ar	7.10–7.24 (*m*)	136.9 (*s*)	
			129.5 (*d*)	
			128.8 (*d*)	
			127.2 (*d*)	

[a]) All assignments were confirmed by DEPT, HMQC, and HMBC.

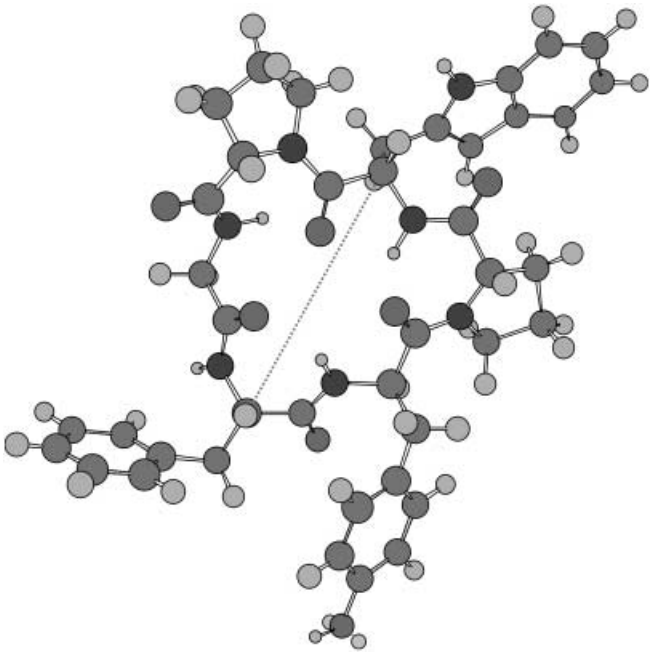

Fig. 2. *Molecular model of diandrine A* (**1**). The straight line indicates the distance (*ca.* 7.18 Å) between Trp[3] C(α) and Phe[6] C(α).

838.4385). IR Absorptions at 3347, 1637, and 1538 cm^{-1} were assignable to amide, carbonyl, and aromatic moieties. The ^{1}H- and ^{13}C-NMR spectra of **2** (*Table 2*) showed seven amide signals and eight carbonyl resonances (δ 174.9, 174.7, 173.4, 173.4, 173.0, 171.2, 170.9, and 169.8), indicating that **2** might be an octapeptide. The presence of amino acid residues Gly, Pro ($2\times$), Trp, Leu ($2\times$), Ser ($2\times$) was deduced by careful analysis of HMBC and TOCSY data. The sequence was assigned from cross-peaks observed in HMBC and ROESY spectra. A small difference of the ^{13}C-NMR chemical shifts of Pro[2] ($\Delta\delta(C(\beta)-C(\gamma))=4.5$ ppm) and Pro[4] ($\Delta\delta(C(\beta)-C(\gamma))=4.7$ ppm) suggested the *trans* geometry of the amide bonds in both Pro residues of **2** [11][12]. Furthermore, the CD spectrum of **2** exhibited the characteristic shape with a minimum, a maximum, and a zero crossing at 219, 190, and 207 nm, respectively, indicating that **2** had an L_{+2} helix conformation [16].

Compound **3** has a molecular formula $C_{34}H_{39}N_7O_7$, as deduced from HR-FAB-MS ($[M+H]^+$ at *m/z* 658.2996, calc. 658.2911). The NMR data (*Table 3*) (five amide signals and six carbonyl resonances) indicates that it is a hexapeptide like **1**. The composition and sequence of the amino acid residues of **3** were established by detailed analysis of 1D and 2D NMR data. These connectivities were further confirmed by ESI-MS^n analysis. The $[M+H]^+$ and $[M+Na]^+$ ions provided the preferential ring opening at the Trp[5]-Pro[6] amide bond, and gave relative B ions of peptide fragments. The fragment ion at *m/z* 472 could be attributed to [Pro[6]-Gly[1]-Gly[2]-Pro[3]-Tyr[4]], followed by the subsequent loss of Tyr[4], Pro[3], Gly[2], and Gly[1]. Acid hydrolysis and *Marfey*'s analysis

Table 2. *^{1}H-NMR* (500 MHz) *and ^{13}C-NMR* (125 MHz) *Data of* **2** *in C_5D_5N.* δ in ppm, J in Hz.

		δ(H)	δ(C)[a]			δ(H)	δ(C)[a]
Gly[1]	C=O	–	169.8 (*s*)	Trp[6]	C=O	–	174.4 (*s*)
	NH	10.10 (*s*)	–		NH	10.70 (*s*)	–
	$CH_2(\alpha)$	3.60, 4.67 (2*m*)	44.5 (*t*)		H–C(α)	4.93 (*m*)	57.6 (*d*)
					$CH_2(\beta)$	3.37, 3.63 (2*m*)	28.3 (*t*)
Pro[2]	C=O	–	173.0 (*s*)				
	H–C(α)	4.79 (*m*)	61.9 (*d*)		Ar	–	110.9 (*s*)
	$CH_2(\beta)$	1.21, 1.98 (2*m*)	30.0 (*t*)			7.41 (*s*)	125.1 (*d*)
						7.60 (*d*, $J=8.0$)	112.4 (*d*)
	$CH_2(\gamma)$	1.64, 1.82 (2*m*)	25.5 (*t*)			7.08 (*t*, $J=8.0$)	119.6 (*d*)
						7.01 (*t*, $J=8.0$)	122.2 (*d*)
	$CH_2(\delta)$	3.54, 3.96 (2*m*)	48.6 (*t*)			7.40 (*d*, $J=8.0$)	119.7 (*d*)
						–	137.8 (*s*)
Leu[3]	C=O	–	174.9 (*s*)			–	128.9 (*s*)
	NH	8.05 (*d*, $J=9.2$)	–			11.90 (*s*)	–
	H–C(α)	5.44 (*m*)	53.1 (*d*)	Ser[7]	C=O	–	171.2 (*s*)
	$CH_2(\beta)$	1.88, 2.06 (2*m*)	40.0 (*t*)		NH	8.21 (*d*, $J=6.4$)	–
					H–C(α)	5.43 (*m*)	52.0 (*d*)
	H–C(γ)	1.90 (*m*)	26.0 (*d*)		$CH_2(\beta)$	4.18, 4.38 (2*m*)	65.0 (*d*)
	Me(δ)	0.94, 0.98 (2*d*, each $J=6.0$)	23.3, 23.7 (2*q*)				
				Ser[8]	C=O	–	170.9 (*s*)
Pro[4]	C=O	–	173.4 (*s*)		NH	8.90 (br. *s*)	–
	H–C(α)	4.64 (*m*)	59.2 (*d*)		H–C(α)	4.10 (*m*)	52.0 (*d*)
	$CH_2(\beta)$	1.32, 2.12 (2*m*)	30.5 (*t*)		$CH_2(\beta)$	4.24 (*m*)	62.4 (*d*)
	$CH_2(\gamma)$	1.98 (*m*)	25.8 (*t*)				
	$CH_2(\delta)$	3.78, 4.11 (2*m*)	48.6 (*t*)				
Leu[5]	C=O	–	174.7 (*s*)				
	NH	8.50 (*d*, $J=9.2$)	–				
	H–C(α)	4.93 (*m*)	50.8 (*d*)				
	$CH_2(\beta)$	1.82, 2.03 (2*m*)	42.8 (*t*)				
	H–C(γ)	2.01 (*m*)	25.1 (*s*)				
	Me(δ)	0.93, 0.81 (2*d*, each $J=6.5$)	22.1, 22.9 (2*q*)				

[a]) All of assignments were based on DEPT, HMQC, and HMBC.

confirmed the L-configuration of the amino acids. The *trans* geometry of the amide bonds in both Pro residues of **3** was deduced from the small difference of the ^{13}C-NMR chemical shifts of Pro[3] ($\Delta\delta$(C(β)–C(γ)) = 4.2 ppm) and Pro[6] ($\Delta\delta$(C(β)–C(γ)) = 3.0 ppm) [11][12]. In addition, the CD spectrum of **3** showed a negative *Cotton* effect at *ca.* 220 nm and two positive bands at *ca.* 195 (max.), 205 (sh), and 240 nm, indicating that cyclopeptide **3** is in a β-pleated sheet conformation [17].

The same composition and sequence of the amino acid residues in **3** and **4** were established by NMR (see *Table 4*), MS, and amino acid analysis. Thus, the 2D ROESY, TOCSY, and HMBC experiments allowed to assign the sequence cyclo(-Gly[1]-Gly[2]-Pro[3]-Tyr[4]-Trp[5]-Pro[6]-) to **4**, and also the ESI-MS^n fragmentation patterns were identical to those of **3**. Although **3** and **4** had the same composition and amino acid sequence, they are stable at room temperature and could be isolated by HPLC, suggesting that they might be conformational isomers. Indeed, the ^{1}H-NMR spectra of **4** were different from those of **3** (*Tables 3* and *4*). For example, the δ(H) and multiplicity of the NH

Table 3. *^{1}H-NMR* (400 MHz) *and ^{13}C-NMR* (100 MHz) *Data of* **3** *in C_5D_5N.* δ in ppm, J in Hz.

		δ(H)	δ(C)[a])	ROESY (δ(H))
Gly[1]	C=O	–	168.4 (*s*)	
	NH	10.37 (*dd*, *J* = 8.4, 3.2)	–	8.90, 4.61, 4.84, 3.79
	$CH_2(\alpha)$	4.84 (*dd*, *J* = 16.8, 8.4)	43.6 (*t*)	3.79, 8.90, 10.37
		3.79 (*dd*, *J* = 16.8, 4.4)		
Gly[2]	C=O	–	169.7 (*s*)	
	NH	8.90 (*d*, *J* = 8.0)	–	10.37, 2.96
	$CH_2(\alpha)$	2.96 (*d*, *J* = 17.5)	41.9 (*t*)	4.41, 4.52, 8.90
		4.52 (*dd*, *J* = 17.5, 8.0)		2.75, 2.96
Pro[3]	C=O	–	172.2 (*s*)	
	H−C(α)	4.41 (*dd*, *J* = 9.2, 4.0)	62.1 (*d*)	1.56, 4.61, 4.52
	$CH_2(\beta)$	1.78, 1.63 (2*m*)	29.0 (*t*)	
	$CH_2(\gamma)$	1.65, 1.56 (2*m*)	25.8 (*t*)	
	$CH_2(\delta)$	2.75 (*t*, *J* = 5.6)	45.7 (*t*)	4.41, 1.63, 4.52
Trp[4]	C=O	–	170.1 (*s*)	
	NH	7.69 (*d*, *J* = 4.0)		3.45, 4.99
	H−C(α)	4.99 (*dd*, *J* = 8.2, 5.2)	53.4 (*d*)	3.35, 3.45, 3.81, 7.69
	$CH_2(\beta)$	3.35, 3.81 (2*m*)	26.1 (*t*)	
	Ar	–	108.5 (*s*)	
		7.92 (*d*, *J* = 2.0)	127.6 (*d*)	3.35, 12.38
		7.59 (*d*, *J* = 7.6)	112.3 (*d*)	7.92, 7.59
		7.75 (*d*, *J* = 7.6)	118.9 (*d*)	
		7.23 (*d*, *J* = 8.0)	119.2 (*d*)	2.75, 12.38
		7.20 (*d*, *J* = 8.0)	121.4 (*d*)	3.35, 3.81, 3.93
		–	136.9 (*s*)	
		–	128.7 (*s*)	
		12.38 (br. *s*)	–	
Tyr[5]	C=O	–	172.8 (*s*)	
	NH	6.23 (br. *d*, *J* = 9.6)	–	4.41, 5.40, 7.48
	H−C(α)	5.40 (*m*)	54.3 (*d*)	3.93, 4.41, 7.48
	$CH_2(\beta)$	3.40 (*m*)	37.4 (*t*)	
		3.93 (*dd*, *J* = 14.2, 3.2)		3.40, 5.40
	Ar	–	129.8 (*s*)	
		7.48 (*d*, *J* = 8.0)	131.5 (*d*)	
		7.26 (*d*, *J* = 8.0)	116.1 (*d*)	
		–	157.7 (*s*)	
Pro[6]	C=O	–	169.7 (*s*)	
	H−C(α)	4.61 (*t*, *J* = 8.0)	62.8 (*d*)	1.64, 2.18, 10.37
	$CH_2(\beta)$	2.18 (*m*)	29.0 (*t*)	
	$CH_2(\gamma)$	1.86, 1.64 (2*m*)	26.0 (*t*)	
	$CH_2(\delta)$	3.45 (*t*, *J* = 8.8)	47.5 (*t*)	4.99, 1.64

[a]) All of assignments were confirmed by DEPT, HMQC, and HMBC.

signals of Tyr[4] and Trp[5] of **3** and **4** were different. Therefore, the major difference in conformations of **3** and **4** might largely be attributed to the H-bonds that influence the NH groups of Tyr[4] and Trp[5]. A *trans* geometry of the Pro amide bonds of **4** could be deduced from the small ^{13}C-NMR-chemical-shift difference of the Pro residues (Pro[3]: $\Delta\delta(C(\beta)-C(\gamma)) = 3.9$; Pro[6]: $\Delta\delta(C(\beta)-C(\gamma)) = 3.1$) and from the ROESY cross-peaks ($Gly^2CH_2(\alpha)/Pro^3CH_2(\delta)$ and $Trp^4H-C(\alpha)/Pro^6CH_2(\delta)$) [11][12]. Furthermore, the CD spectrum of **4** showed a negative *Cotton* effect at *ca.* 219 nm and two positive bands

Table 4. *^{1}H-NMR* (400 MHz) *and ^{13}C-NMR* (100 MHz) *Data of* **4** *in C_5D_5N.* δ in ppm, J in Hz.

		δ(H)	δ(C)[a]	ROESY (δ(H))
Gly[1]	C=O	–	168.4 (*s*)	
	NH	8.94 (*d*, *J* = 8.4)	–	4.41, 4.60, 4.86
	$CH_2(\alpha)$	3.25 (*m*)	42.0 (*t*)	
		4.60 (*dd*, *J* = 17.6, 8.4)		2.80, 4.41
Gly[2]	C=O	–	169.6 (*s*)	
	NH	10.23 (*d*, *J* = 4.0)	–	3.84, 4.69, 4.86
	$CH_2(\alpha)$	3.84 (*dd*, *J* = 17.2, 4.0)	43.6 (*t*)	4.86
		4.86 (*dd*, *J* = 17.2, 8.8)		3.84
Pro[3]	C=O	–	173.0 (*s*)	
	H–C(α)	4.69 (*t*, *J* = 8.0)	62.8 (*d*)	2.17, 1.65
	$CH_2(\beta)$	2.17 (*m*)	29.1 (*t*)	1.65, 1.85, 4.69
	$CH_2(\gamma)$	1.85, 1.65 (2*m*)	25.2 (*t*)	
	$CH_2(\delta)$	3.46 (*m*)	47.5 (*d*)	4.98
Trp[4]	C=O	–	170.8 (*s*)	
	NH	8.00 (br. *s*)	–	5.34
	H–C(α)	4.98 (*dd*, *J* = 7.2, 6.4)	53.6 (*d*)	3.46, 3.78
	$CH_2(\beta)$	3.42 (*dd*, *J* = 6.4, 14.8)	37.6 (*t*)	
		3.78 (*dd*, *J* = 7.2, 14.8)		
	Ar	–	108.5 (*s*)	
		8.06 (*s*)	127.5 (*d*)	
		7.58 (*d*, *J* = 7.2)	112.3 (*d*)	
		7.72 (*d*, *J* = 7.2)	118.9 (*d*)	
		7.24 (*d*, *J* = 7.2, 1.2)	119.2 (*d*)	
		7.18 (*d*, *J* = 7.2, 1.2)	121.5 (*d*)	
		–	137.1 (*s*)	
		–	129.4 (*s*)	
		12.34 (*s*)	–	
Tyr[5]	C=O	–	172.6 (*s*)	
	NH	6.88 (br. *s*)	–	
	H–C(α)	5.34 (*ddd*, *J* = 9.0, 9.0, 6.0)	53.6 (*d*)	4.41
	$CH_2(\beta)$	3.34 (*dd*, *J* = 9.0, 4.8)	37.6 (*t*)	3.92
		3.92 (*dd*, *J* = 13.8, 6.0)		3.34
	Ar	–	128.5 (*s*)	
		7.33 (*d*, *J* = 8.0)	131.4 (*d*)	
		7.11 (*d*, *J* = 8.0)	116.1 (*d*)	
		–	157.7 (*s*)	
Pro[6]	C=O	–	170.0 (*s*)	
	H–C(α)	4.41 (*dd*, *J* = 9.2, 4.4)	62.3 (*d*)	
	$CH_2(\beta)$	1.85, 1.65 (2*m*)	29.2 (*t*)	
	$CH_2(\gamma)$	1.72 (*m*)	26.1 (*t*)	
	$CH_2(\delta)$	2.80 (*dd*, *J* = 16.6, 7.2)	45.8 (*d*)	4.41, 4.60
		3.03 (br. *m*)		

[a]) All assignments were based on DEPT, HMQC, and HMBC.

at *ca*. 200 (max.) and 235 nm, indicating that compound **4** was in a β-pleated-sheet conformation [17].

Since platelet adhesion and subsequent aggregate formation on exposed collagen in the damaged vessel wall play a critical role in hemostasis and thrombosis, the interaction of platelets and collagen could provide a suitable target of antiplatelet agents. It has been reported that collagen is rich in a glycine-proline-hydroxyproline

(-Gly-Pro-Hyp) repeat motif, and that synthetic collagen-like peptides comprising a repeat Gly-Pro-Hyp sequence can induce platelet aggregation through activation of the collagen receptor GP VI [18][19]. Based on the similarity of our peptide sequences, we submitted **1**–**4** to the antiplatelet activity assay and established that compound **1** selectively inhibits collagen-induced platelet aggregation (*Table 5*). To the best of our knowledge, it is the first cyclic peptide with selective inhibition of platelet aggregation induced by collagen.

Table 5. *Inhibitory Effects of Compounds* **1**–**4** *on the Aggregation of Washed Rabbit Platelets*

	Aggregation [%] AA[a]) (100 μM)	Col[a]) (10 μg/ml)	Thr.[a]) (0.1 U/ml)	PAF[a]) (1 ng/ml)
DMSO (Control)	80.3 ± 2.2	84.8 ± 1.6	80.0 ± 1.6	82.3 ± 1.2
1 (100 μM)	70.0 ± 2.0**[b])	21.5 ± 5.2***	75.3 ± 3.2	80.3 ± 1.9
(50 μM)	–	41.0 ± 8.4***	–	–
(20 μM)	–	74.5 ± 3.2*	–	–
2 (100 μM)	81.0 ± 3.5	73.3 ± 5.6	84.0 ± 2.1	83.5 ± 0.4
3 (100 μM)	79.0 ± 6.4	79.3 ± 1.9	71.7 ± 2.0**	80.5 ± 2.5
4 (100 μM)	72.3 ± 2.4*	75.0 ± 4.5	73.4 ± 1.5*	73.7 ± 1.8**

[a]) AA = arachidonic acid, Col = collagen, PAF = platelet-activating factor, Thr. = thrombin. [b]) Values are presented as means ± s. e. m. ($n = 3-4$), * means $P < 0.05$, ** means $P < 0.01$, and *** means $P < 0.001$, as compared with the respective control.

Furthermore, although the amino acid sequences of **2** (-Gly-Pro-Leu-Pro-) and **3** and **4** (-Gly-Pro-Trp-Tyr-Pro-) are similar to that of **1** (Gly-Pro-Trp-Pro-Tyr-), **2**–**4** had no activity on platelet aggregation induced by collagen. Consequently, we conclude that the sequence Gly-Pro-Trp-Pro of **1** may interfere with the interaction of collagen and collagen receptors. Research on the detailed mechanism and structure-activity relationships is in progress.

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Experimental Part

General. For the recording of physical and chemical data, see [20]. HPLC: *Shimadzu LC-10AT*, *Shimadzu LC-8A*, or *LC-10ATvp*; *Shimadzu SPD-10A-* or *SPD-10Avp* UV/VIS detector; *Hypersil-ODS* (5 μm; 250 × 4.6 mm i.d.) and prep. *ODS* (5 μm; 250 × 21.2 mm i.d.) columns. CC = column chromatography. UV Spectra: λ_{max} (log ε) in nm. CD Spectra: *Jasco J-810* spectrometer; λ (mdeg) in nm. IR Spectra: ν in cm^{-1}. ^{1}H-NMR Spectra (400 MHz and 500 MHz). C_5D_5N solns.; *Varian Unity-Plus* or *Bruker AMX-400* NMR spectrometer. ESI-MS^n: *API 3000*™ (*Applied Biosystems*); in *m*/*z* (rel. %).

Plant Material. *D. diandra* BL. was collected at Cao-Chou (Ping-Tung Hsien) in June, 2000. A voucher specimen (TNM-S0773305) is deposited at the National Museum of Natural Science, Taichung, Taiwan.

Extraction and Isolation. The air-dried whole plant (20 kg) of *D. diandra* was extracted with MeOH (3 × 20 l) at r.t. The combined MeOH extracts were evaporated and partitioned between $CHCl_3$ and H_2O to yield an org. (250 g) and an aq. extract. They org. extract was dried and evaporated to give a dark-green viscous residue. The $CHCl_3$ extract (125 g) was further separated by CC (silica gel, $CHCl_3$/MeOH gradient): 10 fractions (*Fr. A*–*J*). *Fr. I* (3.3 g) was further separated by CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:2): 10 subfractions (*Fr. I.1*–*I.10*). *Fr. I.3* was further separated CC (*Sephadex LH-20*) and solid-phase extraction (*Strata C-18* endcaps; *Merck*) and finally purified by HPLC (MeOH/H_2O 50:50, det. at 280 nm): **1** (15.0 mg). From *Fr. I.2*, crude **2** (8.3 mg) was further purified by CC (silica gel, hexane/AcOEt/MeOH of increasing polarity) and HPLC

(MeOH/H_2O 3:2, det. at 280 nm). *Fr. J* (15 g) was further separated by CC (silica gel, AcOEt/MeOH gradient): 10 subfractions (*Fr. J.1–J.10*). *Fr. J.9* was separated by CC (*Sephadex LH-20*) and prep. reversed-phase HPLC (MeOH/H_2O 55:45, det. at 254 nm): **3** (80.1 mg) and **4** (17.0 mg).

Diandrine A (=Cyclo(glycyl-L-phenylalanyl-L-tyrosyl-L-prolyl-L-tryptophyl-L-prolyl) **1**). Pale-yellow powder. $[\alpha]_D^{26} = -67.6$ ($c = 0.14$, MeOH). UV (MeOH): 205 (4.71), 218 (4.70), 279 (3.95), 288 (sh, 3.86), 329 (3.29). CD ($c = 1.9 \cdot 10^{-4}$ M, MeOH): 269 (0.24), 231 (1.21), 209 (sh, −3.71), 196 (−6.46). IR (KBr): 3271, 2950, 2922, 2853, 1630, 1542, 1516, 1448, 1232, 747, 704. ^{1}H- and ^{13}C-NMR: *Table 1*. FAB-MS: 770 (17, $[M+Na]^+$), 748 (46, $[M+H]^+$), 683 (4), 593 (4), 559 (5), 515 (4), 471 (4), 460 (4), 435 (4), 374 (4), 354 (6), 322 (6), 307 (18), 289 (14), 241 (8), 228 (8), 202 (10), 176 (20), 170 (22), 165 (15), 154 (100), 136 (85), 107 (42), 91 (43), 70 (71), 55 (51). HR-FAB-MS: 748.3456 ($[M+H]^+$, $C_{41}H_{46}N_7O_7^+$; calc. 748.3380).

Diandrine B (=Cyclo(glycyl-L-prolyl-L-leucyl-L-prolyl-L-leucyl-L-tryptophyl-L-seryl-L-seryl) **2**). Pale-yellow powder. $[\alpha]_D^{27} = -66.2$ ($c = 0.04$, MeOH). UV (MeOH): 204 (4.83), 218 (sh, 4.61), 271 (3.95). CD ($c = 4.8 \cdot 10^{-5}$ M, MeOH): 263 (0.30), 219.5 (−1.64), 206.5 (0.02), 192 (5.37). IR (KBr): 3347, 2955, 2924, 2852, 1637, 1538, 1455, 1284, 1270, 1055, 745, 700, 619. ^{1}H- and ^{13}C-NMR: *Table 2*. FAB-MS: 860 (100, $[M+Na]^+$), 838 (7, $[M+H]^+$), 730 (4), 687 (3), 604 (2), 460 (2), 386 (5), 371 (4), 330 (5), 315 (6), 307 (5), 289 (3), 253 (5), 227 (3), 185 (6), 176 (15), 154 (39), 136 (32), 70 (69). HR-FAB-MS: 838.4469 ($[M+H]^+$, $C_{41}H_{60}N_9O_{10}^+$; calc. 838.4385).

Diandrine C (=Cyclo(glycylglycyl-L-prolyl-L-tryptophyl-L-tyrosyl-L-prolyl) **3**). Pale-yellow powder. $[\alpha]_D^{26} = +2.2$ ($c = 0.19$, MeOH). UV (MeOH) 205 (4.81), 222 (4.78), 249 (4.01), 255 (sh, 4.06), 261 (4.05), 281 (4.08), 289 (sh, 4.00). CD ($c = 2.9 \cdot 10^{-4}$ M, MeOH): 234.5 (1.62), 220 (−2.23), 212 (0), 201 (7.19), 192.5 (4.97). IR (KBr): 3301, 2930, 1662, 1625, 1540, 1516, 1456, 1340, 1237, 1046, 748. ^{1}H- and ^{13}C-NMR: *Table 3*. FAB-MS: 680 (1, $[M+Na]^+$), 658 (6, $[M+H]^+$), 528 (3), 406 (3), 329 (5), 307 (2), 289 (4), 165 (16), 154 (48), 136 (50), 115 (25), 107 (29), 89 (50), 77 (79), 51 (50). HR-FAB-MS: 658.2996 ($[M+H]^+$, $C_{34}H_{39}N_7O_7^+$; calc. 658.2911).

Diandrine D (=Cyclo(glycylglycyl-L-prolyl-L-tryptophyl-L-tyrosyl-L-prolyl) **4**). Pale-yellow powder. $[\alpha]_D^{26} = +6.8$ ($c = 0.19$, MeOH). UV (MeOH): 206 (4.75), 222 (4.75), 249 (4.00), 255 (sh, 4.06), 261 (4.03), 280 (4.03), 289 (sh, 3.94). CD ($c = 2.9 \cdot 10^{-4}$ M, MeOH): 285 (−0.18), 240 (1.72), 220 (−1.02), 205 (sh, 3.05), 202 (3.86). IR (KBr): 3311, 2875, 1679, 1666, 1625, 1556, 1516, 1435, 1423, 1339, 1234, 1105, 1011, 825, 746. ^{1}H- and ^{13}C-NMR: *Table 4*. FAB-MS: 696 (7, $[M+K]^+$), 680 (3, $[M+Na]^+$), 658 (6, $[M+H]^+$), 528 (3), 474 (3), 345 (1), 329 (1), 307 (2), 289 (3), 242 (6), 212 (7), 192 (19), 165 (12), 154 (56), 136 (62), 115 (17), 107 (25), 89 (53), 77 (64), 51 (51). HR-FAB-MS: 658.2989 ($[M+H]^+$, $C_{34}H_{39}N_7O_7^+$; calc. 658.2911).

Hydrolysis and Derivatization of **1**, **3**, *and* **4** *(*Marfey*'s Procedure)* [9][10]. Compound **1**, **3**, or **4** (0.1 mg) was dissolved in 6N HCl (0.5 ml) in a *MicroVessel*™ (*CEM*) and irradiated in a domestic microwave oven (214 W, *Tatung*) for 10 min. After cooling, the mixture was evaporated, the residue dissolved in H_2O (100 µl), and the soln. treated with 1% FDAA (= N^2-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide) soln. in acetone (250 µl) and 1M $NaHCO_3$ (300 µl). The mixture was heated at 50° for 1 h. HPLC comparison (*Hypersil-ODS*, (5 µ, 250 × 4.6 mm), MeCN (0.01% CF_3COOH)/H_2O (0.1% CF_3COOH 7:3, det. at 340 nm) with FDAA-derived amino acid standards established the L-configuration of the derivatized amino acids in the mixture (except for tyrosine).

Platelet Aggregation Assays. Blood anticoagulated with ethylenediaminetetraacetic acid (EDTA) was collected from New Zealand rabbits. The rabbit platelet suspension was prepared according to the procedure previously described [21]. The platelets, after washing, were finally suspended in *Tyrode*'s soln. containing Ca^{2+} (1 mM), glucose (11.1 mM), and bovine serum albumin (3.5 mg/ml) at a concentration of $3 \cdot 10^8$ platelets/ml. Platelet aggregation was measured turbidimetrically with a light-transmission aggregometer (*Chrono-Log Co.*, U.S.A.). The platelet suspension (400 µl) was incubated with dimethyl sulfoxide (DMSO, vehicle) or test compounds at 37° for 3 min under stirring (1200 r.p.m.); then arachidonic acid (AA, 100 µM), collagen (10 µg/ml), platelet-activating factor (PAF, 1 ng/ml), or thrombin (0.1 U/ml) was added to trigger platelet aggregation. The extent of platelet aggregation was measured as the maximal increase of light transmission within 5 min after the addition of inducers. To avoid the effect of the solvent on platelet aggregation, the final concentration of DMSO in the platelet suspensions was fixed at 0.5%.

Electrospray Ionization Tandem Mass Spectrometry. Each cyclic peptide was directly infused into the mass spectrometer at a flow-rate of 10 µl/ml to acquire full-scan and product-ion MS. A Q1 full-scan spectrum of each sample was first conducted to obtain the corresponding protonated molecular ions. The product-ion scan spectrum was further acquired by transmitting the protonated molecular ion *via* Q1 and scanning for products resulting from fragmentations in the collision cell. The electrospray voltage at the spraying needle was optimized at 4500 V. The *TurboIonSpray* source was operated with N_2 as the nebulizing (set to 10) curtain (set to 10). Low-

energy collision-activated-dissociation (CAD) experiments were performed with N_2 (CAD gas valve set to 4) as collision gas, and a collision energy of 40 eV was used.

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