Salicylihalamides A and B, Novel Cytotoxic Macrolides from the Marine Sponge Haliclona sp.

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Two novel, highly potent, cytotoxic macrolides, salicylihalamides A (1) and B (2), were isolated from the sponge Haliclona sp. This new macrolide class incorporates salicylic acid, a 12-membered lactone ring, and an enamide side chain. COMPARE pattern-recognition analyses of the NCI 60-cell mean graph screening profiles of 1 did not reveal any significant correlations to the profiles of known antitumor compounds in the NCI’s “standard agent database”, thus supporting the conclusion that the salicylihalamides represent a potentially important new class for antitumor lead optimization and in vivo investigations.

Introduction

Sponges of the genus Haliclona are well-known for producing a variety of secondary metabolites, most commonly bioactive alkaloids. In our screening of crude extracts for differential cytotoxicity, the organic extract of an Australian collection of an unidentified species of Haliclona (phylum Porifera, class Demospongiae, order Haplosclerida, family Haliconidaceae) displayed a potent and unique differential cytotoxicity profile in the NCI 60-cell line human tumor assay. Bioassay-guided fractionation of this extract afforded salicylihalamide A (1), a novel salicylate macrolide with a highly unsaturated amide side chain. We report herein the structure elucidation of this unusual metabolite and its geometrical isomer, 2.

Results and Discussion

Salicylihalamide A (1) was an amorphous solid whose molecular formula was established as C26H33NO5 by HREIMS. The 13C and DEPT NMR spectra (Table 1) showed 26 unique resonances: 5 quaternary, 14 methine, 5 methylene, and 2 methyl carbons. Chemical shift values further characterized two ester or amide carbonyls, 14 olefinic or aromatic carbons, and two oxygenated methines. Three exchangeable protons completed the proton count. The IR spectrum confirmed OH and/or NH (3288 cm−1) functionalities and suggested the presence of both an amide (1651 cm−1) and an intramolecularly hydrogen-bonded conjugated ester (1697 cm−1).

Because of the sensitivity of 1 to CD3OD (see Experimental Section), all NMR spectroscopy experiments were carried out in C6D6 or CD3OD. Signals obscured by or unresolved in one solvent were readily discernible in the other. Consequently, 1H−1H COSY, HMOC, and HMBC NMR data were gathered in both solvents. From a combination of HMOC (Table 1) and COSY4 spectral data, the following partial structures were generated:

The enamide functionality in segment C was identified through a 10.8 Hz coupling between the adjacent olefinic

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and amide N–H protons; the latter was characterized by its variable chemical shift values. All 26 carbons were accounted for in partial structures A–C. In the HMBC spectra (Table 1), coupling between the two vinyl hydrogens (δ 5.17 (H-20) and 6.62 (H-21) in C₆D₆; δ 5.68 and 6.87 in CD₃OD) of the hexadiene substructure B and the amide carbonyl (δ 162.9 in C₆D₆ and δ 165.9 in CD₃OD) of substructure C established the connection of B to C through this amide carbonyl carbon. This was confirmed by an HMBC correlation between the amide proton and carbonyl in C₆D₆ and by the base peak in the mass spectrum (m/z 109, C₇H₉O). The IR spectral evidence that the ester carbonyl was both conjugated and imported by the low-field chemical shift of the phenolic OH (δ 11.46, C₆D₆) was supported by the low-field chemical shift of the phenolic OH (δ 11.46, C₆D₆). This demonstrated that the ester carbonyl was ortho to the phenolic OH in substructure A. The remaining open aromatic position would then have to be occupied by the aliphatic carbon of moiety C, giving macrolide 1 as the final planar structure for salicylihalamide.

Confirmation of the aromatic substitution pattern came from HMBC correlations, which were optimized for 8.3 Hz couplings. In CD₃OD, the phenolic carbon and the amide carbonyl carbon were resolved, as were all three aromatic protons and all six aromatic carbons. Moreover, the latter were not obscured by solvent resonances. In CD₃OD, the aromatic proton at δ 7.12 (H-5) showed a correlation to both the oxygen-bearing phenolic carbon at δ 157.1 (C-3) and the quaternary aromatic carbon at δ 140.7 (C-7). As this δ 7.12 proton is a triplet (J = 7.3 Hz) in the ¹H NMR spectrum, the two quaternary aromatic carbons to which it is correlated should be meta to it. The δ 6.65 (H-6) aromatic hydrogen showed correlation to two different aromatic carbons, the methine at δ 115.3 (C-4) and the quaternary carbon at δ 123.0 (C-2). Furthermore, the δ 6.65 proton was correlated to the side-chain aliphatic carbon at δ 38.8 (C-8), necessitating the placement of this side chain ortho to the δ 6.65 hydrogen. To accommodate an ortho substituent and account for its chemical shift, this δ 6.65 hydrogen must be para to the phenolic OH. Confirming this assignment were the correlations observed between the benzyl proton (H-8, δ 3.56) of the side chain and both the quaternary aromatic carbon at δ 140.7 (C-7) and the δ 122.5 (C-6) aromatic carbon bearing the δ 6.65 hydrogen. The only remaining aromatic site for the ester carbonyl attachment is that ortho to the phenolic OH (at δ 123.0), as shown in structure 1. Additional HMBC correlations are found in Table 1.

The relative stereochemistry of salicylihalamide A (1) was deduced from a combination of ¹H–¹H coupling constants and difference NOE spectra (Table 1, Figure 1). Figure 1 illustrates important NOE interactions; most notable were the interactions of the alcohol methine (H-13) with the ester methine (H-15), the methyl-bearing methine (H-12), and the olefinic hydrogen (H-10). All four of these protons must reside on the same face of the molecule, leading to the relative stereochemistry depicted in Figures 1 and 2. The dihedral angle between the ester methine (H-15) and H-14a must be approximately 90°, since no coupling was observed between these two vicinal hydrogens. The dihedral angle between H-15 and H-14b must approach 180° to accommodate both their coupling constant (10.3 Hz) and the lack of a measurable NOE between them. Likewise, H-14b and the alcohol methine (H-13) showed no measurable coupling, indicating a
dihedral angle of approximately 90°, while a large dihedral angle between H-14a and H-13 was needed to accommodate a sizable coupling (8.8 Hz). H-13, in turn, displayed a $J$-value of 3.5 Hz and a substantial NOE with H-12, suggesting a dihedral angle of approximately 60° between these two protons. Molecular modeling studies, generating minimum energy structures with the NOE constraints described above, gave rise to predicted $J$-values that agreed within 1–1.5 Hz of the actual experimental values. Figure 2 depicts one such computer-generated model of salicyclihalamide A.

The question of absolute stereochemistry was addressed through the use of Mosher esters. Both the (R)- and (S)-methoxy trifluoromethylphenyl acetate (MTPA) diesters of 1 were prepared and subjected to $^1$H NMR analysis (Table 2). The C-12 methyl hydrogens (H-26) and H-10 of the (R)-derivative appeared upfield of those of the (S)-derivative in C$_6$D$_6$. Likewise, H-14a, H-15, and H-17 appeared downfield in the (R)-derivative (3a) relative to the (S)-derivative (3b) in C$_6$D$_6$. That H-17 is found on the same face of the molecule as the Mosher ester site could be seen by a strong NOE between H-17 and the ester methine, H-15. Similar results were observed in NMR spectra recorded in CD$_3$OD. These data allowed the absolute configuration at C-13 to be assigned as S. Partial structures 3a and 3b depict the classical Mosher drawings accounting for the observed chemical shift differences. In the (R,S)-diastereomer (3a), the H-10 and Me-26 hydrogens experience shielding from the phenyl group, while in the (S,S)-diastereomer (3b), H-14a, H-15, and H-17 experience shielding from the phenyl group. If C-13 were of (R)-configuration, just the opposite shielding results would be obtained. As the relative stereochemistry of all three chiral centers has been established, assignment of (R)-configuration at C-13 conferred the (R)-configuration at C-12 and C-15, as shown in 1.

Accompanying salicylhalamide A in the sponge extract was a minor component whose spectral data were nearly identical with those of 1. Its structure was determined by careful comparison of $^1$H, $^{13}$C, and $^1$H–$^1$H COSY NMR data in both C$_6$D$_6$ and CD$_3$OD with those of 1. The sequence of proton–carbon connectivities was identical with that of 1. The only significant difference between 1 and its isomer, 2, was the coupling constant for H-17 and H-18, 10.2 Hz, compared to 14.6 Hz for 1. Thus, salicylhalamide B (2) was the 17-Z isomer of 1.

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**Table 2. Mosher Ester Analysis of Salicylhalamide A (1) in C$_6$D$_6$**

<table>
<thead>
<tr>
<th></th>
<th>H-10</th>
<th>H-26</th>
<th>H-14a</th>
<th>H-15</th>
<th>H-17</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-MTPA</td>
<td>4.92</td>
<td>0.52</td>
<td>1.58</td>
<td>5.55</td>
<td>4.64</td>
</tr>
<tr>
<td>(S)-MTPA</td>
<td>5.58</td>
<td>0.63</td>
<td>1.52</td>
<td>5.04</td>
<td>4.44</td>
</tr>
<tr>
<td>$\Delta (\delta_S - \delta_R)$</td>
<td>+0.66</td>
<td>+0.11</td>
<td>-0.06</td>
<td>-0.51</td>
<td>-0.20</td>
</tr>
</tbody>
</table>

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(6) A small amount of the tris-MTPA derivative (diester imide) was formed in each case and removed by preparative TLC prior to NMR analyses (see Experimental Section).
A search of the literature did not reveal any known compounds of this skeletal type. In a sense, salicylihalamides 1 and 2 would seem more typical of fungal metabolites than sponge metabolites. The closest analogues appear to be the fungus-derived orsellinic acid macrolides such as lasiodiplodin (4), which contains a 12-membered benzolacone ring with a methyl substituent at the ester methine. Salicylihalamide also bears some resemblance to zearealenone (5), which contains a 14-membered macrolide. However, these relatively simple macrolides lack the enamide side chain of the kabiramide 10/halichondramide 11/mycalolide 12/jaspisamide 13 and tolytoxin 14 (scytaphycin/sphingolide 15 classes of macrolides. The salicylihalamides, then, are novel medium-sized macrolides bearing a highly unsaturated enamide substituent.

Testing of purified 1 in the NCI 60-cell line human tumor screen gave a striking pattern of differential cytotoxicity. This screen provides a powerful tool to assess whether a new compound has the same or similar, or different, mechanism of antitumor action in reference to any known chemical class. \( ^5 \) \textbf{COMPARE} pattern-recognition analyses of the mean-graph profiles \( \text{of 1 did not reveal any significant correlations to the profiles of known antitumor compounds contained in the NCI's standard agent database,} \) nor did any of the fungal macrolides mentioned above show similar differential cytotoxicity. The mean panel \( G_{10} \) concentration was approximately 15 nM, and the range of differential sensitivity among the 60-cell lines comprising the panel was \( \pm \) 10. As a group, the melanoma cell lines showed the highest average tumor-type subpanel sensitivity (\( G_{10} \) 7 \( \pm \) 2 nM; \( G_{10} \) 60 \( \pm \) 25 nM). The salicylihalamides represent an attractive new target for synthetic and mechanism-of-action studies aimed at lead optimization, in vivo investigations, and potential development of a new class of antitumor compound.

\section*{Experimental Section}

\textbf{Extraction and Isolation.} Halicorda sp. was collected by P. Murphy in Southwestern Australia, 0.7 nautical miles off Rottnest Island, in March, 1989, at a depth of 15 m. The sponge reportedly occurred as a green mass with amorphous lobes, growing on the underside of a rocky overhang, and was identified by P. J.ane from Front of James Cook University. A voucher specimen is on deposit at the Smithsonian Institution Taxonomy and Sorting Center, Suitland, MD. Approximately 450 g frozen wet wt of sponge was ground to a powder with dry ice. The dry ice was allowed to sublime, distilled \( \text{H}_2\text{O} \) was added, and the thawed material was stirred for \( \sim \) 1 h at 3 °C and then centrifuged. The marc was lyophilized and extracted with a mixture of \( \text{CH}_2\text{Cl}_2\)-MeOH (1:1 v/v) at rt overnight. The solvent was removed by filtration and the marc rinsed with MeOH. The combined solutions were evaporated to yield approximately 3.5 g of crude extract.

Initial attempts to isolate the active constituents revealed that the bioactivity was lost from crude extracts and chromatographic fractions thereof in the presence of deuterated chloroform. For example, overnight storage of \( \text{CDCl}_3 \) solutions of partially purified fractions resulted in the formation of a tarry, insoluble residue that was inactive in the in vitro cytotoxicity bioassay. Therefore, all subsequent attempts at isolation avoided the use of chloroform or exposure of samples to \( \text{CDCl}_3 \), and no further problems with decomposition or loss of bioactivity were encountered. Moreover, there was no indication of any instability of the pure isolated compounds in any of the other common organic solvents employed, nor in aqueous or biological media. A typical procedure for efficient isolation and purification of the salicylihalamides from the initial crude extract is as follows.

An aliquot of extract (330 mg) was coated on 2.1 g of diol bonded phase and sequentially batch-eluted with 100 mL aliquots of hexane, \( \text{CH}_2\text{Cl}_2 \), EtOAc, acetone, and MeOH. The cytotoxicity \( \text{CH}_2\text{Cl}_2 \) and EtOAc eluates were combined (107 mg) and permeated through Sephadex LH-20 with hexane–toluene–MeOH (3:2:2 v/v, 1.5 × 45 cm column) to yield four fractions, the second of which (8.4 mg) showed NMR signals corresponding to 1. HPLC on wide-pore C18 (Racicol 1 × 25 cm) using a linear gradient from 70% to 100% MeOH over 20 min yielded 5.5 mg of 1 (retention time 13 min) and a smaller amount (\( \sim \) 0.5 mg) of 2 (retention time 14.5 min). Gratifyingly, TGI- and \( \text{GI}_{50} \) \textbf{Compare} pattern-recognition analyses (see ref 3c for definitions, details of calculation and interpretation) of the NCI 60-cell mean-graph screening profiles of 1 and the crude extract were highly correlated (e.g., Pearson correlation coefficients > 0.7), consistent with the presence of the salicylihalamides in the original extract. The basis for the initial selection of the extract for bioassay-guided fractionation.

\textbf{Salicylihalamide A (1).} Amorphous solid; \( \beta_{20} = -35^\circ \) (c = 0.7, MeOH); \( \lambda_{max} \) (MeOH) 280 nm (\( \epsilon = 34000 \) ) \( \text{vmax} \) (film) 3288, 2964, 1697, 1651, 1606, 1588, 1520, 1464, 1293, 1257, 1247, 1214, 1123, 1068, 972, 869, 735 cm\(^{-1}\); for \( ^1\text{H} \text{NMR} \) and \( ^{13}\text{C} \text{NMR} \), see Table 1; EIMS \( m/z \) 439 [M \( ^+ \)] (43), 421 (41), 410 (5), 409 (4), 392 (2), 370 (7), 318 (3), 313 (3), 312 (3), 296 (10), 289 (12); 278 (9), 231 (12), 191 (4), 184 (17), 129 (10), 109 (10), 108 (11), 107 (9), 106 (3), 91 (10), 83 (31), 82 (50), 81 (87), 79 (35), 56 (27), 55 (24), 43 (14), 18 (19); HR-EIMS \( m/z \) 439.2354 (M \( ^+ \), calc for \( \text{C}_{23}\text{H}_{33}\text{NO}_{3} \) 439.2350).

\textbf{Salicylihalamide B (2).} Amorphous solid; \( \beta_{20} = -73^\circ \) (c = 0.3, MeOH); \( \lambda_{max} \) (MeOH) 280 nm (\( \epsilon = 38000 \) ) \( \text{vmax} \) (film) 3356, 2964, 2923, 1690, 1651, 1503, 1403, 1294, 1246, 1212, 1121, 1092, 972 cm\(^{-1}\); \( ^{1}\text{H} \text{NMR} \) (DMSO-\( \text{d}_6 \)) 0.77 (t, 7.3, 0.83); (3H, d, 6.8), 1.22 (dd, 15.2, 8.8, 1.32 (br s), 1.50 (br q, 6.8), 1.73 (br s), 1.74 (dd, 15.2, 10.5, 1.89 (ddd, 15.2, 7.8, 7.4), 1.95 (2H, quintet, 7.3), 2.05 (ddd, 14.7, 6.8, 6.4), 2.08 (br m), 3.25 (br d, 16.5), 3.27 (br d, 8.8), 3.56 (dd, 16.5, 4.9), 4.52 (dt, 10.2, 8.8), 8.08 (pdd, 15.5, 7.3, 6.4, 5.17 (very br m), 5.20 (very br m), 5.48 (d, 11.2), 5.62 (dt, 10.7, 7.8, 7.3), 6.45 (dd, 8.3, 3.9), 6.62 (dd, 11.2, 10.8), 6.95 (m), 7.29 (t, 10.3), 7.66 (br m), 7.93 (t, 10.8), 11.58 (br s); 13C NMR (DMSO-\( \text{d}_6 \)) \( \delta = 13.8, 14.0, 20.8, 31.5, 36.2, 38.0, 38.4, 39.4, 70.9, 76.1, 103.3, 117.2, 119.5, 123.7, 124.9, 125.4, 126.8, 132.8, 134.6, 137.4, 141.9, 163.0, 172.0 (3 of the quaternary carbons were not observed); HREIMS \( m/z \) 439.2351 (M \( ^+ \), calc for \( \text{C}_{23}\text{H}_{32}\text{NO}_{3} \) 439.2350).

\textbf{Preparation of MTFA Esters.} To a mixture of 0.5 mg of 1 in 10 \( \mu \text{L} \) of anhydrous \( \text{CH}_2\text{Cl}_2 \) and 15 \( \mu \text{L} \) of anhydrous pyridine in a septum-sealed vial were added 1.2 \( \mu \text{L} \) of the enantiomerically pure \( \alpha\)-methoxy-\( \alpha\)-trifluoromethyl phenyl-
acetyl chloride. The reaction mixture was allowed to stand at 25° for 16 h. H2O and CH2Cl2 were added, and the CH2Cl2 layer was washed several times with H2O and then passed through a short column of anhydrous Na2SO4 and evaporated to dryness under N2. The residue was subjected to reverse-phase TLC (C18) with MeOH to give di- and tri-MTPA derivatives.

**Diesters**: HRFABMS m/z 872.324 [(R)-derivative]; 872.323 [(S)-derivative] (MH+, calcd for C46H48F6NO9, 872.322).


**Computer Modeling.** Calculations were performed using Macromodel v3.016 implemented on a VAX 6620. An input model with a flat macrolide structure with relative stereochemistry proposed by Dreiding models was drawn by hand. Distance constraints were applied sequentially, and energy minimization was performed at each step. After constraints corresponding to the prominent observed transannular NOE’s had been applied, the constraints were removed and the structure was minimized without constraint to produce the final model. The graphic (Figure 2) was produced by downloading the structure and printing from the Chem3D program.

**Cytotoxicity Profile in the NCI 60-Cell Line Screen.** Purified 1 was tested in quadruplicate in the NCI in vitro screen,2 mean-graphs were constructed and averaged, and COMPARE analyses were performed as described.3c The averaged, individual negative log GI50 values comprising the GI50 mean-graph, along with the respective subpanel and cell-line identifiers are as follows: [Leukemia] CCRF-CEM (7.89), HL-60-TB (9.04), K-562 (8.41), MOLT-4 (7.96), RPMI-8226 (7.89), SR (8.44); [Lung] A549/ATCC (8.54), EKVX (7.52), HOP-62 (8.38), HOP-92 (7.77), NCI-H226 (8.80), NCI-H23 (6.55), NCI-H322M (6.72), NCI-H460 (9.01), NCI-H522 (7.26); [Colon] COLO 205 (8.07), HCC-2998 (6.74), HCT-116 (8.74), HCT-15 (8.44), HT29 (8.54), KM12 (8.30), SW-620 (7.54); [Brain] SF-268 (7.55), SF-295 (8.96), SF-539 (7.89), SNB-19 (6.47), SNB-75 (6.21), U251 (7.57); [Melanoma] LOX-IMVI (9.11), MALME-3M (7.62), M14 (8.92), SK-MEL-2 (7.47), SK-MEL-28 (7.00), SK-MEL-5 (9.00), UACC-257 (8.47), UACC-62 (8.38); [Ovary] IGROV1 (7.89), OVCAR-3 (7.03), OVCAR-4 (5.30), OVCAR-5 (8.11), OVCAR-8 (8.43), SK-OV-3 (5.54); [Kidney] 786-O (7.92), A498 (6.55), ACHN (8.01), Caki-1 (8.96), RXF-393 (9.07), SN-12C (7.77), TK-10 (5.74), UO-31 (8.31); [Prostate] PC-3 (7.51), DU-145 (8.26); [Breast] MCF-7 (7.12), MCF-7-ADR-RES (8.07), MDA-MB-231/ATCC (6.92), HS-578T (5.85), MDA-MB-435 (7.82), MDA-N (8.00), BT-549 (9.30), T-47D (8.34). The aforementioned screening data are directly amenable to use by investigators who wish to access Compare-search and other pattern-recognition tools and screening databases currently being deployed by the U.S. National Cancer Institute for general use on the Internet (http://epnws1.ncifcrf.gov:2345/dis3d/dtpsearch.html).

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**Supporting Information Available:** Copies of 1H and 13C NMR and mass spectra of 1 and 2 (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.