Total Synthesis and Initial Structure−Function Analysis of the Potent V-ATPase Inhibitors Salicylihalamide A and Related Compounds

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Abstract: Salicylihalamide A is the first member of a growing class of macrocyclic salicylate natural products that induce a variety of interesting phenotypes in cultured mammalian cells. Salicylihalamide A was reported to be a unique and highly differential cytotoxin and a potent inhibitor of the mammalian vacuolar (H$^+$)-ATPase. The total synthesis of both enantiomers of salicylihalamide A, a revision of the absolute configuration assigned to the natural product, and extensive structure−function studies with synthetic salicylihalamide variants are reported. These studies were possible only due to a highly efficient synthetic strategy that features (1) a remarkably *E*-selective ring-closing olefin metathesis to construct the 12-membered benzolactone skeleton **29**, (2) a mild stereocontrolled elaboration to *E*-alkenyl isocyanate **41**, and (3) addition of carbon, oxygen, and sulfur nucleophiles to isocyanate **41** to obtain salicylihalamide A and congeners. We demonstrate for the first time that salicylihalamide A is a potent inhibitor of fully purified reconstituted V-ATPase from bovine brain, and have identified several similarly potent side chain modified derivatives, including salicylihalamide dimers **43**−**45**. In combination, these studies have laid the foundation for ongoing studies aimed at a comprehensive understanding of salicylihalamide's mode-of-action, of potential relevance to the development of lead compounds for the treatment of osteoporosis and cancer.

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).[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*.[2] Notably, these natural products exhibit a unique differential

cytotoxicity profile in the NCI 60-cell line human tumor assay (mean panel GI$_{50}$'s: 1.6−15 nM; range of differential sensitivity ≥10^3). 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.[3] 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.[4] Oximidines I and II are the latest additions to this growing class of enamide-

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(4) Dekker, K. A.; Aiello, R. J.; Hirai, H.; Inagaki, T.; Sakakibara, T.; Suzuki, Y.; Thompson, J. F.; Yamauchi, Y.; Kojima, N. *J. Antibiot.* **1998**, *51*, 14−20.
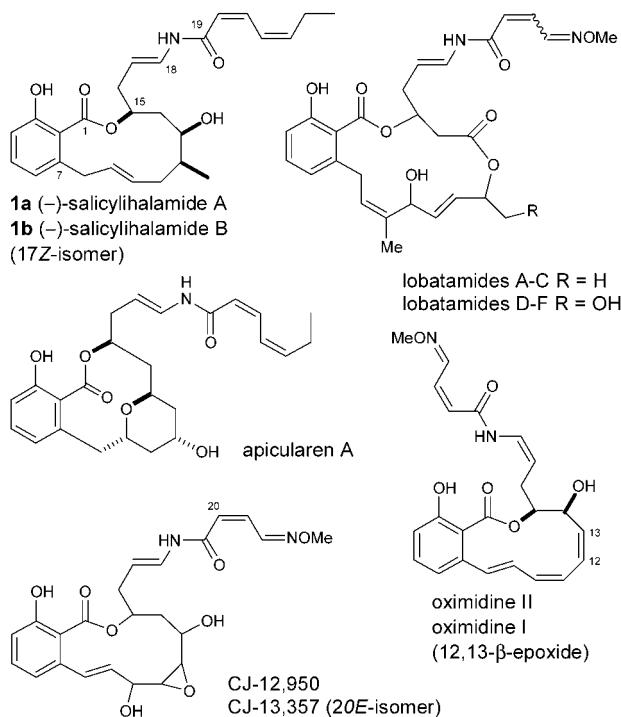
Figure 1. Salicylihalamide A and Structurally Related Natural Products.

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 vacuolar (H$^+$)-ATPase (V-ATPase) inhibitors.[6] A common or similar mode-of-action with bafilomycins was further suggested based on in vitro studies with crude membrane preparations of mammalian V-ATPases.[6] Importantly, these studies have revealed that, unlike bafilomycins, salicylihalamide A exquisitely discriminates between mammalian and nonmammalian V-ATPases. 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 producing sufficient quantities of these natural products as

Scheme 1. Installation of the *N*-Acyl Enamine Side Chain
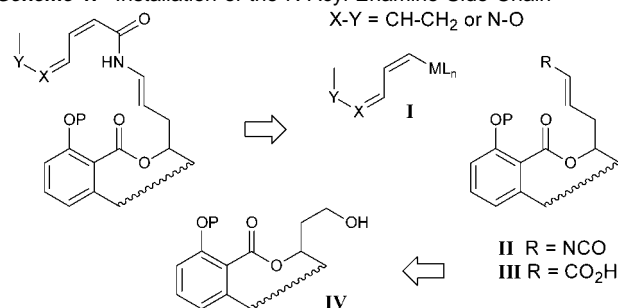
well as derived probe reagents. Herein, we present a full account of our salicylihalamide synthetic venture. Initiated in the context of generating informative structure−function data, these efforts have culminated in a highly efficient total synthesis of salicylihalamide A and modified congeners.[9−11]

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 ^{1}H NMR experiments,[1] was later revised by us to 12*S*,-13*R*,15*S* 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 an unusual, highly unsaturated, *N*-acyl 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−CH$_2$) or the corresponding iminoalkenyl 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).[13,14] 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.
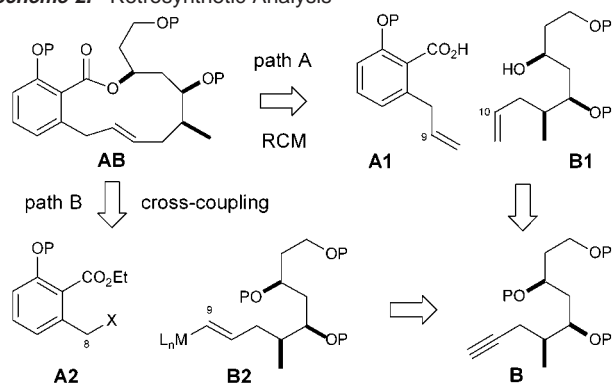
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(6) Boyd, M. R.; Farina, C.; Belfiore, P.; Gagliardi, S.; Kim, J.-W.; Hayakawa, Y.; Beutler, J. A.; McKee, T. C.; Bowman, B. J.; Bowman, E. J. *J. Pharmacol. Exp. Ther.* **2001**, *297*, 114−120.

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(8) For a therapeutic focus, see: Farina, C.; Gagliardi, S. *Drug Discovery Today* **1999**, *4*, 163−172.

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(10) Subsequent total syntheses have appeared: (a) Snider, B. B.; Song, F. *Org. Lett.* **2001**, *3*, 1817−1820. (b) Smith, A. B., III; Zheng, J. *Synlett* **2001**, 1019−1023. (c) Labrecque, D.; Charron, S.; Rej, R.; Blais, C.; Lamothe, S. *Tetrahedron Lett.* **2001**, *42*, 2645−2648. (d) Fürstner, A.; Dierkes, T.; Thiel, O. R.; Blanda, G. *Chem. Eur. J.* **2001**, *7*, 5286−5298. For approaches toward the benzolactone core, see: (e) Fürstner, A.; Thiel, O. R.; Blanda, G. *Org. Lett.* **2000**, *2*, 3731−3734. (f) Feutrill, J. T.; Holloway, G. A.; Hilli, F.; Hügel, H. M.; Rizzacasa, M. A. *Tetrahedron Lett.* **2000**, *41*, 8569−8572. (g) Georg, G. I.; Ahn, Y. M.; Blackman, B.; Farokhi, F.; Flaherty, P. T.; Mossman, C. J.; Roy, S.; Yang, K. *J. Chem. Soc., Chem. Commun.* **2001**, 255−256.

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(12) The revised structure is shown.

(13) For model studies utilizing a conceptually similar approach see: (a) Snider, B. B.; Song, F. *Org. Lett.* **2000**, *2*, 407−408. (b) Kuramochi, K.; Watanabe, H.; Kitahara, T. *Synlett* **2000**, 397−399. (c) Stefanuti, I.; Smith, S. A.; Taylor, R. J. K. *Tetrahedron Lett.* **2000**, *41*, 3735−3738.

(14) For alternative approaches see refs 10b,c and: (a) Shen, R.; Porco, J. A., Jr. *Org. Lett.* **2000**, *2*, 1333−1336. (b) Fürstner, A.; Brehm, C.; Cancho-Grande, Y. *Org. Lett.* **2001**, *3*, 3955−3957.

Scheme 2. Retrosynthetic Analysis

Scheme 3. Initial Synthesis of a C9−C17 Fragment[a]

[a] Reagents and conditions: (a) Et₂BOTf, *i*-Pr₂NEt, CH₂Cl₂, −5 °C; then add **3** at −78 °C (99% of a separable 85:15 diastereomeric mixture); (b) 4-MeOBnOC(=NH)CCl₃, TfOH, Et₂O, room temperature (20−80%); (c) DIBAL-H, CH₂Cl₂, −78 °C; then MeOH, −78 °C → room temperature; (d) **2b**, TiCl₄, *i*-Pr₂NEt, CH₂Cl₂, −78 °C; then add **6** (74%, two steps); (e) TBSOTf, 2,6-lutidine, CH₂Cl₂, −10 °C → room temperature (90%); (f) LiEt₃BH, THF, −78 °C → −40 °C (84%); (g) TsCl, Et₃N, DMAP, CH₂Cl₂ (91%); (h) LiEt₃BH, THF, room temperature (86%).
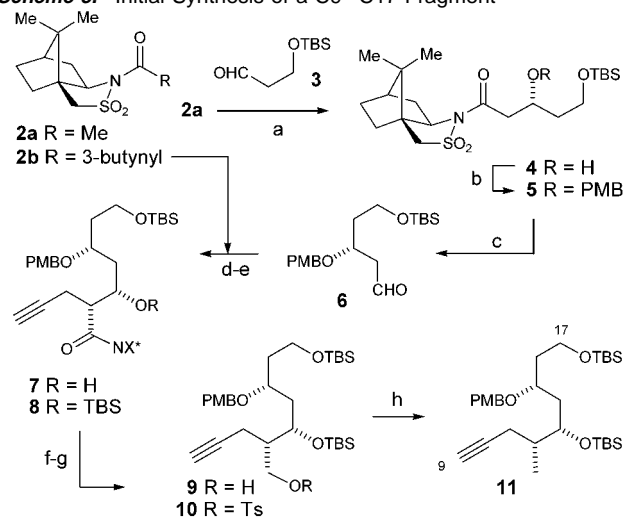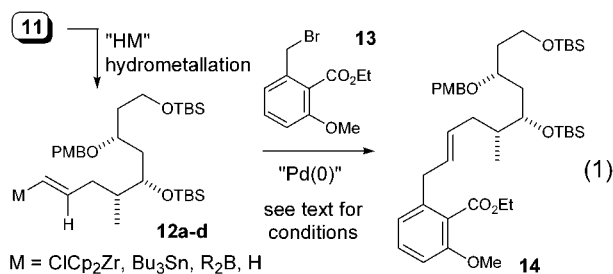
A connectivity analysis of the benzolactone core of salicylihalamide A points to two equally attractive routes for its assembly (Scheme 2). The first one features an esterification/intramolecular olefin metathesis sequence (RCM) to form the C9−C10 bond[15] and offers the advantage of operational simplicity combined with functional group tolerance (**A1** + **B1** → **AB**; Path A).[16] Fürstner 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 synthesis of salicylihalamides if concomitant stereocontrol 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-coupling[19] 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 alkyne 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 **3**[22] with the borylenolate derived from (2*R*)-*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 enolate[27] derived from (2*R*)-*N*-(4-pentynoyl)bornanesultam **2b**, and provided only one diastereoisomer **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*-butyldimethylsilyl (TBS) ether **8** followed by reduction of the *N*-acyl sulfonamide **8** (LiEt₃BH), tosylate formation, and further reduction of **10** (LiEt₃BH) 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 2), this would involve a hydrometalation of the triple bond followed by cross-coupling with benzylic bromide **13**[28] (eq 1). In this context, zirconocene **12a** (M = ZrCp₂Cl) 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
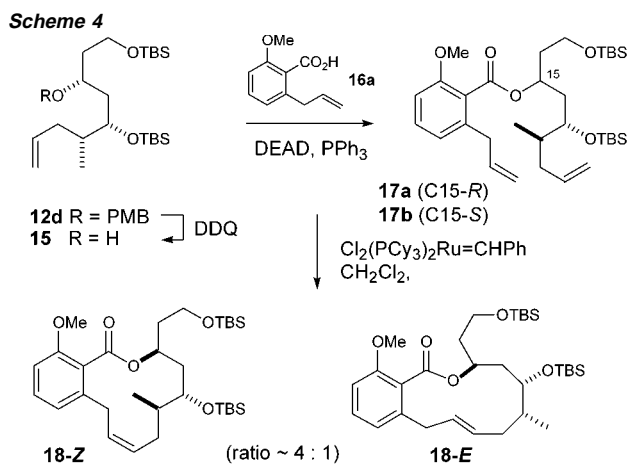
(15) Salicylihalamide numbering[1] will be used throughout this paper.

(16) For selected reviews on ring-closing metathesis, see: (a) Schuster, M.; Blechert, S. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2037−2056. (b) Grubbs, R. H.; Chang, S. *Tetrahedron* **1998**, *54*, 4413−4450. (c) Wright, D. L. *Curr. Org. Chem.* **1999**, *3*, 211−240. (d) Fürstner, A. *Angew. Chem., Int. Ed.* **2000**, *39*, 3012−3043.

(17) A modest selectivity for the *E*-isomer was observed (ratio 2.3:1): Fürstner, A.; Seidel, G.; Kindler, N. *Tetrahedron* **1999**, *55*, 8215−8230.

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

(19) Diederich, F.; Stang, P. J.; Eds. *Metal-Catalyzed, Cross-Coupling Reactions*; Wiley-VCH: Weinheim, 1998.

(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.

(22) Prepared in two steps from 1,3-propanediol: Eppley, A. W.; Totah, N. I. *Tetrahedron* **1997**, *53*, 16545−16552.

(23) Bond, S.; Perlmutter, P. *J. Org. Chem.* **1997**, *62*, 6397−6400.

(24) (a) Nakajima, N.; Horita, K.; Abe, R.; Yonemitsu, O. *Tetrahedron Lett.* **1988**, *29*, 4139−4142. (b) Iversen, T.; Bundle, D. R. *J. Chem. Soc., Chem. Commun.* **1981**, 1240−1241.

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

(26) Oppolzer, W.; Darcel, C.; Rochet, P.; Rosset, S.; De Brabander, J. *Helv. Chim. Acta* **1997**, *80*, 1319−1337.

(27) Evans, D. A.; Rieger, D. L.; Bilodeau, M. T.; Urpí, F. *J. Am. Chem. Soc.* **1991**, *113*, 1047−1049.

(28) Hamada, Y.; Hara, O.; Kawai, A.; Kohno, Y.; Shiori, T. *Tetrahedron* **1991**, *47*, 8635−8652.
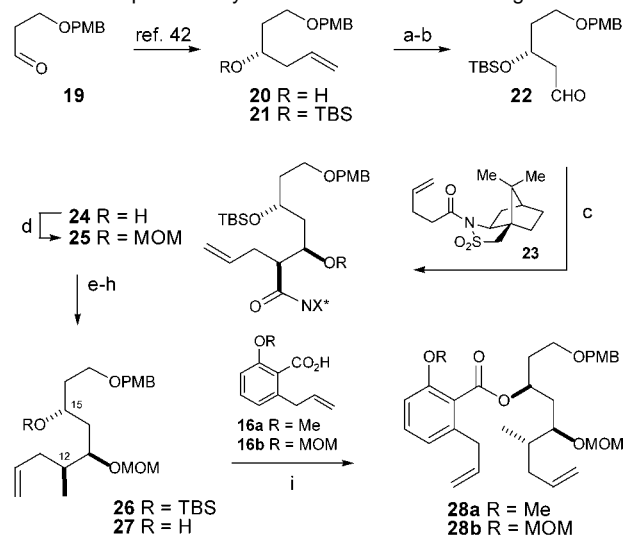
$$M = ClCp_2Zr, Bu_3Sn, R_2B, H \quad \textbf{12a-d}$$

(1)

Scheme 4

18-Z (ratio ~ 4 : 1) **18-E**

Scheme 5. Optimized Synthesis of the C10−C17 Fragment[a]

[a] Reagents and conditions: (a) catalyst OsO₄, NMO, acetone−H₂O; (b) NaIO₄, CH₂Cl₂ (97%, two steps); (c) **23**, TiCl₄, *i*-Pr₂NEt, CH₂Cl₂, −78 °C; then add **22**, −78 °C (92%); (d) MOMCl, NaI, *i*-Pr₂NEt, DME, reflux (91%); (e) LiEt₃BH, THF, −78 °C → room temperature (91%); (f) TsCl, DMAP, Et₃N, CH₂Cl₂, room temperature (91%); (g) LiEt₃BH, THF, room temperature (92%); (h) TBAF, THF, room temperature (98%); (i) **16a** or **16b**, DEAD, PPh₃, Et₂O, reflux (88−92%).

of benzyl halides.[29] We found that benzylic bromide **13** efficiently underwent Pd[PPh₃]₄ mediated cross-coupling with the alkenyl zirconocene derivative in situ prepared by hydrozirconation of 1-heptyne (ClHZrCp₂,[30] THF). Unfortunately, all attempts to obtain zirconocene **12a** via hydrozirconation of alkyne **11** were unsuccessful.[31] In contrast, alkyne **11** did undergo smooth hydrostannylation (Bu₃SnH, AIBN, PhMe → **12b**, M = SnBu₃) as a prelude to an alternative Stille coupling[32] with benzylic bromide **13**. This time, however, the corresponding vinylstannane **12b** was extremely prone to protodestannylation, yielding terminal alkene **12d** (M = H) upon workup.[33] After a lot of experimentation, the desired coupling product **14** could be obtained via a hydroboration (Sia₂BH, THF → **12c**, M = BSia₂)/Suzuki cross-coupling sequence in 15% yield (**13**, CsCO₃, Ph₃As, catalyst PdCl₂(dppf)₂, H₂O/DMF).[34] Again, the culprit seemed to be an inefficient hydrometalation as starting alkyne **11** accounted for the remaining mass balance.[35]

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 alkene **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**[36] (→ **17a**) uniformly met with failure.[37] A reactivity umpolung provided by a Mitsunobu esterification was essential and delivered bis-olefin **17b** in over 90% yield.[38] Gratifyingly, the subsequent RCM with Grubbs' ruthenium carbene complex[39] produced the benzolactones in essentially quantitative yield. 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 (**2** → **5**, Scheme 3). Now was the time to correct these imperfections, and a fully optimized gram-scale synthesis of the C10−C17 fragment was developed (Scheme 5).[40] First, an enantioselective allylation[41] of aldehyde **19** dramatically improved the stereochemical purity at C15, providing the known homoallyl alcohol **20** in 96% yield and 91% ee.[42] Silylation (TBSCl, imidazole, DMF) followed by oxidative double bond-cleavage afforded aldehyde **22** reproducibly in 91% yield (3

(29) Lipshutz, B. H.; Bulow, G.; Lowe, R. F.; Stevens, K. L. *Tetrahedron* **1996**, *52*, 7265−7276.

(30) In situ prepared ClHZrCp₂ was critical for obtaining reproducible results: Lipshutz, B. H.; Keil, R.; Ellsworth, E. L. *Tetrahedron Lett.* **1990**, *31*, 7257−7260.

(31) As a control experiment, a solution of in situ prepared HClZrCp₂ was divided into two portions to which were added **11** and 1-heptyne, respectively. 1-Heptyne underwent smooth hydrozirconation as judged by the subsequent cross-coupling with benzyl bromide **13**. Alkyne **11** on the contrary was recovered unchanged.

(32) For reviews see: (a) ref 19, pp 167−202. (b) Farina, V.; Krishnamurthy, V.; Scott, W. J. *Org. React.* **1997**, *50*, 1−652.

(33) Cross-coupling with the crude vinylstannane mixture was ineffective.

(34) (a) Miyaura, N.; Yano, T.; Suzuki, A. *Tetrahedron Lett.* **1980**, *21*, 2865−2868. (b) Miyaura, N.; Ishiyama, T.; Sasaki, H.; Ishikawa, M.; Satoh, M.; Suzuki, A. *J. Am. Chem. Soc.* **1989**, *111*, 314−321.

(35) A variety of hydroborating agents were tested, including 9-BBN, catecholborane, pinacolborane, freshly prepared Cy₂BH, and freshly prepared disiamylborane. At present, we can offer no explanation as to why this particular alkyne resists hydrozirconation or hydroboration.

(36) For the preparation of **16a** and **16b**, see the Supporting Information.

(37) The following reaction conditions were unsuccessful: (1) in situ activation of acid with TBTU, EDC, or DCC in combination with a variety of catalysts (DMAP, HOBt); (2) in situ activation of acid with 2,4,6-trichlorobenzoyl chloride; (3) direct acylation with the corresponding benzoyl chloride.

(38) Mitsunobu, O. *Synthesis* **1981**, 1−28.

(39) Schwab, P.; Grubbs, R. H.; Ziller, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 100−110.

(40) From this point forward (except Figure 2), the stereochemistry drawn corresponds to that of natural (−)-salicylihalamide A.

(41) Brown, H. C.; Randad, R. S.; Bhat, K. S.; Zaidlewicz, M.; Racherla, U. S. *J. Am. Chem. Soc.* **1990**, *112*, 2389−2392.

(42) Smith, A. B., III; Minbiole, K. P.; Verhoest, P. R.; Schelhaas, M. *J. Am. Chem. Soc.* **2001**, *123*, 10942−10953.

Table 1. Ring-Closing Olefin Metathesis Studies (Eq 2)

substrate	t (h)	products (ratio)[a,b]	
		catalyst **31**	catalyst. **32**
28a	1.3	**29a**:**30a** (90:10)	**29a**:**30a** (66:34)
28a	6.3	**29a**:**30a** (88:12)	**29a**:**30a** (68:32)
28a	18.3	**29a**:**30a** (80:20)	**29a**:**30a** (67:33)
28b	1.3	**29b**:**30b** (90:10)	**29b**:**30b** (64:36)
28b	6.3	**29b**:**30b** (88:12)	**29b**:**30b** (67:33)
28b	18.3	**29b**:**30b** (84:16)	**29b**:**30b** (68:32)

[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 (2*S*)-*N*-(4-pentenoyl) bornanesultam **23**[43] 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[38] coupling of **27** with carboxylic acid **16a**[36] or the corresponding phenolic MOM ether **16b**[36] 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 impressive selectivity of 9:1 when subjected to similar reaction conditions (eq 2; 5 mol % catalyst

(2)

29a R = Me
29b R = MOM

30a R = Me
30b R = MOM
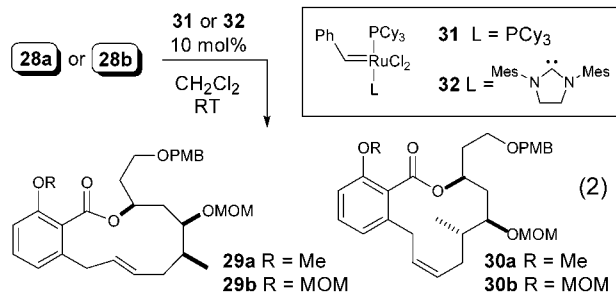
31, CH$_2$Cl$_2$, room temperature).[44] To confuse the issue even more, an initial single experiment with the corresponding phenolic methoxymethyl ether **28b** furnished benzolactones **29b** and **30b** with an eroded selectivity of 3:1 upon exposure to catalyst **31**. In light of the above, a detailed study of the RCM of **28a,b** with Ru-alkylidene precatalysts **31** and **32**[45] was conducted (eq 2 and Table 1).[46] 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 pre-catalyst **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−g] 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 Fürstner 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 salicylihalamide 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

(43) Oppolzer, W.; Osamu, T.; Deerberg, J. *Helv. Chim. Acta* **1992**, *75*, 1965−1978.

(44) For selected examples of remote functionality affecting stereoselectivity, see: (a) Fürstner, A.; Langemann, K. *J. Org. Chem.* **1996**, *61*, 3942−3943. (b) Meng, D.; Su, D.; Balog, A.; Bertinato, P.; Sorensen, E. J.; Danishefsky, S. J.; Zheng, Y.; Chou, T.; He, L.; Horwitz, S. B. *J. Am. Chem. Soc.* **1997**, *119*, 2733−2734.

(45) *N*-Heterocyclic carbene ligated ruthenium alkylidene catalysts have been independently reported by three groups, cf.: (a) Huang, J.; Stevens, E. D.; Nolan, S. P.; Petersen, J. L. *J. Am. Chem. Soc.* **1999**, *121*, 2674−2678. (b) Scholl, M.; Trnka, T. M.; Morgan, J. P.; Grubbs, R. H. *Tetrahedron Lett.* **1999**, *40*, 2247−2250. (c) Ackermann, L.; Fürstner, A.; Weskamp, T.; Kohl, F. J.; Herrmann, W. A. *Tetrahedron Lett.* **1999**, *40*, 4787−4790.

(46) Identical reaction conditions (solvent, concentration, catalyst loading, temperature, same batch of catalyst) were employed for all reactions of Table 1.

(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.

(48) For selected examples of reversible olefin metathesis reactions, see: (a) Marsella, M. J.; Maynard, H. D.; Grubbs, R. H. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 1101−1103. (b) Xu, Z.; Johannes, C. W.; Houri, A. F.; La, D. S.; Cogan, D. A.; Hofilena, G. E.; Hoveyda, A. H. *J. Am. Chem. Soc.* **1997**, *119*, 10302−10316. (c) Hamilton, D. G.; Feeder, N.; Teat, S. J.; Sanders, J. K. M. *New J. Chem.* **1998**, *22*, 1019−1021. (d) Smith, A. B., III; Adams, C. M.; Kozmin, S. A.; Paone, D. V. *J. Am. Chem. Soc.* **2001**, *123*, 5925−5937. (e) Lee, W. C.; Grubbs, R. H. *Org. Lett.* **2000**, *2*, 2145−2147; correction: *Org. Lett.* **2000**, *2*, 2559. (f) Fürstner, A.; Thiel, O. R.; Ackermann *Org. Lett.* **2001**, *3*, 449−451. (g) Lee, W. C.; Grubbs, R. H. *J. Org. Chem.* **2001**, *66*, 7155−7158. (h) Wright, D. L.; Usher, L. C.; Estrella-Jimenez, M. *Org. Lett.* In press.

(49) For the mechanism and activity of ruthenium olefin metathesis catalysts, see: Sanford, M. S.; Love, J. A.; Grubbs, R. H. *J. Am. Chem. Soc.* **2001**, *123*, 6543−6554 and references therein.

(50) For stability and decomposition studies, see: Ulman, M.; Grubbs, R. H. *J. Org. Chem.* **1999**, *64*, 7202−7207.

(51) Fürstner and co-workers recently reported results concerning the RCM of compounds that differ from **28a,b** only by an additional gem-dimethyl substitution at the C9 olefin terminus.[10d,e] Limited to the use of a more competent second-generation catalyst, compounds **29a**/**30a** and **29b**/**30b** were obtained in an identical ratio of 67:33.

(52) Dess, D. B.; Martin, J. C. *J. Am. Chem. Soc.* **1991**, *113*, 7277−7287.

Scheme 6 *a*
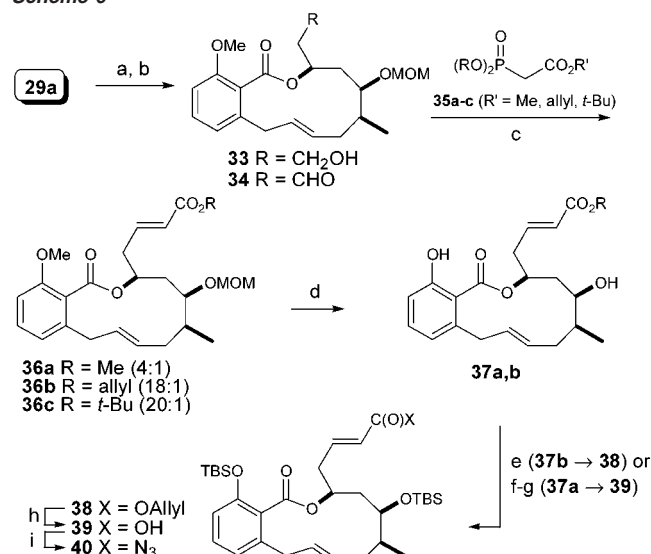
a Reagents and conditions: (a) DDQ, CH₂Cl₂−H₂O, room temperature (96%); (b) Dess−Martin periodinane, CH₂Cl₂, room temperature (95%); (c) **35a−c**, NaH, THF, 0 °C (85−95%); (d) BBr₃, CH₂Cl₂, −78 °C (81%); (e) TBSCl, imidazole, DMAP, DMF, room temperature (92% of **38**); (f) Ba(OH)₂·8H₂O, THF, room temperature; (g) TBSCl, imidazole DMAP, DMF, room tmeperature (50% of **39**, two steps); (h) catalyst Pd(PPh₃)₄, morpholine, THF, room temperature (96%); (i) (PhO)₂P(O)N₃, Et₃N, PhH, room temperature (92−96%).

removal of the ether protecting groups with BBr₃, the corresponding *E*-methyl ester **37a** was separated by flash chromatography,[53] hydrolyzed (Ba(OH)₂·8H₂O) and extensively silylated with excess TBSCl to deliver carboxylic acid **39**.[54] The overall yield for the transformation of **34** to **39** was a disappointing 20−30%. Therefore, a series of optimization experiments were performed that quickly led to the use of allyl diethylphosphonoacetate, delivering allyl ester **36b** with 18:1 selectivity for the *E*-isomer.[55] Subsequent deprotection, bissilylation, and Pd-catalyzed removal of the allyl ester improved the overall yield of **39** dramatically (67% from aldehyde **34**). Transformation into acyl azide **40** ((PhO)₂P(O)N₃, NEt₃, PhH)[56] preceded a Curtius rearrangement induced by heat (PhH, 80 °C, 6 h). Although the corresponding isocyanate **41** was stable to chromatographic purification, it was usually engaged in the next reaction without purification (Scheme 7).

Final carbon−carbon bond formation proceeded smoothly via the addition of hexadienyllithium, prepared in situ from bromide **42**[57] via metal−halogen exchange (*t*-BuLi, Et₂O), to a −78 °C solution of isocyanate **41** (Scheme 7). The desired compound was obtained as an inseparable mixture of 22*Z* and 22*E* isomers together with a chromatographically (silicagel) more mobile fraction. 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

(53) 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 protecting groups that could be removed after installation of the acid-labile acylated enamine functionality.
(55) The use of *tert*-butyl dimethylphosphonoacetate **35c** provided *tert*-butyl ester **36c** with 20:1 *E*:*Z*-selectivity. However, **36c** decomposed to unidentifiable products upon BBr₃ treatment (CH₂Cl₂, −78 °C).
(56) Ninomiya, K.; Shioiri, T.; Yamada, S. *Tetrahedron* **1974**, *30*, 2151−2157.
(57) (1*Z*,3*Z*)-1-Bromo-1,3-hexadiene (**42**) was contaminated with 20−50% of (1*Z*,3*E*)-1-bromo-1,3-hexadiene and used as such. For the preparation of **42**, see the Supporting Information.

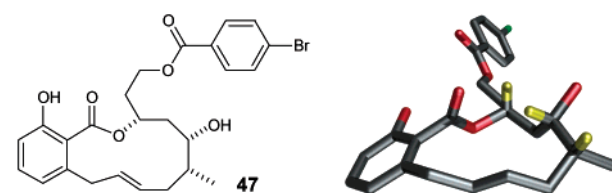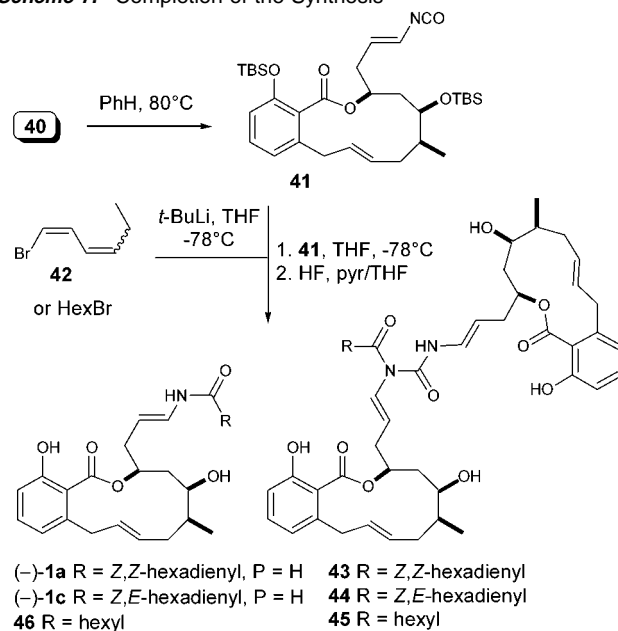**Scheme 7.** Completion of the Synthesis

Figure 2. Rasmol Representation of the X-ray Structure of *p*-Bromobenzoate **47**.

by semipreparative HPLC.[58] 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.[59]

In contrast to the current study, we had initially synthesized *ent*-**1a** based on the absolute configuration reported for the natural product.[9a] 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 HPLC (two different solvent systems), and TLC (three different solvent systems),[60,61] the signs of the optical rotations of synthetic *ent*-**1a** ([α]²³_D +20.8; *c* 0.12; MeOH) and the natural product (reported: [α]²³_D −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.[61] 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).[9a] Based on all the available evidence, the absolute configuration of natural (−)-salicylihalamide A was assigned

(58) The ratios of **1a**:**1c** and **43**:**44** are indicative of the isomeric ratio of bromide **42**.
(59) These dimers are presumably formed through reaction of the intermediate lithiated amide with a second molecule of isocyanate **41**. For a similar observation, see ref 13b.
(60) Careful examination of the ¹H NMR spectra of natural salicylihalamide A indicates the presence of a minor contaminant with an identical spectroscopic signature to our synthetic *Z*,*E*-isomer **1c** (see spectra provided in the Supporting Information). This contaminant could be a natural isomer of salicylihalamide A, or the result of isomerization during the isolation/purification procedure.
(61) We thank Dr. Michael Boyd (NCI) for providing a sample of natural salicylihalamide A and for testing synthetic **1a,c** in the 60-cell line screen.

Scheme 8. Synthesis of Side Chain Modified Salicylihalamides[a]
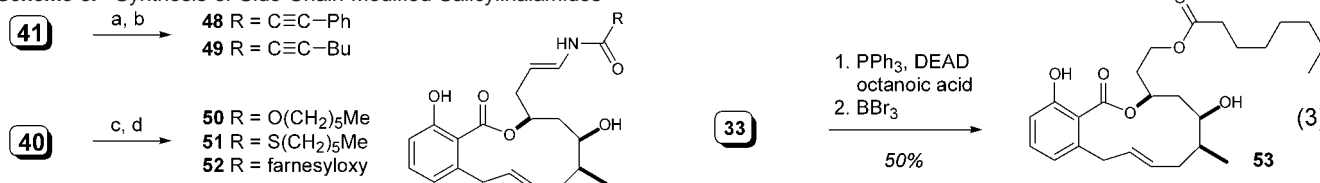
[a] Reagents and conditions: (a) phenylacetylene or 1-hexyne, *n*-BuLi, −78 °C; then add **41**, −78 °C; (b) HF·Py, Py-THF, room temperature (**48**: 57%, **49**: 50%; two steps); (c) 1-pentanol or 1-pentanethiol or farnesol, **40**, PhH, 80 °C; (d) HF·Py, Py-THF, room temperature (**50**: 47%, **51**: 80%, **52**: 50%; two steps).

to be as drawn in **1a**. Unequivocal proof for the absolute stereochemistry of natural salicylihalamide ultimately came from biological 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 hexyl-lithium (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 detected (Scheme 8). Alternatively, carbamates or thiocarbamates **50**−**52** are obtained in an operationally simplified procedure by heating the acyl azide **40** in the presence of the appropriate alcohol or thiol. In light of the fact that V-ATPase is a membrane-bound protein, we also prepared analogue **52** incorporating a lipophilic farnesyl anchor hoping to target this derivative to the membrane (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 Mitsunobu[38] 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 endocyclic 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 radio-active 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 brain[62] to completely eliminate potential effects arising from contaminating nonvacuolar ATPase activities present in the crude membrane preparations utilized in the initial[6] 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 IC_{50} 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

(62) (a) Xie, X.-S.; Tsai, S.-J.; Stone, D. K. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8913−8917. (b) Mattsson, J. P.; Schlesinger, P. H.; Keeling, D. J.; Teitelbaum, S. L.; Stone, D. K.; Xie, X.-S. *J. Biol. Chem.* **1994**, *269*, 24479−24982.

(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.

Scheme 9. Synthesis of Reduced Salicylihalamides[a]
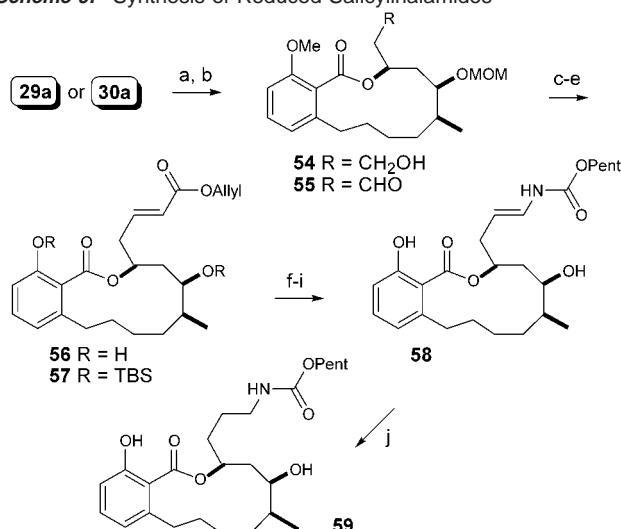
[a] Reagents and conditions: (a) **29a**, 10% Pd/C, H$_2$, MeOH, room temperature (82%); or **30a**, 10% Pd/C, NH$_4$O$_2$CH, MeOH, reflux (67%); (b) Dess–Martin periodinane, CH$_2$Cl$_2$, room temperature (81%); (c) CH$_2$=CHCH$_2$OP(O)(OEt)$_2$, NaH, THF, 0 °C (93%); (d) BBr$_3$, CH$_2$Cl$_2$, −78 °C (97%); (e) TBSCl, imidazole, DMAP, DMF, room temperature (67%); (f) catalyst Pd(PPh$_3$)$_4$, morpholine, THF, room temperature (92%); (g) (PhO)$_2$P(O)N$_3$, Et$_3$N, PhH, room temperature; (h) 1-pentanol, PhH, 80 °C; (i) HF·Py, Py-THF, room temperature (43%; three steps); (j) 10% Pd/C, H$_2$, MeOH, room temperature (25%).

Scheme 10. Synthesis of Hydroxy-Derivatized Salicylihalamides[a]
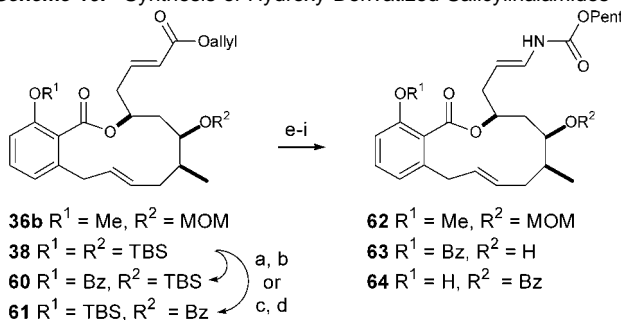
[a] Reagents and conditions: (a) TBAF, THF, room temperature (84%); (b) BzCl, Py, room temperature (78%); (c) concentrated HCl, THF, room temperature (91%); (d) BzCl, Py, room temperature (50%); (e) catalyst Pd(PPh$_3$)$_4$, morpholine, THF, room temperature (85−97%); (f) (PhO)$_2$P(O)N$_3$, Et$_3$N, PhH, room temperature (68−96%); (h) 1-pentanol, PhH, 80 °C (**62**: 55%; benzoates: not purified) (i) HF·Py, Py-THF, room temperature (**63**: 55%, **64**: 33%; two steps).

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

compd	IC$_{50}$ (nM)	compound	IC$_{50}$ (nM)
Bafilomycin A	3.1	**49**	1
1a	<1	**50**	1.6
ent-**1a**	270	**51**	1.8
37b	230	**52**	>1000
43	3.7	**53**	1000
44	1.2	**58**	3
45	3	**59**	30
46	1	**63**	300
48	<1	**64**	180

[a] IC$_{50}$ values (measured according to ref 63) are the average of at least three experiments (error ±5%).

concentrations similar to those of the parent compound. This indicates that the hexadienoyl fragment is not crucial for biological activity and that substantial sterically demanding modifications emanating off the amide nitrogen can be accom-

modated 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 farnesylated 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.[64] Moreover, whereas the *N*-carbamoyl enamine derivative **58** retained a significant capacity to inhibit proton pumping in the V-ATPase assay, the enamide 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.[65] Although the structure−activity relationships found in this assay [IC$_{50}$ (μM): **1a/c**, 0.06; **43**, 0.04; **44**, 0.1; **45**, 0.6; **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 or the function of an alternative cellular target as the mechanism by which salicylihalamides induce differential cytotoxicity 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 isocyanate **41** through Horner−Wadsworth−Emmons homologation of aldehyde **34**, followed by Curtius rearrangement of acyl azide **40**. Final addition of hexadienyllithium to a cold solution of vinyl isocyanate **41**, followed by a mild deprotection delivered

(64) However, an intact side chain is not sufficient for biological activity given the inactivity of the unnatural enantiomer of salicylihalamide A.

(65) This cell line was reported to be particularly sensitive to natural salicylihalamide A.[1]

(66) A detailed study of the cytotoxic effects of salicylihalamide A and analogues against a panel of human breast and lung cancer cell lines, including drug-resistant cell lines, will be published elsewhere.

salicylihalamide A (**1a**), the 22*E*-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.

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.

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