Introduction

During the past decades, products of secondary metabolic pathways have fueled the pharmaceutical research enterprise and, for example, have provided valuable lead compounds for the treatment of various human diseases. Furthermore, small molecule ligands that elicit specific and unique biological responses in mammalian cells also constitute extremely valuable research tools in discovery biology efforts. In this context, a unique opportunity is provided by the discovery of a variety of unusual macrocyclic salicylate natural products that were isolated from both terrestrial and marine sources based on their ability to induce a particular phenotype in mammalian cells.

In 1997, scientists at the Laboratory of Drug Discovery Research and Development at the National Cancer Institute reported the bioassay-guided isolation of salicylihalamides A and B (1a,b) from an unidentified species of the marine sponge Haliclona (Figure 1). Shortly afterward, the same group identified a family of related metabolites, termed lobatamides A–F, in three different tunicate species of the genus Aplidium. Notably, these natural products exhibit a unique differential cytotoxicity profile in the NCI 60-cell line human tumor assay (mean panel GI50’s: 1.6–15 nM; range of differential sensitivity ≥ 103). Subsequent years witnessed the isolation of structurally related compounds from a variety of terrestrial microorganisms. Apicularen A was isolated from a myxobacteria based on its extremely potent cytostatic activity against human cancer cell lines including a multi-drug-resistant cervix carcinoma cell line. The same report mentions anecdotally that apicularen A induces several abnormal effects including the formation of mitotic spindles with multiple spindle poles and clusters of bundled actin from the cytoskeleton and the induction of an apoptotic-like cell death. Pfizer Pharmaceuticals isolated the fungal metabolites CJ-12,950 and CJ-13,357 based on their ability to induce Low-Density Lipoprotein (LDL) receptor gene expression, an observation that has potential relevance to the treatment of hypercholesterolemia and hyperlipidemia. OXIMIDINES I and II are the latest additions to this growing class of enamide-
substituted cyclic salicylates and were shown to induce selective growth inhibition of oncogene-transformed rat fibroblasts (3Y1 cells) at 15- to 30-fold lower concentrations than for the parent cell line.5

Salicylihalamides evoked particular interest due to the provocative observation that pattern-recognition analysis (COMPARE) of their activity profiles in the NCI 60-cell line screen initially did not reveal any significant correlation to the profiles acquired for other antitumor compounds contained within the NCI standard agent database.1 Such unique signature profiles are indicative of a potentially novel mechanism of antineoplastic activity. Using a more extensive database analysis, the NCI team subsequently found that the 60-cell profiles of salicylihalamides, lobatamides, and oximidines gave consistently high correlations with the historical database profiles of bafilomycins, prototypical lobatamides, and oximidines. The implication of aberrant V-ATPase function in many different human diseases, such as osteoporosis and cancer, warrants the development of synthetically tractable and biologically selective pharmacological modulators of V-ATPases.7,8 To study the molecular pharmacology of this unique class of macrocyclic salicylates, we initiated a synthetic program aimed at understanding the mechanism of action of salicylihalamides as well as derived probe reagents. Herein, we present a full account of our salicylihalamide synthetic venture. Initiated in the context of generating informative structure–function data, these efforts have culminated in a highly efficient total synthesis of salicylihalamide A and modified congeners.9–11

**Architecture and Synthetic Strategy**

The structure and relative stereochemistry of salicylihalamide A was assigned based on extensive data gathered from a variety of high-field NMR experiments.1 The absolute configuration of (−)-salicylihalamide A, initially assigned through Mosher ester 1H NMR experiments, was later revised by us to 12S,-13R,15S through total synthesis.9,12 Salicylihalamides as well as the biosynthetically related macrocyclic salicylates represent a structurally novel class of macrocyclic benzolactones incorporating salicylic acid, and are decorated with a unique, highly unsaturated, N-acetyl enamine side chain. Given the sensitive nature of this common construct, we deemed it crucial to introduce this at a late stage in the synthesis utilizing a unifying and mild strategy. Considering the options, we felt that the addition of 1-metallo-1,3-hexadiene (I, X–Y = CH–CH2) or the corresponding iminoalkenyl isocyanate derivative (I, X–Y = N=O) to a stereodefined E-alkenyl isocyanate II would offer the distinct advantage of mild reaction conditions and control of stereochemistry (Scheme 1).1,3,14 Isocyanate II is to be derived from the corresponding α,β-unsaturated carboxylic acid III (acyl azide formation/Curtius rearrangement), in turn accessible from alcohol IV via oxidation/Horner–Wadsworth–Emmons homologation.


(12) The revised structure is shown.


A connectivity analysis of the benzolactone core of salicylihalamide A points to two equally attractive routes for its assembly (Scheme 2). The first one features an esterification/intramolecular olefin metathesis sequence (RCM) to form the C9–C10 bond and offers the advantage of operational simplicity combined with functional group tolerance (A1 + B1 → AB; Path A).16 Fürsten and co-workers demonstrated the feasibility of this approach to form a 12-membered resorcylic benzolactone related to lasiodiplodin while avoiding isomerization of the endocyclic double bond to the styryl position.17 Despite the robustness of the RCM in carbon–carbon bond formation, it can only be implemented successfully for the desired E-isomer can be exerted. As detailed in full later in this paper, we have identified a highly E-selective RCM avenue to salicylihalamide A.18 At the outset of our work, cross-coupling19 of a stereodefined E-alkenyl organometallic fragment B2 with a benzyl halide A2 was envisioned as a more robust alternative and would join the C8–C9 bond with control and maintenance of olefin geometry (Path B).20 Importantly, both strategies converge to a common alkene precursor B, adding flexibility to the synthesis.

**Results and Discussion**

The synthesis of alkyne 11 (fragment B) starts from readily available aldehyde 3 as described in Scheme 3.21 Aldol reaction of aldehyde 322 with the borylenolate derived from (2R)-N-acetylbornanesultam 2a gave a separable mixture of two aldol products, 4 and its epimer, in an 85:15 ratio.23 Protection of the β-hydroxy amide 4 as the p-methoxybenzyl (PMB) ether 5 exploited the Bundle trichloroacetimidate protocol,24 but proved to be highly capricious in our hands, providing irreproducible yields ranging from 20 to 80%.25 Aldehyde 6, obtained via a one-step reduction of protected aldol derivative 5 (DIBAL-H, CH2Cl2, −78 °C; MeOH quench at −78 °C),26 was subjected to a stereoselective syn-aldol reaction with the titanium enolate27 derived from (2R)-N-(4-pentynyl)bornanesultam 2b, and provided only one distereoisomer 7 as judged by 1H NMR analysis of the crude reaction mixture. Our major motivation to single out an aldol tactic to form the C12–C13 bond was the opportunity to introduce a molecular handle at a site that would otherwise not be accessible for derivatization. Such a molecular handle could prove extremely useful for probing reagent development and/or analogue synthesis. For the completion of the natural product, however, an oxidation state adjustment was required. To this end, the β-hydroxy amide 7 was protected as the tert-butylmethylsilyl (TBS) ether 8 followed by reduction of the N-acetyl sulfonamide 8 (LiEt2BH), tosylate formation, and further reduction of 10 (LiEt2BH) to complete the synthesis of fragment 11.

With alkyne 11 in hand, the stage was set to explore its assembly with the aryl sector. In one of two approaches (path B, Scheme 2b), this would involve a hydrometallation of the triple bond followed by cross-coupling with benzyl bromide 1328 (eq 1). In this context, zirconocene 12a (M = ZrCp3Cl) was considered the most appealing nucleophilic coupling partner. Indeed, stereodefined 1-(E)-alkenyl zirconocenes are readily accessible via a functional group tolerant hydrozirconation of 1-alkynes and engage in cross-coupling chemistry with a variety of aldehydes.

(15) Salicylihalamide numbering14 will be used throughout this paper.


(18) After our total synthesis, all successful syntheses of salicylihalamide so far have relied on a RCM approach: see refs 10a–d.


(20) For an application of this approach in a model system see ref 10f.

(21) Because of the erroneous assignment of absolute stereochemistry to (−)-salicylihalamide A, our explorative studies and initial total synthesis engaged materials that reflect an antipodal relationship to those that would be required for the natural enantiomer.


(25) We later changed our protecting group strategy. At this point, enough material could be obtained to continue exploratory work.


of benzyl halides. We found that benzylc bromide 13 efficiently underwent Pd[PPh3]4 mediated cross-coupling with the alkenyl zirconocene derivative in situ prepared by hydrozirconation of 1-heptyne (CH2ZrCp2, THF). Unfortunately, all attempts to obtain zirconocene 12a via hydrozirconation of alkyne 11 were unsuccessful. In contrast, alkyne 11 did undergo smooth hydrostannylation (Bu3SnH, AIBN, PhMe → 12b, M = SnBu3) as a prelude to an alternative Stille coupling with benzylc bromide 13. This time, however, the corresponding vinylstannane 12b was extremely prone to protodeamination, yielding terminal alkyne 12d (M = H) upon workup. After a lot of experimentation, the desired coupling product 12b with benzylic bromide 13 could be obtained via a hydroboration (Si2BH, THF → 12c, M = Si2Mes)/Suzuki cross-coupling sequence in 15% yield (13, Cs2CO3, PhAs, catalyst PdCl2(dppf), H2O/DMF). Again, the culprit seemed to be an inefficient hydrometalation as starting alkyne 11 accounted for the remaining mass balance.

At this point, the validity of planning a flexible strategy became apparent and it was time to explore the RCM path to salicylihalamides (Path A, Scheme 2). Toward this end, the p-methoxybenzyl protecting group of alkyne 12d was oxidatively (DDQ) deprotected (Scheme 4). Unfortunately, attempts to acylate the resulting alcohol 15 with an activated ester derived from benzoic acid derivative 16a (−17a) uniformly met with failure. A reactivity umpolung provided by a Mitsunobu esterification was essential and delivered bis-olefin 16b as a result of a substrate en route to salicylihalamides. To address this point, C15-epimeric compounds had to be prepared.

Our initial route for the synthesis of the C10–C17 fragment was plagued by a less than optimal diastereoselection during the installation of the C15 stereocenter and an irreproducible protection of the corresponding aldol product as the PMB derivative 15 underwent smooth hydrozirconation as judged by 1H NMR. A 4:1 mixture of separable isomers resulted with the Z-isomer 18-Z predominating. However, this material was improperly configured at C15, and as such, this ratio does not per se reflect E/Z-selectivity for an eventual RCM of a substrate en route to salicylihalamides. To address this point, C15-epimeric compounds had to be prepared.

Our initial route for the synthesis of the C10–C17 fragment was plagued by a less than optimal diastereoselection during the installation of the C15 stereocenter and an irreproducible protection of the corresponding aldol product as the PMB derivative 16a (Scheme 5). First, an enantioselective alkylation of aldehyde 19 dramatically improved the stereochemical purity at C15, providing the known homoaal alcohol 20 in 96% yield and 91% ee. Silylation (TBSCl, imidazole, DMF) followed by oxidative double bond-decleavage afforded aldehyde 22 reproducibly in 91% yield.
Table 1. Ring-Closing Olefin Metathesis Studies (Eq 2)

<table>
<thead>
<tr>
<th>substrate</th>
<th>( t (h) )</th>
<th>29a:30a (ratio)</th>
<th>catalyst 31</th>
<th>catalyst 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>28a</td>
<td>1.3</td>
<td>99:10</td>
<td>29a:30a:66:34</td>
<td></td>
</tr>
<tr>
<td>28a</td>
<td>6.3</td>
<td>88:12</td>
<td>29a:30a:68:32</td>
<td></td>
</tr>
<tr>
<td>28a</td>
<td>18.3</td>
<td>80:20</td>
<td>29a:30a:67:33</td>
<td></td>
</tr>
<tr>
<td>28b</td>
<td>1.3</td>
<td>90:10</td>
<td>29b:30b:64:36</td>
<td></td>
</tr>
<tr>
<td>28b</td>
<td>6.3</td>
<td>88:12</td>
<td>29b:30b:67:33</td>
<td></td>
</tr>
<tr>
<td>28b</td>
<td>18.3</td>
<td>84:16</td>
<td>29b:30b:68:32</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) Ratio determined by \(^1\)H NMR analysis. \(^b \) In each individual experiment, the combined isolated yield was >93%.

steps). Treatment of this aldehyde with the \( Z(O) \)-titanium enolate derived from \((Z\text{-}N)-(4\text{-}pentenoyl) \) bornanesultam \(^{23b} \) produced exclusively one diastereomeric aldol product \( 24 \) in 92% yield. As before (Scheme 3), a three-step sequence starting from the methoxymethyl ether \( 25 \) accomplished the oxidation state readjustment to deliver the \( C12 \) methyl-substituted fragment \( 26 \). Fluoride-assisted liberation of the \( C15 \) alcohol and subsequent Mitsunobu\(^{28} \) coupling of \( 27 \) with carboxylic acid \( 16a \) or the corresponding phenolic MOM ether \( 16b \) set the stage for a detailed study of the crucial ring-closing olefin metathesis of bis-olefins \( 28a, b \).

The lack of a stereopredictive model for the formation of large rings via RCM is exemplified by our results with the metathetical ring closure of substrates \( 28a, b \). Whereas the diastereomeric substrate \( 17b \) gave the \( Z \)-olefin \( 18-Z \) (vide supra, Scheme 4) as the major isomer, \( 28a \) fortuitously produced the \( E \)-benzolactone \( 29a \) with an impressively high selectivity of 9:1 when subjected to similar reaction conditions (eq 2; 5 mol % catalyst 31 or 32).

\( 28a \) or \( 28b \)

31, \( CH_{2}Cl_{2} \), room temperature.\(^{44} \) To confuse the issue even more, an initial single experiment with the corresponding phenolic methoxymethyl ether \( 28b \) furnished benzolactones \( 29b \) and \( 30b \) with an eroded selectivity of 3:1 upon exposure to catalyst 31. In light of the above, a detailed study of the RCM of \( 28a, b \) with Ru-alkylidene precatalysts \( 31 \) and \( 32 \) was conducted (eq 2 and Table 1).\(^{45} \) It is clear from these results that both catalysts are equally efficient in performing the desired transformation, albeit with different degrees of \( E/Z \)-selectivity. Also, both differentially protected substrates \( 28a, b \) gave identical \( E/Z \)-ratios under identical reaction conditions.\(^{47} \) Whereas precatalyst 31 provided a 9:1 (\( E/Z \)) ratio with only a slight erosion of selectivity over time, precatalyst 32 produced a lower 67:33 ratio, which remained constant over time.

In principle, olefin metathesis will produce a thermodynamic distribution of products if secondary metathetical isomerizations compete on the time scale of the experiment.\(^{48} \) There are two primary factors that will affect the efficiency of secondary metathetical isomerizations: (1) the activity of the propagating Ru-methylidene species and (2) catalyst decomposition rates.\(^{49, 50} \) In light of this, the more stable and more active “second generation” catalysts (e.g. 32) were shown to enrich initially formed products to the thermodynamic equilibrium ring closure product.\(^{48d} \) In contrast, kinetic product ratios cannot be ruled out with the “first generation” Ru-alkylidene catalysts due to their shorter lifetime (thermal instability)\(^{50} \) and less efficient reaction with 1,2-disubstituted olefins (reaction products).\(^{16} \) We conclude from our results that RCM with catalyst 32 rapidly produces a thermodynamic ratio of products \( 29-30 \) on a time scale that consumed all the starting material based on the following observations: (1) the product ratio did not change after prolonged exposure indicating that equilibrium was established, (2) an identical product ratio is observed for the formation of benzolactones \( 29-30 \) from both our precursors \( 28a, b \) and the nonidentical precursors described by Fürsten et al.,\(^{51} \) and (3) upon exposure of either geometrically pure \( 29a \) or \( 30a \) to catalyst 32, an identical 67:33 mixture of \( 29a, 30a \) was formed. In contrast, the first generation Grubbs’ catalyst 31 kinetically induced the formation of the desired \( E-isomers 29a, b \) with relatively high selectivity and a thermodynamic product ratio was never reached, even after prolonged reaction times.

Having secured a viable sequence to the benzolactone core of salicylhalamide A, we turned our attention to the installation of the acylated enamine side chain. Toward this end, the \( p \)-methoxybenzyl ether in \( 29a \) was oxidatively removed (DDQ) and the resulting alcohol \( 33 \) was oxidized with Dess–Martin periodinane (Scheme 6).\(^{52} \) Engagement of the resulting aldehyde \( 34 \) in a Horner–Wadsworth–Emmons (HWE) homologation with trimethyl phosphonoacetate provided methyl ester \( 36a \) as an inseparable mixture of \( E/Z \)-isomers in a ratio of 4:1. After

\( ^{47} \) This indicates that the differences in \( E/Z \)-selectivity for the RCM of \( 28a \) vs \( 28b \) observed during our initial experiments with catalyst 31 must be due to subtle variations in reaction conditions.

diethylphosphonoacetate, delivering allyl ester experiments were performed that quickly led to the use of allyl 

(55) The use of tert-butyl dimethylphosphonoacetate 35c provided tert-butyl ester 36c with 20:1 E/Z-selectivity. However, 36c decomposed to unidentified products upon BBr₃ treatment (CH₂Cl₂, −78 °C).

(55) The minor Z-isomer would be the desired one for the synthesis of salicylihalamide B.

(54) The phenolic methyl ether and MOM ether had to be replaced with protection groups that could be removed after installation of the acid-labile acylated enamine functionality.

(53) The phenolic methyl ether and MOM ether had to be replaced with protection groups that could be removed after installation of the acid-labile acylated enamine functionality.

(53) The minor Z-isomer would be the desired one for the synthesis of salicylihalamide B.

(52) Careful fluoride-assisted desilylation with a buffered solution of commercially available HF/pyridine in THF/pyridine provided geometrical isomers 1a/1c, which could be separated by semipreparative HPLC. The chromatographically more mobile fraction was treated in a similar fashion and provided a mixture of the corresponding salicylihalamide dimers 43/44, again separable by semipreparative HPLC.

In contrast to the current study, we had initially synthesized enantio-1a based on the absolute configuration reported for the natural product. Although this synthetic material was found to be identical to natural salicylihalamide A according to NMR ([D₆]benzene and [D₄]methanol), IR, UV, and coelution on TLC (three different solvent systems), the signs of the optical rotations of synthetic ent-1a ([α]D²³[z] +20.8; c 0.12; MeOH) and the natural product (reported: [α]D²³[z] −35; c 0.7; MeOH) were opposite. Moreover, synthetic ent-1a was devoid of growth inhibitory activity when screened against the NCI 60-cell line panel. At this point we were fortunate that p-bromobenzoate derivative 47 provided crystals suitable for X-ray diffraction studies, confirming the absolute configuration of our synthetic lactones (Figure 2). Based on all the available evidence, the absolute configuration of natural (−)-salicylihalamide A was assigned by semipreparative HPLC. The chromatographically more mobile fraction was treated in a similar fashion and provided a mixture of the corresponding salicylihalamide dimers 43/44, again separable by semipreparative HPLC.

In contrast to the current study, we had initially synthesized ent-1a based on the absolute configuration reported for the natural product. Although this synthetic material was found to be identical to natural salicylihalamide A according to NMR ([D₆]benzene and [D₄]methanol), IR, UV, and coelution on TLC (three different solvent systems), the signs of the optical rotations of synthetic ent-1a ([α]D²³[z] +20.8; c 0.12; MeOH) and the natural product (reported: [α]D²³[z] −35; c 0.7; MeOH) were opposite. Moreover, synthetic ent-1a was devoid of growth inhibitory activity when screened against the NCI 60-cell line panel. At this point we were fortunate that p-bromobenzoate derivative 47 provided crystals suitable for X-ray diffraction studies, confirming the absolute configuration of our synthetic lactones (Figure 2). Based on all the available evidence, the absolute configuration of natural (−)-salicylihalamide A was assigned by semipreparative HPLC. The chromatographically more mobile fraction was treated in a similar fashion and provided a mixture of the corresponding salicylihalamide dimers 43/44, again separable by semipreparative HPLC.

In contrast to the current study, we had initially synthesized ent-1a based on the absolute configuration reported for the natural product. Although this synthetic material was found to be identical to natural salicylihalamide A according to NMR ([D₆]benzene and [D₄]methanol), IR, UV, and coelution on TLC (three different solvent systems), the signs of the optical rotations of synthetic ent-1a ([α]D²³[z] +20.8; c 0.12; MeOH) and the natural product (reported: [α]D²³[z] −35; c 0.7; MeOH) were opposite. Moreover, synthetic ent-1a was devoid of growth inhibitory activity when screened against the NCI 60-cell line panel. At this point we were fortunate that p-bromobenzoate derivative 47 provided crystals suitable for X-ray diffraction studies, confirming the absolute configuration of our synthetic lactones (Figure 2). Based on all the available evidence, the absolute configuration of natural (−)-salicylihalamide A was assigned by semipreparative HPLC. The chromatographically more mobile fraction was treated in a similar fashion and provided a mixture of the corresponding salicylihalamide dimers 43/44, again separable by semipreparative HPLC.
to be as drawn in 1a. Unequivocal proof for the absolute stereochemistry of natural salicylihalamide ultimately came from biochemical characterization of synthetic 1a, which provided a differential cytotoxicity profile undistinguishable from the natural product in the NCI-60 cell line panel.61

With issues related to absolute stereochemistry resolved, we decided to initiate a detailed study to unravel salicylihalamide’s mode-of-action at the cellular and biochemical level. During the course of this work, the Vacuolar ATPase (V-ATPase) was identified as a putative target of salicylihalamide A.6 In contrast to bafilomycins and other prototypical V-ATPase inhibitors, salicylihalamide A exquisitely discriminates between mammalian and nonmammalian V-ATPases.6 To fully exploit this unique feature of salicylihalamide, it is imperative to map its binding site on V-ATPase in molecular detail. To identify regions in the molecule that would tolerate the obligate structural changes that will accompany the introduction of the prerequisite probes, we prepared a variety of salicylihalamide analogues. Ideally, such reporter constructs would be available from late stage intermediates, avoiding extensive chemical sequences. Our initial efforts were therefore orchestrated toward side chain modifications, emanating from a common, naturally configured isocyanate 41, now accessible in 20 steps (longest linear sequence) and 20% overall yield. Thus, compound 46 and the corresponding dimer 45 were prepared by addition of hexyllithium (instead of hexadienyllithium) to isocyanate 41 followed by final deprotection (Scheme 7). An inverse addition of isocyanate 41 to a cold solution (−78 °C) of organolithium nucleophiles was next explored to suppress dimer formation.59 Indeed, preparation of alkynoyl enamine derivatives 48/49 followed this procedure, and no trace of dimer formation was observed (Scheme 8).

Side chain modified analogues that lack salicylihalamide’s characteristic N-acyl enamine functionality are attractive candidates for the following reasons: (1) they are expected to confer increased acid stability; (2) they can potentially be prepared via shorter sequences; and (3) they would answer an important question related to the functional role of the N-acyl enamine moiety. Octanoate 53, a compound with identical chain length and hydrophobicity similar to those of salicylihalamide, is representative of this class of compounds and was prepared from alcohol 33 via a Mitsunobu6 esterification followed by deprotection (eq 3).

A minimally perturbed side chain that would retain the potentially hydrogen bonding characteristics (donor and/or acceptor) of the N-acyl functionality was envisioned to arise from a simple saturation of the enamine double bond of biologically active (vide infra) salicylihalamide derivative 50. However, direct hydrogenation of 50 also saturated the endo-cyclic double bond to produce 59 (Scheme 9). Because there was no obvious short solution to this chemoselectivity problem, a control reagent 58 was prepared as a probe to investigate independently the effect of endocyclic double bond saturation on biological activity (Scheme 9). Our point of departure entailed a hydrogenation of 29a or 30a with concomitant removal of the p-methoxybenzyl (PMB) ether. Subsequent conversion of 54 to 58 took full advantage of the chemistry outlined for the preparation of 50 without complication. Hydrogenation of this material also yielded the fully saturated salicylihalamide derivative 59.

Although the potent in vivo and in vitro biological activity of salicylihalamide-based dimers 43–45 (vide infra) pointed to a potential site for attachment of a photoactivatable radioactive probe, the phenolic and secondary hydroxyls were also investigated as a handle for derivatization (Scheme 10). Starting with bis-TBS derivative 38, selective deprotection of the phenolic (TBAF, THF, 0 °C, 91%) or secondary TBS ether (aqueous HCl, 91%) was followed by benzoylation to furnish benzoates 60 (78%) and 61 (50%), respectively. Together with compound 36b, these materials were independently elaborated to bis- and monoprotected forms (62–64) of salicylihalamide derivative 50.

The in vitro inhibition of V-ATPase activity by salicylihalamide A, its enantiomer, and the other synthetic derivatives is summarized in Table 2. For our studies, we utilized a reconstituted, fully purified V-ATPase from bovine brain62 to completely eliminate potential effects arising from contaminating nonvacuolar ATPase activities present in the crude membrane preparations utilized in the initial6 study. Here, we demonstrate for the first time that synthetic (−)-salicylihalamide A inhibits ATP-energized proton pumping of the intact, reconstitutively active V-ATPase with an IC50 value of <1.0 nM, as compared to 3.1 nM for bafilomycin A (Table 2, entries 1–2).63 The unnatural enantiomer (+)-salicylihalamide A was 300-fold less potent (Table 2, entry 3), further confirming the absolute configuration of natural salicylihalamide A.

Side chain modified analogues 43–46 and 48–51 all retain the ability to potently inhibit proton pumping activity at


(63) In contrast to the initial study,6 which measures inhibition of ATPase activity, we utilized a “H” transport (pumping) assay that is a far more specific and accurate measure for V-ATPases than the ATPase assay. The pumping assay requires every part of the enzyme functioning. The ATPase activity, on the other hand, only tells its partial activity, i.e., ATP hydrolysis, which may or may not be coupled to proton pumping. For a description of the proton pumping assay, see: Crider, B. P.; Xie, X.-S.; Stone, D. K. J. Biol. Chem. 1994, 269, 17379–17381.
mediated without abrogating biological activity (43–45). However, there is a size restriction associated with the N-acyl fragment indicated by the 1000-fold drop in activity of farne-sylated analogue 52. This result also demonstrates that the side chain is not merely functioning as a lipophilic membrane anchor.

The seriously compromised potency of octanoate 53 and allyl ester 37b in the in vitro V-ATPase assay demonstrated the importance of the N-acyl enamine functionality. 62 Moreover, whereas the N-carbamoyl enamine derivative 58 retained a significant capacity to inhibit proton pumping in the V-ATPase assay, the enamine to amine permutation (58−59) substantially attenuated inhibitory potential. Also, benzoylation of the C15 alcohol (64) or the C3 phenol (63) led to a significant drop in biological activity, thereby virtually eliminating the possibility of exploiting the most obvious functional handles to develop a useful salicylihalamide-based probe reagent.

Initially, we guided our chemical work with a cell-based assay looking for growth inhibition of the SK-MEL 5 human melanoma cell line. 55 Although the structure−activity relationships found in this assay (IC50 (μM); 1a/c, 0.06; 43, 0.04; 44, 0.1; 45, 0.6, 0.46, 0.38; 48, 0.3; 49, 0.3; 50, 0.5; 51, 0.45; 52, 1.5; 53, >20; 37b, >20; 58, 8; 59, >20, 63, 1, 64, >20] mirror those of the in vitro V-ATPase assay, they do not per se indicate a link between inhibition of the vacuolar ATPase and cytotoxicity. 66 Further biochemical and cell biological studies will be necessary to implicate modulation of V-ATPase function as or the function of an alternative cellular target as the mechanism by which salicylihalamides induce differential cytoxicity in cultured mammalian cells. These studies will require the development of relevant fluorescent and/or radioactive photoactivatable probe reagents. The structure−function data presented here point to the side chain nitrogen as a point for attachment or modification of the N-acyl terminus as the most promising avenue for obtaining these probe reagents.

Conclusions

Herein, we have detailed in full our synthetic efforts toward salicylihalamide A, the first example of a structurally novel class of natural products that induce unique biological effects in cultured mammalian cells. The synthesis of the structure originally assigned to natural salicylihalamide A as well as the corresponding enantiomer have unambiguously established the absolute configuration of the natural product as presented by 1a. The advantage of a flexible synthetic strategy was highlighted by our ability to quickly advance the ring-closing olefin metathesis approach as the method of choice to build the 12-membered benzolactone core of salicylihalamides. Notably, a detailed study of this key step identified conditions that kinetically favor formation of the desired E-benzolactone 29a with high levels of stereocontrol. The end game included elaboration of this material to a stereodefined E-alkenyl isocyane 41 through Horner−Wadsworth−Emmons homolysis of aldehyde 34, followed by Curtius rearrangement of acyl azide 40. Final addition of hexadienyllithium to a cold solution of vinyl isocyane 41, followed by a mild deprotection delivered

Table 2. Inhibition of the H+ Transport Activity of Reconstituted V-ATPase from Bovine Brain by Synthetic Salicylihalamides

<table>
<thead>
<tr>
<th>compd</th>
<th>IC50 (nM)</th>
<th>compound</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bafilomycin A</td>
<td>3.1</td>
<td>43</td>
<td>1</td>
</tr>
<tr>
<td>1a</td>
<td>&lt;1</td>
<td>50</td>
<td>1.6</td>
</tr>
<tr>
<td>ent-1a</td>
<td>270</td>
<td>51</td>
<td>1.8</td>
</tr>
<tr>
<td>37b</td>
<td>230</td>
<td>52</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>43</td>
<td>3.7</td>
<td>53</td>
<td>1000</td>
</tr>
<tr>
<td>44</td>
<td>1.2</td>
<td>58</td>
<td>3</td>
</tr>
<tr>
<td>45</td>
<td>1</td>
<td>59</td>
<td>30</td>
</tr>
<tr>
<td>46</td>
<td>1</td>
<td>63</td>
<td>300</td>
</tr>
<tr>
<td>48</td>
<td>&lt;1</td>
<td>64</td>
<td>180</td>
</tr>
</tbody>
</table>

* IC50 values (measured according to ref 63) are the average of at least three experiments (error ±5%).
salicylihalamide A (1a), the 22E-isomer 1c and the corresponding salicylihalamide dimers 43 and 44.

The highly efficient synthesis of acyl azide 40 (20–25% overall yield; 19 steps longest linear sequence from aldehyde 19) was a crucial asset in investigating salicylihalamide structure–activity relationships. A collection of 17 salicylihalamide-based structures have been synthesized and evaluated as inhibitors of the Vacuolar (H⁺)-ATPase. These investigations have identified several highly potent analogues of salicylihalamide A, and provided the framework for the development of probe reagents. These reagents will be valuable tools for mapping the salicylihalamide-binding site on V-ATPase and investigating the mechanism(s) by which salicylihalamide induces differential cytotoxicity in mammalian cells.

Acknowledgment. Financial support provided by the Robert A. Welch Foundation, the National Institutes of Health through grants CA 90349 (J. K. De Brabander) and DK 33627 (X.-S. Xie), and junior faculty awards administered through the Howard Hughes Medical Institute and the University of Texas Southwestern Medical Center are gratefully acknowledged. HRMS analyses were performed at the NIH regional mass spectrometry facility at the University of Washington.; St. Louis, MO. J. K. De Brabander is a fellow of the Alfred P. Sloan Foundation.

Supporting Information Available: Experimental procedures and characterization data and NMR spectra of synthetic and natural salicylihalamide and compounds 43–46, 48–52, 58–59, and 62–64 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.