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Isohalitulin and Haliclorensins B and C, Three Marine Alkaloids from *Haliclona tulearensis*†

Hagit Sorek,‡ Amira Rudi,‡ Maurice Aknin,§ Emile M. Gaydou,⊥ and Yoel Kashman*,‡

School of Chemistry, Tel-Aviv University, Ramat Aviv, 69978, Israel, Laboratoire de Chimîe des Substances Naturelles et des Aliments, Faculté des Sciences et Techniques, Université de la Réunion, 15 Avenue Rene Cassin, B.P 7151, 97715 Saint Denis, Cedex 9, France, and Equipe Phytochimie, UMR 6171, Faculté des Sciences et Techniques de Saint-Jérôme, Université Paul Cézanne (Aix-Marseille III), Avenue Escadrille Normandie Niémen, 13397, Marseille Cedex 20, France

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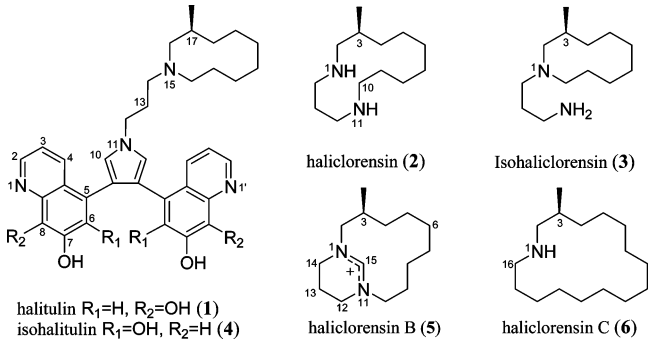
Three new alkaloids, designated isohalitulin (**4**), haliclorensin B (**5**), and haliclorensin C (**6**), were isolated from two specimens of the Madagascan sponge *Haliclona tulearensis*, collected at two locations in Salary Bay, north of Tulear. Their structures were elucidated by extensive spectroscopic means. Alkaloids **4**−**6** exhibited mild toxicity in the brine shrimp test.

Halitulin (**1**) and haliclorensin (**2**) are two unique alkaloids isolated in our group from the marine sponge *Haliclona tulearensis* collected in Sodwana Bay, Durban, South Africa.[1,2] The significant cytotoxicity of haliclorensin against P-388 mouse leukemia cells and that of halitulin against several tumor cell lines has stimulated studies toward the total syntheses of both molecules.[1,2] Steglich and Banwell's syntheses of haliclorensin (**2**) allowed the revision of its structure, from structure **3** to **2**, and the initially assigned structure for haliclorensin was subsequently renamed isohaliclorensin (**3**).[3,4] Furthermore, it was suggested that both compounds **2** and **3** (a precursor of halitulin, **1**) originate from a common 1,11-diazabicyclo[8.4]tetradecane.[3] Two recent reports on the total synthesis of halitulin confirmed the previously assigned structure **1** and allowed the determination of its absolute (17*S*) configuration.[5,6]

Together with halitulin and haliclorensin, an additional related compound was isolated from the same sponge.[1,2] Because this compound was isolated in minute amounts and was highly sensitive to light and air, the elucidation was not accomplished. As part of our ongoing search for novel bioactive substances from marine invertebrates,[7,8] we resumed our work on *Haliclona* sponges. The constituents of two Madagascan *Haliclona tulearensis* sponge specimens were examined for the purpose of finding additional interesting metabolites and hopefully to once again isolate the above-mentioned sensitive compound and complete its elucidation.



The present report describes the isolation and structure elucidation of three new alkaloids, designated isohalitulin (**4**) (the related unstable compound) and haliclorensin B (**5**) and haliclorensin C (**6**), from two collections of *Haliclona tulearensis* collected at Madagascar (referred to below as specimens 1 and 2). Both sponges were collected in Salary Bay, ca. 100 km north of Tulear. The $CHCl_3$−MeOH (2:1) extract of each frozen sample was subjected to solvent partitioning, i.e., aqueous MeOH against hexanes and $CHCl_3$. The $CHCl_3$ fraction of each extract was repeatedly chromatographed over a Sephadex LH-20 column. *H. tulearensis* 1 yielded isohalitulin (**4**) and *H. tulearensis* 2 yielded haliclorensins B (**5**) and C (**6**) as well as the known haliclorensin (**2**).

The EI mass spectrum of isohalitulin (**4**) exhibited a molecular ion $[M]^+$ at *m*/*z* 580, for which the molecular formula $C_{35}H_{40}N_4O_4$ was determined by HREIMS. As the ^{13}C NMR spectrum showed only 24 resonances i.e., 13 sp^3 carbons (one methyl, 11 methylenes, and one methine) and 11 sp^2 carbons (five methines and six quaternary carbons) exactly as in halitulin (**1**), 11 carbon atoms have to be doubled, suggesting compound **4** to be a structural isomer of halitulin. Comparing the NMR spectroscopic data of **4** with halitulin (**1**),[2] including COSY, TOCSY, and HMBC data, determined the identity of the structures with the exception of the two 7,8-dihydroxyquinoline systems. The ^1H and ^{13}C chemical shifts of the aromatic part of **4** implied a different substitution pattern from that in halitulin. A characteristic three-proton spin system (δ and *J* values) confirmed by a COSY experiment [δ_H 8.46 d (J = 3.2 Hz), 6.93 dd (J = 8.0, 3.2 Hz), and 8.14 d (J = 8.0 Hz)] indicated the existence of an unsubstituted pyridine ring of the quinoline system. On the other hand, the adjacent benzene ring, carrying a single proton (δ 7.15 s), has to be trisubstituted. That is, in addition to the connection to the pyrrole ring, the benzene has to carry two OH groups. Hence, it was suggested that compound **4** differs from halitulin (the 7,8-dihydroxy isomer) only in the position of the two OH groups. Two alternative substitutions of the quinoline system were either 6,8- or 6,7-dihydroxyquinoline. Empirical calculations of the carbon chemical shifts for both options[9] excluded the 6,8-dihydroxyquinoline isomer and gave a better match with a 6,7-dihydroxyquinoline, which is the suggested tentative structure.

The small available amounts of isohalitulin and especially its instability prevented further experiments to confirm its structure unequivocally. Unfortunately, halitulin (**1**) was not present in the two Madagascan specimen extracts; however, NMR spectra for both **1** and **4** were recorded in 1999[2] using the same solvent (see Supporting Information) and clearly established the differences in the chemical shifts between both structures. Support for the suggested 6(6′)-hydroxy position came from the change in the proton NMR appearance of the azacyclodecane ring of **4**, in comparison to **1**. The 6(6′) hydroxy group of **4** is expected to form atropisomers around the 5−9(5′−9′) bond (not likely for halitulin), bringing about a nonplanar twisted structure of the aromatic 9,9′-diquinolino pyrrole system. The latter bending may cause a different

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* To whom correspondence should be addressed. Tel: +972-3-6408419. Fax: +972-3-6409293. E-mail: kashman@post.tau.ac.il.

‡ Tel-Aviv University.

§ Université de la Réunion.

⊥ Université Paul Cézanne (Aix-Marseille III).

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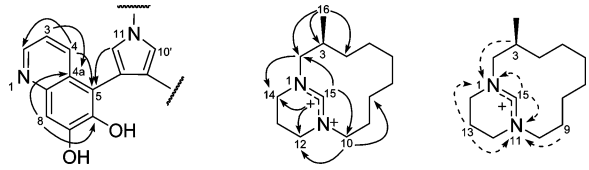

Figure 1. Key ^{13}CH-HMBC (↷) correlations in **4** and **5** and ^{15}NH-HMBC (dashed arrow) correlations in **5**.

conformation of the azacyclodecane ring, thence, change in the appearance of its proton NMR spectrum.

The EIMS spectrum of haliclorensin B (**5**) exhibited a molecular ion $[M]^+$ at *m/z* 223. A molecular formula of $C_{14}H_{27}N_2$ was determined by HREIMS (2.5 degrees of unsaturation), indicating **5** to be positively charged. Comparison of the NMR spectroscopic data of haliclorensin B (**5**) with that of haliclorensin (**2**) showed a resemblance, apart from an additional sp^2 carbon atom (C-15, 153.7 d), bearing an extremely low-field singlet proton resonating at 8.94 ppm. The $^1J_{CH}$ value of this methine (197 Hz, measured from the HMBC experiment) suggested it to be between two nitrogen atoms.[10] In addition, the ^{13}C chemical shifts of the aliphatic C-12 (δ 42.7), C-14 (δ 42.0), C-2 (δ 60.3), and C-10 (δ 54.5) carbons indicated that each one of these four carbons was vicinal to a nitrogen atom. Comparison with haliclorensin (**2**) indicated that the chemical shifts of both C-2 and C-10 were downfield shifted by ca. 10 ppm. HMBC correlations observed from the low-field H-15 to C-2, C-10, C-12, and C-14 (Figure 1) determined the position of CH-15. Further support for the location of the immonium cation came from ^{13}CH-HMBC correlations from H_2-2 to C-14 and from H_2-10 to C-12. The proton and carbon low-field resonances of both CH_2-2 and CH_2-10 suggested that the positive charge of the immonium ion is, as could be expected, spread over the two nitrogen atoms. Further support for the structure came from a ^{15}NH-HMBC experiment. In previous papers we demonstrated the benefit of using ^{15}N NMR data (measured from ^{15}NH-HMBC correlations; $^2J_{NH}$ and $^3J_{NH}$) for the structure determination of various N-atom-containing compounds.[10,11] The ^{15}NH-HMBC correlations between H-3, H_2-9, H_2-13, and H-15 to a single resonance signal at 113.7 ppm determined that both N-1 and N-11 displayed the same low-field chemical shift due to the similar partial positive charge on both nitrogen atoms. Haliclorensin (**2**), on the other hand, revealed correlations from H-3, H_2-9, H_2-13, and H_2-2 to a broad signal at 43.5 ppm, representing both amines N-1 and N-11. To the best of our knowledge, the only naturally reported tetrahydropyrimidinium ring, as in **5**, is the pyrrole-derived alkaloid *N*-methylmanzacidin C isolated from the sponge *Axinella brevistyla*.[12] However, the tetrahydropyrimidinium ring is known synthetically.[13]

The third isolated compound, haliclorensin C (**6**), was obtained in minute amounts (2 mg, 0.002 wt %). Its ^{1}H and ^{13}C NMR spectroscopic data were similar to those of haliclorensin (**2**); however, compound **6** possesses 12 overlapping methylenes, deduced from the ^{13}C NMR and MS data, and only one nitrogen atom. The mass spectrum of compound **6** suggested the formula $C_{16}H_{33}N$ (established by HREIMS), indicating a single degree of unsaturation as in haliclorensin and one amino group. In addition, in the ^{13}C NMR spectrum there were only two methylenes α to nitrogen, C-2 (δ 50.5) and C-16 (δ 45.1). The HMBC experiment showed correlations between the methyl protons and their neighboring C atoms (C-2, C-3, and C-4) and also correlations between both methylenes, H_2-2 and H_2-4, and the doublet methyl. In the COSY spectrum, correlations were observed between H_2-2 and H-3 and between H-3 and the doublet methyl. Hence, the structure of **6** was assigned as 3-methylazacyclohexadecane. Haliclorensin C (**6**) joins two other marine naturally occurring aza-cycloalkanes, i.e., keramaphidine C (6*Z*-azacycloundecene), the first reported marine azamacrocycle,[14] and haliclorensin (**2**), 7-methyl-1,5-diazacyclotetradecane.[1] On the grounds of common biogenetic precursors it is tentatively suggested that isohalitulin (**4**) and haliclorensins B (**5**) and C (**6**) have the same absolute configuration of the single stereogenic center (*S*) as determined for halitulin (**1**) and haliclorensin (**2**).

Obtaining different secondary metabolites from the two Salary Bay collections of *H. tulearensis* (−1 and −2, *vide supra*) and from a sample collected on the other side of the Mozambique Canal raises the question of the real source of the compounds, namely, the sponge or guest microorganisms. Isohalitulin (**4**) and haliclorensins B (**5**) and C (**6**) were tested for toxicity to brine shrimp (*Artemia salina*)[15] and were found to be moderately active. Isohalitulin (**4**) shows a greater potency, with a LD_{50} value of 0.9 mM, while haliclorensins B (**5**) and C (**6**) have LD_{50} values of 2.2 and 2.1 mM, respectively.

Experimental Section

General Experimental Procedures. Optical rotations were obtained with a Jasco P-1010 polarimeter. IR spectra were obtained with a Bruker FTIR Vector 22 spectrometer. NMR spectra were acquired on a Bruker Avance-500 spectrometer (using standard Bruker pulse sequences) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C using the residual solvent signals as an internal reference ($CDCl_3$ δ_H 7.26 ppm, δ_C 77.0). The ^{15}NH-HMBC experiment was optimized for a delay of 65 ms, and the ^{15}N chemical shift is reported with respect to liquid NH_3 as the reference standard. High-resolution mass spectrometric data were obtained on a Fisons, Autospec Q instrument.

Biological Material. *Haliclona tulearensis* (Vasseur and Lévi, 1976) class Demospongiae, order Haplosclerida, family Chalinidae, is a clear brown sponge with oscules of 7−8 mm. Both *H. tulearensis* 1 and 2 were collected in Madagascar, at Salary Bay, ca. 100 km north of Tulear. *H. tulearensis* 1 was collected in January 2007 by scuba at a depth of 30 m, and *H. tulearensis* 2 was collected in January 2006 in the zone spurs grooves at a depth of 9 to 22 m. A voucher specimen from specimen 1 is deposited at Station Marine d'Endoume, Marseille (voucher number MHNM.16030).

Extraction and Isolation. A frozen wet sample of *H. tulearensis* 1 (AMSA-111; 93 g) was homogenized and extracted with $CHCl_3$−MeOH (2:1) to give after evaporation a crude extract (1.2 g). Partitioning using a modified Kupchan procedure[16] yielded the $CHCl_3$ fraction (240 mg). The $CHCl_3$ fraction was chromatographed on Sephadex LH-20, eluting with hexanes−MeOH−CH_2Cl_2 (2:1:1), to afford isohalitulin (**4**, 5 mg, 0.005 wt %).

A frozen wet sample of *H. tulearensis* 2 (AGK-134; 86 g) was homogenized and extracted with $CHCl_3$−MeOH (2:1) to give, after evaporation, a crude extract (0.82 g). Partitioning using a modified Kupchan procedure[16] yielded the $CHCl_3$ fraction (775 mg). The $CHCl_3$ fraction was chromatographed on Sephadex LH-20, eluting with hexanes−MeOH−CH_2Cl_2 (2:1:1), to afford haliclorensin (**2**, 12 mg, 0.01 wt %), haliclorensin B (**5**, 6 mg, 0.007 wt %), and haliclorensin C (**6**, 2 mg, 0.002 wt %).

Haliclorensin (2):[1] brown-orange oil; $[\alpha]^{20}_D$ −19 (*c* 0.57, MeOH); IR (KBr) ν_{max} 3397, 2930, 1617, 1507, 1473 cm^{-1}; ^{13}C NMR (DMSO-d_6, 125 MHz) δ 53.1 (CH_2, C-2), 30.7 (CH, C-3), 29.7 (CH_2, C-4), 24.9 (CH_2, C-5), 24.4 (CH_2, C-6), 25.3 (CH_2, C-7), 23.9 (CH_2, C-8), 22.2 (CH_2, C-9), 48.0 (CH_2, C-10), 45.7 (CH_2, C-12), 21.5 (CH_2, C-13), 47.1 (CH_2, C-14), 18.5 (CH_3, 3-Me); EIMS *m/z* 212 $[M]^+$; HREIMS *m/z* 212.2254 (calcd for $C_{13}H_{28}N_2$, 212.2252). In this context it should be mentioned that the NMR chemical shifts of **2** were shifted downfield (to the reported values; ca. up to 5 ppm) probably because of acidic pH.

Isohalitulin (4): brown-orange oil; $[\alpha]^{20}_D$ +18 (*c* 0.28, MeOH); IR (KBr) ν_{max} 3397, 2930, 1617, 1507, 1473 cm^{-1}; ^{1}H NMR ($CDCl_3$ + MeOH-d_4, 4:1, 500 MHz) δ 8.46 (2H, d, J = 3.2, H-2), 6.93 (2H, dd, J = 8.0, 3.2, H-3), 8.14 (2H, d, J = 8.0, H-4), 7.15 s (2H, s, H-8), 6.88 (2H, s, H-10), 4.08 (2H, m, H-12), 1.67 (1H, m, H-13a) 1.44 (1H, m, H-13b), 2.61 (2H, m, H-14), 2.44 (1H, m, H-16a), 2.21 (1H, m, H-16b), 1.90 (1H, m, H-17), 1.55 (2H, m, H-18), 1.35−1.70 (10H, m, H-19−23), 2.80 (1H, m, H-24a), 2.30 (1H, m, H-24b), 0.83 (3H, d, J = 6.8, 17-Me); ^{13}C NMR (125 MHz) δ 147.4 (CH, C-2), 117.8 (CH, C-3), 135.5 (CH, C-4), 122.5 (C, C-4a), 125.3 (c, C-5), 135.2 (C, C-6), 141.8 (C, C-7) 120.6 (CH, C-8), 138.6 (C, C-8a), 120.8 (C, C-9), 121.0 (CH, C-10), 48.1 (CH_2, C-12), 31.9 (CH_2, C-13), 52.3 (CH_2, C-14),

60.5 (CH, C-16), 29.9 (CH, C-17), 32.0 (CH_2, C-18), 22.2−26.4 (CH_2, C-19−23), 53.1 (CH_2, C-24), 19.4 (CH_3, 17-Me); EIMS *m/z* 580 $[M]^+$; HREIMS *m/z* 580.3049 (calcd for $C_{35}H_{40}N_4O_4$, 580.3049).

Haliclorensin B (5): brown oil; $[\alpha]^{20}_D$ −87 (*c* 0.20, MeOH); IR (KBr) ν_{max} 3320, 2930, 1680, 1465 cm^{-1}; ^1H NMR ($CDCl_3$ + MeOH-d_4, 4:1, 500 MHz) δ 3.68 (1H, t, J = 4.5, H-2a), 3.45 (1H, dt, J = 14.4, 3.6, H-2b), 1.91 (1H, m, H-3), 1.26−1.40 (10H, m, H-4−8), 1.64 (2H, m, H-9), 3.90 (1H, br quin, H-10a), 3.56 (1H, t, J = 12.4, H-10b), 3.30 (2H, m, H-12), 2.17 (2H, m, H-13), 3.30 (2H, m, H-14), 8.94 (1H, s, H-15), 0.93 (3H, d, J = 6.9, 3-Me); ^{13}C NMR (125 MHz) δ 60.3 (CH_2, C-2), 29.5 (CH, C-3), 31.0 (CH_2, C-4), 21.6−26.7 (CH_2, C-5−8), 23.6 (CH_2, C-9), 54.5 (CH_2, C-10), 42.0 (CH_2, C-12), 18.5 (CH_2, C-13), 42.7 (CH_2, C-14), 153.7 (CH, C-15), 17.8 (CH_3, 3-Me); EIMS *m/z* 223 $[M]^+$, HREIMS *m/z* 223.2174 (calcd for $C_{14}H_{27}N_2$, 223.2174).

Haliclorensin C (6): brown oil; $[\alpha]^{20}_D$ +53 (*c* 0.15, MeOH); IR (KBr) ν_{max} 3330, 2938, 2850, 1460 cm^{-1}; ^1H NMR ($CDCl_3$ + MeOH-d_4, 4:1, 500 MHz) δ 2.85 (1H, m, H-2a), 2.75 (1H, m, H-2b), 1.85 (1H, m, H-3), 1.72 (2H, m, H-15), 2.94 (2H, m, H-16); ^{13}C NMR (125 MHz) δ 50.5 (CH_2, C-2), 28.8 (CH, C-3), 32.3 (CH_2, C-4), 23.4 (CH_2, C-15), 45.1 (CH_2, C-16), 17.6 (CH_3, 3-Me); EIMS *m/z* 239 $[M]^+$; HREIMS *m/z* 239.2613 (calcd for $C_{16}H_{33}N$, 239.2613).

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Supporting Information Available: ^1H and ^{13} C NMR spectra for **4**−**6** and ^1H NMR spectra for **1** and **4**, which were recorded (during 1999) using the same conditions for comparison between the two compounds and tabulated spectroscopic data for **4** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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