Vasorelaxing Alkaloids and Flavonoids from *Cassytha filiformis*

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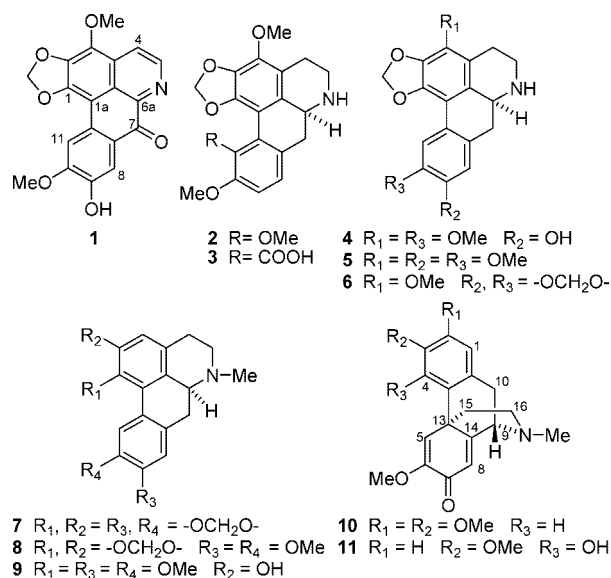
Two new aporphine alkaloids, isofiliformine (**1**) and cassythic acid (**3**), along with 22 known compounds were isolated from whole herb of *Cassytha filiformis*. Cassythic acid (**3**), cassythine (**4**), neolitsine (**7**), and dicentrine (**8**) had potent vasorelaxing effects on precontracted rat aortic preparations with mean IC$_{50}$ values between 0.08 and 2.48 μM. Compounds **1**, 1,2-methylenedioxy-3,10,11-trimethoxyaporphine (**2**), (−)-*O*-methylflavinatine (**10**), (−)-salutaridine (**11**), isohamnetin-3-*O*-β-glucoside, and isohamnetin-3-*O*-rutinoside exerted moderate vessel-relaxing activities with IC$_{50}$ values from 16.50 to 32.81 μM at the test concentrations.

Cassytha filiformis Linn. (Lauraceae) is a parasitic herb that grows at low elevations, commonly along seashores, in Taiwan.[1] It is used in Taiwan folk medicine as a diuretic and antibiotic agent.[2] Previous chemical studies of *C. filiformis* have shown the presence of a variety of aporphine alkaloids and lignans.[3–5] In screening for biological activity, we found that an EtOH extract of the whole plant caused significant vasorelaxation of precontracted rat aortic preparations. Thus, a systemic study was conducted to identify the active compounds. In this study, we report the isolation of a series of aporphine alkaloids, morphinan alkaloids, flavonoids, and some other compounds from *C. filiformis*. The results of vasorelaxation assays of individual compounds are also presented.

Isofiliformine (**1**) was obtained as a red, amorphous powder having the molecular formula C$_{19}$H$_{13}$O$_6$N (HREIMS). The IR spectrum showed OH (3374 cm^{-1}), carbonyl (1653 cm^{-1}), and aromatic ring (1558, 1539 cm^{-1}) absorptions. Its UV spectrum had absorption bands at 250, 281, 356, and 461 nm, and a bathochromic shift observed upon the addition of alkali suggested that it was a phenolic 1,2,3,9,10-oxygenated oxoaporphine.[6] The ^{1}H NMR spectrum indicated two methoxy groups (δ 4.16 and 4.43) and had a methylenedioxyl signal at δ 6.59 (2H, s). In the aromatic region, a pair of AB doublets at δ 8.72 (d, *J* = 6.0 Hz) and 8.84 (d, *J* = 6.0 Hz) were characteristic signals of H-5 and H-6 of oxoaporphine derivatives. Two singlets (δ 7.99 and δ 8.24) were ascribed to H-8 and H-11, and their assignments were confirmed by NOESY and HMBC spectra. The NOESY spectrum of **1** showed correlation of the methoxy group (δ 4.43) with H-4 (δ 8.84) and the methylene-dioxy signal (δ 6.59), and H-11 (δ 8.24) with the methylenedioxy signal (δ 6.59) and the C-10 methoxy group (δ 4.16), which suggested a 3,10-dimethoxy-1,2-methylenedioxy oxoaporphine structure. HMBC correlations of **1** from C-7 (δ 178.2) to H-8 (δ 7.99) indicated the positions of H-8 and OH-9. Thus, isofiliformine must be represented by structure **1**. Chemical shifts of H-5 and H-4, from NOESY and HMBC experiments, established that H-5 was at higher field than H-4, which reversed the assignments in previous reports of oxoaporphine derivatives.[6]

Compound **2** had the molecular formula C$_{20}$H$_{21}$O$_5$N, as confirmed by ^{13}C NMR, DEPT, and ESIMS *m/z* 356 [M + H]$^+$. The UV spectrum of **2** in MeOH showed absorption maxima at 226 and 277 nm characteristic of a 1,2,10,11-substituted aporphine.[7] The ^{1}H NMR spectrum of **2** had signals indicating three methoxy groups, a methylenedioxy group, three sets of methylene protons, and a

1

2 R= OMe
3 R= COOH

4 R$_1$ = R$_3$ = OMe   R$_2$ = OH
5 R$_1$ = R$_2$ = R$_3$ = OMe
6 R$_1$ = OMe   R$_2$, R$_3$ = -OCH$_2$O-

7 R$_1$, R$_2$ = R$_3$, R$_4$ = -OCH$_2$O-
8 R$_1$, R$_2$ = -OCH$_2$O-   R$_3$ = R$_4$ = OMe
9 R$_1$ = R$_3$ = R$_4$ = OMe   R$_2$ = OH

10 R$_1$ = R$_2$ = OMe   R$_3$ = H
11 R$_1$ = H   R$_2$ = OMe   R$_3$ = OH

methine proton. A pair of AB doublets at δ 6.98 and 7.04 (each 1H, d, *J* = 8.0 Hz) was assigned to H-9 and H-8 in the aromatic region. The NOESY spectrum of **2** showed correlations of H-8 with H-7 (δ 3.02) and H-9, H-9 with OMe-10 (δ 3.88), OMe-11 (δ 3.78) with −OCH$_2$O− (δ 5.94), and OMe-3 with H-4 (δ 2.97). According to the above data, **2** was determined to be 1,2-methylenedioxy-3,10,11-trimethoxyaporphine, which was confirmed by ^{13}C NMR, COSY, HMQC, and HMBC spectra. Compound **2** had been registered (CAS registry No. 14050-90-9), but no spectroscopic data were recorded.

Cassythic acid (**3**) had the molecular formula C$_{20}$H$_{19}$O$_6$N, as determined by ^{13}C NMR, DEPT, and HREIMS data. The IR spectrum showed OH (3389 cm^{-1}), carbonyl (1614 cm^{-1}), and aromatic ring (1482, 1423 cm^{-1}) absorptions. The UV spectrum of **2** in MeOH showed absorption maxima at 227, 277, and 310 (sh) nm characteristic of a 1,2,10,11-substituted aporphine.[7] The ^{1}H NMR spectrum showed the presence of two methoxy groups, a methylenedioxy signal, three sets of methylene protons, a methine proton, and a pair of AB doublets (Table 1). The ^{1}H and ^{13}C NMR spectra of **3** were very similar to those of **2**, except for the absence of a signal for a methoxy group at C-11 and the presence of a carboxylic acid moiety (δ_C 170.1). On comparison of ^{13}C NMR data of **3** with those of **2**, the chemical shift of C-11 was upfield (−3.8 ppm) and those of C-10 and C-8 were shifted upfield (−4.6 and −4.4 ppm, respectively), due to the carboxylic acid group at C-11. Furthermore, HMBC correlations were observed from the

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Table 1. ^{13}C and ^{1}H NMR Data (δ) of Compounds **1–3**

no.	1[a] ^{13}C	1[a] ^{1}H	2[b] ^{13}C	2[b] ^{1}H	3[b] ^{13}C	3[b] ^{1}H
1	157.6		148.0		146.4	
1a	104.9		109.8		109.5	
1b	123.7		126.1		125.4	
2	145.3		137.3		137.1	
3	139.1		141.5		141.2	
3a	137.0		117.1		117.4	
4	125.1	8.84 (1H, d, 6.0)	21.9	2.97 (2H, m)	22.0	2.93(2H, m)
5	134.8	8.72 (1H, d, 6.0)	42.3	3.28 (1H, m)/3.71(1H, m)	42.3	3.19 (1H, m)/3.68 (1H, m)
6a	140.1		54.7	4.14 (1H, d, 10.0)	54.7	4.05 (1H, m)
7	178.2		35.3	2.73 (1H, t, 13.0)/3.02 (1H, dd, 13.5, 4.0)	35.5	2.70 (1H, t, 13.5)/2.98 (1H, dd, 13.5, 4.0)
7a	124.4		127.3		127.3	
8	115.9	7.99 (1H, s)	123.8	7.04 (1H, d, 8.0)	119.4	6.77(1H, d, 8.5)
9	148.4		113.2	6.98 (1H, d, 8.0)	111.5	6.86(1H, d, 8.0)
10	158.8		154.2		149.6	
11	111.2	8.24 (1H, s)	148.4		144.6	
11a	133.0		125.4		118.8	
3-OMe	62.3	4.43 (3H, s)	60.1	4.10 (3H, s)	60.8	4.07 (3H, s)
10-OMe	58.1	4.16 (3H, s)	56.5	3.88 (3H, s)	56.6	3.88 (3H, s)
11-OMe			61.2	3.78 (3H, s)		
1,2-OCH$_2$O—	107.5	6.59 (2H, s)	102.2	5.94 (1H, s)/6.08 (1H, s)	102.2	5.94 (1H, s)/6.06 (1H, s)
COOH					170.1	

[a] Measured in CF$_3$COOH-d_1. [b] Measured in MeOH-d_4.

C-10 to OMe-10 (δ 3.88), H-9, and H-8, from C-8 to H-7, and from C-3 to OMe-3 (δ 4.07) and H-4, indirectly indicating attachment of the carboxylic acid group at C-11. The absolute configuration of C-6a was assumed to be *S* according to the optical rotation at $[\alpha]_D^{24}$ +105.[4] Therefore, cassythic acid was assigned structure **3**.

Compounds **10** and **11** were identified as the morphinandienone alkaloids (−)-*O*-methylflavinatine[8] and (−)-salutaridine,[9] respectively. Due to the small amount of compound available, the low-intensity signals of C-6 and C-14 of **11** could not be identified in ref 9. Our 1D and 2D NMR studies of **11** assigned the chemical shifts of C-6 and C-14 to signals at δ 151.9 and 162.5, respectively.

The other known compounds were characterized as aporphine alkaloids [cassythine (**4**),[10] *O*-methylcassythine (**5**),[11] cassythidine (**6**),[11] neolitsine (**7**),[12] dicentrine (**8**),[13] and norpredicentrine (**9**)[14]], flavonoids [isohamnetin,[15] isohamnetin-3-*O*-β-glucoside,[15] isohamnetin-3-*O*-rutinoside,[15] isohamnetin-3-*O*-robinobioside,[16] quercetin-3-*O*-β-galactoside,[17] quercetin-3-*O*-rutinoside,[15] quercetin-3-*O*-robinobioside,[16] and kaempferol-3-*O*-robinobioside[16]], phenylethanol glycosides [acteoside[18] and 3,4-dihydroxyphenylethyl alcohol 8-*O*-β-glucoside[19]], adenosine, nicotinic acid, and β-sitosterol glycoside.

In an attempt to evaluate their potential as vasorelaxing agents, 18 of the major compounds were tested for their potential as vasorelaxing agents in rat aortic preparations according to our previous report.[20] Compounds **3**, **4**, **7**, and **8** had the most potent vessel-relaxing activities, with mean IC$_{50}$ values of 2.48 ± 0.59, 0.09 ± 0.03, 0.29 ± 0.19, and 0.08 ± 0.03 μM, respectively. Compounds **1**, **2**, **10**, **11**, isohamnetin-3-*O*-β-glucoside, and isohamnetin-3-*O*-rutinoside exerted moderate vasorelaxing effects, with IC$_{50}$ values of 32.0 ± 18.6, 16.5 ± 3.0, 28.9 ± 4.1, 32.8 ± 9.8, 23.6 ± 6.0, and 16.8 ± 5.5 μM, respectively. Isohamnetin produced a slight vasorelaxation effect. None of the other compounds significantly affected the vascular tone of precontracted rat aortic rings, as they all had with IC$_{50}$ values higher than 100 μM. These results suggest that the aporphine alkaloids play a significant role in the vasorelaxing effect of *C. filiformis*.

Earlier chemical studies[3,4,11] almost always focused on the aporphine alkaloids. In this study, we found that flavonoids are also important constituents in the title plant and identified isohamnetin-3-*O*-robinobioside as a major component. Flavonoid diglycosides are common natural products, and rutinoside usually appears more frequently than robinobioside. Morphinandienone alkaloids and phenylethanol glycosides are minor substances in *C. filiformis*

and were first isolated from the title plant. Among the compounds tested in isolated rat aortas, the aporphine and morphinandienone alkaloids and some isohamnetin derivatives all possess significant vasorelaxing effects.

Experimental Section

General Experimental Procedures. Melting points were determined with a Yanagimoto micromelting point apparatus and are uncorrected. IR spectra were obtained as KBr pellets on a Nicolet Avatar 320 IR spectrometer. UV spectra were obtained on a Hitachi U-3200 spectrophotometer in MeOH. ^{1}H, ^{13}C, and 2D NMR spectra were measured with a Varian Inova-500 spectrometer with deuterated solvent as internal standard. EIMS, HREIMS, and APCIMS were recorded on a Finnigan MAT 95S and Finnigan LCQ spectrometer, respectively.

Plant Material. The whole plant of *Cassytha filiformis* was collected at Shan-Chih, Taipei, Taiwan, in July 2006. A voucher specimen (No.142655) has been deposited in the herbarium of the Department of Botany of the National Taiwan University.

Extraction and Isolation. Whole plants of *C. filiformis* (1.37 kg) were extracted with 95% EtOH (50 L × 3). The ethanolic extracts were combined and concentrated under vacuum to a volume of 1.5 L. A precipitate from the concentrated ethanolic extracts was removed, and the filtrate was concentrated to dryness (390 g). It was then dissolved in water and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH (each 1 L × 3). The *n*-BuOH extract (82 g) was subjected to silica gel column chromatography (4.8 × 60 cm) eluted with CHCl$_3$/MeOH/H$_2$O (7:2:0.2) and seven fractions (1–7) were collected. A solid precipitate was separated from fraction 3 and recrystallized from MeOH to give **4** (1.6 g). The filtrate of fraction 3 (9.8 g) was chromatographed on a Sephadex LH-20 column eluted with MeOH to remove pigments and further purified by Sephadex LH-20 (50% MeOH) and then silica gel (12–26 μM, performed at ~10 bar, 2% MeOH/CHCl$_3$) to give **1** (81 mg), **2** (34 mg), **5** (12 mg), **6** (20 mg), **7** (72 mg), and **8** (315 mg). Fraction 4 (10.2 g) was rechromatographed over silica gel (230–400 mesh, 2% MeOH/CHCl$_3$) and further purified by preparative TLC to give compounds **3** (4 mg), **9** (21 mg), **10** (53 mg), **11** (25 mg), nicotinic acid (4 mg), and β-sitosterol glycoside (6 mg). Repeated chromatography of fraction 5 (2.2 g) over Sephadex LH-20 (MeOH) yielded acteoside (112 mg) and 3,4-dihydroxyphenylethyl alcohol 8-*O*-β-glucoside (179 mg). Fraction 6 (27 g) was rechromatographed over Sephadex LH-20 eluted with MeOH and six subfractions were collected. A precipitate from fraction 6-5 (15 g) was recrystallized (MeOH) to give isohamnetin-3-*O*-robinobioside (9.2 g). The filtrate from fraction 6-5 was separated by semipreparative HPLC (column: Cosmosil C-18, 5 μM, 25 × 250 mm; mobile phase: MeOH/CH$_3$CN/1% HOAc, 35:5:60; flow rate: 15 mL/min, detector: UV 254 nm) to give isohamnetin-3-*O*-rutinoside (92 mg), isohamnetin-3-*O*-robinobioside

(138 mg), quercetin-3-*O*-rutinoside (4 mg), quercetin-3-*O*-robinobioside (5 mg), and kaempferol-3-*O*-robinobioside (5 mg). Fraction 10-6 (0.5 g) gave isohamnetin (34 mg), isohamnetin-3-*O*-β-glucoside (36 mg), quercetin-3-*O*-β-galactoside (3 mg), and adenosine (4 mg) after Sephadex LH-20 (MeOH) column chromatography.

Isofiliformine (1): red, amorphous powder (CHCl$_3$/MeOH); mp >300 °C; UV (MeOH) λ_{max} (log ϵ) 461 (2.96), 356 (3.16), 281 (3.73), 250 (3.67) nm; (+NaOH) 536 (3.04), 389 (3.32), 294 (4.03), 257 (3.90) nm; IR (KBr) λ_{max} 3374 (OH), 1653 (C=O), 1558, 1539 (C=C) cm^{-1}; ^{1}H NMR (CF$_3$COOD, 500 MHz) and ^{13}C NMR (CF$_3$COOD, 125 MHz) see Table 1; EIMS *m/z* (rel int) 351 [M]$^+$ (100), 336 (57); HREIMS *m/z* 351.0743 [M]$^+$ (calcd 351.0744 for C$_{19}$H$_{13}$O$_6$N).

Cassythic acid (3): brown syrup; [α]$_D^{24}$ 105 (MeOH, *c* 1.0); UV (MeOH) λ_{max} (log ϵ) 310 (sh. 3.63), 277 (4.06), 227 (4.31) nm; IR (KBr) λ_{max} 3389 (OH), 1614 (C=O), 1482, 1423 (C=C) cm^{-1}; ^{1}H NMR (CD$_3$OD, 500 MHz) and ^{13}C NMR (CD$_3$OD, 125 MHz) see Table 1; EIMS *m/z* (rel int) 369 [M]$^+$ (12), 341 (100); HREIMS *m/z* 369.1214 [M]$^+$ (calcd 369.1213 for C$_{20}$H$_{19}$O$_6$N).

Biological Studies. Adult male Sprague–Dawley rats, weighing 280–320 g (National Laboratory Animal Center, Taipei, Taiwan), were tested. The rats were allowed to become accustomed to environmentally controlled quarters with a constant temperature of 20–22 °C, relative humidity 55%, and light with 12:12 h light−dark cycles. Standard laboratory fodder (Purina Mills, Richmond, IN) and drinking water were provided *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committees of National Research Institute of Chinese Medicine and were conducted in accordance with the National Institutes of Health Animal Care standards.

Vascular Tension Experiments. The methods were similar to those published previously.[21] Briefly, aortic rings from male Sprague–Dawley rats were fixed in organ chambers isometrically under passive tension of 1.8 g for 60 min. The functional integrity of endothelium was confirmed by observation of more than 98% relaxation in response to acetylcholine (3 μM) in tissues precontracted with phenylephrine (0.3 μM). For the evaluation of vasorelaxation, one of the isolated compounds (0.01–100 μM) was added in a cumulative manner during the tonic phase of contraction (considered as 100%) induced by phenylephrine (0.3 μM). The samples were tested for their vasorelaxation effects on rat aortic rings with the endothelium to determine their potency. Construction of concentration–response curves was based on the percent of relaxation of the phenylephrine-induced contraction. Complete relaxation was considered attained when the precontracted rings returned to the baseline position. In each preparation only one concentration–response curve was examined.

Statistical Aanalysis. For each experiment, data are given as mean $\pm$ SE and *n* represents the number of independently performed experiments. All data were analyzed by an IBM-compatible statistical software package (SPSS for Windows, Ver. 10.0). The significance of the concentrations and sample treatments was determined by two-way analysis of variance (ANOVA) with repeated measures. If there were significant interactions, the simple main effect of each factor was assessed using the Student–Newman–Keuls test. A *P* value less than 0.05 was considered to indicate a statistically significant difference.

Supporting Information Available: 1D and 2D NMR data of compounds **1** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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