Two New Cytotoxic Carbonimidic Dichlorides from the Nudibranch *Reticulidia fungia*

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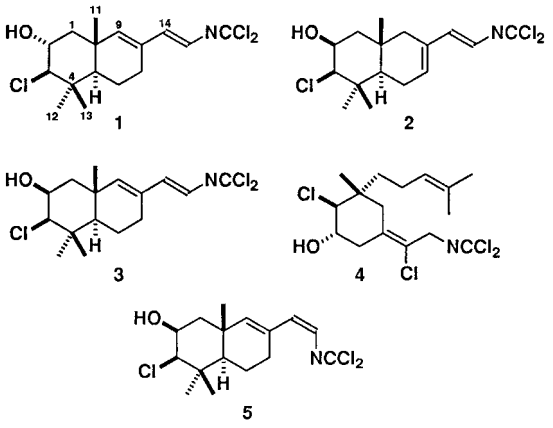
Two new sesquiterpenes, reticulidins A (**1**) and B (**2**), containing a rare functional group, N=CCl_2, have been isolated together with known congeners from the nudibranch *Reticulidia fungia* and their structures elucidated by spectroscopic data. Both **1** and **2** were cytotoxic against KB and L1210 cells.

In 1977 Faulkner reported sesquiterpenes containing an unusual functional group N=CCl_2, the first naturally occurring carbonimidic dichlorides.[1] The presence of this functionality was proved by comparison of spectroscopic and chemical properties with a synthetic compound and then by X-ray analysis. Faulkner's group subsequently isolated six sesquiterpene carbonimidic dichlorides altogether from the sponge *Pseudoaxinyssa pitys*.[2,3] More recently four additional sesquiterpenes of this class have been reported by two other groups, one by Garson's group from the sponge *Stylotella aurantium*[4] and three by Fusetani's group from a sponge, *Axinyssa* sp.[5] To date only 10 naturally occurring carbonimidic dichlorides, all sesquiterpenes, have been described from three sponges. By feeding experiments with ^{14}C-labeled cyanide Garson suggested their biogenesis through corresponding isonitriles and isothiocyanates.[4,6,7] Fusetani's group reported them to have potent antifouling properties.

In a chemoecological study of marine invertebrates we recently found two new carbonimidic dichlorides, reticulidins A (**1**) and B (**2**), together with two known compounds (**3**, **4**) from the nudibranch *Reticulidia fungia* (family Phyllidiidae).[8] The new compounds exhibited moderate cytotoxicity. In this report we describe the isolation and the structures of these interesting metabolites.



Four individuals of *R. fungia*, collected at Irabu Island, Okinawa, were extracted with acetone. The CH_2Cl_2 soluble portion of the extract was separated on silica gel followed by preparative silica TLC to give four sesquiterpenes **1**−**4**. The known compounds **3** and **4** were readily identified by comparing their spectral data with those reported in the literature.[1,3] EIMS of reticulidin A (**1**) gave a cluster of molecular ions at *m/z* 349, 351, 353, and 355, indicating the presence of three chlorine atoms. The HREIMS established the molecular formula $C_{16}H_{22}Cl_3NO$. Comparison of the IR (1643 cm^{-1}), UV (λ_{max} 289 nm), and NMR data with those of **3** and **5**[2,3] revealed that it was composed of virtually the same structural elements including an $NCCl_2$ (δ 124.3 s) group conjugated with a diene [δ 129.2 d, 130.8 s, 137.5 d, 145.3 d; δ 5.64 s, 6.52 (d, J = 13.1 Hz), 6.72 (d, J = 13.1 Hz)] as in **3**. The bicyclic portion of the structure was established by 2D NMR (COSY, HMQC, HMBC) analysis (see Experimental Section for the signal assignment). Placement of a hydroxyl group at C-2 [δ 68.6; δ 3.91 (ddd, J = 12.2, 10.1, 4.3 Hz)] rather than at C-3 [δ 80.6; δ 3.61 (d, J = 10.1 Hz)] was confirmed by a deuterium-induced shift experiment.[9] When a ^{13}C NMR spectrum was taken in $CDCl_3 + D_2O$, the signals at δ 68.8 and 80.6 were observed shifted to slightly higher field. The $\Delta\delta$ values were 0.124 and 0.033, respectively, indicating the presence of the hydroxyl at the C-2 and thus the chlorine atom at the C-3. The large coupling constant (10.1 Hz) between H-2 and H-3 revealed their trans diaxial relationship. The NOE observed between H-3 and H-5 and between H-2 and H-11 pointed to the relative configuration of the molecule as depicted. A somewhat small coupling constant (J = 13.1 Hz) between H-14 and H-15 was consistent with the trans geometry observed with **3** (J = 13 Hz).

Reticulidin B (**2**) had the same molecular formula $C_{16}H_{22}Cl_3NO$ with those of **1** and **3**, as deduced from HREIMS, indicating that it was a related isomer. The presence of the $NCCl_2$ function and its extended conjugation with two double bonds (δ 128.4 d, 131.9 s, 133.8 d, 137.8 d; δ 5.99 m, 6.60 d, 6.67 d) were revealed by similar spectral features, i.e., IR (1641 cm^{-1}), UV (λ_{max} 291 nm) and ^{13}C NMR signal at δ 124.0 (C-16). 2D NMR (COSY, HMQC, HMBC) analysis established the planar structure of **2** including the position of the endocyclic double bond at C-7. The position of the hydroxyl group at C-2 was again confirmed by a deuterium-induced shift experiment, i.e., $\Delta\delta$ 0.115 for C-2 and 0.016 for C-3. The same relative stereochemistry with that of **3** was determined by the observation of a small coupling constant (J = 3.0 Hz) between H-2 and H-3 and of the positive NOEs (H-2/H-3, H-3/H-12, H-12/H-5, H-13/H-11). The C-14/C-15 double bond was trans (J = 13.4 Hz) as in **1** and **3**.

Both reticulidins A and B were moderately cytotoxic with IC_{50} 0.41 and 0.42 μg/mL against KB cells and 0.59 and 0.11 μg/mL against L1210 cells, respectively.

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Experimental Section

General Experimental Procedures. IR spectra were measured on a Jasco FT/IR 300 and UV spectra on a Jasco UVIDEC 610 spectrophotometer. NMR spectra were recorded on a JEOL A500 instrument at 500 MHz (^{1}H) and 125 MHz (^{13}C). LR and HREIMS were taken on a Hitachi M-2500 mass spectrometer and optical rotation on a Jasco DIP-1000 polarimeter.

Animal Material. Four specimens (4.2 g wet wt) of *R. fungia* were collected by hand using scuba at a depth of 25 m in Irabu Island, Okinawa, June, 1997. A voucher specimen (JT-43-71) is deposited at Department of Chemistry, Biology, and Marine Science, University of the Ryukyus. Taxonomic identification was made by consulting with Prof. R. F. Bolland, University of Maryland/Asian Division.

Extraction and Isolation. Freshly collected specimens were kept frozen until extraction. The whole animals were extracted with acetone (30 mL). Extraction was repeated with fresh solvent three times. The combined extracts were concentrated in vacuo, and the resulting residue was extracted with CH_2Cl_2 to give 38.3 mg of an oil. Most of the oil (37.2 mg) was separated on a small silica gel column to give three fractions. The first fraction was further separated by preparative TLC (silica, heptane−CH_2Cl_2, 1:1) to give compounds **2** (1.1 mg), **3** (0.1 mg), and **4** (0.4 mg). Similar separation of the second fraction on preparative TLC (CH_2Cl_2) gave compounds **1** (1.8 mg) and **4** (7.0 mg).

Reticulidin A (1): colorless glass; $[\alpha]^{26}_D$ +11° (*c* 0.32, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 289 nm (4.3); IR (neat) ν_{max} 3426, 1643, 1575, 897 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.97 (3H, s, H-13), 1.08 (3H, s, H-11), 1.14 (3H, s, H-12), 1.29 (1H, dd, J = 12.5, 2.1 Hz, H-5), 1.35 (1H, t, J = 12.2 Hz, H-1α), 1.62 (1H, m, H-6β), 1.89 (1H, brdd, J = 13.4, 7.0 Hz, H-6α), 2.03 (1H, dd, J = 12.2, 4.3 Hz, H-1β), 2.21 (1H, m, H-7α), 2.40 (1H, m, H-7β), 2.48 (brs, OH), 3.61 (1H, d, J = 10.1 Hz, H-3), 3.91 (1H, ddd, J = 12.2, 10.1, 4.3 Hz, H-2), 5,64 (1H, s, H-9), 6.52 (1H, d, J = 13.1 Hz, H-14), 6.72 (1H, d, J = 13.1 Hz, H-15); ^{13}C NMR ($CDCl_3$) δ 17.9 q (C-13), 19.2 t (C-6), 21.9 q (C-11), 26.1 t (C-7), 29.3 q (C-12), 36.5 s (C-10), 40.3 s (C-4), 45.2 t (C-1), 50.9 d (C-5), 68.8 d (C-2), 80.6 d (C-3), 124.3 s (C-16), 129.2 d (C-15), 130.8 s (C-8), 137.5 d (C-14), 145.3 d (C-9); EIMS *m*/*z* 355 (2, [M$^+$]), 353 (18), 351 (54), 349 (57), 318 (18), 316 (73), 314 (100), 300 (11), 298 (38), 296 (42), 282 (7), 280 (29), 278 (53); HREIMS *m*/*z* 349.0735 (calcd for $C_{16}H_{22}{}^{35}Cl_3NO$, 349.0765).

Reticulidin B (2): colorless glass; $[\alpha]^{26}_D$ −26° (*c* 0.092, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 291 nm (4.2); IR (neat) ν_{max} 3451, 1641, 899 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.06 (3H, s, H-12), 1.16 (3H, s, H-13), 1.19 (3H, s, H-11), 1.46 (1H, dd, J = 11.7, 5.5 Hz, H-5), 1.53 (1H, m, H-1α), 1.91 (1H, brd, J = 15.9 Hz, H-9α), 2.02 (1H, d, J = 15.9 Hz, H-9β), 2.21 (1H, dd, J = 14.7, 3.1 Hz, H-1β), 2.26 (1H, m, H-6β), 2.34 (1H, m, H-6α), 3.94 (1H, d, J = 3.0 Hz, H-3), 4.20 (1H, m, H-2), 5.99 (1H, m, H-7), 6.60 (1H, d, J = 13.4 Hz, H-14), 6.67 (1H, d, J = 13.4 Hz, H-15); ^{13}C NMR ($CDCl_3$) δ 17.7 q (C-13), 20.7 q (C-11), 25.4 t (C-6), 30.0 q (C-12), 31.9 s (C-10), 39.0 s (C-4), 44.8 t (C-9), 45.3 t (C-1), 49.2 d (C-5), 72.1 d (C-2), 76.5 d (C-3), 124.0 s (C-16), 128.4 d (C-15), 131.9 s (C-8), 133.8 d (C-7), 137.8 d (C-14); EIMS *m*/*z* 355 (4, [M$^+$]), 353 (32), 351 (95), 349 (100), 318 (12), 316 (63), 314 (95), 278 (38); HREIMS *m*/*z* 351.0730 (calcd for $C_{16}H_{22}{}^{35}Cl_2{}^{37}ClNO$, 351.0736).

Cytotoxicity Test. The assay was conducted by Prof. M. Kobayashi and Dr. S. Aoki at Faculty of Pharmaceutical Sciences, Osaka University. KB and L1210 cells were seeded in 100 μL of RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and kanamycin (50 μg/mL). Cells were exposed to graded concentrations of reticulidins at 37 °C for 72h in triplicate. Cytotoxicity was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Results were expressed as IC_{50} determined by the concentration that reduced by 50% the optical density of treated cells with respect to the optical density of untreated controls.

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