New Nitrogenous Bisabolene-Type Sesquiterpenes from a Hainan Sponge *Axinyssa* aff. *variabilis*

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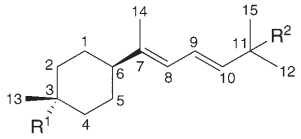
Two new uncommon nitrogenous sesquiterpenes, 11-ethoxy-3-formamidotheonellin (**1**) and 7-ethoxy-3-formamidobisabolane-8,10-diene (**2**), together with two known related sesquiterpenes, 3-formamidotheonellin (= theonellin formamide; **3**) and theonellin isothiocyanate (**4**), were isolated from the Hainan sponge *Axinyssa* aff. *variabilis*. Their structures were determined on the basis of extensive spectroscopic analysis and by comparison of their NMR data with those of known compounds.

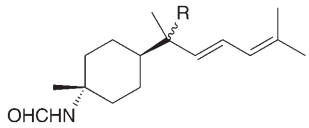
Introduction. – Over the one decade since *Faulkner*, *Clardy*, and co-workers first reported the chemical investigation of an *Axinyssa* species of sponge [1], marine sponges of the genus *Axinyssa* (order Halichondrida, family Halichodriidae) have attracted considerable research interest mainly due to the presence of sesquiterpenes containing unusual nitrogenous functional groups, such as isothiocyanate, formamide, isonitrile, and thiocyanate [2–6]. Recently, a guaiane-type sesquiterpene hydroperoxide, sesquiterpene carbonimide dichlorides [7], a diterpene hydrocarbon [8], and nitrogenous bisabolene- [9], eudesmane- [10], and germacrane-type [11] sesquiterpenes have also been reported from sponges of this genus. Interestingly, many nitrogenous-containing terpenes have been found to possess activity in anthelmintic [2], antimalarial [6], antitumor [10], and antifouling assays [7][12].

As part of our ongoing research on the biologically active substances from Chinese marine invertebrates [13–16], we made a collection of the sponge *Axinyssa* aff. *variabilis* off the Lingshui Bay, Hainan Province, China. On separation of the Et_2O-soluble fraction of an acetone extract of this sponge, two new uncommon bisabolene-type sesquiterpene formamides, 11-ethoxy-3-formamidotheonellin (**1**) and 7-ethoxy-3-formamidobisabolane-8,10-diene (**2**), together with two known related sesquiterpenes, 3-formamidotheonellin (= theonellin formamide; **3**) [9][17][18] and theonellin isothiocyanate (**4**) [17], were isolated (bisabolane = 1-(1,5-dimethylhexyl)-4-methylcyclohexane; theonellin = *trans*-4-[(1*E*,3*E*)-1,5-dimethylhexa-1,3-dienyl]-1-methylcyclohexane). Herein, we report the isolation and structure elucidation of the two new compounds.

Results and Discussion. – Freshly collected animals were immediately chilled to $-20°$ and kept frozen until used. Frozen material was extracted exhaustively with



1 R^1 = NHCHO, R^2 = EtO
3 R^1 = NHCHO, R^2 = H
4 R^1 = NCS, R^2 = H



2 R = EtO
5 R = H

acetone. The acetone extract was then partitioned between Et_2O and H_2O. The Et_2O extract was repeatedly subjected to silica gel and *Sephadex LH-20* column chromatography followed by HPLC (*C-18*) purification to afford pure compounds **1**–**4**.

Compounds **3** and **4** were readily identified as 3-formamidotheonellin (**3**) [9][17][18] and theonellin isothiocyanate (**4**) [17], respectively, by comparison of their spectral data with those reported in the literature. As reported in [2][9] [17], the observed doubling of many of the NMR signals in the ^{1}H- and ^{13}C-NMR spectra of **3** is due to the occurrence of rotational isomers of the formamide group. This same phenomenon was also observed in the NMR spectra of the new compounds **1** and **2**, indicating that both of them contained a formamide moiety.

Compound **1** was isolated as colorless oil whose molecular formula $C_{18}H_{31}NO_2$ was established on the basis of EI-MS and ^{13}C-NMR (DEPT) spectra and confirmed by HR-EI-MS (M^+ at m/z 293.2336). Thus, the structure of **1** possesses four degrees of unsaturation. The characteristic bands at 3273 and 1686 cm^{-1} in the IR spectrum were assigned to a secondary-amide functionality, in agreement with the doubling of most ^{1}H- and ^{13}C-NMR peaks (*Table*) due to a formamide group as noted above. The structure of **1** was established by its spectroscopic data and comparison with those of 3-formamidotheonellin (**3**)[1]).

Taking into account the peak-doubling phenomenon, the formamide-group NH ^{1}H-NMR signals of **1** were identified as broad, exchangeable (CD_3OD) proton resonances at δ 5.21 (br. *s*) and 5.75 (br. *d*, J = 9.3 Hz), which were coupled (COSY), to downfield signals at δ 8.05 (*d*, J = 2.4 Hz) and 8.32 (*d*, J = 12.1 Hz), respectively, which in turn were one-bond coupled to downfield ^{13}C-NMR signals at δ 160.3 and 162.5, respectively. The ^{13}C-NMR spectrum also provided evidence for one EtO (δ 57.9 (*t*), 16.1 (*q*)) and two C=C bonds, as well as for four Me, four CH_2, and one CH group, and two heteroatom-bearing quaternary sp^3 C-atoms, implying, from the required degrees of unsaturation, a monocyclic sesquiterpene framework. These NMR data were strongly reminiscent of those of the co-occurring sesquiterpene 3-formamidotheonellin (**3**) [9][17][18]. A comparison of the ^{1}H- and ^{13}C-NMR data (*Table*) revealed that **1** differs from **3** only by the presence of an EtO group, in agreement with the observed molecular-mass difference of 40 mass units. The EtO group linked at C(11) was deduced from the HMBC correlation between the CH_2 signals of the EtO group at δ 3.35 (*q*, J = 6.9 Hz) and that of C(11)[1]) at δ 74.9. These spectroscopic finding disclosed that compound **1** had the structure of a bisabolene-type sesquiterpene with a formamide moiety at C(3) and an EtO group at C(11). The relative configuration of **1** is proposed to be the same as in **3** because, in the two compounds the ^{13}C-NMR chemical shifts of the sp^3 C-atoms of the six-membered ring, including that of Me(13) [9][17], are almost the same (δ 22.3 and 24.7 for **1** *vs.* 22.1 and 24.3 for **3**), as are the shapes of the H–C(6) ^{1}H-NMR signals.

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[1]) Trivial atom numbering; for systematic names, see *Exper. Part*.

Table. *^{1}H- and ^{13}C-NMR Data* ($CDCl_3$) *for Compounds* **1** *and* **2**[a])[b])[1]). δ in ppm, J in Hz.

	1[c])		**3**[d])	**2**[e])	
	δ(H), (multiplicity, J in Hz)	δ(C)	δ(C)	δ(H)	δ(C)
CH_2(1)	1.43 (*dddd*, J = 13.9, 12.1, 11.8, 3.9, 1 H, H_β), 1.66–1.68 (*m*, 1 H, H_α)	27.2, 27.3 (2*t*)	27.07, 27.14 (2*t*)	1.41–1.43 (*m*, 1 H, H_α), 1.66–1.68 (*m*, 1 H, H_β)	27.2, 27.3 (2*t*)
CH_2(2)	1.57, 1.71 (2*m*, each 1/2 H, H_β), 1.83, 2.20 (*m*, each 1/2 H)	36.9, 39.4 (2*t*)	36.7, 39.0 (2*t*)	1.48, 1.55 (2*m*, each 1/2 H, H_β), 1.78, 2.20 (2*m*, each 1/2 H, H_α)	37.4, 39.7 (2*t*)
C(3)	–	52.5, 53.8 (2*s*)	52.4, 53.6 (2*s*)	–	52.8, 54.1 (2*s*)
CH_2(4)	1.57, 1.72 (2*m*, each 1/2 H, H_β), 1.83, 2.21 (2*m*, each 1/2 H, H_α)	36.9, 39.4 (2*t*)	36.7, 39.0 (2*t*)	1.48, 1.55 (2*m*, each 1/2 H, H_β), 1.78, 2.20 (2*m*, each 1/2 H, H_α)	37.4, 39.7 (2*t*)
CH_2(5)	1.43 (*dddd*, J = 13.9, 12.1, 11.8, 3.9, 1 H, H_β), 1.66–1.68 (*m*, 1 H, H_α)	27.2, 27.3 (2*t*)	27.2 (2*t*)	1.41–1.43 (*m*, 1 H), 1.66–1.68 (*m*, 1 H)	27.2, 27.3 (2*t*)
H–C(6)	1.93–1.95 (*m*, 1 H)	46.2, 46.4 (2*d*)	46.0, 46.2 (2*d*)	2.00–2.02 (*m*, 1 H)	47.4, 47.5 (2*d*)
C(7)	–	141.5, 142.2 (2*s*)	139.3, 139.9 (2*s*)	–	78.8, 79.0 (2*s*)
H–C(8)	5.85 (br. *d*, J = 10.8, 1 H)	122.8, 123.1 (2*d*)	123.1, 123.3 (2*d*)	5.44 (*d*, J = 15.6, 1 H)	134.6, 134.8 (2*d*)
H–C(9)	6.35 (*dd*, J = 15.6, 10.8, 1 H)	125.0, 125.1 (2*d*)	123.35, 123.37 (2*d*)	6.35 (*dd*, J = 15.6, 10.8, 1 H)	127.0, 127.2 (2*d*)
H–C(10)	5.61 (*dd*, J = 15.6, 3.6, 1 H)	137.8, 138.2 (2*d*)	140.1, 140.3 (2*d*)	5.85 (br. *d*, J = 10.8, 1 H)	124.7, 125.3 (2*d*)
C(11) or H–C(11)	–	74.9 (*s*)	31.2 (*d*)	–	135.0, 135.2 (2*s*)
Me(12)	1.30 (*s*, 3 H)	26.5 (*q*)	22.39, 22.41 (2*q*)	1.79 (*s*, 3 H)	26.0 (*q*)
Me(13)	1.37, 1.43 (2*s*, each 3/2 H)	22.3, 24.7 (2*q*)	22.1, 24.3 (2*q*)	1.31, 1.39 (2*s*, each 3/2 H)	22.1, 24.7 (2*q*)
Me(14)	1.76 (*s*, 3 H)	15.2 (*q*)	14.9 (*q*)	1.21, 1.22 (2*s*, each 3/2 H)	18.8 (*q*)
Me(15)	1.30 (*s*, 3 H)	26.5 (*q*)	22.4 (*q*)	1.77 (*s*, 3 H)	18.4 (*q*)
NH	5.21 (br. *s*, 1/2 H), 5.75 (*d*, J = 9.3, 1/2 H)			5.18 (br. *s*, 1/2 H), 5.61 (*d*, J = 9.3, 1/2 H)	
CHO	8.05 (*d*, J = 2.4, 1/2 H), 8.32 (*d*, J = 12.1, 1/2 H)	160.3, 162.5 (2*d*)	160.5, 162.9 (2*d*)	8.02 (*d*, J = 1.8, 1/2 H), 8.30 (*d*, J = 12.3, 1/2 H)	160.2, 162.6 (2*d*)
EtO	3.35 (*q*, J = 6.9, 2 H), 1.16 (*t*, J = 6.9, 3 H)	57.9 (*t*), 16.1 (*q*)		3.32 (*q*, J = 6.9, 2 H), 1.12 (*t*, J = 6.9, 3 H)	57.3 (*t*), 16.4 (*q*)

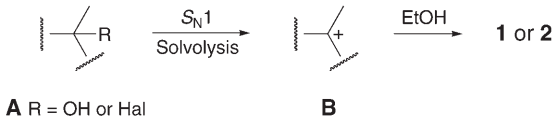
[a]) Most ^{1}H- and ^{13}C-NMR signals for **1** and **2** were doubled due to the presence of a formamide group. [b]) δ Values referenced to $CDCl_3$ (δ(H) 7.26, δ(C) 77.0) as internal standard. Assignments deduced from the analysis of mononuclear and heteronuclear spectra. [c]) Data recorded with a *Varian Inova*-600-MHz (^{1}H, COSY, NOESY) and *Varian Inova*-400-MHz spectrometer (^{13}C, DEPT, HMQC, HMBC). [d]) Data from [9]. [e]) Data recorded with a *Varian Inova*-300-MHz (^{1}H) and *Bruker DRX*-400-MHz spectrometer (^{13}C, DEPT, COSY, NOESY, HSQC, HMBC).

Compound **2** was also obtained as a colorless oil. Its molecular formula, $C_{18}H_{31}NO_2$, established by HR-EI-MS (M^+ at m/z 293.2338), is identical to that of **1**. The ^{1}H- and ^{13}C-NMR data of **2** (*Table*) were very similar to those of **1** and the model compound **5**, a sesquiterpene formamide previously isolated from Palauan sponge *Halichondria* cf. *lendenfeldi* [18]. The structure of **2** was established by its spectroscopic data and comparison with those of **1** and **5**. The configuration at C(6) was tentatively assigned to be the same as that of **1** and **5** based on biogenetic considerations [17][19][20]. The relative configuration at C(7) remains to be defined.

The UV spectrum fo **2** with an absorption at 238 nm ($\log \varepsilon = 4.12$) pointed to a conjugated-diene moiety. Like compound **5**, the ^{1}H-NMR spectrum of **2** contained four Me signals at δ 1.79 (*s*, Me(12)), 1.77 (*s*, Me(15)), 1.39 and 1.31 (2*s*, each 3/2 H, Me(13)), and 1.22 and 1.21 (2*s*, each 3/2 H, Me(14))[1]. In addition, in the ^{1}H,^{1}H-COSY plot, an olefinic signal at δ 6.35 (*dd*, J = 15.6, 10.8 Hz, 1 H) was coupled (J = 15.6 Hz; (*E*) configuration) to both the signal at δ 5.44 (*d*, J = 15.6 Hz, 1 H) and the signal at δ 5.85 (br. *d*, J = 10.8 Hz, 1 H). The latter signal was broadened by allylic coupling to the Me groups at δ 1.79 and 1.77. Moreover, the presence of an EtO group in **2** as in **1** was evident by the typical ^{1}H-NMR signals at δ 3.32 (*q*, J = 6.9 Hz, 2 H) and 1.12 (*t*, J = 6.9 Hz, 3 H). The EtO group attached to C(7) was determined by the HMBC correlation between the CH_2 signal of the EtO group at δ 3.32 and that of C(7) (δ 78.8, 79.0). In fact, careful comparison of the NMR data of **2** with those of **5** revealed that the former differs from the latter only by the substituent at C(7). Due to the replacement of the methine H–C(7) (in **5**) by the EtO group (in **2**), the signal of Me(14) was significantly downfield shifted from δ 1.02 (*d*, J = 7.0 Hz) in **5** to 1.21 and 1.22 (2*s*) in **2**. All NMR data of **2** were unambiguously assigned by ^{1}H,^{1}H-COSY, HMQC, and HMBC experiments as reported in the *Table*. Analogously to **1**, the relative configuration at C(3) was determined by comparing ^{13}C-NMR data of **2** with those of **1** and **5**. The nearly identical chemical shifts of the signals assigned to the formamide C-atom (δ 160.2 and 162.6 in **2**; 160.3 and 162.5 in **1**), to C(3) (δ 52.8 and 54.1 in **2**; 52.5 and 53.8 in **1**), and to Me(13) (δ 22.1 and 24.7 in **2**; 22.3 and 24.7 in **1**) indicated that the relative configuration at C(3) of the six-membered ring is the same in both compounds.

Bisabolene-type sesquiterpenes constitute a diverse assembly of terrestrial- and marine-derived metabolites. Within the marine environment alone, numerous bioactive bisabolene sesquiterpenes having rare functionalities such as halogenated, nitrogenous, acetylated, and heterocyclic derivatives have been discovered. However, to the best of our knowledge, this is the first report on the isolation of an ethoxylated bisabolene-type sesquiterpene from a natural source. It may be worth to point out that we could not rule out the possibility that compounds **1** and **2** are probably artifacts. We propose a hypothesis to explain the possible origin of compounds **1** and **2**. As outlined in the *Scheme*, the common precursor of compounds **1** and **2** could be an allylic tertiary alcohol or halogenide **A** which upon solvolysis (S_N1) produces a fairly stable carbenium **B**, and the latter, in turn, is quenched by a sufficiently good nucleophile, like EtOH which might have been present as an impurity in the acetone needed for the first extraction, to yield **1** or **2**.

Scheme. *Hypothesis of Formation of* **1** *and* **2**



A R = OH or Hal **B**

Although many bisabolene-type sesquiterpenes exhibited broad bioactivities [2][6][7][10][12], the new compounds **1** and **2** were found to be inactive against the fungus *Cladosporium cucumerinum*. Further studies should be conducted to test other bioactivities, such as cytotoxic or anti-inflammatory properties, *etc.* of these new compounds, as well as to understand the true biological/ecological role of these metabolites in the life cycle of the sponge.

This work was financially supported by the *Natural Science Foundation of China* (No. 20572116), *STCSM Project* (No. 054307062), and *MST Marine '863' Project* (No. 2006AA09Z447 and 2006AA09Z412).

Experimental Part

General. Column chromatography (CC): silica gel 200–300 and 400–600 mesh from *Qing Dao Hai Yang Chemical Group Co.* TLC. silica gel plates *G60 F-254* from *Yan Tai Zi Fu Chemical Group Co.* Reversed-phase HPLC: *Agilent 1100* chromatographe; *VWD-G1314A* detector at 210 nm; a semi-prep. *ODS-HG-5* (5 μm, 10 mm (i.d.) × 25 cm) column. Optical rotations: *Perkin-Elmer 241MC* polarimeter; in $CHCl_3$. UV Spectra: *Varian Cary-300 Bio* spectrophotometer. IR Spectra: *Nicolet Magna-FT-IR-750* spectrometer; $\tilde{\nu}_{max}$ in cm^{-1}. NMR Spectra: *Bruker DRX-400* spectrometer; δ in ppm rel. to residual $CHCl_3$ (δ(H) 7.26, δ(C) 77.0) as internal standard, *J* in Hz. MS: *Finnigan MAT-95* spectrometer; in *m*/*z*.

Animal Material. Specimens of *Axinyssa* aff. *variabilis*, identified by Prof. *Rob van Soest* of the Zoological Museum, University of Amsterdam, were collected in February, 2004 by scuba techniques at a depth of −10 m off Sanya, Hainan Province, China, in the South China Sea. A voucher specimen is available for inspection at the Shanghai Institute of Materia Medica, CAS, under the registration No. 04LS-146.

Extraction and Isolation. The frozen animals (150 g dry weight) were cut into small pieces and exhaustively extracted with acetone (1.5 l × 3) at r.t. The extract was concentrated, and the resulting residue was extracted with Et_2O and BuOH. The Et_2O-soluble portion was fractionated by CC (silica gel, light petroleum ether with increasing amounts of acetone): nine fractions. The fraction eluted with petroleum ether/acetone 8.5:1.5 was subjected to CC (silica gel, petroleum ether/Et_2O gradient): theonellin isothiocyanate (**4**; 23.7 mg). The fraction eluted with petroleum ether/acetone 8:2 was subjected to CC (silica gel, petroleum ether/Et_2O; then *Sephadex LH-20*, petroleum ether/$CHCl_3$/MeOH 2:1:1): theonellin formamide (**3**; 105.8 mg). The fraction eluted with petroleum ether/acetone 7.5:2.5 was purified by reversed-phase HPLC (MeOH/H_2O 87:13): **1** (5.5 mg) and **2** (2.3 mg).

11-Ethoxy-3-formamidotheonellin (= N-*{*trans-*4-[(1*E*,3*E*)-1,5-Dimethylhexa-1,3-dienyl]-1-methylcyclohexyl}formamide*; **1**): Colorless oil. $[\alpha]_D^{24} = 0.0$ ($c = 0.25$, $CHCl_3$). UV (MeOH): 238 (4.18). IR (KBr): 3273, 1686, 1475, 1325, 1037. ^{1}H- and ^{13}C-NMR: *Table.* HR-EI-MS: 293.2336 (M^+, $C_{18}H_{31}NO_2^+$; calc. 293.2355).

7-Ethoxy-3-formamidobisabolane-8,10-diene (= N-*{*trans-*4-[(2*E*)-1,5-Dimethylhexa-2,4-dienyl]-1-methylcyclohexyl}formamide*; **2**): Colorless oil. $[\alpha]_D^{24} = 3.0$ ($c = 0.10$, $CHCl_3$). UV (MeOH): 238 (4.12). IR (KBr): 3271, 1675, 1475, 1320, 1022. ^{1}H- and ^{13}C-NMR: *Table.* HR-EI-MS: 293.2338 ($C_{18}H_{31}NO_2^+$; calc. 293.2355).

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Received November 8, 2006