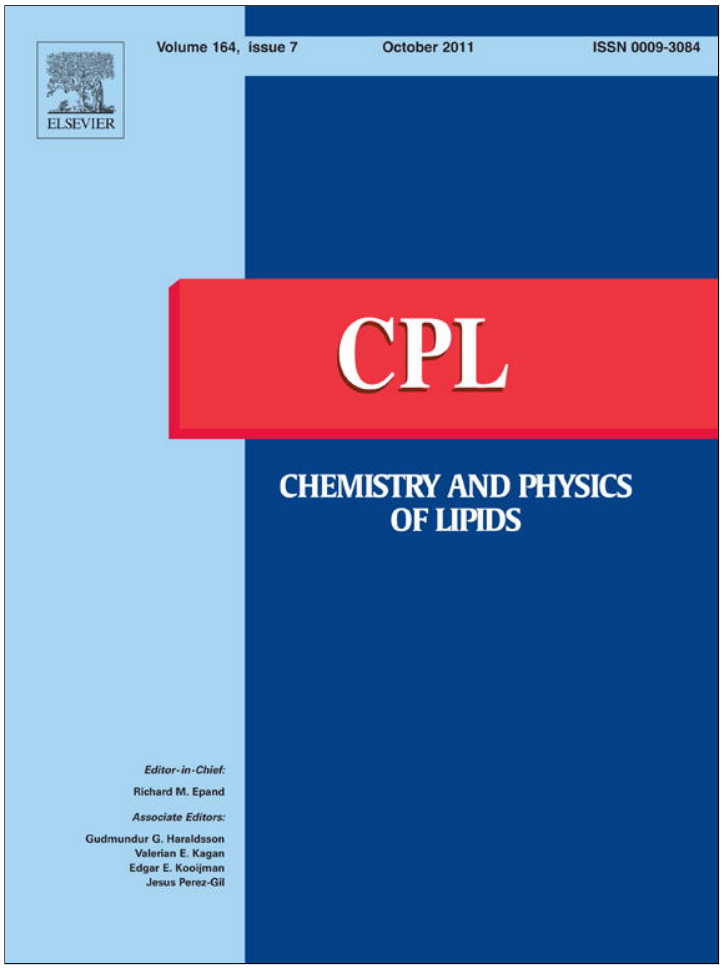

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Brominated aliphatic hydrocarbons and sterols from the sponge *Xestospongia testudinaria* with their bioactivities

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ABSTRACT

Four brominated aliphatic hydrocarbons (**1**–**4**), including a novel brominated ene-tetrahydrofuran named as mutafuran H (**1**), and five sterols (**5**–**9**) were isolated from the South China Sea sponge *Xestospongia testudinaria*. The structure of **1** was determined on the basis of NMR (^{1}H, ^{13}C NMR, HSQC, HMBC, ^{1}H–^{1}H COSY, and NOESY), MS, and optical rotation analysis. Known compounds were identified by comparison of their NMR data with those reported in the literature. Compounds **1**–**4**, and **6**–**9** were evaluated for their toxicity against *Artemia salina* larvae, and anti-acetylcholinesterase activity.

1. Introduction

Sponges (phylum Porifera) are soft bodied and sessile animals, which lack spine and shell. In order to defend against fouling organisms, predators and neighbours competing for space, sponges rely mainly on bioactive natural products instead (Proksch et al., 2002). Sponges of the genus *Xestospongia* (class Desmospongia, order Haplosclerida, family Petrosiidae), known as barrel sponges, are large and common members of the coral reef communities at depths greater than 10 m, all over the Indo-Pacific Ocean and the Caribbean Sea. Although *Xestospongia* species are among the richest resources of pharmacologically active chemicals isolated from marine organisms, their chemical and biological characterization, such as in the South China Sea, is still lacking (Zhou et al., 2010).

Acetylcholinesterase (AChE) is a key component of cholinergic brain synapses and neuromuscular junctions. AChE inhibitors (AChEIs) are usually employed mostly for correcting the effects of insufficient levels of acetylcholine. The usefulness of AChEIs as a treatment for symptoms of the early stages of Alzheimer's disease has stimulated much research in recent years into finding natural products displaying this activity (Houghton et al., 2006; Pohanka et al., 2009; Williams et al., 2011). On the other hand, AChEIs are also attractive in agriculture and ecology since they may affect the nervous system of vertebrate and invertebrate organisms (e.g. fish, shellfish, and insects) by inhibiting the cholinesterase enzymes *in vivo* (Tsukamoto et al., 2005). AChE inhibition is also considered one of the most important mechanisms of chemical defence of some plants and marine organisms (Key and Fulton, 2006).

During the course of our search for bioactive constituents from South China Sea marine sponges, a rapid TLC bioautographic method to detect the anti acetylcholinesterase activity (Marston et al., 2002) showed positive results for several extracts, including the alcohol extract of the sponge *Xestospongia testudinaria*. In present study, a novel brominated ene-tetrahydrofuran named as mutafuran H (**1**) and other three brominated aliphatic hydrocarbons (**2**–**4**), together with five known sterols (**5**–**9**) (Fig. 1), were isolated from the alcohol extract of the sponge *X. testudinaria*, collected off the coast of Sanya, north of South China Sea. Compounds **1**–**4**, and **6**–**9** were evaluated for their toxicity against brine shrimp (*Artemia salina*) larvae and anti-acetylcholinesterase activity. Herein, we report the isolation, structure elucidation and bioactivities of those compounds from *X. testudinaria*.

Abbreviations: BSL, brine shrimp lethality; AChE, acetylcholinesterase; *X. testudinariaXestospongia testudinaria*; PE, petroleum ether; BPFC, brominated polyunsaturated fatty acids.

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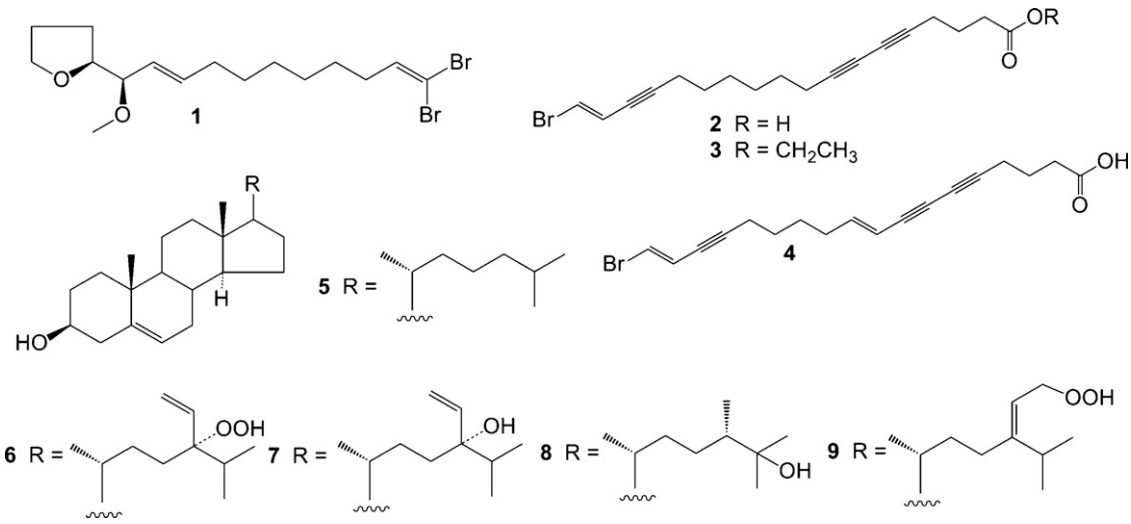

Fig. 1. Structures of compounds **1**–**9**.

2. Materials and methods

2.1. General

NMR spectra were measured on Bruker AVANCE-500 spectrometer. ESI-MS and HRESI-MS were obtained from Thremo LCQ-DECA-XP LC-MS and Q-Tof Micro mass spectrometers, respectively. Silica gel (100–200, 200–300 mesh, Qingdao Marine Chemical Group Co., Qingdao, China), Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and ODS (500/400 mesh, YMC, Kyoto, Japan) were used for column chromatography.

2.2. Animal material

The sponge was collected at a depth 7–10 m off the coast of Sanya (South China Sea), Hainan province of China, in May 2008. Animal material was stored in a −20 °C freezer prior to extraction. The specimen was identified as *X. testudinaria* by Dr. Kyung Jin Lee, Wildlife Genetic Resources Center, National Institute of Biological Resources, Environmental Research Complex, Incheon, Korea, and a voucher (Xt200805) was deposited at the Key Laboratory of Marine Bio-resources Sustainable Utilization, South China Sea Institute of Oceanology.

2.3. Extraction and isolation

The sponge (7 kg, wet wt) was crushed and extracted with 75% alcohol (3× 10 L) at room temperature. The combined alcohol extracts were concentrated in vacuo. The residue was suspended in H_2O (5 L) and partitioned with petroleum ether (PE, 3× 5 L), EtOAc (3× 5 L), and *n*-BuOH (3× 5 L) successively. The PE fraction (30 g) was chromatographed on silica gel column (6× 45 cm) using a gradient of PE/EtOAc to obtain subfractions 1–7 (pure PE, PE/EtOAc 100:1, 50:1, 20:1, 10:1, 4:1, 1:1, and pure EtOAc). Subfraction 3 (PE/EtOAc 50:1) was chromatographed successively on silica gel [PE/acetone (12:1)] and Sephadex LH-20 columns (MeOH) to obtain **1** (19 mg), **5** (256 mg), **6** (44 mg), **7** (23 mg), **8** (5.3 mg), and **9** (15 mg). Compounds **2** (23 mg), **3** (4.5 mg), and **4** (43 mg) were obtained from the EtOAc fraction (10 g) by repeated Sephadex LH-20 (MeOH) and silica gel columns [PE/acetone (10:1)] chromatography.

2.4. Mutafuran H (*1*)

Colorless oil; $[\alpha]_D^{20}$ −56.7° (*c* 0.075, $CHCl_3$); IR (CCl_4) ν_{max} 2845, 1630, 1120, 965 cm^{-1}; ESI(+)-MS: *m*/*z* (%): 409 (62), 411 ($[M+H]^+$, 100), 413 (47); HRESI-MS: *m*/*z* 411.1929 (calcd for $C_{16}H_{27}O_2Br_2$ 411.1924), precise isotopic composition of $[M+H]^+$; ^1H and ^{13}C NMR, see Table 1.

2.5. Brine shrimp larvae toxicity test

The brine shrimp larvae (BSL) test was performed as described previously (Ortlepp et al., 2007).

2.6. Acetylcholinesterase inhibitory activity test

The AChE inhibitory activity was measured according to Ellman's coupled enzyme assay (Ellman et al., 1961), modified as follows. 0.2 Units of AChE were dissolved in 0.1 M potassium phosphate buffer (pH 7.4), and purified compounds dissolved in DMSO (the series of final concentrations as 8, 4, 2, 1, 0.5, 0.25 μM) were added to each well of a 96-well plate. Then, acetylthiocholine iodide and 5,5′-dithiobis(2-nitrobenzoic acid) dissolved in 0.1 M potassium phosphate buffer (pH 7.4) were added to reach a final concentration of 50 μM both. The reaction was carried out at 30 °C for 30 min. The absorbance was measured at 410 nm using a spectrophotometer and the half maximum inhibitory concentration (IC_{50}) was calculated.

Table 1
^{13}C and ^1H NMR data of **1** (500 MHz, in $CDCl_3$, *J* in Hz).

No.	δ_C	δ_H (multiplicity)
1	88.7	
2	138.7	6.38 (t, 6.5)
3	32.9	2.09 (dt, 6.5, 6)
4–7	26–29	1.2–1.5 (m) 8H
8	32.2	2.09 (dt, 6.5, 6)
9	137.6	5.77 (ddd, 15.0, 8.0, 6.5)
10	125.2	5.35 (dd, 15.0, 8.5)
11	83.9	3.61 (dd, 8.5, 8.5)
12	81.6	4.50 (br.dd, 12.5, 8.5)
13	23.9	2.09 (m) 2.20 (m)
14	27.6	1.83 (m)
15	71.8	3.69 (t, 6.0)
16	56.5	3.30 (s)

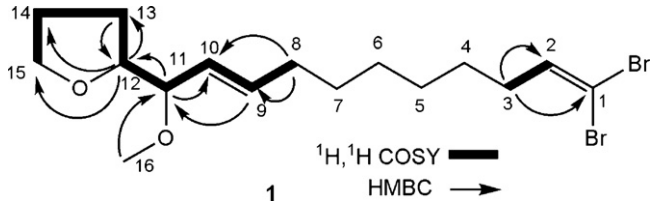

Fig. 2. Key HMBC and ^{1}H, ^{1}H COSY correlations of **1**.

3. Results and discussion

Compound **1** was obtained as colorless oil. The ESI(+)-MS of **1** showed molecular ion peaks at *m*/*z* (%): 409 (62), 411 (100), 413 (47), corresponding to a characteristic isotope pattern for two bromine atoms. The molecular formula of **1** was established as $C_{16}H_{26}O_2Br_2$ by HRESI-MS from the pseudo-molecular ion $[M+H]^+$ at *m*/*z* 411.1929. The ^{1}H and ^{13}C NMR data (Table 1) of **1** indicated the presence of eight methylene groups and two C=C double bonds, one of which showing characteristic resonances (δ_C 138.7, 88.7; δ_H 6.38 (t, J = 6.5)) for a terminal ω,ω-dibromovinylidene Br_2C=CH (Brantley et al., 1995; Morinaka et al., 2007). In the HMBC spectrum, the correlations of H-12 (δ_H 4.50, br.dd, J = 12.5, 8.5), linked to the oxymethine C-12 (δ_C 81.6, CHO), with C-13 (δ_C 23.9, CH_2), C-14 (δ_C 27.6, CH_2), and oxygenated methylene C-15 (δ_C 71.8, CH_2O), suggested the presence of a monosubstituted tetrahydrofuranyl moiety at the other end of the structure (Fig. 2). This assignment was corroborated by the ^{1}H, ^{1}H COSY correlations of H-12 and H-13a, 13b (δ_H 2.09, 2.20), H-13a, 13b and H-14 (δ_H 1.83), H-14 and H-15 (δ_H 3.69, t, J = 6.0). Furthermore, both HMBC and COSY correlations confirmed that an oxymethine carbon (δ_C 83.9; δ_H 3.61) bearing a methoxy group (δ_C 56.5; δ_H 3.30, 3H, s), was linked to tetrahydrofuranyl at C-12 (δ_C 81.6; δ_H 4.50) (Fig. 2). The location of the other double bond group (δ_C 137.6, 125.2; δ_H 5.77, 5.35) was also determined by HMBC and ^{1}H, ^{1}H COSY correlations, and its configuration was suggested to be *E* by the coupling constant ($J_{CH=CH}$ = 15.0) of the two olefinic protons. Thus, the planar structure of this brominated ene-tetrahydrofuran was determined as in Fig. 2.

The absolute configuration of mutafuran H was deduced from the value of its specific optical rotation, and the NOESY spectrum. Comparison of the specific optical rotation of **1** ($[\alpha]_D^{20}$ −56.7), with those of sapinofuranone B (Clough et al., 2000), *ent*-sapinofuranone B (Kumar et al., 2005), and sapinofuranone A (Kumar et al., 2005) (Fig. 3), led us to consider the alternative 11*R*,12*S* or 11*R*,12*R* configurations for **1**. Moreover, observed NOESY correlations between H-9 and H-11, H-10 and H-11, H-10 and H-12, H-11 and H-12, H-12 and H-13a (δ_H 2.09), H-12 and H-13b (δ_H 2.20), and the absence of a cross peak between H-11 and H-13a or H-13b (Fig. 4), suggested the 11*R*,12*S* absolute configuration for **1**.

Compounds **2**–**9** were identified as xestospongic acid (**2**) (Bourguet-Kondracki et al., 1992), xestospongic acid ethyl ester (**3**) (Bourguet-Kondracki et al., 1992), 18-brornooctadeca-(9*E*,17*E*)-diene-5,7,15-triynoic acid (**4**) (Patil et al., 1992), cholesterol (**5**) (Rubinstein et al., 1976), 24-hydroperoxy-24-vinylcholesterol (**6**) (Guyot et al., 1982), saringosterol (**7**) (Guyot et al., 1982), 24-methylcholest-5-ene-3β,25-diol (**8**) (Kobayashi et al., 1991), and 29-hydroperoxystigmasta-5,24(28)-dien-3β-ol (**9**) (Sheu et al., 1997) by NMR data comparison with those in the literature. This is the first reported occurrence of compounds **4**, and **6**–**9** in *X. testudinaria*.

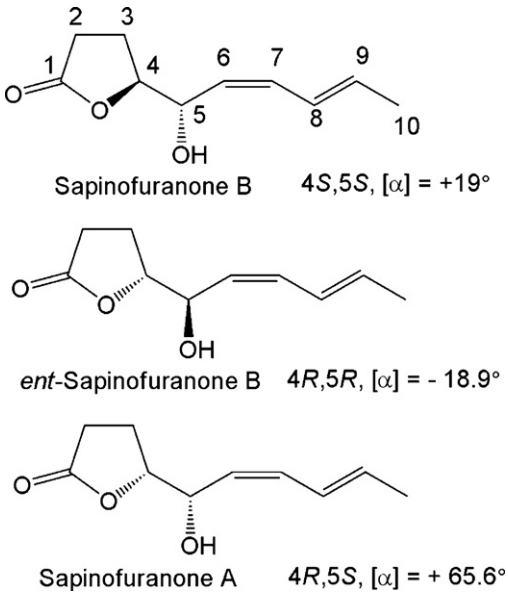

Fig. 3. Structures and specific optical rotations of sapinofuranone B, *ent*-sapinofuranone B and sapinofuranone A.

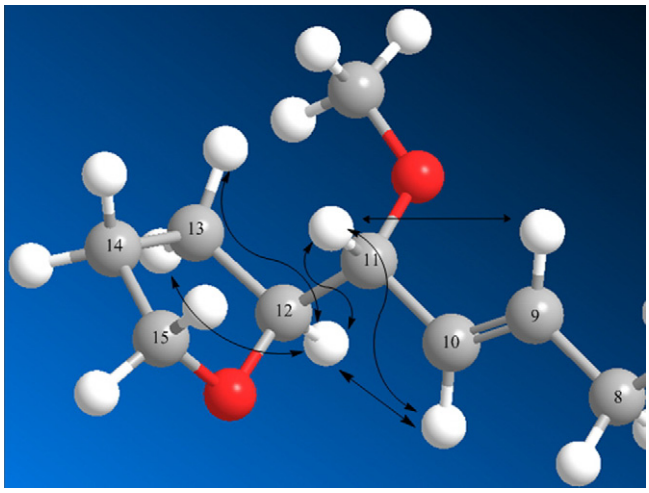

Fig. 4. Absolute configuration (part) and key NOESY correlations of **1** (drawn by ChemDraw Ultra 10.0 and Chem3D Ultra 10.0).

Compounds **1**, **2**, **6**, **7** and **9** showed significant or moderate toxicities against *A. salina* larvae, with LC_{50} values ranging from 0.56 to 6.99 μM. Mutafuran H (**1**) displayed significant AChE inhibitory activity (IC_{50} 0.64 μM), whereas xestospongic acid (**2**),

Table 2
BSL toxicities and AChE inhibitory activities of compounds **1**–**4**, and **6**–**9**.

	Compounds								
	1	**2**	**3**	**4**	**6**	**7**	**8**	**9**	Tacrine[a]
BSL toxicities (LC_{50}, μM)	2.60	6.99	26.21	10.52	0.56	5.89	12.45	0.63	1.25
AChE inhibition (IC_{50}, μM)	0.64	12.65	–[b]	–[b]	11.45	–[b]	–[b]	14.51	0.41

[a] Tacrine was considered as positive control.
[b] Not active at 50 μM.

and hydroperoxyl steroids (**6** and **9**) revealed weak activities (Table 2).

As previously reported, brominated polyunsaturated fatty acids (BPFC) and sterols are the main chemical constituents of *X. testudinaria*, and BPFC appear to be hallmark metabolites of *X. testudinaria* and *X. muta* (Zhou et al., 2010). Brominated ene-tetrahydrofurans are rare in natural resources, and only nine of them have been reported so far in marine sponge. These include mutafurans A–G isolated from the Bahamian sponge *X. muta* (Morinaka et al., 2007), and xestospongiene Z from *X. testudinaria* (Jiang et al., 2011). Our finding of mutafuran H (**1**) in *X. testudinaria*, with its chemotaxonomic significance, suggests a biogenetic affinity between this species and *X. muta*.

Our study also showed that brominated aliphatic hydrocarbons, in particular brominated ene-tetrahydrofurans, are the toxic constituents of *X. testudinaria*, as it is the case for numerous brominated sponge-derived compounds involved in chemical defences (Ortlepp et al., 2007; Paul and Ritson-Williams, 2008). In addition, mutafuran H is the first reported brominated ene-tetrahydrofuran showing AChE inhibitory activity.

Oxidized steroids containing a hydroperoxyl group, such as 24-hydroperoxy-24-vinylcholesterol (**6**) and 29-hydroperoxystigmasta-5,24(28)-dien-3β-ol (**9**), were found in *Xestospongia* for the first time. Although several hydroperoxysterols have been isolated from tunicates (Sung et al., 2007), seaweeds (Teixeira et al., 2006), algae (Sheu et al., 1997), and some plants (Kato et al., 1996), they were still uncommon as marine natural products. Our results showed significant toxicities against *A. salina* larvae and weak AChE inhibitory activities for compounds **6** and **9**, which suggest that these may be involved in chemical defence of the sponge.

Acknowledgements

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