ORGANIC LETTERS

2011
Vol. 13, No. 10
2516–2519

Structure and Cytotoxicity of Phidianidines A and B: First Finding of 1,2,4-Oxadiazole System in a Marine Natural Product

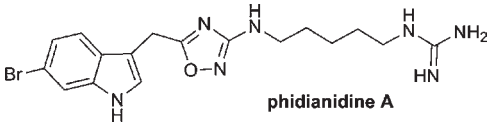
Marianna Carbone,[†] Yan Li,[‡] Carlo Irace,[§] Ernesto Mollo,[†] Francesco Castelluccio,[†] Antonio Di Pascale,[§] Guido Cimino,[†] Rita Santamaria,[§] Yue-Wei Guo,*[,‡] and Margherita Gavagnin*[,†]

Consiglio Nazionale delle Ricerche (CNR), Istituto di Chimica Biomolecolare (ICB), Via Campi Flegrei, 34, 80078 Pozzuoli (Na), Italy, Dipartimento di Farmacologia Sperimentale, Facoltà di Farmacia, Università 'Federico II' di Napoli, Italy, and State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, PR China

yw_guo58@yahoo.com; mgavagnin@icb.cnr.it

Received January 25, 2011

ABSTRACT



phidianidine A

Two indole alkaloids, phidianidines A (1) and B (2), exhibiting an uncommon 1,2,4-oxadiazole ring linked to the indole system, have been isolated from the marine opisthobranch mollusk *Phidiana militaris*. The structures of the two metabolites have been elucidated by spectroscopic techniques. Phidianidines exhibit high cytotoxicity against tumor and nontumor mammalian cell lines in *in vitro* assays.

Marine organisms represent a unique and vast resource for the discovery of bioactive molecules.[1] In particular, many marine alkaloids have generated interest for their various and often striking pharmacological activities as well as for challenging problems in structure elucidation and synthesis.[1]A large class of marine alkaloids is related to the metabolism of tryptophan and displays differently substituted indole rings.[2−6] The substituent is commonly an additional heterocyclic ring: e.g. imidazole (topsentin),[7] piperazine (dragmacidin),[8] pyrimidine (meridianin),[9] and oxazole (martefragin).[10]

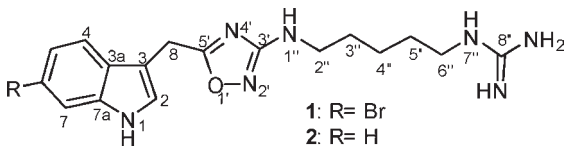
During our continuing search for new bioactive compounds from marine organisms,[11,12] we have isolated two unprecedented 3-substituted indole alkaloids, phidianidines A (**1**) and B (**2**), from the aeolid opisthobranch *Phidiana militaris* (Alder & Hancock, 1864). This shell-less mollusk, belonging to the family Glaucidae, has never

[†] Consiglio Nazionale delle Ricerche (CNR).
[‡] Chinese Academy of Sciences.
[§] Università `Federico II' di Napoli.

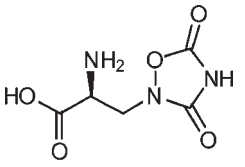
(1) Blunt, J. W.; Copp, B. R.; Munro, M. H. G.; Northcote, P. T.; Prinsep, M. R. *Nat. Prod. Rep.* **2010**, *27*, 165–237 and previous issues in this series.
(2) Somei, M.; Yamanda, F. *Nat. Prod. Rep.* **2005**, *22*, 73–103 and previous issues in this series.
(3) Aygün, A.; Pindur, U. *Curr. Med. Chem.* **2003**, *10*, 1113–1127.
(4) Kuramoto, M.; Arimoto, H.; Uemura, D. *Mar. Drugs* **2004**, *2*, 39–54.
(5) Gul, W.; Hamann, M. T. *Life Science* **2005**, *78*, 442–453.
(6) Kochanowska-Karamyan, A. J.; Hamann, M. T. *Chem. Rev.* **2010**, *110*, 4489–4497.
(7) Bartik, K.; Braekman, J.-C.; Daloze, D.; Stoller, C.; Huysecom, J.; Vandevyver, G.; Ottinger, R. *Can. J. Chem.* **1987**, *65*, 2118–2121.
(8) Kohmoto, S.; Kashman, Y.; McConnell, O. J.; Rinehart, K. L.; Wright, A.; Koehn, F. *J. Org. Chem.* **1988**, *53*, 3116–3118.
(9) Franco, L. H.; Joffè, E. B. K.; Puricelli, L.; Tatian, M.; Seldes, A. M.; Palermo, J. A. *J. Nat. Prod.* **1998**, *61*, 1130–1132.
(10) Takahashi, S.; Matsunaga, T.; Hasegawa, C.; Saito, H.; Frujita, D.; Kiuchi, F.; Tsuda, Y. *Chem. Pharm. Bull.* **1998**, *46*, 1527–1529.
(11) Li, Y.; Carbone, M.; Vitale, R. M.; Amodeo, P.; Castelluccio, F.; Sicilia, G.; Mollo, E.; Nappo, M.; Cimino, G.; Guo, Y.-W.; Gavagnin, M. *J. Nat. Prod.* **2010**, *73*, 133–138.
(12) Carbone, M.; Irace, C.; Costagliola, F.; Castelluccio, F.; Villani, G.; Calado, G.; Padula, V.; Cimino, G.; Cervera, J. L.; Santamaria, R.; Gavagnin, M. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2668–2670.

10.1021/ol200234r
Published on Web 04/20/2011

been studied to date. The structures of phidianidines are characterized by the presence of a 1,2,4-oxadiazole ring linking the indole system through a methylene bridge and displaying an aminoalkylguanidine group at C-3′.



To the best of our knowledge, the only known natural product possessing a ring related to the 1,2,4-oxadiazole core is quisqualic acid (**3**), which was first reported from the seeds of *Quisqualis indica* and *Q. fructus*.[13,14] This metabolite is a strong agonist for both AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and group I metabotropic glutamate receptors.[15]



quisqualic acid (**3**)

Herein we report the isolation and the structural characterization of compounds **1** and **2** as well as the evaluation of their *in vitro* cytotoxicity on various tumor and non-tumor mammalian cells.

Twelve individuals of *P. militaris* were collected by scuba diving along the coast of Hainan island (South China Sea) during February 2006 and frozen at −20 °C. Subsequently, the animals were extracted with acetone. The butanolic soluble portion of the acetone extract was fractionated by Sephadex LH20 chromatography, followed by reversed-phase silica HPLC (0.1% TFA, MeOH/H_2O) to afford phidianidines A (**1**, 12.5 mg, 11%) and B (**2**, 7.8 mg, 7%), isolated as their protonated forms.

Phidianidine A[16] (**1**) was characterized first. The molecular formula $C_{17}H_{22}BrN_7O$, which was deduced by analysis of both HRESIMS and carbon data, implied ten degrees of unsaturation. In the HRESIMS spectrum, the molecular ion $[M + H]^+$ was observed as a 1:1 ion cluster at *m*/*z* 420.1144/422.1105, indicating the presence of a bromine. Consistently with the molecular formula, the ^{13}C NMR spectrum showed seventeen signals which were assigned to six CH_2, four CH, and seven quaternary carbons by DEPT sequence (Table 1).

Table 1. NMR Data[a,b] of Phidianidine A (**1**)[c]

	δ_C	m[d]	δ_H, mult (*J* in Hz)	significant HMBC correlations
1	-	-	11.15, bs	
2	125.0	*CH*	7.35, bs	NH-1; H_2-8
3	106.8	*C*	-	NH-1; H-2; H_2-8
3a	125.2	*C*	-	H-5; H-7; H_2-8
4	120.0	*CH*	7.46, d (8)	
5	121.3	*CH*	7.13, dd (8 and 1)	
6	113.5	*C*	-	H-7
7	114.1	*CH*	7.56, d (1)	H-4; H-2
7a	137.0	*C*	-	H-4; H-7
8	22.5	CH_2	4.20, s	
3′	168.5	*C*	-	H_2-2″
5′	176.7	*C*	-	H_2-8
1″	-	-	6.72	
2″	40.5	CH_2	2.99, m	
3″	28.1	CH_2	1.36−1.56, m	
4″	23.4	CH_2	1.28, m	
5″	28.1	CH_2	1.36−1.56, m	
6″	41.9	CH_2	3.02, m	
7″	-	-	7.41	
8″	156.6	*C*	-	H_2-6″

[a] The spectra were recorded in d_6-DMSO at 300 and 400 MHz. [b] Assignments made by $^1H-^1H$ COSY, HSQC, and HMBC (*J* = 10 and 6 Hz) experiments. [c] Protonated form. [d] By DEPT sequence.

The ^1H NMR spectrum of phidianidine A was characterized by a set of aromatic signals, including a pair of ortho-coupled protons at δ 7.46 (H-4, d, *J* = 8 Hz) and 7.13 (H-5, dd, *J* = 8 and 1 Hz), a proton at δ 7.56 (H-7, d, *J* = 1 Hz) displaying a weak meta coupling to H-5, and a broad singlet at δ 7.35 (H-2) coupled with an exchangeable proton at δ 11.15 (1-NH), that were assigned to a disubstituted indole. The presence of an indole moiety, accounting for six unsaturation degrees, was supported by the typical UV absorption at 225 nm (log ε = 4.37). The proton chemical shift pattern of ring A of the indole system was consistent with the placement of the bromine atom at C-6 (δ 113.5).[17] This assignment was supported by the diagnostic HMBC correlation observed between C-3a and H-5 (Table 1). A significant HMBC correlation between C-2 (δ 125.0) and the 2H singlet at δ 4.20 (H_2-8) led us to place the isolated methylene C-8 at C-3 thus defining the substructure **a** (Figure 1).

Two additional exchangeable protons and multiplets assigned to five methylene groups completed the ^1H NMR spectrum of compound **1** (Table 1). Analysis of the $^1H-^1H$ COSY experiment showed that they formed an isolated spin system. The protons at both the ends of this chain at δ 2.99 (H_2-2″) and δ 3.02 (H_2-6″), coupled with -NH protons at δ 6.72 (1″-NH) and δ 7.41 (7″-NH), respectively, were correlated in the HSQC spectrum with nitrogen-bearing carbons (δ 40.5, C-2″; δ 41.9, C-6″). These data were consistent with a 1,5-diamino alkyl residue (substructure **b**, Figure 1).

(13) Takemoto, T.; Takagi, N.; Nakajima, T.; Koike, K. *Yakuffaku Zasshi* **1975**, *95*, 176–179.

(14) Flippen, J. L.; Gilardi, R. D. *Acta Crystallogr.* **1976**, *B32*, 951–953.

(15) Jin, R.; Horning, M.; Mayer, M. L.; Gouaux, E. *Biochemistry* **2002**, *41*, 15635–15643.

(16) Phidianidine A (**1**): IR (liquid film): 3199, 2937, 2875, 1687, 1590, 1200, 1142 cm^{-1}; UV (MeOH) (log ε): 225 (4.37); ^1H and ^{13}C NMR values in CD_3OD are reported in the Supporting Information; ESIMS *m*/*z* (rel. intensity) 420/421 (1:1) $(M + H)^+$; HR ESIMS calcd for $C_{17}H_{23}{}^{79}BrN_7O$: 420.1147. Found 420.1144. Phidianidine B (**2**): IR (liquid film): 3191, 2937, 2860, 1675, 1602, 1208, 1135 cm^{-1}; UV (MeOH) (log ε): 225 (4.12); ^1H and ^{13}C NMR values in CD_3OD are reported in the Supporting Information; ESIMS *m*/*z* 342 $(M + H)^+$; HR ESIMS calcd for $C_{17}H_{24}N_7O$: 342.2042. Found 342.2059.

(17) Swersey, J.; Ireland, C. M. *J. Nat. Prod.* **1994**, *57*, 842–845.

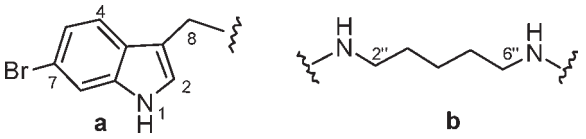


Figure 1. Partial structures **a** and **b**.

Having defined the two substructures **a** and **b**, the molecular formula and the spectroscopic data of **1** required that the remaining parts of the structure had to include 3 sp^2 carbons, 4 nitrogen, and 1 oxygen atoms. Analysis of HMBC spectra aided us in connecting **a** and **b** and constructing the structure framework. First, diagnostic correlations were observed between terminal methylenes H_2-2″ and H_2-6″ in moiety **b** with two sp^2 carbon resonances at δ 168.5 (C-3′) and 156.6 (C-8″), respectively. The close analogy of NMR values of the C-2″/C-8″ fragment with those reported for eusynstyelamide[17] suggested to locate a guanidino group at one of the extremities of the aminoalkyl chain, consistent with the assignment of C-8″ as the aliphatic carbon of this terminal group.[17] Second, the methylene H_2-8 in substructure **a** showed a diagnostic HMBC correlation with the third sp^2 carbon at δ 176.7 (C-5′). The carbon value of C-8 (δ 22.5) excluded the connection of this methylene with an heteroatom thus implying a direct linkage to C-5′. At this point, it remained to connect C-5′ to C-3′ and to place an oxygen and two nitrogen atoms in a structural arrangement accounting for three unsaturations. The presence of an oxadiazole core including C-3′ and C-5′ was thus suggested. According to their carbon values, it was assumed that (a) C-3′ and C-5′ were not directly connected and (b) C-5′ was necessarily linked to oxygen. Consequently, alternative possible structures 1,2,4- (**i**) and 1,3,4-oxadiazole (**ii**) were considered (Figure 2).

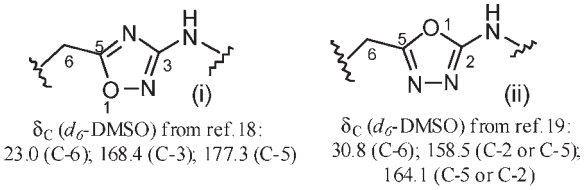


Figure 2. Alternative possible oxadiazole rings.

Comparison of the carbon value pattern of the oxadiazole core in **1** with the corresponding carbon resonances in different synthetic literature models[18,19] (Figure 2) enabled us to set the 1,2,4-oxadiazole arrangement (**i**) for structure **1**. NMR assignments of phidianidine A were made by analysis of 1D and 2D experiments, as reported in Table 1.

(18) Chen, C.; Senanayake, C. H.; Bill, T. J.; Larsen, R. D.; Verhoeven, T. R; Reider, P. J. *J. Org. Chem.* **1994**, *59*, 3738–3741.

Phidianidine B (**2**)[16] was determined as a debromo derivative of phidianidine A (**1**). The HRESIMS ion peak at m/z 342.2042 $[M + H]^+$ indicated a molecular formula of $C_{17}H_{23}N_7O$ with 78 mass units less with respect to compound **1** according to the absence of the bromine atom. The ^{1}H NMR spectrum of phidianidine B (**2**) was substantially similar to that of phidianidine A (**1**) with regard to the amino pentyl guanidine moiety. The only differences were observed in the aromatic region displaying an additional signal with respect to **1**. Four aromatic signals were coupled forming an isolated spin system, clearly indicating that ring A of the indole moiety was nonsubstituted. Accordingly, in the ^{13}C NMR spectrum of phidianidine B (**2**), the most remarkable difference was observed for the C-6 value, which shifted from δ 113.5 in compound **1** to δ 120.2 in compound **2**. The complete proton and carbon resonance assignment was achieved by analysis of 1D and 2D NMR experiments (Table 2).

Table 2. NMR Data[a,b] of Phidianidine B (**2**)[c]

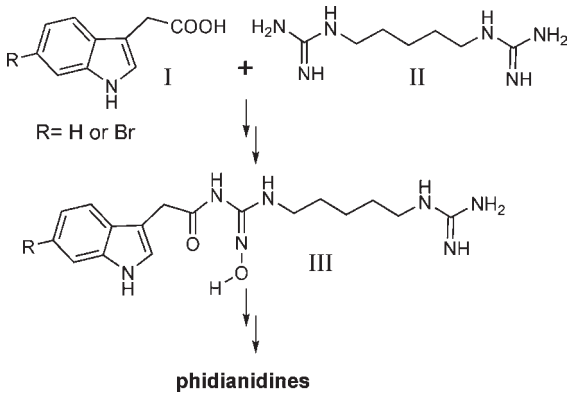
	δ_C	m[d]	δ_H, mult (J in Hz)	significant HMBC correlations
1	-	-	11.00, bs	
2	125.3	*CH*	7.31, bs	NH-1; H_2-8
3	106.9	*C*	-	NH-1; H-2; H_2-8
3a	126.7	*C*	-	H-2; H-5; H-7; H_2-8
4	118.3	*CH*	7.50, d (8)	H-6
5	118.6	*CH*	6.99. app. t (8)	H-7
6	120.2	*CH*	7.09, app. t (8)	H-4
7	111.5	*CH*	7.36, d (8)	H-5
7a	136.1	*C*	-	H-2; H-4; H-6
8	22.7	CH_2	4.20, s	
3′	168.5	*C*	-	H_2-2″
5′	176.9	*C*	-	H_2-8
1″	-	-	6.71, t (5)	
2″	40.7	CH_2	3.00, m	
3″	28.1	CH_2	1.36–1.56, m	
4″	23.4	CH_2	1.28, m	
5″	28.1	CH_2	1.36–1.56, m	
6″	42.3	CH_2	3.05, m	
7″	-	-	7.43, m	
8″	156.6	*C*	-	H_2-6″

[a] The spectra were recorded in d_6-DMSO at 300 and 400 MHz. [b] Assignments made by ^{1}H–^{1}H COSY, HSQC, and HMBC (J = 10 and 6 Hz) experiments. [c] Protonated form. [d] By DEPT sequence.

Phidianidine-free bases were obtained by filtration on Sephadex LH20 using 0.2% triethylamine in MeOH as eluent. The ^{1}H NMR spectra were substantially similar to those of the corresponding protonated forms except for the value of 7″-NH (see Supporting Information).

A plausible biogenetic pathway leading to phidianidines should involve the coupling of an indole acetic acid precursor I with a suitable alkyl bis-guanidine II to give an amido derivative. Subsequent oxidation of the guanidino residue could lead to a hydroxyl derivative III, which could favor an intramolecular addition to the adjacent amide carbonyl followed by dehydration to form the 1,2,4-oxadiazole ring (Scheme 1).

Scheme 1. Hypothetical Biogenetic Pathway



Even though the 1,2,4-oxadiazole is extremely rare in nature, there is a wide interest in the synthesis of compounds containing this scaffold. It has been extensively used in the design of potent, metabolically stable, and bioavailable compounds in many research programs, being a hydrolysis-resisting bioisosteric alternative for the ester moiety.[20−22] The broad spectrum of biological properties possessed by 1,2,4-oxadiazole-containing compounds includes tyrosine kinase inhibition,[23] muscarinic agonism,[24] histamine H3 antagonism,[25] anti-inflammation activity,[26] monoamine oxidase inhibition,[27] and anticancer activity.[28,29]

The cytotoxicity of phidianidines A (**1**) and B (**2**) has been investigated on a panel of tumor (C6 rat glioma cells, HeLa human epithelial cervical cancer cells and CaCo-2 human epithelial colorectal adenocarcinoma cells) and nontumor (H9c2 rat embryonic cardiac myoblasts and 3T3-L1 murine embryonic fibroblasts) cell lines by evaluation of cell growth and viability. The results are summarized in Table 3 and clearly show that both compounds are highly cytotoxic and exhibit specificity toward some cell types relative to others. In particular, they are strongly active against both tumor (C6 and HeLa) and nontumor (fibroblasts) cells, as indicated by the IC_{50} values within the nanomolar range. This might suggest the existence of specific interactions with biological targets that are present only in the sensitive cell lines. However, like other antiproliferative drugs, phidianidines show activity against both highly proliferating tumor cells and embryonic cells such as fibroblasts and myoblasts. This is consistent with the partial lack of activity against CaCo-2 cells because of their reduced replicative potential after confluence and the beginning of differentiation. Further SAR studies will be planned in order to clarify these aspects.

Table 3. Cytotoxicity Profile of Compounds **1** and **2** against Tumor and Nontumor Cell Lines, IC_{50} (μM)[a]

cell line	phidianidine A (**1**)	phidianidine B (**2**)
C6	**0.642 ± 0.2**	**0.98 ± 0.3**
HeLa	1.52 ± 0.3	**0.417 ± 0.4**
CaCo-2	35.5 ± 4	100.2 ± 8.5
3T3-L1	**0.14 ± 0.2**	**0.786 ± 0.3**
H9c2	2.26 ± 0.6	5.42 ± 0.8

[a] IC_{50} values are expressed as mean ± SEM (n = 24) of three independent experiments. Bold values show IC_{50} of less than 1 μM.

Acknowledgment. The NMR spectra were recorded at the ICB NMR Facility, the staff of which is gratefully acknowledged. Particular thanks are due to Mr. V. Mirra for performing 2D NMR experiments. This research work was financially supported by a national S & T major project (2009ZX09301-001), a national marine 863 project (2007AA09Z447 and 2007AA09Z402), NSFC grants (Nos. 40976048, 30730108, 20721003, and 20772136), CAS key projects (grant KSX2-YW-R-18 and SIMM0907KF-09), and PRIN-MIUR 2007 Project "Antitumor natural products and synthetic analogues". The authors are deeply grateful to the anonymous referees for their precious suggestions.

Supporting Information Available. Full experimental procedures, 1D and 2D NMR spectra of phidianidines A (**1**) and B (**2**). This material is avaiable free of charge via Internet at http://pubs.acs.org.

(19) Levins, C. G.; Wan, Z.-K. *Org. Lett.* **2008**, *10*, 1755–1758.
(20) Diana, G. D.; Volkots, D. L.; Nitz, T. J.; Bailey, T. R.; Long, M. A.; Vescio, N.; Aldous, S.; Peveur, D. C.; Dutko, F. J. *J. Med. Chem.* **1994**, *37*, 2421–2436.
(21) Andersen, K. E.; Jørgensen, A. S.; Braestrup, C. *Eur. J. Med. Chem.* **1994**, *29*, 393–399.
(22) Andersen, K. E.; Lundt, B. F.; Jørgensen, A. S.; Braestrup, C. *Eur. J. Med. Chem.* **1996**, *31*, 417–425.
(23) Vu, C. B.; Corpuz, E. G.; Merry, T. J.; Pradeepan, S. G.; Bartlett, C.; Bohacek, R. S.; Botfield, M. C.; Eyermann, C. J.; Lynch, B. A.; MacNeil, I. A.; Ram, M. K.; Van Schravendijk, M. R.; Violette, S.; Sawyer, T. K. *J. Med. Chem.* **1999**, *42*, 4088–4098.
(24) Orlek, B. S.; Blaney, F. E.; Brown, F.; Clark, M. S.; Hadley, M. S.; Hatcher, J.; Riley, G. J.; Rosenberg, H. E.; Wadsworth, H. J.; Wyman, P. *J. Med. Chem.* **1991**, *34*, 2726–2735.
(25) Clitherow, J. W.; Beswick, P.; Irving, W. J.; Scopes, D. I. C.; Barnes, J. C.; Clapham, J.; Brown, J. D.; Evans, D. J.; Hayes, A. G. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 833–838.
(26) Nicolaides, D. N.; Fylaktakidou, K. C.; Litinas, K. E.; Hadjipavlou-Litina, D. *Eur. J. Med. Chem.* **1998**, *33*, 715–724.
(27) Chimirri, A.; Grasso, S.; Montforte, A.-M.; Rao, A.; Zappala, M. *Farmaco* **1996**, *51*, 125–129.
(28) Zhang, H. Z.; Kasibhatla, S.; Kuemmerle, J.; Kemnitzer, W.; Ollis-Mason, K.; Qiu, L.; Crogan-Grundy, C.; Tseng, B.; Drewe, J.; Cai, S. X. *J. Med. Chem.* **2005**, *48*, 5215–5223.
(29) Kumar, D.; Patel, G.; Johnson, E. O.; Shah, K. *Bioorg. Chem. Lett.* **2009**, *19*, 2739–2741.