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New vasorelaxant indole alkaloids, villocarines A–D from *Uncaria villosa*

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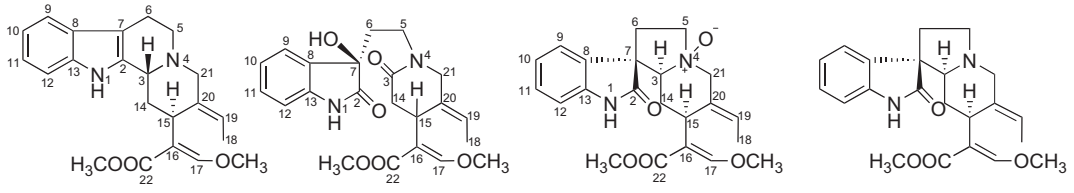
ABSTRACT

Villocarines A–D (**1**–**4**), four new indole alkaloids have been isolated from the leaves of *Uncaria villosa* (Rubiaceae) and their structures were elucidated by 2D NMR methods and chemical correlations. Villocarine A (**1**) showed vasorelaxation activity against rat aortic ring and showed inhibition effect on vasocontraction of depolarized aorta with high concentration potassium, and also inhibition effect on phenylephrine (PE)-induced contraction in the presence of nicardipine in a Ca^{2+} concentration-dependent manner. The vasorelaxant effect by **1** might be attributed mainly to inhibition of calcium influx from extracellular space through voltage-dependent calcium channels (VDC) and/or receptor-operated Ca^{2+}-channels (ROC), and also partly mediated through the increased release of NO from endothelial cells and opening of voltage-gated K^+-channels.

1. Introduction

The vasodilators are useful for treatment of cerebral vasospasm and hypertension, and for improvement of peripheral circulation. Several endothelium-dependent vasodilators, such as bradykinin, acetylcholine, and histamine, have been reported to elevate Ca^{2+} levels in endothelial cells and activate NO release, leading to vasorelaxation.[1] On the other hand, contractile response in smooth muscle is caused by an influx of Ca^{2+} through voltage-dependent Ca^{2+}-channels (VDC) and/or receptor-operated Ca^{2+}-channels (ROC).[2] The endothelium-independent vasodilators, such as nicardipine, niphedipine, dirtiazem, and verapamil, have been reported to inhibit VDC and led to decrease in the intracellular Ca^{2+} concentration in smooth muscle, leading to vasorelaxation.[2]



1 **2** **3** **4**

Recently, we have reported that some cyclic peptides such as dichotomin J from *Stellaria dichotoma* var. *lanceolata*[3] and cyclosquamosin B from *Annona squamosa*[4] and some alkaloids such as cassiarin A from *Cassia siamea*[5] and bisnicalaterine B from *Hunteria zeylanica*[6] showed a vasorelaxant effect on rat aorta. During our search for bioactive compounds targeting aortic smooth muscle from medicinal plants, we found that the extract from the leaves of *Uncaria villosa* showed a vasorelaxant effect on rat aorta. *Uncaria* species belonging to Rubiaceae are known to contain corynanthe and heteroyohimbine type indole and oxindole alkaloids.[7] Some corynanthe and oxindole type alkaloids such as geissoschizine methyl ether,[8] hirsutine,[9] and rhynchophylline[10] showed vasorelaxant effects against isolated strips of rat aorta. *Uncaria* species have been used in traditional medicine by the native people of the Chinese for the treatment of antihypertensive agents.[11] We report here the isolation and structure elucidation of four new indole alkaloids, villocarines A–D (**1**–**4**) from *Uncaria villosa* and their vasodilator effects on isolated rat aorta.

2. Results and discussion

2.1. Structures of villocarines A–D (1–4)

Leaves of *Uncaria villosa* were extracted with MeOH, and the extract was partitioned between EtOAc and 3% aqueous tartaric acid. Water-soluble materials, adjusted to pH 9 with satd aq Na_2CO_3,

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were extracted with $CHCl_3$. The $CHCl_3$-soluble materials were subjected to an amino silica gel column ($CHCl_3$/MeOH) followed by a silica gel column ($CHCl_3$/MeOH). The eluted fractions were further separated by ODS HPLC (MeOH/H_2O) to afford villocarines A–D (**1**–**4**) together with a known alkaloid, pseudoyohimbine[10] (**5**).

Villocarine A {**1**, $[\alpha]_D^{29}$ −12 (*c* 0.2, $CHCl_3$)} was revealed to have the molecular formula $C_{22}H_{26}N_2O_3$, by HRESITOFMS [*m*/*z* 367.2014 $(M+H)^+$, Δ −0.8 mmu]. The ^1H NMR data (Table 1) showed the presence of four aromatic protons, an ethylidene side chain, a methyl ester function, and a methoxy group. The gross structure of **1** was deduced from detailed analyses of two-dimensional NMR data, including ^1H–^1H COSY, HSQC, and HMBC spectra in $CDCl_3$ (Fig. 1). The ^1H–^1H COSY and HSQC spectra revealed the presence of four partial structures **a** (C-9–C-12), **b** (C-5–C-6), **c** (C-3, C-14–C-15), and **d** (C-18–C-19) as shown in Figure 1. The connectivity between an indole ring including partial structures **a** and **b** was revealed by the HMBC correlations of H-5 to C-7 (δ_C 108.3) and H-6 to C-2 (δ_C 134.8). HMBC correlations of H-5 to C-3 (δ_C 56.0) and H_2-14 to C-2 established the connection between partial structures **b** and **c**, and the indole ring. The HMBC cross-peaks of H_2-21 to C-19 and H_3-18 to C-20 indicated the ethylidene side chain at C-20. The presence and position of the methoxy and methoxy carbonyl groups were confirmed by HMBC correlations as shown in Figure 1. ^1H and ^{13}C NMR data (Tables 1 and 2) suggested the connection among C-3, C-5, and C-21 through a nitrogen atom. Thus, the gross structure of **1** was assigned as shown in Figure 1. Based on the proposed structure, **1** could be implied to be corynanthe type indole alkaloid such as geissoschizine methyl ether[12] and hirsutine.[13] Villocarine A (**1**) showed similar ^1H and ^{13}C NMR chemical shifts to those of geissoschizine methyl ether except for the existence of a proton at C-10 and ^3J coupling constant (12.0 Hz) between H-3 and H-14a. Therefore, the relative stereochemistry of H-3 was assigned to be β. Absolute configurations at C-3 and C-15 could be assigned to be 3*R*,15*S* by negative Cotton effect (θ −1000) around 270 nm in the CD spectrum.[14]

Table 1
^1H NMR data [δ_H (*J*, Hz)] of villocarines A–D (**1**–**4**)[a]

	1	2	3	4
3	3.69 (dd, 12.0, 2.0)		3.96 (dd, 13.7, 4.0)	4.21 (m)
5a	3.10 (dd, 11.0, 5.5)	3.30 (m)	3.89 (m)	3.75 (m)
5b	2.65 (ddd, 11.0, 4.2)	3.48 (m)	4.22 (m)	3.96 (m)
6a	3.00 (m)	2.10 (m)	2.28 (dd, 13.7, 5.9)	2.24 (dd, 13.5, 6.0)
6b	2.73 (br d, 15.0)	2.10 (m)	3.18 (ddd, 13.7, 13.7, 5.9)	2.76 (ddd, 13.5, 13.5, 6.0)
9	7.45 (d, 7.2)	7.36 (d, 7.4)	7.61 (d, 7.2)	7.63 (d, 7.5)
10	7.07 (td, 7.2, 1.0)	7.00 (dd, 7.5, 7.4)	7.09 (t, 7.2)	7.10 (t, 7.5)
11	7.11 (td, 7.2, 1.0)	7.18 (dd, 7.7, 7.5)	7.22 (t, 7.2)	7.24 (t, 7.5)
12	7.28 (d, 7.2)	6.85 (d, 7.7)	6.86 (d, 7.2)	6.94 (d, 7.5)
14a	1.90 (ddd, 13.4, 12.0, 7.2)	2.27 (m)	1.39 (ddd, 13.7, 4.8, 4.0)	1.41 (ddd, 14.1, 4.2, 4.2)
14b	2.19 (br d, 13.4)	2.56 (m)	2.41 (q, 13.7)	2.52 (q, 14.1)
15	4.05 (d, 7.2)	3.81 (m)	3.70 (m)	3.65 (m)
17a	7.36 (s)	7.26 (s)	7.25 (s)	7.23 (s)
18	1.55 (dd, 6.8, 1.7)	1.46 (d, 6.3)	1.48 (d, 6.7)	1.47 (d, 7.1)
19	5.45 (q, 6.8)	5.33 (m)	5.73 (q, 6.7)	5.73 (q, 7.1)
21a	3.35 (br d, 13.2)	3.35 (m)	4.06 (m)	3.82 (m)
21b	3.62 (d, 13.2)	4.25 (br d, 13.4)	4.61 (m)	4.18 (br s)
CO_2Me	3.73 (s)	3.65 (s)	3.66 (s)	3.63 (s)
17-O–Me	3.82 (s)	3.80 (s)	3.79 (s)	3.80 (s)
NH	7.71 (s)	8.91 (m)	8.34 (br s)	8.45 (br s)

[a] Free base in $CDCl_3$.

Villocarine B {**2**, $[\alpha]_D^{29}$ −142 (*c* 1.9, $CHCl_3$)} was revealed to have the molecular formula $C_{22}H_{26}N_2O_6$, by HRESITOFMS [*m*/*z* 415.1868 $(M+H)^+$, Δ −0.1 mmu]. The UV absorptions at 242 and 287 nm indicated that **2** had a β-alkoxyacrylic ester moiety in addition to the oxindole chromophore. The structure was clarified by means of ^1H and ^{13}C NMR spectra as shown below. The ^{13}C NMR signals with particular significance were two singlet signals at δ 75.2 (C-7) and 171.9 (C-3). The gross structure of **2** was deduced from detailed analyses of two-dimensional NMR data as shown in Figure 2. The HMBC spectra revealed connectivities of four partial structures **a**–**d**. The absolute stereochemistry at C-7 was clarified to be *R* by the negative cotton effect (θ −1000) at 235 nm in CD spectrum which was quite consistent with the data of 3-oxo-7*R*-hydroxy-3,7-secorhynchophylline.[15] The stereochemistry at C-15 was not determined yet.

Villocarine C {**3**, $[\alpha]_D^{29}$ −12 (*c* 0.8, $CHCl_3$)} was obtained as a brown amorphous solid and was revealed to have the molecular formula $C_{22}H_{26}N_2O_5$, by HRESITOFMS [*m*/*z* 399.1923 $(M+H)^+$, Δ +0.3 mmu], which was larger than that of corynoxeine[15] by an oxygen unit. The ^1H NMR data (Table 1) showed the presence of four aromatic protons, an ethylidene side chain, and a methyl ester function. Partial structures C-9–C-12, C-5–C-6, C-3, C-14–C-15, and C-18–C-19 were deduced from a detailed analysis of ^1H–^1H COSY spectrum of **3**. The HMBC cross-peaks of H_3-18 to C-20 and H-19 to C-21 indicated the presence of an ethylidene side chain at C-20 (Fig. 3). And the presence of an oxindoline ring was elucidated by HMBC correlations for H-3, H-6, and H-9 to C-7, and H-3 and H-6 to C-2. These HMBC correlations indicated villocarine C possessed corynantheine type oxindole skeleton. Comparison of ^{13}C chemical shifts of C-3, C-5, and C-21 (δ_C 85.3, 67.8, and 69.8, respectively) in **3** with those (δ_C 80.4, 67.3, and 66.4, respectively) of corynoxeine *N*-oxide[16] indicated the presence of an *N*-oxide functionality at *N*-4. The ^1H chemical shift of H-9 (δ_H 7.61) indicated that the oxygen atom of *N*-oxide functionality and the benzene ring oriented opposite side of the molecule.[16] The relative stereochemistry of **3** was elucidated by ^3J coupling constants and NOESY correlations as shown in Figure 3.

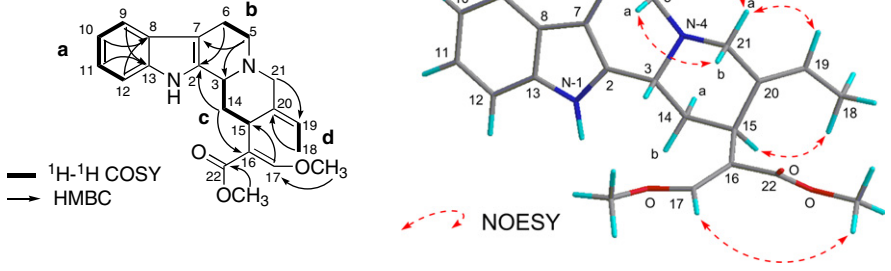


Figure 1. Selected 2D NMR correlations for villocarine A (**1**).

Table 2
^{13}C NMR data (δ_C) of villocarines A–D (**1**–**4**)[a]

	1	2	3	4
2	134.8	180.0	178.4	178.3
3	56.0	171.9	85.3	65.8
5	52.5	41.5	67.8	53.6
6	21.4	35.8	34.6	35.3
7	108.3	75.2	55.7	56.1
8	127.3	131.1	128.7	128.2
9	118.0	124.1	123.7	124.3
10	119.2	122.8	123.1	123.7
11	121.1	129.4	129.2	129.3
12	110.7	110.6	110.4	110.0
13	136.0	140.6	141.1	140.6
14	35.5	36.4	26.9	25.1
15	30.9	30.4	30.8	30.4
16	112.3	111.4	111.3	111.2
17	158.8	159.1	159.2	159.1
18	12.8	13.2	13.5	13.4
19	122.5	121.3	130.8	129.7
20	134.2	134.5	128.1	129.7
21	61.7	54.6	69.8	55.1
22	168.8	167.4	167.1	167.1
CO_2Me	51.4	51.2	51.3	51.2
17-O-Me	61.5	61.7	61.8	61.8

[a] Free base in $CDCl_3$.

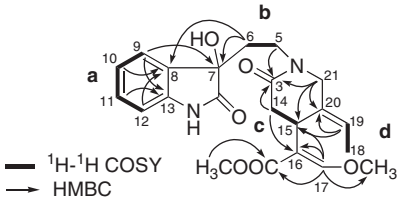


Figure 2. Selected 2D NMR correlations for villocarine B (**2**).

NOESY correlations of H_3-18 to H-15 indicated that the geometry of the ethylidene side chain was *E*. The NOESY correlations of H-3/H-15 indicated that both H-3 and H-15 were α-orientated. The relative configuration of C-7 was established to be R^* by the NOESY correlation of H-3/H-9.

Absolute configurations at C-3 and C-7 could be assigned to be 3*S*,7*R* by the Cotton pattern [211 (θ −17600), 254 (θ −12500), 282 (θ 1200)] in the CD spectrum in comparison with that of rhynchophylline.[17,18]

Villocarine D {**4**, $[\alpha]_D^{29}$ −3 (*c* 0.8, $CHCl_3$)} was obtained as a brown amorphous solid and was revealed to have the molecular formula $C_{22}H_{26}N_2O_4$, by HRESITOFMS [*m*/*z* 383.1988 $(M+H)^+$, Δ +1.7 mmu], which was smaller than that of villocarine C (**3**) by an oxygen unit. The NMR data of **4** were analogous to those of villocarine C except for the chemical shift values around an *N*-4 atom. Treatment of villocarine C (**3**) with Na_2SO_3 in aqueous MeOH/H_2O afforded the reductive derivative, whose spectroscopic data and specific rotation were identical with those of villocarine D (**4**). Thus, the structure of **4** was confirmed.

2.2. Vasorelaxant activity in ex vivo

All isolated compounds were tested for vasorelaxant activity against rat aorta (Fig. 4). When PE (0.3 μM) was applied to thoracic aortic rings with endothelium after achieving a maximal response, a series of villocarines was added and villocarine A (**1**) showed potent vasorelaxant effects at 30 μM (Fig. 4). The excellent activity could be observed for **1** at early stage within 10–30 min after injection. Furthermore, vasorelaxant effect was examined by using endothelium-denuded aortic tissues for **1** (Fig. 5).

In endothelium-denuded aortic tissues, **1** caused slightly less vascular relaxation (Fig. 5). Treatment with N^G-monomethyl-L-arginine (L-NMMA, 100 μM),[19] an inhibitor of nitric oxide (NO) synthase, also slightly inhibited villocarine A (**1**)-induced vasorelaxation (Fig. 5). The vasorelaxant effect of **1** may be slightly mediated through the increased release of NO from endothelial cells.

In an attempt to elucidate the possible mechanisms involved in the vasorelaxant effects, the effects of **1** was examined with preincubation of tetraethylammonium chloride (TEA, 1 mM)[20] as an inhibitor of K^+ channel in PE-contracted endothelium-denuded rings. Incubation of endothelium-denuded rings with TEA slightly shifted the concentration-response curve for **1** to the right as shown in Figure 6.

Ca^{2+} can contract aortic rings concentration dependently in Ca^{2+}-free KHS after depolarization with isotonic high K^+ (60 mM) by Ca^{2+} influx via VDCs; this contraction was significantly inhibited by villocarine A (**1**) at 30 μM (Fig. 7).

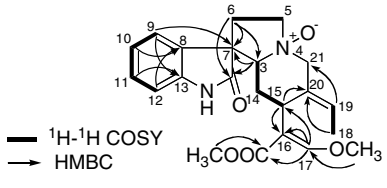
In addition, the PE (1 μM)-induced contractions of the aortic rings in the presence of nicardipine (1 μM) in Ca^{2+}-free KHS occurred in Ca^{2+} (10 μM to 1 mM) concentration-dependent manner, presumably due to Ca^{2+} influx via ROCs. Villocarine A (**1**) showed moderate inhibition of this contraction at 30 μM, suggesting that **1** exerted inhibitory effects on Ca^{2+} influx via ROCs (Fig. 8).

In conclusion, we isolated four new indole alkaloids, villocarines A–D (**1**–**4**) from the leaves of *Uncaria villosa* (Rubiaceae) and their structures were elucidated by 2D NMR methods and chemical correlations. Villocarine A (**1**) showed vasorelaxation activity against rat aortic ring and showed inhibition effect on vasocontraction of depolarized aorta with high concentration potassium, and also inhibition effect on PE-induced contraction in the presence of nicardipine in a Ca^{2+} concentration-dependent manner. The vasorelaxant effect by **1** might be attributed mainly to inhibition of calcium influx from extracellular space through voltage-dependent calcium channels (VDC) and/or receptor-operated Ca^{2+}-channels (ROC), and also partly mediated through the increased release of NO from endotheliul cells and opening of voltage-gated K^+-channels.

3. Experimental section

3.1. General methods

^{1}H and 2D NMR spectra were recorded on a Bruker AV 400 spectrometer and chemical shifts were reported using residual $CDCl_3$



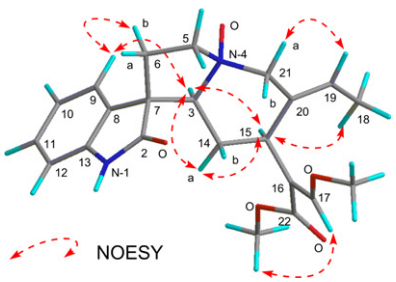


Figure 3. Selected 2D NMR correlations for villocarine C (**3**).

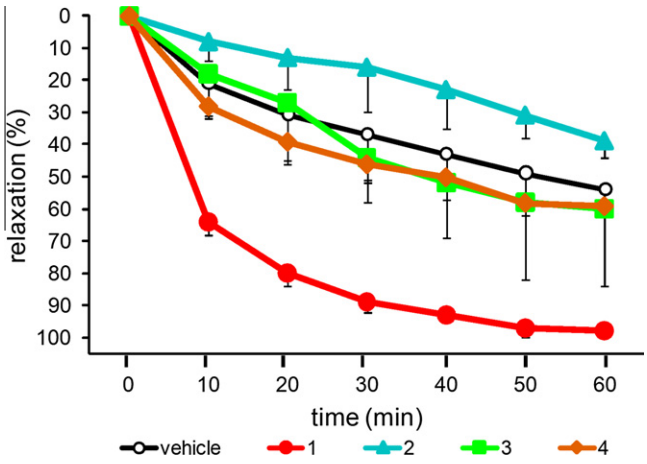


Figure 4. Vasorelaxant effects of villocarines A–D (**1**–**4**, 30 μM) on the rat aortic rings pre-contracted with PE (0.3 μM). Values are the mean ± S.D. (n = 3).

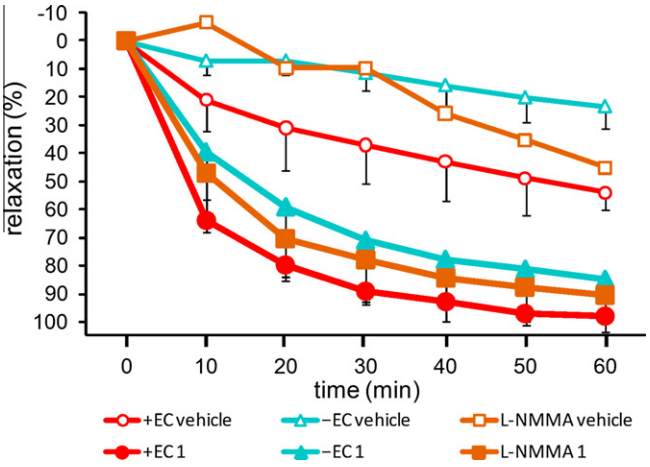


Figure 5. Vasorelaxant effects of villocarine A (**1**) on isolated rat aortic rings pre-contracted with PE (0.3 μM) in presence or absence of endothelium. Vasorelaxant effect of **1** on isolated rat aortic rings pre-contracted with PE (0.3 μM) in the presence of L-NMMA (100 μM). Values are the mean ± S.D. (n = 3).

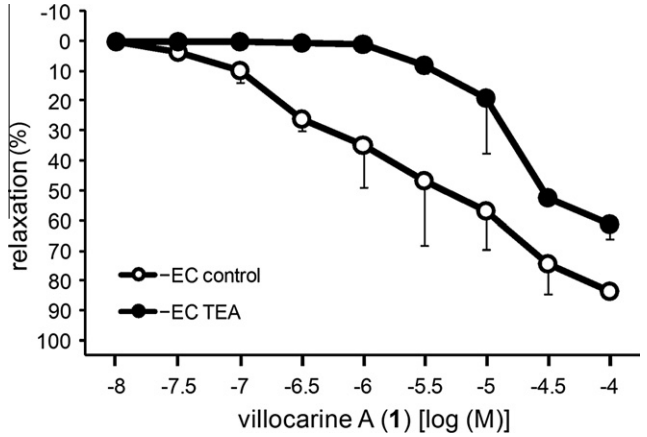


Figure 6. Vasorelaxant effects of **1** on endothelium-denuded rat aortic rings pre-contracted with PE (0.3 μM) in the presence of TEA (1 mM). Values are the mean ± S.D. (n = 3).

(δ_H 7.26 and δ_C 77.0) as internal standards. Standard pulse sequences were employed for the 2D NMR experiments. ^{1}H–^{1}H COSY, HOHAHA, and NOESY spectra were measured with spectral widths

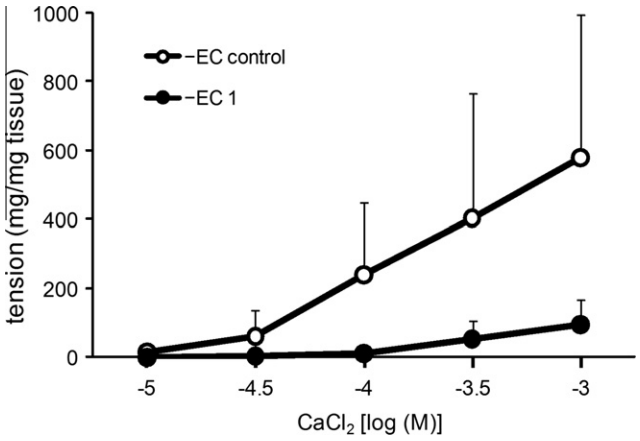


Figure 7. Effects of **1** at 30 μM on the concentration-response curves of $CaCl_2$ in endothelium-denuded rat aortic rings in Ca^{2+}-free K^+-rich (60 mM KCl) medium. Values are the mean ± S.D. (n = 3).

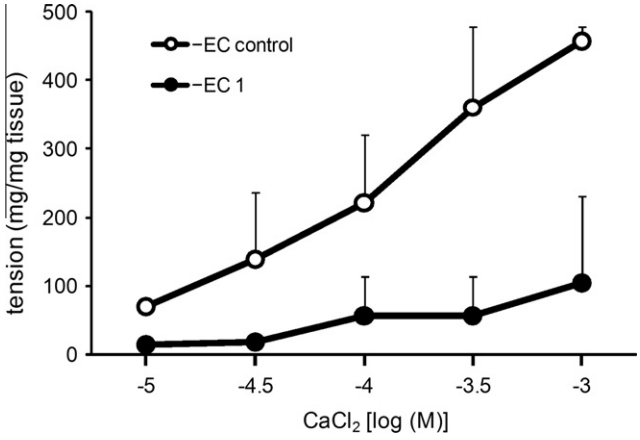


Figure 8. Effects of **1** at 30 μM on the concentration-response curves of $CaCl_2$ in endothelium-denuded rat aortic rings in Ca^{2+}-free medium pre-incubated with PE (1 μM) and nicardipine (1 μM). Values are the mean ± S.D. (n = 3).

of both dimensions of 4800 Hz, and 32 scans with two dummy scans were accumulated into 1 K data points for each of 256 t_1 increments. NOESY spectra in the phase sensitive mode were measured with a mixing time of 800 ms. For HMQC spectra in the phase sensitive mode and HMBC spectra, a total of 256 increments of 1 K data points were collected. For HMBC spectra with Z-axis PFG, a 50 ms delay time was used for long-range C–H coupling. Zero-filling to 1 K for F_1 and multiplication with squared cosine-bell windows shifted in both dimensions were performed prior to 2D Fourier transformation.

3.2. Material

The leaves of *Uncaria villosa* were collected in Sekayu, Terengganu, Malaysia in August, 2008. The botanical identification was made by Mr. Syamsul Khamis the Institute of Bioscience, University Putra Malaysia. A voucher specimen (Herbarium No. SK1571-08) is deposited at the Herbarium of Laboratory of Biodiversity, Institute of Bioscience, Universiti Putra, Malaysia.

3.3. Extraction and isolation

The leaves of *Uncaria villosa* (90.7 g) were extracted with MeOH. The MeOH extract (22 g) was treated with 3% tartaric acid (pH 2)

and then partitioned with EtOAc. The aqueous layer was treated with satd aq Na_2CO_3 aq to pH 9 and extracted with $CHCl_3$ to give alkaloidal fraction (2.06 g). The alkaloidal fraction was purified by an amino silica column ($CHCl_3$/MeOH, 1:0) and a SiO_2 column ($CHCl_3$/MeOH, 1:0→0:1) and the fraction eluted by $CHCl_3$/MeOH (9:1) was purified by an ODS HPLC (MeOH/H_2O, 45:55, flow rate, 2 mL/min; UV detection at 254 nm) to afford villocamines A (**1**, 1.8 mg, 0.0020% yield), B (**2**, 1.3 mg, 0.0014%), C (**3**, 10.4 mg, 0.0011%), and D (**4**, 3.6 mg, 0.0040%), together with a known alkaloid, pseudoyohimbine[10] (**5**, 10.4 mg, 0.0011%).

3.3.1. Villocarine A (1)

Brown amorphous solid; $[\alpha]_D^{29}$ −12 (*c* 0.2, $CHCl_3$); IR (film) ν_{max} 2880, 1700, and 1635 cm^{-1}; UV (MeOH) λ_{max} 204 (log ε 3.86) and 209 (3.83) nm; CD (MeOH) λ_{max} 205 (θ 4300), 224 (θ −2300), 243 (θ 2300), and 270 (θ −1000) nm; ^1H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m*/*z* 367 $(M+H)^+$; HRESITOFMS *m*/*z* 367.2014 [$(M+H)^+$, Δ −0.8 mmu, calcd for $C_{22}H_{27}N_2O_3$, 367.2022].

3.3.2. Villocarine B (2)

Brown amorphous solid; $[\alpha]_D^{29}$ −142 (*c* 1.9, $CHCl_3$); IR (film) ν_{max} 3255, 2940, 1715, 1620, and 1245 cm^{-1}; UV (MeOH) λ_{max} 287 (log ε 3.30) and 242 (4.03) nm; CD (MeOH) λ_{max} 202 (θ 4800), 235 (θ −1000), and 256 (θ 400) nm; ^1H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m*/*z* 415 $(M+H)^+$; HRESITOFMS *m*/*z* 415.1868 [$(M+H)^+$, Δ −0.1 mmu, calcd for $C_{22}H_{27}N_2O_6$, 415.1869].

3.3.3. Villocarine C (3)

Brown amorphous solid; $[\alpha]_D^{29}$ −12 (*c* 0.8, $CHCl_3$); IR (film) ν_{max} 2880, 1700, 1640, 1620, 1240, and 1110 cm^{-1}; UV (MeOH) λ_{max} 282 (log ε 3.31), 241 (4.05), and 206 (4.35) nm; CD (MeOH) λ_{max} 200 (θ −12400), 204 (θ −8100), 211 (θ −17600), 237 (θ 1300), 254 (θ −12500), and 282 (θ 1200) nm; ^1H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m*/*z* 399 $(M+H)^+$; HRESITOFMS *m*/*z* 399.1923 [$(M+H)^+$, Δ +0.3 mmu, calcd for $C_{22}H_{27}N_2O_5$, 399.1920].

3.3.4. Villocarine D (4)

Brown amorphous solid; $[\alpha]_D^{29}$ −3 (*c* 0.8, $CHCl_3$); IR (film) ν_{max} 2880, 1710, 1640, 1620, 1245, and 1120 cm^{-1}; UV (MeOH) λ_{max} 288 (log ε 3.26) and 242 (3.91) nm; CD (MeOH) λ_{max} 200 (θ 3900), 211 (θ −5400), 236 (θ 1300), and 254 (θ −3500) nm; ^1H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m*/*z* 383 $(M+H)^+$; HRESITOFMS *m*/*z* 383.1988 [$(M+H)^+$, Δ +1.7 mmu, calcd for $C_{22}H_{27}N_2O_4$, 383.1971].

3.4. Chemical transformation of villocarine C (3) into villocarine D (4)

To a solution of villocarine C (**3**, 1.0 mg) in aqueous MeOH/H_2O (0.2 mL) was added Na_2SO_3 (1.0 mg) and the mixture was kept at room temperature for 30 min. After evaporation, the residue was applied to a silica gel column ($CHCl_3$/MeOH, 4:1) to give a compound (0.4 mg), whose spectroscopic data including $[\alpha]_D^{27}$ −4 (*c* 0.5, $CHCl_3$) was identical to that of natural villocarine D (**4**).

3.5. Vasodilation assay[4]

A male Wistar rat weighting 260 g was sacrificed by bleeding from carotid arteries under an anesthetization. A section of the thoracic aorta between the aortic arch and the diaphragm was removed and placed in oxygenated, modified Krebs–Henseleit solution (KHS: 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, and 11.0 mM glucose). The aorta was cleaned of loosely adhering fat and connective tissue and cut into ring preparations 3 mm in length. The tissue was placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 5 mL KHS solution at 37 °C with one end connected to a tissue holder and the other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g. During this time the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic ring was contracted by treatment with 3×10^{-7} M PE. The presence of functional endothelial cells was confirmed by demonstrating relaxation to 10^{-5} M acetylcholine (ACh), and aortic ring in which 80% relaxation occurred, were regarded as tissues with endothelium. When the PE-induced contraction reached a plateau, each sample (1×10^{-6}–3×10^{-5} M) was added.

Data are expressed as means ± S.D. Statistical comparisons between time-response curves were made using a one-way analysis of variance (ANOVA), with Bonferroni's correction for multiple comparisons being performed post hoc (P <0.05 being considered significant).

These animal experimental studies were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University and under the supervision of the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Science, Sports Culture, and Technology of Japan.

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