ภาคผนวก

ผลงานตีพิมพ์ที่เป็นผลผลิตของโครงการวิจัยนี้ รวมถึงผลงานที่ได้รับการตอบรับและอยู่ระหว่างการจัดพิมพ์โดยสำนักพิมพ์ (ยกเว้นรายงานที่อยู่ระหว่างการพิจารณา ซึ่งยังไม่สามารถนำมาเผยแพร่ได้ ณ ขณะนี้ ทั้งนี้ จะได้แจ้งต่อ สกว. ต่อไป เมื่อได้รับการตอบรับ และผลงานได้รับการตีพิมพ์เรียบร้อยแล้ว)

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4-Methyl-3*H*-pyrrolo[2,3-c]quinoline

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Key indicators

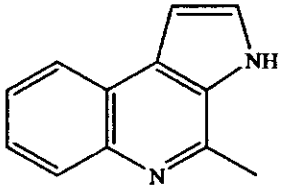
Single-crystal X-ray study
T = 100 K
Mean σ(C–C) = 0.003 Å
R factor = 0.037
wR factor = 0.099
Data-to-parameter ratio = 7.7

For details of how these key indicators were automatically derived from the article, see http://journals.iucr.org/e.

The title compound, $C_{12}H_{10}N_2$, is a new marine natural product which was isolated for the first time from a novel marine gliding bacterium. The asymmetric unit contains a pair of achiral molecules. Both the molecules are essentially planar and they form a dihedral angle of 83.81 (3)°. In the crystal structure, the molecules exist as N—H···N hydrogen-bonded tetramers.

Comment

During the course of screening for biologically active substances and new secondary metabolites from marine natural products, the title compound, (I), which we named as Marinoquinoline A, was isolated for the first time from a novel marine gliding bacterium GB009, obtained from seaweed collected from Yong Ling beach, Trang province, on the southern coast of Thailand. The 16 s rRNA sequence of this gliding bacterium suggested that this gliding bacterium should be classified in a new genus because the similarity of the nucleotide sequence with the closest match (*Flexibacter aggregran*) was less than 92%. The complete identification of this bacterium is currently under investigation. The biological activity of (I) will be published elsewhere. We report here the crystal structure of (I). This is also the first report of the X-ray crystal structure of a marine natural product obtained from this bacterium.



(I)

There are two molecules of (I) (*A* and *B*) in the asymmetric unit of a non-centrosymmetric space group (Fig. 1). The corresponding bond distances and angles in the two molecules agree with each other (Table 1) and show normal values (Allen *et al.*, 1987). Both the independent molecules are essentially planar with the maximum deviation of 0.023 (2) Å for atom N1*A* in molecule *A* and 0.027 (2) Å for atom C2*B* in molecule *B*. The dihedral angle between the least-squares planes of molecules *A* and *B* is 83.81 (3)°.

In the crystal structure, pairs of molecules are linked *via* intermolecular N—H···N hydrogen bonds (Table 2) to form a tetramer (Fig. 2). These tetramers are stacked along the *c* axis. In addition, the molecular packing is stabilized by C—H···π interactions.

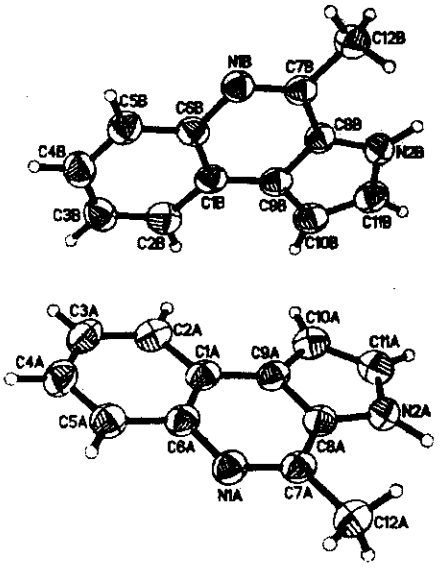


Figure 1
The asymmetric unit of (I), showing 80% probability displacement ellipsoids and the atomic numbering.

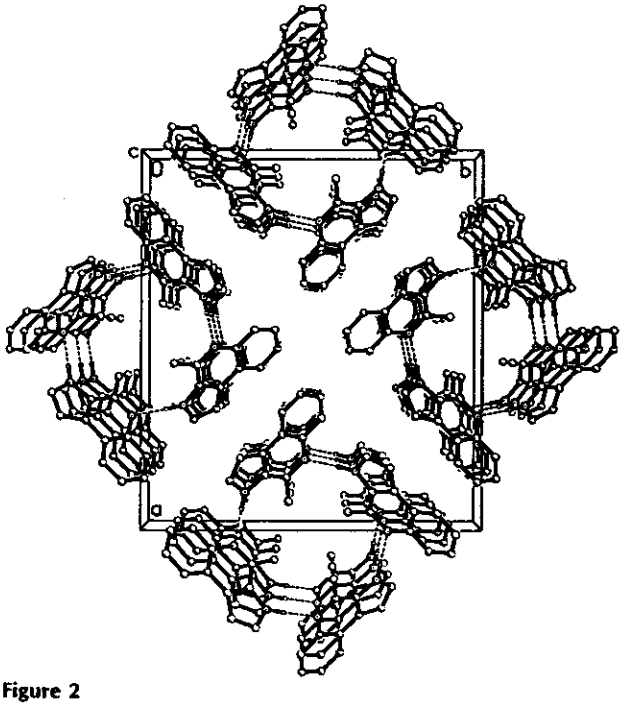


Figure 2
The crystal packing of (I), viewed down the *c* axis. Hydrogen bonds are shown as dashed lines. The C-bound H atoms have been omitted for clarity.

Experimental

A marine gliding bacterium GB009 was cultivated in 40 flasks of 100 ml of SK liquid medium containing natural seawater and 0.2% of XAD-16 amberlite resins for 7 d. The resins were removed from the culture broth and rinsed twice with deionized water before extracting with MeOH (4 l). The crude extract (1.07 g) obtained after evaporation of solvent was subjected to column chromatography over Sephadex LH-20 and silica gel to afford 15 mg of compound (I). Colourless needle-shaped single crystals of (I) were obtained by recrystallization from an acetone–chloroform–hexane (1:1.5:1.5) mixture after several days.

Crystal data

$C_{12}H_{10}N_2$
M_r = 182.22
Orthorhombic, $P2_12_12$
a = 20.2212 (5) Å
b = 18.0889 (4) Å
c = 5.1003 (1) Å
V = 1865.58 (7) Å^3
Z = 8
D_x = 1.298 Mg m^{-3}
Mo $K\alpha$ radiation
μ = 0.08 mm^{-1}
T = 100.0 (1) K
Needle, colourless
0.52 × 0.13 × 0.09 mm

Data collection

Bruker SMART APEX2 CCD area-detector diffractometer
ω scans
Absorption correction: multi-scan (*SADABS*; Bruker, 2005)
T_{min} = 0.988, T_{max} = 0.993
19204 measured reflections
2037 independent reflections
1847 reflections with $I > 2\sigma(I)$
R_{int} = 0.038
θ_{max} = 25.5°

Refinement

Refinement on F^2
$R[F^2 > 2\sigma(F^2)]$ = 0.037
$wR(F^2)$ = 0.099
S = 1.07
2037 reflections
263 parameters
H atoms treated by a mixture of independent and constrained refinement
$w = 1/[\sigma^2(F_o^2) + (0.0569P)^2 + 0.4629P]$ where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max}$ = 0.001
$\Delta\rho_{max}$ = 0.16 e Å^{-3}
$\Delta\rho_{min}$ = −0.22 e Å^{-3}

Table 1
Selected geometric parameters (Å, °).

N1*A*—C7*A*	1.325 (3)	N1*B*—C7*B*	1.324 (3)
N1*A*—C6*A*	1.384 (3)	N1*B*—C6*B*	1.392 (3)
N2*A*—C11*A*	1.367 (3)	N2*B*—C11*B*	1.364 (3)
N2*A*—C8*A*	1.377 (3)	N2*B*—C8*B*	1.377 (3)
C10*A*—C11*A*	1.369 (3)	C10*B*—C11*B*	1.372 (3)
C7*A*—N1*A*—C6*A*	119.72 (19)	C7*B*—N1*B*—C6*B*	119.44 (19)
C11*A*—N2*A*—C8*A*	107.68 (19)	C11*B*—N2*B*—C8*B*	107.93 (19)
N1*A*—C7*A*—C12*A*	118.4 (2)	N1*B*—C7*B*—C12*B*	118.7 (2)
C6*A*—N1*A*—C7*A*—C12*A*	178.10 (19)	C6*B*—N1*B*—C7*B*—C12*B*	−177.90 (19)
C12*A*—C7*A*—C8*A*—N2*A*	2.5 (4)	C12*B*—C7*B*—C8*B*—N2*B*	−2.4 (4)

Table 2
Hydrogen-bond geometry (Å, °).

D—H···*A*	*D*—H	H···*A*	*D*···*A*	*D*—H···*A*
N2*A*—H1*NA*···N1*B*^{i}	0.97 (3)	1.91 (3)	2.878 (3)	178 (3)
N2*B*—H1*NB*···N1*A*^{ii}	0.93 (3)	1.95 (3)	2.875 (3)	173 (3)
C12*A*—H12*B*···*Cg*1^{iii}	0.96	2.66	3.434 (3)	138
C12*B*—H12*F*···*Cg*2^{iii}	0.96	2.64	3.459 (3)	143

Symmetry codes: (i) $-x+\frac{1}{2}, y+\frac{1}{2}, -z$; (ii) $x+\frac{1}{2}, -y+\frac{1}{2}, -z$; (iii) $x, y, z-1$. *Cg*1 and *Cg*2 denote the centroids of the C8*A*–C11*A*/N2*A* and C8*B*–C11*B*/N2*B* rings, respectively.

H atoms attached to N2*A* and N2*B* were located in a difference map and isotropically refined. The remaining H atoms were placed in

calculated positions, with C—H distances of 0.93–0.96 Å. The U_{iso}(H) values were constrained to be $1.5U_{eq}$ of the carrier atom for the methyl H atoms and $1.2U_{eq}$ for the remaining H atoms. In the absence of significant anomalous dispersion effects, 396 Friedel pairs were merged before the final refinement. The molecule is achiral.

Data collection: *APEX2* (Bruker, 2005); cell refinement: *APEX2*; data reduction: *SAINT* (Bruker, 2005); program(s) used to solve structure: *SHELXTL* (Sheldrick, 1998); program(s) used to refine structure: *SHELXTL*; molecular graphics: *SHELXTL*; software used to prepare material for publication: *SHELXTL* and *PLATON* (Spek, 2003).

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ว. Langjae, R.; Bussarawit, S.; Yuenyongsawad, S.; Ingkaninan, K.; Plubrukarn, A. Acetylcholinesterase-Inhibiting Steroidal Alkaloid from the Sponge *Corticium* sp. Steroids. (2007) 72, in press (accepted manuscript).; Journal impact factor (2005) 2.416

Abstract

A new stigmastane-type steroidal alkaloid, 4-acetoxy-plakinamine B (**1**), was isolated from the Thai sponge *Corticium* sp. The compound was subjected to the acetylcholinesterase inhibitory activity determination to reveal a high inhibitory activity (IC_{50} 3.75±1.69 μM). The kinetics of enzyme inhibition showed a decrease in V_{max}, whereas K_m was increased, thus suggesting an unusual mixed-competitive mode of inhibition. Compound **1** is the first steroidal alkaloid bearing a stigmastane skeleton ever been reported to exhibited such good potency in the acetylcholinesterase inhibition bioassay.

1. Introduction

Using acetylchlinesterase (AChE) inhibitors is among the best accepted approaches towards the treatment of Alzheimer's disease (AD). The concept of such remedy is based on the "cholinergic hypothesis," stating the deficit of cholinergic neurotransmitters, particularly in cholinergic neurons in the basal forebrains, as a neurochemical characteristic in AD patients. Retaining the neurotransmitters especially at the synaptic terminals via the inhibition of the hydrolytic enzymes, i.e., the cholinesterases, in order to compensate the deficiency of in the cholinergic neurotransmitters would lead to the improved cognitive activities of the patients. To date, only 4 AChE inhibitors, donepezil, metrifonate, rivastigmine, and galantamine, are available clinically. Despite being claimed as a mere symptom intervention, clinical evidences however indicated that the positive effects of AChE inhibitors could be maintained for at least 1 year, and in certain studies, beneficial effects had been observed for up to 36 months [1-3].

Various groups of natural products have been determined for the cholinesterase-inhibiting activities and were reported to possess such activities in various extents. Among these, the steroidal

alkaloids, particularly those with the pregnane skeleton from the plants of the genus *Sarcococca* (family Buxaceae), are among the extensively investigated ones [4]. Here, we report the isolation and structure elucidation of a new steroidal alkaloid, 4-acetoxy-plakinamine B (**1**), from the marine sponge of the genus *Corticium*. The compound is not only the first marine-derived acetylcholinesterase-inhibiting steroidal alkaloid, but also the first non-pregnane type steroid ever been reported to possess such activity in a comparable extent.

2. Experimental

2.1. General

Specific rotation was recorded on a Jasco J-810 spectropolarimeter. IR spectrum was obtained from on a Jasco IR-810 infrared spectrometer. UV spectrum was measured on a Spectronic Genesys 5 spectrophotometer. Mass spectra, both low and high resolution ones, were operated on a Micromass LCT mass spectrometer. All NMR experiments were performed on an FTNMR, Varian Unity Inova 500 spectrometer using solvent signals of C_6D_6 as references. For the chromatographic separation, the chromatographic supporting materials were as followed; Merck SiO_2 (230-400 mesh) for flash chromatography; Pharmacia Sephadex LH-20 for size exclusion chromatography; Merck C-18 bonded phase SiO_2 (230-400 mesh) for reversed phase chromatography; and Merck pre-coated SiO_2 60 F_{254} (layer thickness 0.20 mm) for TLC.

2.2. Animal material

The sponge *Corticium* sp. (family Plakinidae) was collected in April, 2003 and 2004, at the depth of 18-30 m in the vicinity of Koh-Tao, Surat-Thani Province. Upon surfacing, the specimens appeared as a small flat colonial sponge, with colony size ranging from 3- to 15- cm wide and 0.2- to 0.4-cm thick. The texture was leathery, with dark brownish grey color outside and paler inside. The taxonomic identification was carried out by one of us (SB) and the voucher specimen (PMBC21360) was deposited at Phuket Marine Biological Center, Phuket, Thailand.

2.3. Extraction and isolation

The freeze-dried sponge, weighed 260 g, was consecutively and exhaustively macerated in a series of solvents, started from hexane, to CH_2Cl_2, and to MeOH. The MeOH-extracts, which showed the most potent AChE inhibiting activity, was subjected to further consecutive chromatographic separation as followed; a SiO_2 flash chromatography (10% MeOH in EtOAc); Sephadex LH20 (MeOH); C-18 RP flash chromatography (40% aq MeCN with 0.1% ethanolamine); and repeated preparative TLC (MeOH:acetone:CH_2Cl_2 1.5:1:7.5), from which compound **1** was obtained (2.8 mg). 4-Acetoxy-plakinamine B (**1**); $[\alpha]_D$ +21.9° (*c* 0.0014, MeOH); UV (MeOH) λ_{max} (log ε) 242 (4.29) nm; IR (thin film) ν_{max} 3400, 2925, 1740, 1240 cm^{-1}; ^1H and ^{13}C NMR (C_6D_6, 500 MHz for ^1H) see Table 1; EIMS *m/z* (relative intensity) 508 [M^+] (63), 493 (10), 433 (10), 164 (36), 136 (41); HR-EIMS *m/z* 508.4001 (calcd for $C_{33}H_{52}N_2O_2$ 508.4029).

2.4. Acetylcholinesterase inhibitory activity

The AChE inhibitory assay and inhibition kinetics analysis were conducted (all triplicate) according to the protocol described in reference [1]. Briefly described, to a solution of 125 μL 5,5′-dithiobis[2-nitrobenzoic acid] (3 mM), 25 μL acetylthiocholine iodide (1.5 mM), 50 μL Tris-HCl buffer (pH 8.0; 50 μM), and 25 μL of **1** in Tris-HCl buffer was added 25 μL electric eel AChE (0.28 $U.mL^{-1}$; type VI-S, EC 3.1.1.7; Sigma®). The developing yellow color was measured at 405 nm over 2 min with a 5-s interval. The resulting velocity was calculated and used for the determination of the enzyme activity and inhibitory activity. The IC_{50}, K_m, and V_{max} were calculated using software package Prism (Graph Pad Inc, San Diego, CA). The potency of **1** was referred to that of the standard galantamine.

3. Results and discussion

Using bioassay-guided fractionation, the Thai sponge *Corticium* sp., whose MeOH-extracts showed a good AChE inhibiting activity (>90% inhibition of AChE at 0.1 mg/mL), was subjected to the further chemical investigation to yield compound **1** as white glass (2.8 mg). Analyses of the EI mass spectra (*m/z* 508, [M^+]) and NMR spectra (signals equivalent to 33 carbons and 51 protons) led to the proposed molecular formula of $C_{33}H_{52}N_2O_2$. This was confirmed by the HR-EI mass spectral

analysis (*m/z* 508.4001 calcd for $C_{33}H_{52}N_2O_2$ 508.4029). The molecular formula as proposed yielded an unsaturation degree of 9, belonging to 3 olefinic double bonds, 1 ester carbonyl, and 5 ring systems. An IR absorption band at 1740 cm^{-1} confirmed the presence of the ester functionality. The characteristic absorption band of a secondary amine (ν_{max} 3400 cm^{-1}) was also observed.

Apart from the overwhelming methylene signals typical of a steroid skeleton, a spin system of an *E*-olefin was observed in the ^1H and ^{13}C NMR spectra (500 MHz for ^1H, C_6D_6; δ_H 5.49,dd, J = 15.3, 8.9 Hz, H-22; 6.54, d, J = 5.3 Hz, H-23; δ_C 134.6, C-22; 125.6, C-23; see Table 1). Extending this functionality by means of HMBC spectral analysis led to the elucidation of a 4-(1,3-dimethyl)-3,4-didehydropiperidinyl moiety (C-24 – C-29) attached on C-23, and a methyl-substituted methine (C-20 – C-21) attached on C-22.

Elucidation of the remaining signals as a steroid skeleton relied heavily on the analysis of $^1H,^1H$-COSY and HMBC spectra. This steroid skeleton possesses 3 major functionalities. An *N*-methyl group was placed on C-3 as deducible from the characteristic methyl signals at δ_H 2.25 (s) and δ_C 34.9. The signal of H-3, found at δ 2.62 (br d, J = 2.6 Hz), showed a $^1H,^1H$-correlation observable in COSY spectrum with that of H-4 (δ 5.07, br s). According to such down-field chemical shift, and to HMBC correlations observable throughout the entire moiety, an acetoxy group (δ_C 169.6, C-4-$OCOCH_3$; 20.8, C-4-$OCOCH_3$) was then placed on C-4. On ring B, a trisubstituted olefinic moiety was placed. This was composed of 1 proton resonating at δ 5.29 (br s, H-7), and 2 carbons at δ 118.4 (C-7) and 139.6 (C-8). The remaining signals were typical of steroid nucleus and were elucidated according to the correlations observed in the $^1H,^1H$-COSY and HMBC spectra, as well as to ^{13}C chemical shift comparison with those reported for various steroidal compounds. The chemical structure of **1** was then proposed as 4-acetoxy-plakinamine B, a new derivative of stigmastane-type steroidal alkaloids in the plakinamine family.

The relative stereochemistry of **1** was proposed primarily on the basis of NMR analysis. Peak broadening in the signals of H-3 and H-4 (δ 2.62, br d; and 5.07, br s, respectively) was attributed to the minute and non-resolvable coupling constant (J < 1Hz) between H-3 and H-4, suggesting the

equatorial orientation of both protons; the orientation of the amino group on C-3 and the acetoxy one on C-4 therefore were both axial. The chemical shifts of the two angular methyl groups, i.e., C-18 and C-19 (δ 12.3 and 15.1, respectively), were in the typical ranges to those of the axial methyl groups in most steroids and triterpenoids (as compared to lower-fielded equatorial methyls of cardenolides and triterpenes; for examples, see [5-7]). A skeleton of all-trans tetracyclic steroid ring junction was hence deduced from the orientation of the two axial methyls.

Similar argument was applied to the elucidation for the stereochemistry of C-17 side chain. The chemical shifts, both ^{1}H and ^{13}C, of the positions 17, 20, and 21 (δ_H 1.25, m, H-17; 2.18, m, H-20, and 1.14, d, J = 5.8 Hz, H-21; δ_C 52.6, C-17; 41.6, C-20; 21.3, C-21, respectively), were all in agreement to those reported for related steroidal alkaloids from *Plakina* and *Corticium* sponge [8-10]. Based on the parent structures of plakinamine B (**2**) [8] and its related analogs [10], the relative configurations of C-17 side chain was proposed to adopt a β orientation, with an *anti* methyl (i.e., C-21) substituted on C-20 as shown.

It should be mentioned here that, due to the peculiarity in the solubility and chromatographic behavior of chemical constituents in the extracts, we were unable to retrieve other compounds that could be related to **1**. Most of the compounds that were active to our bioassay were irreversibly trapped by the packing materials, presumably due to the interaction between their basic nitrogenated functional groups and the acidic SiO_2. The remaining components, although chromatographically separable, were recovered in the amounts too small for the further chemical analyses.

Compound **1** was subjected to an AChE inhibiting assay, using electric eel AChE as the enzyme target. The high inhibiting activity (IC_{50} 3.75$\pm$1.69 μM) was observed. The inhibition of **1** against AChE was independent from the incubation time (up to 60 min, data not shown), suggesting that **1** inhibited AChE reversibly. In order to determine the inhibition mode of compound **1**, kinetics analysis of enzyme inhibition was conducted, and V_{max} and K_m were calculated from a non-linear regression using software package Prism. The contrasted decrease in V_{max} and increase in K_m

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(Table 2) upon addition of **1** indicated that the compound inhibited the targeted enzyme in a mixed-competitive manner.

Structurally, the plakinamines and *Sarcococca* steroidal alkaloids are closely related, particularly at C-3 substituted groups; therefore, it is not quite surprising that both groups of alkaloids exhibit similar AChE inhibiting activity. In fact, the potency of compound **1** as discussed above is in a comparable range to, or even better than, those of the most potent members of the *Sarcococca* alkaloids. For examples, axillaridine A (**3**) and sarsalignenone (**4**), representing the most potent *Sarcococca* alkaloids, exhibited the IC_{50}s of 5.21 ± 0.11 and 5.83 ± 0.07 μM, respectively (IC_{50} of galantamine reported therein 0.45 ± 0.02 μM) [11]. However, the modes of inhibition among the two groups of alkaloids are different. The inhibitory mode of **1**, as discussed earlier, is mixed-type, i.e., expressing a combining mode between competitive and uncompetitive ones. Most of *Sarcococca* alkaloids, on the contrary, inhibit the enzyme primarily in a noncompetitive fashion [11].

According to the 3-D QSAR studies on the AChE inhibiting activity of the *Sarcococca* alkaloids, the inhibitory activity was primarily favored by the negative density surrounding C-3 and C-4, whereas the side chain on C-17 evidently did not affect the activity [12]. Such statement is in a good agreement to our observation with compound **1**, of which the stigmastane skeleton distances the nitrogen atom on the side chain with a 4-carbon bridge farther than those in the pregnane skeleton. Although it is too early for any concrete conclusion to be made from only one member of the stigmastane-type steroidal alkaloids, it is certainly interesting to explore the further evidences that could unify the structure activity relationship for the skeletons of the two groups of alkaloids.

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