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Symbioimine Exhibiting Inhibitory Effect of Osteoclast Differentiation, from the Symbiotic Marine Dinoflagellate *Symbiodinium* sp.

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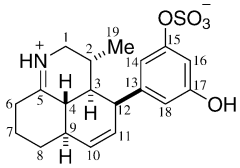
Marine organisms produce various molecules with remarkable physiological activities. In particular, large polyol and polyether compounds, such as palytoxin, halichondrin, ciguatoxin, and maitotoxin, are some of the most attractive molecules in natural products chemistry.[1] These compounds are composed of a long carbon backbone functionalized by oxygen and have been called "super-carbon-chain compounds".[1a] It has been suggested that most of these bioactive metabolites are biosynthesized by marine microorganisms, i.e., bacteria and blue-green algae, that are in the food chain of, or in a symbiotic relationship with, their host animals.

The symbiotic marine dinoflagellate *Symbiodinium* sp., which is a type of zooxanthellae, is found in a wide range of marine invertebrates[2] and produces several super-carbon-chain compounds, such as zooxanthellatoxins[3] and zooxanthellamides.[4] In our continuing search for biologically active compounds, we have isolated a unique amphoteric iminium compound, named symbioimine (**1**), from this dinoflagellate. We describe here the isolation, structure elucidation, and biological activities of **1**.



Symbioimine (**1**)

The cultivated dinoflagellate (36 g), isolated from the marine acoel flatworm *Amphiscolops* sp., was extracted with 80% aqueous ethanol. The concentrated extract was partitioned with ethyl acetate and water, and the aqueous layer was chromatographed on TSK G-3000S polystyrene gel and DEAE-Sephadex. Final purification was achieved by reversed-phase HPLC to give symbioimine (**1**) [5.7 mg; $[\alpha]^{27}_{D}$ 245° (*c* 0.10, DMSO)]. Symbioimine (**1**) inhibited osteoclastogenesis of the murine monocytic cell line RAW264, which can differentiate into osteoclasts following treatment with receptor activator of nuclear factor κB ligand, (RANKL, EC_{50} = 44 μg/mL),[5,6] whereas its cell viability was not affected even at 100 μg/mL. Thus, symbioimine (**1**) is an antiresorptive drug candidate for the prevention and treatment of osteoporosis in postmenopausal women.

The molecular formula of **1** was found to be $C_{19}H_{23}NO_5S$ [(M + H)$^+$, *m*/*z* 378.1368, Δ −0.7 mmu; (M − H)$^-$, *m*/*z* 376.1213, Δ −0.6 mmu] by HRESIMS. Negative ESIMS also showed a characteristic fragment ion [(M − SO_3H)$^-$, *m*/*z* 296] that suggested

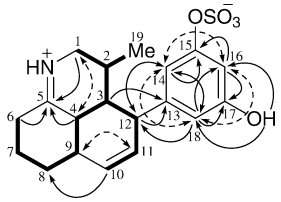


Figure 1. Gross structure of **1** determined by 2D-NMR spectroscopy (bold lines, COSY; arrows, selected HMBC correlations; dotted arrows, ^{1}H−^{1}H long-range couplings).

the presence of a sulfate moiety. A detailed analysis of the ^{1}H, ^{13}C NMR, COSY, HMQC, and HMBC spectra in DMSO-d_6 showed that **1** contained one methyl group, four methylenes, 10 methines, four quaternary carbons, and two protons on heteroatoms (δ_{NH} 12.82, δ_{OH} 9.35). In addition to supporting the presence of hydroxyl groups (3450 cm^{-1}), the IR (KBr) spectrum of **1** showed absorption bands for iminium (1690 cm^{-1}) and sulfate (1240, 1140, 1050 cm^{-1}) groups. The ^{13}C NMR signal at 188.0 (C-5) implied the presence of an iminium functionality in this water-soluble amphoteric compound.

A detailed analysis of the COSY spectrum of **1** suggested partial carbon−carbon connectivities: C-1 to C-12 except for C-5 and a methyl group (C-19) (Figure 1). Homoallylic coupling between H-1/H-4 and HMBC correlations between H-1/C-5, H-4/C-5, and H-6$_b$/C-5 were observed, which suggested that C-5 could be assigned to be the carbon of the imine group.[7] Moreover, the allylic coupling between H-9/H-11 and the HMBC correlation between H-10/C-8 indicated that **1** possessed a 6,6,6-tricyclic ring system that included C-1 to C-12 and a nitrogen atom. The aromatic ring structure of **1** was established by HMBC correlations H-14/C-15 and C-18, H-16/C-15 and C-17, H-18/C-14 and C-17. Three aromatic protons could be placed on a 3,5-dioxygenated benzene based on the magnitudes of meta-coupling ($J_{14,16}$ = $J_{14,18}$ = $J_{16,18}$ = 1.8 Hz) and the ^{13}C NMR chemical shifts (δ_{C-15} 154.1 and δ_{C-17} 157.2). The HMBC correlations between O*H*/C-16 and C-18 confirmed that the hydroxyl group was directly connected to the aryl carbon (C-17). Due to the possible asymmetry of the aryl moiety (H-14 and H-18, C-14 and C-18, and C-15 and C-17), the remaining oxygenated aryl carbon (C-15) may be linked to a sulfate group. The HMBC correlations H-3/C-13, H-12/C-13 and C-14, H-14/C-12, and H-18/C-12 suggested that the aryl carbon (C-13) was linked to the methine carbon (C-12). Thus, symbioimine (**1**) was confirmed to be a 6,6,6-tricyclic iminium ring compound possessing an aryl sulfate moiety, as shown in Figure 1.

The relative stereochemistry of **1** was deduced as follows. The large magnitude of $J_{1a,2}$ = 12.0 Hz, $J_{2,3}$ = 11.1 Hz, $J_{3,4}$ = 11.1 Hz, $J_{4,9}$ = 11.9 Hz, $J_{7a,8a}$ = 12.9 Hz, and $J_{8a,9}$ = 12.9 Hz suggested that all seven of these protons, H-1a, H-2, H-3, H-4, H-7a, H-8a,

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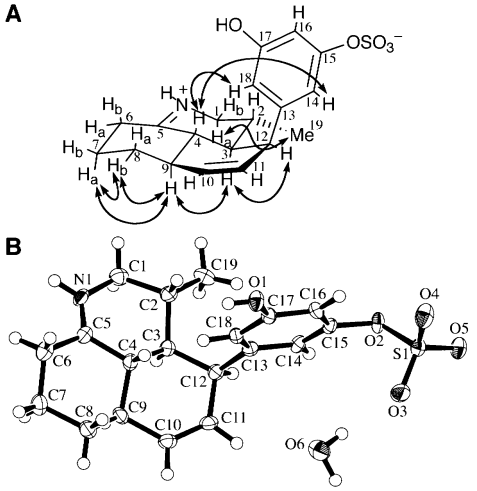

Figure 2. (A) Relative stereochemistry of **1** determined by 2D-NMR spectroscopy (arrows, selected NOESY correlations). Higher-field methylene proton signals were labeled "a" and lower-field signals were "b". (B) ORTEP drawing of **1**. The dihedral angle (deg) between C3−C12−C11 and the aromatic ring = 86.73(9); selected torsion angles (deg): C3−C12−C13−C18 = 53.0(5), C11−C12−C13−C18 = −74.4(4).

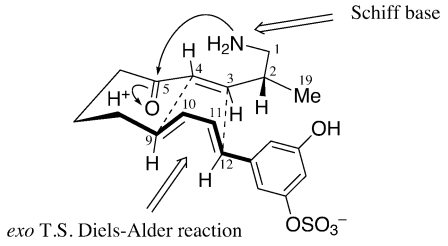

Figure 3. Plausible biogenetic pathway for the carbon framework of **1**.

and H-9a, were oriented in anti arrangements with respect to the tricyclic ring (Figure 2A). In NOE experiments (800 MHz) on **1**, NOEs were observed for H-1a/H_3-19, H-3/H-9, H-3/H-12, H-7a/H-8b, H-7a/H-9, and H-8b/H-9. These results suggested that these three six-membered rings may show trans ring fusion with each other and that the methyl group (C-19) may be oriented in a pseudoequatorial conformation with respect to the six-membered iminium ring with a twist-boat conformation. NOEs were also observed for H-4/H-14 and H-4/H-18, suggesting that the aryl moiety may be oriented in a pseudoaxial conformation with respect to the cyclohexene ring with a twist-boat conformation.

The stereostructure of **1** was confirmed by X-ray crystallographic analysis (Figure 2B),[8] and it completely coincided with those determined by the spectroscopic analysis described above. Symbioimine (**1**) was recrystallized from water to give well-formed, monocyclic colorless crystals as monohydrate: mp 214−215 °C (dec). The absolute stereochemistry of **1** was confirmed to be 2*R*, 3*R*, 4*S*, 9*R*, 12*S*, from the value of the Flack parameter 0.03(13). Notably, the tricyclic iminium ring was oriented almost orthogonal to the aromatic ring.

This unique structure of **1**, including a 6,6,6-tricyclic iminium ring, can be explained by the plausible biogenetic pathway shown in Figure 3. An intramolecular *exo* transition-state Diels−Alder reaction followed by imine cyclization could form the carbon framework of **1** stereospecifically, as in the case of pinnatoxins.[9] Various piperidine alkaloids have been reported in terrestrial organisms,[10] and their biosynthesis has been well-studied.[11] A large group of piperidine alkaloids have been shown to be derived from lysine or acetic acid based on experiments using labeled precursors. Various marine metabolites with an imine moiety have also been described, but no natural compounds with a tricyclic iminium ring structure, such as in **1**, have been reported in either terrestrial or marine organisms. Further biological studies on symbioimine (**1**) are in progress.

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Supporting Information Available: Experimental procedures, a table of NMR data, 1D and 2D NMR spectra, results of osteoclast differentiation assay, and crystallographic information for **1** (PDF and CIF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Under the HMBC measurement of **1** (DMSO-d_6, $^{2,3}J_{CH}$ = 6 Hz), correlations between H-4/C-5 and H-6_b/C-5 were not observed. These cross-peaks were observed when the $^{2,3}J_{CH}$ value was changed to 8 Hz.

(8) Crystal data for **1**: orthorhombic, $P2_12_12_1$, a = 7.055(2) Å, b = 15.043-(5) Å, c = 17.448(6) Å, V = 1851(1) Å^3, Z = 4, D_{calcd} = 1.418 g/cm^3. T = −150 °C, R (Rw) = 0.080 (0.083) based on 4135 reflections {$I > 0.00\sigma(I)$} and 256 variable parameters.

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