New Nitrogenous Germacranes from a Thai Marine Sponge, *Axinyssa* n. sp.

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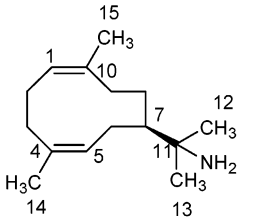
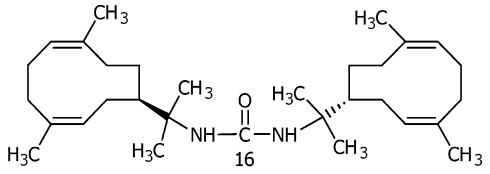
Two new germacrane sesquiterpenes, including (1*Z*,4*Z*)-7α*H*-11-aminogermacra-1(10),4-diene (**1**) and *N*,*N*′-11-bis[(1*Z*,4*Z*)-7α*H*-germacra-1(10),4-dienyl]urea (**2**), were isolated from a Thai marine sponge *Axinyssa* n. sp. collected from the Andaman Sea. The structure elucidations of **1** and **2** were accomplished by means of 1D and 2D NMR, MS, and IR spectroscopy. Only **1** exhibited strong antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Candida albicans*.

Marine sponges of the genus *Axinyssa* (order Halichondrida, family Halichondriidae) have attracted considerable research interest mainly due to the presence of sesquiterpenes containing unusual nitrogenous functional groups, such as isothiocyanate, formamide, isonitrile, and thiocyanate.[1] A guaiane-type sesquiterpene hydroperoxide, sesquiterpene carbonimide dichlorides,[2] and nitrogenous bisabolene-[3] and eudesmane-type[4] sesquiterpenes have also been reported from sponges of this genus. Many nitrogenous-containing terpenes have been found to possess potency in anthelmintic,[1c] antimalarial,[1g] and antifouling[2,5] assays. In our continuing search for bioactive substances from Thai marine sponges, we have found strong antimicrobial activity in the EtOAc extract of an *Axinyssa* n. sp., a reddish brown sponge collected from the Andaman Sea. Purification of the EtOAc extract led to the discovery of two new nitrogenous germacrane compounds, **1** and **2**. We describe here the isolation, structure elucidation, and antimicrobial activity of both new compounds.



[1]



[2]

The EtOAc extract of the sponge *Axinyssa* n. sp. (15 kg, wet wt) was subjected to bioassay-guided fractionation using antimicrobial activity against *S. aureus*, *B. subtilis*, and *C. albicans*. The antimicrobial active extract was repeatedly chromatographed on Si gel and Sephadex LH-20 columns to obtain two fractions containing common ^{1}H NMR signals of the olefinic protons at about δ 5 ppm. These fractions were further purified to obtain two new germacrane-type compounds, **1** (17 mg, 1×10^{-4}% wet wt) and **2** (32 mg, 2×10^{-4}% wet wt).

Compound **1** was obtained as a yellow oil, and its ESITOFMS showed an accurate mass $[M + H]^+$ at *m*/*z* 222.2232, corresponding to a molecular formula of $C_{15}H_{27}N$ and therefore possessing three degrees of unsaturation. The ^{1}H NMR spectrum of **1** displayed signals of four singlet methyls (δ 1.25, 1.58, 1.61, and 1.63), two olefinic methines (δ 5.05 and 5.32), and a bundle of methine and methylene signals resonating at δ 1.77−2.05. The ^{13}C NMR spectrum of **1** displayed 15 carbon signals which were classified into four methyl, five methylene, three methine, and three quaternary carbons by DEPT experiments. The presence of four sp^2 carbon resonances in the ^{13}C NMR spectrum at δ 123.4 (C-1), 133.6 (C-4), 119.9 (C-5), and 131.8 (C-10) represented two degrees of unsaturation, and hence compound **1** must contain a monocyclic ring. The germacrane skeleton, the position of substituents, and unsaturation were analyzed by 2D NMR experiments. The H,H-COSY (Table 1) and HMQC NMR spectra indicated the partial structures $=CH-CH_2-CH_2-$, $=CH-CH_2-CH-$, and $-CH_2-CH_2-$ in the molecule. The connectivity of these fragments with two allylic methyls (δ_C 23.3/δ_H 1.61, 14-CH_3 and δ_C 25.7/δ_H 1.63, 15-CH_3) and the olefinic quaternary carbons (δ 133.6, C-4 and δ 131.8, C-10) to construct a 10-membered ring of the germacrane skeleton was deduced by the following long-range H−C correlations in the HMBC spectrum (Table 1): H_2-8 to C-6; H_3-14 to C-3, C-4, and C-5; and H_3-15 to C-1, C-9, and C-10. The *Z*-orientation of both double bonds was assigned on the basis of the following NOESY correlations: H_3-14 to H-5 and H_3-15 to H-1. Furthermore, the downfield resonances of the allylic methyl carbons at δ 23.3 (C-14) and 25.7 (C-15) also confirmed the designated *Z*-double bonds.[6] The aminoisopropyl side chain was readily identified by the NMR signals of two tertiary methyls (δ_C 22.2/δ_H 1.25, 12-CH_3 and δ_C 17.9/δ_H 1.58, 13-CH_3) and a quaternary carbon attached to the amino group (δ 58.1, C-11). This was supported by the presence of the primary amine band at 3360 and 3212 cm^{-1} in its IR spectrum and the ion peaks at *m*/*z* 205 $[(M + H) - NH_3]^+$ in the TOFMS. The placement of the side chain at C-7 was based on the HMBC correlations of H-7 to C-13 and H_3-12 and H_3-13 to C-7.

Attempts to form *p*-bromobenzoyl amide and dinitrobenzoyl amide derivatives of **1** for X-ray crystallographic study led to decomposed products. Therefore, the stereochemistry of C-7 in **1** was mainly proposed by $[\alpha]_D$ comparison with similar known germacranes containing a single chiral

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Table 1. H,H-COSY, HMBC, and NOESY Correlations of Compounds **1** and **2**

compound **1**				compound **2**			
^{1}H	H,H-COSY	HMBC	NOESY	^{1}H	H,H-COSY	HMBC	NOESY
1	H_2-2	C-15	H_3-15, H_2-2	1 (1′)	H_2-2 (H_2-2′)	C-15 (C-15′)	H_3-15 (H_3-15′)
2	H-1, H_2-3		H_2-3, H-1	2 (2′)	H-1 (H-1′), H_2-3 (H_2-3′)		
3	H_2-2	C-1, C-10	H_2-2	3 (3′)	H_2-2 (H_2-2′)	C-1 (C-1′)	
5	H_2-6		H_3-14, H_2-6	5 (5′)	H_2-6 (H_2-6′)		H_3-14 (H_3-14′)
6	H-5, H-7			6 (6′)	H-5 (H-5′), H-7 (H-7′)	C-7 (C-7′), C-8 (C-8′), C-11 (C-11′)	
7	H_2-6	C-13		7 (7′)	H_2-6 (H_2-6′)		
8	H_2-9	C-6		8 (8′)	H_2-9 (H_2-9′)		
9	H_2-8			9 (9′)	H_2-8 (H_2-8′)	C-15 (C-15′)	
12		C-7, C-11		12 (12′)		C-7 (C-7′), C-11 (C-11′)	
13		C-7		13 (13′)		C-11 (C-11′)	
14		C-3, C-4, C-5	H-5	14 (14′)		C-4 (C-4′), C-5 (C-5′)	H-5 (H-5′)
15		C-1, C-9, C-10	H-1	15 (15′)		C-1 (C-1′), C-10 (C-10′)	H-1 (H-1′)

center at C-7. The germacranes with H-7 in a β-orientation (for example, (−)-germacrene A,[7] (*E*,*S*)-germacra-1,4(15),-11-triene-5-one,[8] and 7*S*-1*E*,4*E*-germacra-1(10),4-diene)[9] have been reported to show negative $[\alpha]_D$ of −3.2°, −167.0°, and −2.8°, respectively. In contrast, the H-7α germacranes (for example, hedycaryol,[10] bacchascandon,[11] and 6-oxogermacra-1(10)*E*,4(15)-diene)[12] have been reported to show positive $[\alpha]_D$ of +24.2°, +28.5°, and +53.0°, respectively. In addition, replacement of H-11 in the isopropyl side chain by a heteroatom (e.g., dilophol[13] and hydroxydilophol[14]) and alteration of *E*- or *Z*-double bonds (e.g., kikkanol D and kikkanol F[15]) affected only the magnitude of $[\alpha]_D$. From the above observation, **1**, which exhibited a positive $[\alpha]_D$ of +28.0°, was established as (1*Z*,4*Z*)-7α*H*-11-aminogermacra-1(10),4-diene, on the basis of the germacranolide nomenclature proposed by Rogers et al.[16] To our knowledge, **1** is the first natural germacrane sesquiterpene containing an amino functional group and possessing *Z*-configuration at both C-1(10) and C-4 double bonds. Few *Z*,*Z*-germacranes have been reported in nature.[17] Metabolite **1** may be biogenetically synthesized from the unusual precursor *Z*,*Z*-farnesyl pyrophosphate.

Compound **2** was isolated as colorless needles. The molecular formula of $C_{31}H_{52}N_2O$ was established by an accurate mass from the ESITOFMS of **2** showing the $[M + H]^+$ peak at *m*/*z* 469.4151. The ^{1}H and ^{13}C NMR spectra of **2** were quite similar to those of **1**, suggesting that **2** was composed of two identical units of 1*Z*,4*Z*-11-aminogermacra-1(10),4-diene. Further NMR studies, including H,H-COSY, HMQC, HMBC, and NOESY experiments (Table 1), confirmed this observation. Finally, the presence of the carbonyl carbon at δ 155.9 in the ^{13}C NMR spectrum and the absorption bands at 3368 and 1642 cm^{-1} in the IR spectrum indicated that the two identical germacrane units were connected through a urea functionality. Compound **2** was subjected to crystallization in several solvents; however, the obtained crystals were not suitable for X-ray crystallographic study. Since **2** exhibited an $[\alpha]_D$ of the same sign and similar magnitude (+32.5°), the stereochemistry at C-7 and C-7′ must be the same as in **1**. Compound **2** was therefore identified as *N*,*N*′-11-bis[(1*Z*,4*Z*)-7α*H*-germacra-1(10),4-dienyl]urea.

Both **1** and **2** were directly detected from the EtOAc crude extract as brown spots on a Si gel TLC plate with hR_f values of 25 and 80, respectively (solvent system: C_6H_{14}−EtOAc (2:3), visualizing agent: anisaldehyde in H_2SO_4). This result ruled out that **2** was a urea artifact of **1** and phosgene from chloroform decomposition during the purification process.

Compound **1** displayed significant antifungal activity against *C. albicans* with an inhibition zone of 27 mm and antibacterial activity against *S. aureus* and *B. subtilis* with inhibition zones of 23 and 22 mm, respectively (all at 500 μg/disk). In contrast to **1**, compound **2** showed no antimicrobial activity at this test level.

Experimental Section

General Experimental Procedures. The optical rotations and IR spectra were measured on a Perkin-Elmer 341 polarimeter and a Perkin-Elmer FT-IR 1760 X, respectively. ^{1}H and ^{13}C, DEPT, H,H-COSY, HMQC, HMBC, and NOESY experiments were obtained from a Bruker AVANCE DPX-300 FT-NMR spectrometer operating at 300 MHz for protons and 75 MHz for carbons. The ESITOFMS were measured on a Micromass LCT mass spectrometer, and the lock mass calibration was applied for the determination of accurate mass.

Animal Material. The sponges were collected from the Andaman Sea, Trang Province, Thailand, at a depth of 10−15 m in April 2002, frozen on site, and stored at −20 °C before extraction. The sponge was identified as *Axinyssa* n. sp. (class Demospongiae, order Halichondrida, family Halichondriidae) by Dr. John N. A. Hooper of Queensland Museum, South Brisbane, Australia. The voucher specimens of this sponge (TR98−16) and the underwater photo are available from our laboratory. A voucher specimen is also deposited at Queensland Museum under the specimen number QM G320224.

Extraction and Isolation. Freshly thawed specimens of the sponge (15 kg wet wt) were cut into small pieces and macerated three times with MeOH (14 L each). The combined extracts were concentrated in vacuo, and the residue was partitioned between EtOAc and H_2O to obtain the crude EtOAc extract (18 g). The crude EtOAc extract was chromatographed on a Si gel column by eluting stepwise with C_6H_{14}−$CHCl_3$, $CHCl_3$, $CHCl_3$−MeOH, and MeOH to give five fractions. The second fraction was repeatedly chromatographed on a Si gel column using C_6H_{14}−$CHCl_3$ (1:4) as an eluent to yield **2** (32 mg). The fourth fraction was successively separated on a Si gel column by eluting with $CHCl_3$−MeOH (10:1) and on a Sephadex LH-20 column by eluting with MeOH to yield **1** (17 mg).

(1*Z*,4*Z*)-7α*H*-11-Aminogermacra-1(10),4-diene (1): yellow oil; $[\alpha]^{25}_D$ +28.0° (*c* 0.2, $CHCl_3$); IR (film) ν_{max} 3360, 3212, 2914, 1617 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.25 (3H, s, H_3-12), 1.58 (3H, s, H_3-13), 1.60 (2H, m, H_2-3), 1.61 (3H, s, H_3-14), 1.63 (3H, s, H_3-15), 1.77 (1H, m, H-7), 1.81 (2H, m, H_2-8), 1.95 (2H, m, H_2-9), 2.00 (2H, m, H_2-6), 2.05 (2H, m, H_2-2), 5.05 (1H, br t, J = 6.0 Hz, H-1), and 5.32 (1H, br s, H-5); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 17.9 (CH_3, C-13), 22.2 (CH_3, C-12), 22.7 (CH_2, C-2), 23.3 (CH_3, C-14), 23.8 (CH_2, C-8), 25.7 (CH_3, C-15), 26.3 (CH_2, C-6), 30.9 (CH_2, C-9), 37.2 (CH_2, C-3), 41.1 (CH, C-7), 58.1 (C, C-11), 119.9 (CH, C-5), 123.4 (CH, C-1), 131.8 (C, C-10), 133.6 (C, C-4); H,H-COSY, HMBC, and NOESY data, see Table 1; ESITOFMS *m*/*z* 222.2232 $[M + H]^+$ (calcd for $C_{15}H_{28}N$, 222.2222).

***N*,*N*′-11-Bis[(1*Z*,4*Z*)-7α*H*-germacra-1(10),4-dienyl]urea (2):** colorless needles; $[\alpha]^{25}_D$ +32.5° (*c* 0.2, $CHCl_3$); IR (film)

ν_{max} 3368, 2921, 1642, 1557 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.05 (2 × 3H, s, H_3-12/ H_3-12′), 1.17 (2 × 2H, m, H_2-8/H_2-8′), 1.50 (2 × 1H, m, H-3a/H-3a′), 1.57 (2 × 3H, s, H_3-13/H_3-13′), 1.59 (2 × 3H, s, H_3-14/H_3-14′), 1.65 (2 × 3H, s, H_3-15/H_3-15′), 1.87 (2 × 2H, m, H_2-9/H_2-9′), 1.89 (2 × 1H, m, H-3b/H-3b′), 1.97 (2 × 2H, m, H_2-6/H_2-6′), 2.00 (2 × 2H, m, H_2-2/H_2-2′), 2.17 (2 × 1H, br t, J = 12.3 Hz, H-7/H-7′), 3.84 (2 × 1H, br s, NH/N′H), 5.10 (2 × 1H, br t, J = 6.2 Hz, H-1/H-1′), and 5.33 (2 × 1H, br s, H-5/H-5′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 17.7 (CH_3, C-13/C-13′), 21.4 (CH_3, C-12/C-12′), 22.7 (CH_2, C-2/C-2′), 23.4 (CH_3, C-14/C-14′), 24.6 (CH_2, C-8/C-8′), 25.8 (CH_3, C-15/C-15′), 26.6 (CH_2, C-6/C-6′), 31.5 (CH_2, C-9/-9′), 36.8 (CH_2, C-3/C-3′), 41.2 (CH, C-7/C-7′), 57.4 (C, C-11/C-11′), 120.7 (CH, C-5/C-5′), 124.6 (CH, C-1/C-1′), 131.1 (C, C-10/C-10′), 133.7 (C, C-4/C-4′), and 155.9 (C, C-16); H,H-COSY, HMBC, and NOESY data, see Table 1; ESITOFMS m/z 469.4151 $[M + H]^+$ (calcd for $C_{31}H_{53}N_2O$, 469.4168).

Antimicrobial Activity. The antimicrobial activity was tested using the agar disk diffusion method[18] against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 10231. All tested bacteria were cultivated on tryptic soy agar slants, TSA (Difco), and the yeast, *C. albicans*, was cultivated on Sabouraud dextrose agar slants, SDA (Difco), at 37 °C for 24 h. The cell cultures were washed from the agar surface and suspended with sterile normal saline solution and standardized to match a 0.5 turbidity standard of MacFarland No. 1, providing approximately 1×10^8 CFU (colony forming units/mL). A loopful of each tested microorganisms was swabbed on the surface of TSA and SDA plates. All tested samples were dissolved in the suitable solvent and then applied on sterile paper disks for disk diffusion assay. The dried paper disks were placed on the surface of the swabbed plates and incubated at 37 °C for 24 h. The diameters of inhibition zones were measured.

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Supporting Information Available: ^1H NMR, ^{13}C NMR, HMQC, HMBC, NOESY, and MS spectra of (1*Z*,4*Z*)-7α*H*-11-aminogermacra-1(10),4-diene (**1**) and *N*,*N*′-11-bis[(1*Z*,4*Z*)-7α*H*-germacra-1(10),4-dienyl]-urea (**2**) and the underwater photo of the sponge *Axinyssa* n. sp. These materials are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (a) He, H.; Faulkner, D. J.; Shumsky, J. S.; Hong, K.; Clardy, J. *J. Org. Chem.* **1989**, *54*, 2511−2514. (b) Marcus, A. H.; Molinski, T. F.; Fahy, E.; Faulkner, D. J.; Xu, C.; Clardy, J. *J. Org. Chem.* **1989**, *54*, 5184−5186. (c) Alvi, K. A.; Tenenbaum, L.; Crews, P. *J. Nat. Prod.* **1991**, *54*, 71−78. (d) He, H.; Salva, J.; Catalos, R. F.; Faulkner, D. J. *J. Org. Chem.* **1992**, *57*, 3191−3194. (e) Compagnone, R. S.; Faulkner, D. J. *J. Nat. Prod.* **1995**, *58*, 145−148. (f) Patil, A. D.; Freyer, A. J.; Reichwein, R.; Bear, M. F.; Faucette, L.; Johnson, R. K.; Haltiwanger, R. C.; Eggleston, D. S. *J. Nat. Prod.* **1997**, *60*, 507−510. (g) Simpson, J. S.; Garson, M. J.; Hooper, J. N. A.; Cline, E. I.; Angerhofer, C. K. *Aust. J. Chem.* **1997**, *50*, 1123−1127.
(2) Hirota, H.; Okino, T.; Yoshimura, E.; Fusetani, N. *Tetrahedron* **1998**, *54*, 13971−13980.
(3) Li, C. J.; Schmitz, F. J.; Kelly, M. *J. Nat. Prod.* **1999**, *62*, 1330−1332.
(4) Petrichtcheva, N. V.; Duque, C.; Duenas, A.; Zea, S.; Hara, N.; Fujimoto, Y. *J. Nat. Prod.* **2002**, *65*, 851−855.
(5) Hirota, H.; Tomono, Y.; Fusetani, N. *Tetrahedron* **1996**, *52*, 2359−2368.
(6) Ganber, D.; Pollak, F. C.; Berger, R. G. *J. Nat. Prod.* **1995**, *58*, 1790−1793.
(7) Weinheimer, A. J.; Youngblood, W. W.; Washecheck, P. H.; Karns, T. K. B.; Ciereszko, L. S. *Tetrahedron Lett.* **1970**, *7*, 497−500.
(8) Suzuki, M.; Kowata, N.; Kobayashi, H.; Tanaka, I. *Chem. Lett.* **1990**, 2187−2190.
(9) Segawa, M.; Yamano, K.; Shirahama, H. *Phytochemistry* **1990**, *29*, 973−974.
(10) Minnaard, A. J.; Wijnberg, J. B. P. A.; de Groot, A. *Tetrahedron* **1994**, *50*, 4755−4764.
(11) Bohlmann, F.; Zdero, C.; Robinson, H.; King, R. M. *Phytochemistry* **1979**, *18*, 1993−1996.
(12) Zdero, C.; Bohlmann, F.; Solomon, J. C.; King, R M.; Robinson, H. *Phytochemistry* **1989**, *28*, 531−542.
(13) Ishitsuka, M.; Kusumi, T.; Kakisawa, H.; Kawakami, Y.; Nagai, Y.; Sato, T. *Tetrahedron Lett.* **1986**, *27*, 2639−2642.
(14) Sun, H. H.; Fenical, W. *J. Org. Chem.* **1979**, *44*, 1354−1356.
(15) Yoshikawa, M.; Morikawa, T.; Toguchida, I.; Harima, S.; Matsuda, H. *Chem. Pharm. Bull.* **2000**, *48*, 651−656.
(16) Rogers, D.; Moss, G. P.; Neidle, S. *J. Chem. Soc., Chem. Commun.* **1972**, 142−143.
(17) Bohlmann, F.; Ehlers, D. *Phytochemistry* **1977**, *16*, 137−138.
(18) Lorian, V. *Antibiotics in Laboratory Medicine*; The Williams & Wilkins Co.: Baltimore, 1980; pp 161−207.

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