Design, Synthesis, and Biological Evaluation of Fluorinated Analogues of Salicylihalamide

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Received October 7, 2008

Salicylihalamide A (SA), a benzolactone enamide compound, possesses potent cytotoxicity against human tumor cell lines. SA is a selective inhibitor of mammalian vacuolar type H^+-ATPase (V-ATPase), and is distinct from previously known V-ATPase inhibitors such as bafilomycins and concanamycins that do not discriminate between mammalian and nonmammalian V-ATPases. Because of its potent antitumor activity and structural simplicity, SA is a promising candidate for an anticancer drug. Although a number of structure−activity relation studies using synthetic analogues have been reported, no fluorinated derivative of SA has been evaluated even though selective addition of a fluorine atom into a therapeutic small molecule candidate often enhances pharmacokinetic and physicochemical properties. We designed and synthesized fluorinated analogues of SA and evaluated their V-ATPase inhibitory activities. Compared to the natural product, the synthetic analogues were potent V-ATPase inhibitors, suggesting that these analogues are potential drug candidates and potential molecular probes for mode-of-action studies using fluorine-based analytical methods such as ^{19}F-NMR spectroscopy.

Introduction

Development of antitumor drugs with limited side effects is important for cancer patients undergoing chemotherapy. Although combinatorial chemistry has greatly influenced the drug discovery process,[1] isolation of novel natural products remains important for discovery of lead compounds possessing promising biological profiles.[2] Salicylihalamide A (SA[a], **1a**) was isolated from the sponge *Haliclona* sp. (southwestern Australian coast) by Boyd and co-workers in 1997 with the minor component salicylihalamide B (SB, **1b**),[3] containing a labile enamide linkage connecting a salicylic acid-derived macrolactone core (Figure 1). Since the discovery of SA, a number of structurally similar bioactive compounds that possess a benzolactone enamide moiety have been isolated (Figure 2),[4] e.g., lobatamides A (**2**),[5] apicularenes A (**3**),[6] **4** (CJ-12,950),[7] and oximidines I (**5**).[8] SA elicited a unique differential cytotoxicity profile in the human tumor NCI 60-cell line, with a mean panel GI_{50} value of 15 nM, and melanoma cell lines showed the highest average sensitivity (GI_{50} = 7 nM).[3] Furthermore, SA was a potent inhibitor of vacuolar H^+-ATPases (V-ATPases), with an IC_{50} value less than 1.0 nM against bovine brain V-ATPase.[9] The V-ATPases are ubiquitous proton-translocating pumps of eukaryotic cells located on the membranes of vacuoles, lysosomes, and other components of the endomembrane system, which regulate the pH of the compartments. Numerous physiological processes depend on the activity of V-ATPases, which are implicated as a contributing factor in multiple diseases including osteoporosis and cancer.[10] SA is distinct from previously known V-ATPase inhibitors such as bafilomycins and concanamycins, which do not discriminate between mammalian and nonmam-

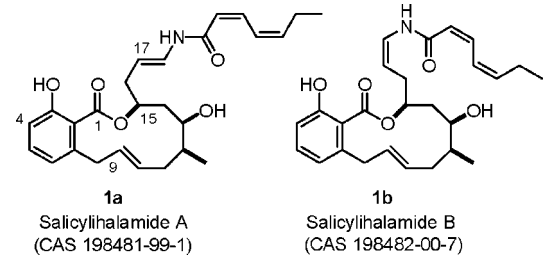

Figure 1. Structures of salicylihalamide A and B.

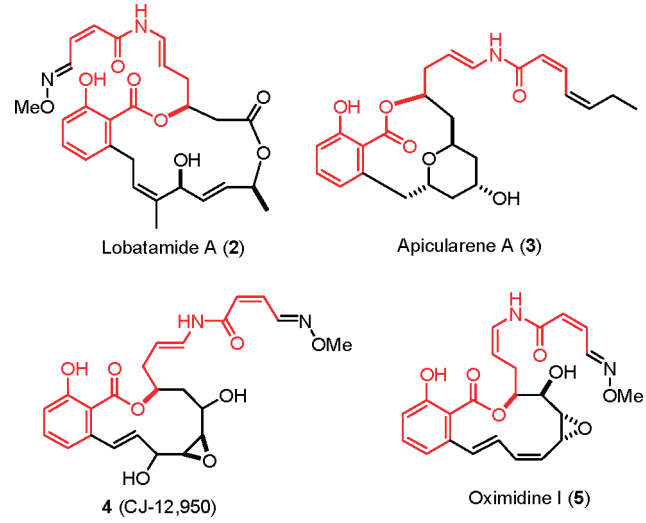

Figure 2. Structures of benzolactone enamide class compounds.

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[a] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; ATP, adenosine 5′-triphosphate; BCA, bicinchoninic acid; CGM, chromaffin granule membranes; CuTC; copper thiophene-2-carboxylate; dba, dibenzylideneacetone; DDQ, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone; DEAD, diethyl azodicarboxylate; DMA, *N*,*N*-dimethylacetamide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; F-SA, 4-^{19}F-salicylihalamide A; F-SB, 4-^{19}F-salicylihalamide B; GI_{50}, 50% growth inhibition; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; IC_{50}, 50% inhibition concentration; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NCI, National Cancer Institute; NMP, *N*-methylpyrrolidone; NMR, nuclear magnetic resonance; PMB, *p*-methoxybenzyl; SA, salicylihalamide A; SB, salicylihalamide B; Tf, trifluoromethanesulfonyl; TFP, 2-trifurylphosphine; THF, tetrahydrofuran; V-ATPase, vacuolar type H^+-ATPase.

10.1021/jm801265e CCC: $40.75
Published on Web 12/31/2008

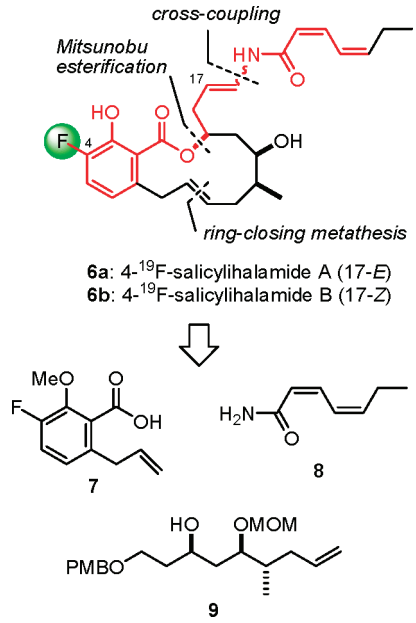


Figure 3. Design and synthetic strategy of fluorinated salicylihalamides.

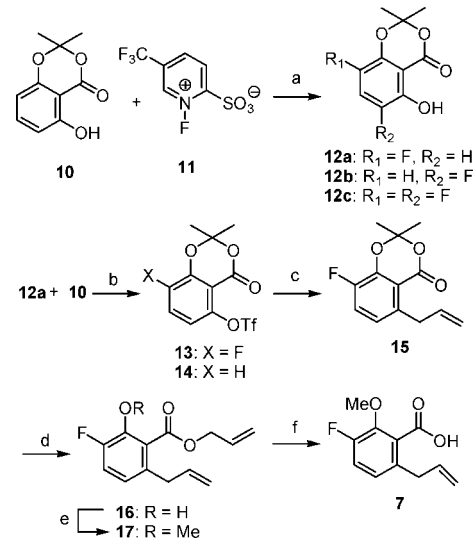
malian V-ATPases. SA binds to the transmembrane (V_0) domain of V-ATPase at a site different from bafilomycin A_1 and concanamycin A,[11] while the precise mode-of-action of SA and structure of the ligand−protein complex have not been elucidated.

Because of the potent antitumor activity and structural simplicity compared with other V-ATPase inhibitors, SA is expected to be a promising anticancer drug candidate.[12] Although a number of synthetic studies and structure−activity relation studies have been reported,[13] no fluorinated derivatives of SA have been evaluated, while examination of the role of fluorine in medicinal chemistry reveals that selective addition of fluorine into a therapeutic small molecule candidate can enhance pharmacokinetic and physicochemical properties such as metabolic stability and membrane permeability and can increase binding affinity of drug candidates to the target protein.[14] Therefore, fluorinated SA may be a promising drug candidate, as well as a possible molecular probe for elucidating the mode-of-action when using fluorine-based analytical methods such as ^{19}F-NMR spectroscopy. The properties of ^{19}F (nuclear spin of 1/2, high gyromagnetic ratio, 100% natural abundance, and low background signals in biological systems) make it useful for investigating biological systems.[15] Here, we report the design and synthesis of fluorinated salicylihalamides and evaluation of the V-ATPase inhibitory activity.

Results and Discussion

Chemistry. The compounds, 4-^{19}F-salicylihalamide A (F-SA, **6a**) and B (F-SB, **6b**), were designed as target molecules (Figure 3) because the salicylate moiety is a common structural feature of natural benzoenamide class products, which is considered to be a binding motif toward the target protein, and electrophilic fluorination of phenol derivatives is a versatile synthetic method.[16] However, the utility of introduction of fluorine at C4 position was uncertain because of its proximity to the hydroxy group at C3, which might be involved in hydrogen bonding with the target protein. Fluorinated analogues were synthesized based on previous reports of the total synthesis and synthetic studies of SA[13] and benzolactone enamide class natural products (Figure 3).[17−19] The enamide side chain, the most labile moiety of the molecule, was planned to be introduced

Scheme 1[a]



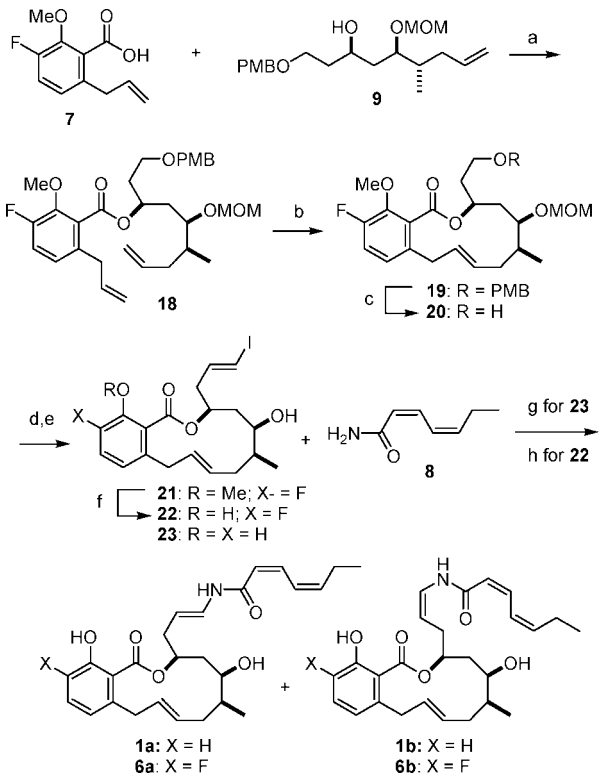
[a] Reagents and conditions: (a) $Cl_2CHCHCl_2$, 146 °C, 3 h, **12a**:**12b**:**12c**:**10** = 5:9:1:10; (b) Tf_2O, pyridine, 0 °C, 1 h, **13** (16%, two steps), **14** (27%, two steps); (c) *n*-$Bu_3SnCH_2CH{=}CH_2$, $[Pd_2(dba)_3]\cdot CHCl_3$, TFP, LiCl, NMP, rt, 18 h, 84%; (d) EtMgBr, $CH_2{=}CHCH_2OH$, THF, 0 °C to rt, 18 h, 81%; (e) MeI, K_2CO_3, acetone, 24 h, 92%; (f) $Pd(PPh_3)_4$, morpholine, THF, 1 h, 85%.

at the final stage by cross-coupling using amide **8**,[13d] and the macrocyclic core was to be constructed by ring-closing metathesis of the diene derived from salicylate derivative **7** and alcohol **9**[13g,] by Mitsunobu esterification.[20]

For synthesis of building block **7**, electrophilic fluorination of the salicylate derivative **10**[21] was conducted as shown in Scheme 1. Treatment of **10** with *N*-fluoro-5-(trifluoromethyl)-pyridinium-2-sulfonate **11**[22] in 1,1,2,2-tetrachloroethane at 146 °C for 3 h afforded a mixture of monofluorinated **12a** and **12b**, difluorinated **12c**, and recovered **10** in ca. 5:9:1:10 ratio. The mixture was separated by silica gel column chromatography as a mixture of **12a** and **10**, and **12b** and **12c**, respectively. The mixture of **12a** and **10** was treated with Tf_2O in pyridine to afford triflate **13** (16%, 2 steps), which was separated from **14**.[23] According to De Brabander's procedure,[13a] triflate **13g** was converted to carboxylic acid **7** via (i) Stille coupling of triflate **13** with allylstannane, (ii) transesterification of **15** with removal of the acetonide by treatment with alkoxide generated from allyl alcohol and ethylmagnesium bromide, (iii) protection of the resulting phenol **16** as a methyl ether **17**, and (iv) conversion of the allyl ester to carboxylic acid **7** through the action of a palladium catalyst.

Then, the coupling of the fragments was performed as shown in Scheme 2. Condensation of carboxylic acid **7** with secondary alcohol **9** under Mitsunobu esterification conditions using DEAD/PPh_3 afforded diene **18**. Ring-closing metathesis of the diene by the action of Grubbs catalyst[24] resulted in the formation of macrocycle **19** (*E*:*Z* = 10:1). Removal of the PMB group followed by Dess−Martin oxidation of the resulting alcohol **20** gave an aldehyde, which was converted to iodoolefin **21** by Takai olefination[25] under modified conditions reported by Evans.[26] Removal of the methyl group with BBr_3 gave key intermediate **22** for the coupling reaction to construct the enamide moiety,[27] which is the most critical step in the present synthesis. Prior to the synthesis of fluorinated analogues, coupling of counterpart **23**[13f] with amide **8** giving natural SA was examined. After considerable experimentation according to Fürstner's procedure[13f] and modification by Panek,[18a] coupling of iodoolefin **23** with amide **8** was achieved by treatment

Scheme 2[a]



[a] Reagents and conditions: (a) DEAD, PPh_3, Et_2O, rt, 36 min, 91%; (b) $(PCy_3)_2Cl_2Ru{=}CHPh$, CH_2Cl_2, rt, 21 min, 90%; (c) DDQ, CH_2Cl_2/H_2O, 0 °C to rt, 53 min, 97%; (d) Dess−Martin periodinane, CH_2Cl_2, 0 °C to rt, 65 min; (e) $CrCl_2$, CHI_3, dioxane, THF, rt, 26 h 80% (two steps); (f) BBr_3, CH_2Cl_2, −78 °C, 1 h, 74%; (g) **8**, CuTC, 2,2′-bipyridine, Rb_2CO_3, DMA, 90 °C, 60 min, **1a** (23%), **1b** (26%); (h) **8**, CuTC, 2,2′-bipyridine, Rb_2CO_3, DMA, 90 °C, 60 min, **6a** (15%), **6b** (22%).

with copper thiophene-2-carboxylate (CuTC)[28] in the presence of Rb_2CO_3 and 2,2′-bipyridine in *N*,*N*-dimethylacetamide (DMA) at 90 °C for 60 min to afford a mixture of *E*,*Z*-isomers of the enamide moiety in ca. 1:1 ratio. The mixture of enamide moiety isomers was purified by HPLC to afford SA (**1a**, 23%) and SB (**1b**, 26%), whose physical data were identical to reported values.[3] Note that the use of exhaustively degassed solvent (DMA) is essential for a successful coupling reaction. Although nearly equal amounts of isomers formed in contrast to the previous report,[13f,18a,27] it is advantageous for structure−activity relation studies to obtain a significant amount of SB, which was isolated as a minor compound. In an analogous sequence, fluorinated analogues were synthesized and purified by HPLC to afford F-SA (**6a**, 15%) and F-SB (**6b**, 22%).

Biological Results and Discussion. V-ATPase inhibitory activity of fluorinated analogues F-SA (**6a**) and F-SB (**6b**), as well as synthetic SA (**1a**) and SB (**1b**), were evaluated in cultured simian renal cells (COS-7) and chromaffin granule membranes prepared from porcine adrenal glands by monitoring a pH change visualized by fluorescent dyes.

1. Imaging Assays Using Simian Renal Cells (COS-7). The effect of salicylihalamides was examined on the luminal pH of intracellular acidic compartments (lysosomes), which were acidified prominently by the proton pumping activity of V-ATPase. Simian renal cells (COS-7)[29] were incubated with salicylihalamides SA (**1a**) and SB (**1b**), and 4-^{19}F-salicylihalamides, F-SA (**6a**) and F-SB (**6b**), at a concentration of 1 μM for 5 h. Then, the cells were incubated with the pH indicator LysoSensor Green (1 μM) for 1 min.[30] As shown in Figure 4A, lysosomes of intact cells emitted significant fluorescence,

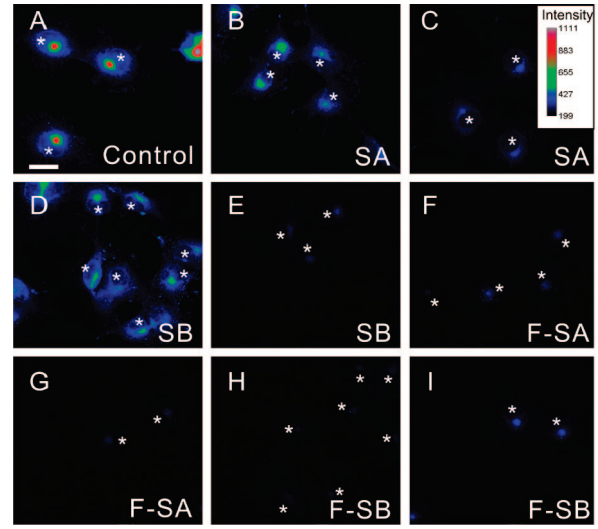


Figure 4. Change in pH of acidic organelles (lysosomes) of simian renal cells (COS-7) induced by salicylihalamides: (A) control; (B) salicyclihalamide A (SA, **1a**), 1 μM; (C) SA; (D) salicyclihalamide B (SB, **1b**), 1 μM; (E) SB; (F) 4-^{19}F-salicylihalamide A (F-SA, **6a**), 1 μM; (G) F-SA; (H) 4-^{19}F-salicylihalamide B (F-SB, **6b**), 1 μM; (I) F-SB; Change in pH was visualized by fluorescence microscopy using LysoSensor Green. The panels B and C, D and E, F and G, and H and I were the results of duplicate experiments. The fluorescent intensity (arbitrary unit) is indicated in pseudocolor as shown in the color scale on the top-right. Asterisks denote the nuclei of the cells. Scale bar, 20 μm.

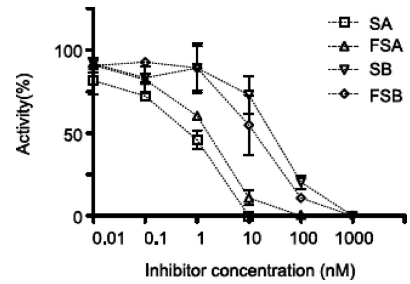


Figure 5. Concentration-dependent inhibition of H^+ transport activity of V-ATPase (from porcine adrenal glands) by synthetic salicylihalamides.

indicating that these organelles were highly acidified. When the cells were treated with SA (Figure 4B,C) or SB (Figure 4D,E), fluorescence diminished, reflecting an increase in pH in these organelles by inhibition of V-ATPase. To assess the viability, the trypan blue dye exclusion test was also performed after the drug treatment, and the results suggested that the viability was not affected by the drug treatment at this time point (data not shown). As expected, not only F-SA (Figure 4F,G) but also F-SB (Figure 4H,I) also actively inhibited the proton pump.

2. Quantitative Assays Using Chromaffin Granule Membranes. Although V-ATPase inhibitory activity is commonly evaluated by measuring ATP-dependent proton uptake into isolated chromaffin granules from bovine adrenal glands,[31] chromaffin granules from porcine adrenal glands were used in this study due to short supply of fresh bovine tissue. Proton uptake into the membrane vesicles was measured by monitoring the fluorescence quenching of the 9-amino-6-chloro-2-methoxyacridine (ACMA)[31,32] employed as an indicator. The activity of synthetic SA and SB, as well as fluorinated analogues F-SA and F-SB, were evaluated (Figure 5), and the results are summarized in Table 1. SA elicited potent inhibitory activity with an IC_{50} value of approximately <1 (0.7) nM, comparable

Table 1. Inhibition of H^+ Transport Activity of V-ATPase (from Porcine Adrenal Glands) by Synthetic Salicylihalamides[a]

entry	compound	IC_{50}/nM
1	**1a** (SA)	<1 (0.7)
2	**6a** (F-SA)	2
3	**1b** (SB)	30
4	**6b** (F-SB)	10

[a] IC_{50} values are the average of three experiments.

to reported values. As expected, F-SA acted as a potent inhibitor of V-ATPase (IC_{50} = 2 nM), but was slightly less potent than SA. Since the V-ATPase inhibitory activity of SB has not been reported (SB was isolated as minor compound: ~0.5 mg), the IC_{50} value of be approximately 30 nM was determined for the first time. This value indicates that SB is 30 times less potent than SA. Note that the activity of F-SB was comparable to that of the corresponding natural product (IC_{50} = 10 nM).

Conclusions

In conclusion, 4-^{19}F-salicylihalamide A (F-SA) and B (F-SB) were synthesized through electrophilic fluorination of the salicylate derivative, ring-closing metathesis for the construction of the macrocyclic core, and cross-coupling reaction for installation of the enamide side chain. The V-ATPase inhibitory activity of synthetic salicylihalamides was evaluated both in vivo and in vitro using cultured simian renal cells (COS-7) and chromaffin granule membranes prepared from porcine adrenal glands, respectively. The F-SA was a potent V-ATPase inhibitor (IC_{50} = 2 nM) comparable to SA (<1 nM), suggesting that the fluorinated analogue is a promising drug candidate and a potential probe for mode-of-action studies using fluorine-based analytical methods. In this study, the previously unknown IC_{50} value of SB was estimated to be 30 nM and that of F-SB (10 nM) was comparable to SB. Further studies on the antitumor activity of fluorinated salicylihalamides and mode-of-action studies based on ^{19}F-NMR are currently in progress in our laboratory.

Experimental Section

Chemistry. General Procedures. All the solvents and chemicals were used without further treatment unless otherwise stated. The following solvents and chemicals used were purified by distillation over the drying agents indicated in parentheses and were transferred under argon atmosphere: THF, Et_2O and 1,4-dioxane (Mg/benzophenone), 1,1,2,2-tetrachloroethane, pyridine, triethylamine, and morpholine (CaH_2), dichloromethane ($LiAlH_4$), allyl alcohol (Mg). *N*-methylpyrrolidone (NMP) was dried over activated MS4A for 3 days and distilled. *N*,*N*′-dimethyl acetamide (DMA) was distilled over di-*n*-butyltin dilaurilate, redistilled over CaH_2 under reduced pressure, and stocked in the presence of activated MS4A. Before use, the DMA was degassed by freeze−thaw method over 10 times. Analytical thin-layer chromatography (TLC) was performed using E. Merck silica gel 60 F_{254} precoated plates (0.25-mm thickness). For column chromatography, Kanto silica gel 60N (spherical, neutral, 100−210 μm), and Merck silica gel 60 (40−63 μm, for flash column chromatography) were used. Optical rotations were recorded on a JASCO P-1010 polarimeter. IR spectra were recorded on a JASCO FT-IR-300E Fourier transform infrared spectrometer. ^{1}H, ^{13}C, and ^{19}F-NMR spectra were recorded on a JEOL JNM-GSX500 or JNM-ECA500 spectrometer. Chemical shifts of ^{1}H and ^{13}C NMR are reported in ppm from tetramethylsilane with reference to internal residual solvent [^{1}H NMR, $CHCl_3$ (7.24), C_6HD_5 (7.15), $CHDCl_2$ (5.29); ^{13}C NMR, $CDCl_3$ (77.0), C_6D_6 (128)], chemical shifts of ^{19}F NMR are reported in ppm with reference to CF_3COOH (−76.5) in D_2O. The following abbreviations are used to designate the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. High resolution mass spectra (HRMS) were recorded on an AB QSTAR Elite under ESI-TOF conditions. Combustion elemental analyses were performed using Yanaco CHN CORDER MT-3.

8-Fluoro-5-trifluoromethanesulfonyloxy-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one (13). To the 300 mL two-necked flask charged with *N*-fluoro-5-(trifluoromethyl)pyridinium-2-sulfonate **11** (3.98 g, 16.2 mmol) was added a solution of 5-hydroxy-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one **10** (3.73 g, 19.4 mmol) in dry 1,1,2,2-tetrachloroethane (49 mL), and the resulting mixture was refluxed for 4 h with light shielding. After cooling to room temperature, the reaction mixture was diluted with dichloromethane (90 mL), quenched with saturated aqueous $Na_2S_2O_3$ at 0 °C, and extracted with ether. The organic layer was washed with saturated aqueous $Na_2S_2O_3$, pH 7.0 phosphate buffer solution and brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (50:1 to 5:1 hexane/ethyl acetate) to afford a mixture of **12a** and recovered **10** containing 1,1,2,2-tetrachloroethane, and a mixture of **12b** and **12c**. **12a**: R_f = 0.40 (10:1 hexane/ethyl acetate, developed in twice); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.99 (1H, br s), 7.26 (1H, dd, J = 9.7, 9.7 Hz), 5.98 (1H, dd, J = 9.2, 3.4 Hz), 1.78 (6H, s). **12b**: R_f 0.36 (10:1 hexane/ethyl acetate, developed in twice); ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.3 (1H, br s), 7.24 (1H, dd, J = 9.7, 9.7 Hz), 6.37 (1H, dd, J = 8.6, 2.9 Hz), 1.73 (6H, s); ^{1}H NMR (500 MHz, CD_2Cl_2) δ 10.3 (1H, br s), 7.25 (1H, dd, J = 10.9, 6.0 Hz), 6.39 (1H, dd, J = 9.2, 3.4 Hz), 1.70 (6H, s). **8c**: R_f 0.36 (hexane/ethyl acetate = 10/1, developed in twice); ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.98 (1H, br s), 7.18 (1H, dd, J = 10.0, 10.0 Hz), 1.78 (6H, s); ^{1}H NMR (500 MHz, CD_2Cl_2) δ 9.96 (1H, br s), 7.20 (1H, dd, J = 10.3, 10.3 Hz), 1.76 (6H, s).

To a solution of **12a** and **10** in pyridine (18 mL), trifluoromethanesulfonic anhydride (11.0 mL, 2.09 mmol) was added dropwise at 0 °C, and the mixture was stirred at 0 °C for 1 h. The reaction mixture was quenched with saturated aqueous $NaHCO_3$ at 0 °C and extracted with ether. The organic layer was washed with saturated aqueous NH_4Cl, water, and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (30:1 to 10:1 hexane/ethyl acetate) to afford **13** (1.07 g, 3.11 mmol, 16%, two steps) as a colorless solid which was separated from **14** (1.71 g, 5.25 mmol, 27%, two steps). **13**: R_f = 0.18 (10:1 hexane/ethyl acetate); IR (film) 3092, 2986, 1749, 1632, 1497, 1419, 1403, 1395, 1381, 1319, 1301, 1286, 1264, 1229, 1219, 1182, 1141, 1068, 1033, 979, 907, 856, 828, 649, 593 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39 (1H, t, J = 9.0 Hz), 6.95 (1H, dd, J = 9.2, 3.6 Hz), 1.80 (6H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.1 (d, J_{CF} = 4 Hz), 150.4 (d, $^1J_{CF}$ = 254 Hz), 145.7 (d, J_{CF} = 15 Hz), 143.9 (d, J_{CF} = 4 Hz), 122.2 (d, J_{CF} = 19 Hz), 118.7 (d, $^1J_{CF}$ = 312 Hz, -CF_3), 110.0, 108.0, 25.5; ^{19}F NMR (470 MHz, $CDCl_3$) δ 132.4 (dd, J = 8.9, 4.0 Hz); HRMS (ESI-TOF) m/z calcd for $C_{11}H_8F_4NaO_6S$ (M + Na^+) 366.9875, found 366.9877.

8-Fluoro-5-(2-propenyl)-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one (15). Lithium chloride (395 mg, 9.33 mmol) in a Schlenk flask was dried by being heated under reduced pressure. Tris(dibenzylideneacetone)dipalladium(0)−methylene chloride complex (166 mg, 0.16 mmol), tris(2-furyl)phosphine (74.3 mg, 0.32 mmol), and *N*-methylpyrrolidone (2.2 mL) were added to the flask, and a solution of **13** (1.07 g, 3.11 mmol) in *N*-methylpyrrolidone (4.0 mL) was added and then stirred at room temperature for 12 min. To the stirred solution, allyl tri-*n*-buthyltin (1.14 mL, 3.73 mmol) was added dropwise at 0 °C. After being stirred at room temperature for 8 h, the reaction mixture was quenched with saturated aqueous KF at 0 °C, the resulting precipitates were removed filtration, and the filtrate was extracted with ether. The organic layer was washed with saturated aqueous KF, water, brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (20:1 to 10:1 hexane/ethyl acetate) to afford **15** (620 mg, 2.62 mmol, 84%) as a pale greenish-yellow oil. R_f = 0.50 (5:1 hexane/ethyl acetate); IR (film) 2956, 2924, 1740, 1639, 1616, 1594, 1503, 1465, 1429, 1391, 1381, 1281, 1262, 1204, 1150, 1065, 1030, 997, 982, 911, 823 cm^{-1}; ^{1}H

NMR (500 MHz, $CDCl_3$) δ 7.24 (1H, dd. J = 9.6, 8.6 Hz), 6.88 (1H, dd, J = 8.6, 4.7 Hz), 5.98 (1H, ddt, J = 16.9, 10.3, 6.5 Hz), 5.04 (1H, ddd, J = 10.2, 2.9, 1.3 Hz), 5.01 (1H, ddd, J = 17.1, 3.3, 1.7 Hz), 3.82 (2H, d, J = 6.4 Hz), 1.73 (6H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.2 (d, J_{CF} = 4 Hz), 149.7 (d, $^1J_{CF}$ = 247 Hz), 145.1 (d, J_{CF} = 13 Hz), 140.1 (d, J_{CF} = 5 Hz), 136.4, 124.0 (d, J_{CF} = 6 Hz), 121.6 (d, J_{CF} = 17 Hz), 116.2, 113.6, 106.3, 37.7, 25.6; ^{19}F NMR (470 MHz, $CDCl_3$) δ −138.5 (dd, J = 9.9, 4.5 Hz); HRMS (ESI-TOF) m/z calcd for $C_{13}H_{13}F_4NaO_3$ (M + Na^+) 259.0746, found 259.0747.

3-Fluoro-2-hydroxy-6-(2-propenyl)benzoic Acid 2-Propenyl Ester (16). To a solution of 2-propene-1-ol (1.40 mL, 20.6 mmol) in THF (1 mL), a solution of ethylmagnesium bromide in THF (0.49 M, 21.1 mL, 10.3 mmol) was added dropwise at 0 °C, and the resulting solution was stirred at room temperature for 20 min. To the reaction mixture, a solution of **15** (610 mg, 2.58 mmol) in THF (3 mL) was added dropwise at 0 °C. After being stirred at room temperature for 17 h, the reaction mixture was diluted with ether, quenched with aqueous NH_4Cl at 0 °C, and extracted with ether. The organic layer was washed with saturated aqueous NH_4Cl, water, and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (40:1 to 10:1 hexane/ethyl acetate) to afford **16** (494 mg, 2.09 mmol, 81%) as a pale greenish-yellow oil. R_f = 0.37 (20:1 hexane/ethyl acetate, developed in twice); IR (film) 3080, 2982, 1734, 1669, 1615, 1595, 1490, 1432, 1373, 1307, 1251, 1171, 1143, 983, 918, 822, 811, 797, 767 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.1 (1H, s), 7.14 (1H, dd, J = 9.8, 8.7 Hz), 6.66 (1H, dd, J = 8.4, 5.0 Hz), 6.01 (1H, ddt, J = 17.2, 10.5, 6.0 Hz), 5.92 (1H, ddt, J = 17.0, 10.2, 6.3 Hz), 5.42 (1H, dd, J = 17.0, 2.6 Hz), 5.34 (1H, d, J = 10.5 Hz), 5.00 (1H, dd, J = 10.2, 3.0 Hz), 4.92 (1H, dd, J = 17.0, 3.4 Hz), 4.86 (2H, dt, J = 6.0, 1.4 Hz), 3.64 (2H, br d, J = 6.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.4 (d, J_{CF} = 2 Hz), 151.0 (d, J_{CF} = 12 Hz), 150.4 (d, $^1J_{CF}$ = 243 Hz), 137.7 (d, J_{CF} = 5 Hz), 137.3, 131.0, 121.2 (d, J_{CF} = 6 Hz), 121.2 (d, J_{CF} = 6 Hz), 120.1 (d, J_{CF} = 13 Hz), 120.0, 115.6, 66.8, 39.7; ^{19}F NMR (470 MHz, $CDCl_3$) δ −138.8 (dd, J = 9.9, 4.5 Hz); HRMS (ESI-TOF) m/z calcd for $C_{13}H_{13}F_4NaO_3$ (M + Na^+) 259.0746, found 259.0759.

3-Fluoro-2-methoxy-6-(2-propenyl)benzoic Acid 2-Propenyl Ester (17). To a solution of **16** (481 mg, 2.04 mol) in acetone (4.0 mL), potassium carbonate (366 mg, 2.65 mmol), and methyl iodide (1.27 mL, 20.4 mmol) were added. After being stirred at room temperature for 23 h, the reaction mixture was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (20:1 hexane/ethyl acetate) to afford **17** (471 mg, 1.88 mmol, 93%) as a pale greenish-yellow oil. R_f = 0.65 (20:1 hexane/ethyl acetate); IR (film) 2982, 2944, 1734, 1640, 1606, 1490, 1458, 1421, 1359, 1277, 1195, 1160, 1134, 1105, 1043, 986, 922, 820, 734 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.05 (1H, dd, J = 11.2, 8.5 Hz), 6.86 (1H, dd, J = 8.5, 4.5 Hz), 5.99 (1H, ddt, J = 17.2, 10.5, 5.9 Hz), 5.85 (1H, ddt, J = 16.9, 10.2, 6.6 Hz), 5.41 (1H, ddd, J = 17.2, 2.9, 1.4 Hz), 5.28 (1H, ddd, J = 10.3, 2.4, 1.3 Hz), 5.04 (1H, ddd, J = 10.2, 3.0, 1.3 Hz), 5.02 (1H, ddd, J = 16.9, 3.4, 1.7 Hz), 4.79 (2H, dd, J = 5.9, 1.4 Hz), 3.92 (3H, d, J = 1.9 Hz, -OMe), 3.32 (2H, br d, J = 6.7 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.5 (d, J_{CF} = 4 Hz), 153.6 (d, $^1J_{CF}$ = 246 Hz), 144.7 (d, J_{CF} = 12 Hz), 136.0, 133.7 (d, J_{CF} = 5 Hz), 131.7, 129.1, 124.7 (d, J_{CF} = 7 Hz), 119.1, 117.9 (d, J_{CF} = 19 Hz), 116.5, 66.1, 62.0 (d, J_{CF} = 5 Hz), 37.0; ^{19}F NMR (470 MHz, $CDCl_3$) δ −133.5 (br ddq, J = 11.1, 4.5, 1.8 Hz); HRMS (ESI-TOF) m/z calcd for $C_{14}H_{15}FNaO_3$ (M + Na^+) 273.0903, found 273.0929.

3-Fluoro-2-methoxy-6-(2-propenyl)benzoic Acid (7). To a flask charged with tetrakis(triphenylphosphine)palladium (8.3 mg, 7.2 μmol), a solution of **17** (18.0 mg, 71.9 μmol) in THF (2.3 mL), followed by morpholine (62.7 μL, 0.72 mmol), was added dropwise. After being stirred at room tempeature for 1 h, the reaction mixture was concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (1:1 hexane/ethyl acetate, containing 0.05% HCOOH) to afford **7** (12.9 mg, 61.2 μmol, 85%) as a colorless oil. R_f = 0.44 (1:1 hexane/ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.10 (2H, dd, J = 11.2, 8.3 Hz), 6.92 (2H, dd, J = 8.6, 4.6 Hz), 5.90 (1H, ddt, J = 17.0, 10.0, 6.3 Hz), 5.05 (1H, d, J = 10.3 Hz), 5.04 (1H, d, J = 17.2 Hz), 3.98 (3H, d, J = 1.7 Hz), 3.47 (2H, br d, J = 6.9 Hz); IR (film) 3503, 2946, 2654, 2361, 1707, 1640, 1606, 1559, 1491, 1462, 1425, 1280, 1199, 1164, 1141, 1042, 994, 968, 921, 820, 768, 708, 668 cm^{-1}; ^{13}C NMR (125 MHz, $CDCl_3$) δ171.7, 153.7 (d, $^1J_{CF}$ = 246 Hz), 145.1 (d, J_{CF} = 13 Hz), 136.0, 134.4 (d, J_{CF} = 4 Hz), 127.6, 125.1 (d, J_{CF} = 7 Hz), 118.6 (d, J_{CF} = 18 Hz), 116.7, 62.2 (d, J_{CF} = 6 Hz), 37.3; ^{19}F NMR (470 MHz, $CDCl_3$) δ −133.1 (br dq, J = 10.8, 2.2 Hz); HRMS (ESI-TOF) m/z calculated for $C_{11}H_{10}FO_3$ (M-H^-) 209.0613, found 209.0639.

3-Fluoro-2-methoxy-6-(2-propenyl)-benzoic acid (1*S*,3*R*,4*S*)-3-(methoxymethoxy)-1-[2-[(4-methoxyphenyl)methoxy]ethyl]-4-methyl-6-heptenyl Ester (18). To a stirred solution of triphenylphosphine (254 mg, 0.97 mmol), **7** (0.86 mmol), and **9** (254 mg, 0.72 mmol) in ether (3.6 mL), DEAD (40% in toluene, 0.44 mL, 0.97 mmol) was added at 0 °C. After being stirred at room temperature for 36 min, the reaction mixture was quenched with saturated aqueous NH_4Cl at 0 °C and extracted with ether. The organic layer was washed with saturated aqueous NH_4Cl, water, and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (10:1 to 5:1 hexane/ethyl acetate) to afford **18** (368 mg, 0.68 mmol, 91%) as a pale greenish-yellow oil. $[\alpha]_D^{27}$ +3.63 (c 0.32, $CHCl_3$); R_f = 0.50 (3:1 hexane/ethyl acetate); IR (film) 2935, 1727, 1640, 1613, 1514, 1490, 1458, 1442, 1421, 1362, 1275, 1248, 1197, 1173, 1155, 1139, 1097, 1039, 995, 975, 917, 820 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25 (2H, d. J = 8.6 Hz), 7.03 (1H, dd, J = 11.2, 8.5 Hz), 6.87−6.82 (3H, m), 5.88 (1H, ddt, J = 17.0, 10.2, 6.4 Hz), 5.71 (1H, ddt, J = 17.0, 10.0, 7.0 Hz), 5.42 (1H, dddd, J = 6.2, 6.0, 6.0, 6.0, 6.0 Hz, H15), 5.05 (1H, dd, J = 10.2, 3.0 Hz), 5.00 (1H, dd, J = 17.0, 3.3 Hz), 4.94 (1H, dd, J = 17.2, 3.2 Hz), 4.90 (1H, dd, J = 10.0, 1.9 Hz), 4.68 (1H, d, J = 6.9 Hz), 4.66 (1H, d, J = 6.9 Hz), 4.45 (1H, d, J = 11.5 Hz), 4.41 (1H, d, J = 11.5 Hz), 3.90 (3H, d, J = 2.0 Hz, -OMe), 3.78 (3H, s), 3.61 (1H, ddd, J = 9.5, 8.4, 5.9 Hz, H17a), 3.60 (1H, dt, J = 10.0, 3.6 Hz, H13), 3.55 (1H, ddd, J = 9.5, 6.9, 6.9 Hz, H17b), 3.38 (3H, s), 3.30 (2H, br d, J = 6.4 Hz), 3.30 (2H, br d, J = 6.4 Hz), 2.05 (1H, ddd, J = 13.9, 6.6 Hz, H11a), 2.03 (1H, ddd, J = 14.0, 6.0 6.0 Hz, H16a), 1.97 (1H, ddd, J = 14.0, 6.9, 5.7 Hz, H16b), 1.93−1.85 (1H, m, H12), 1.80 (1H, ddd, J = 13.9, 8.6, 7.9 Hz, H11b), 1.73−1.69 (2H, m, H14), 0.87 (3H, d, J = 6.7 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.6, 159.1, 153.4 (d, $^1J_{CF}$ = 246 Hz), 144.3 (d, J_{CF} = 12 Hz), 137.1, 136.2, 133.3 (d, J_{CF} = 5 Hz), 130.5, 129.6, 129.3, 124.5 (d, J_{CF} = 7 Hz), 117.7 (d, J_{CF} = 17 Hz), 116.6, 115.9, 113.7, 96.7, 78.2, 72.7, 71.3, 66.4, 61.7 (d, J_{CF} = 7 Hz), 55.8, 55.3, 37.5, 36.7, 36.3, 35.3, 35.1, 13.7; ^{19}F NMR (470 MHz, $CDCl_3$) δ −133.6 (br ddq, J = 10.8, 4.5, 1.8 Hz); HRMS (ESI-TOF) m/z calcd for $C_{31}H_{41}FNaO_7$ (M + Na^+) 567.2734, found 567.2731.

(3*S*,5*R*,6*S*,8*E*)-3,4,5,6,7,10-Hexahydro-13-fluoro-14-methoxy-5-(methoxymethoxy)-3-[2-[(4-methoxyphenyl)methoxy]ethyl]-6-methyl-1*H*-2-benzoxacyclododecin-1-one (19). To a flask charged with dichloromethane (18 mL) under vigorous stirring, a solution of **18** (358 mg, 0.66 mmol) in dichloromethane (67 mL) and $(Cy_3P)_2Cl_2Ru$=CHPh (41 mg, 0.0495 mmol) in dichloromethane (63 mL) were added simultaneously at room temperature over 26 min, and the resulting solution was stirred at room temperature for 21 min. The reaction was quenched with triethylamine (3.2 mL), exposed to air for 13 h with vigorous stirring, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (10:1 to 5:1 hexane/ethyl acetate) to afford **19** (306 mg, 0.59 mmol, 90%) as a pale-gray oil (10:1 mixture of *E*:*Z* isomers at C9). $[\alpha]_D^{26}$ −44.5 (c 0.21, $CHCl_3$); R_f = 0.28 (3:1 hexane/ethyl acetate); IR (film) 2954, 2933, 1725, 1613, 1587, 1514, 1489, 1456, 1417, 1359, 1275, 1248, 1207, 1173, 1155, 1142, 1099, 1067, 1040, 975, 917, 822 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25 (2H, d. J = 8.6 Hz), 6.97 (1H, dd, J = 11.3, 8.3 Hz), 6.85 (2H, d. J = 8.6 Hz), 6.78 (1H, dd, J = 8.4, 4.5 Hz), 5.50−5.40 (2H, m, H15 and H10), 5.29 (1H, ddt, J = 15.2, 9.5,

2.2 Hz, H9), 4.84 (1H, d, $J = 6.9$ Hz), 4.76 (1H, d, $J = 6.9$ Hz), 4.45 (2H, s), 4.06 (1H, dd, $J = 9.5$, 3.7 Hz, H13), 3.82 (3H, d, $J = 1.9$ Hz), 3.78 (3H, s), 3.63 (1H, br dd, $J = 16.4$, 10.0 Hz, H8a), 3.61 (2H, t,dd, $J = 6.7$, 6.7 Hz, H17), 3.41 (3H, s), 3.28 (1H, ddt, $J = 16.4$, 4.6, 2.6 Hz, H8b), 2.28 (1H, br ddd, $J = 14.0$, 5.7, 3.3 Hz, H11a), 2.17−2.06 (1H, m, H12), 2.01 (1H, dddd, $J = 14.2$, 8.3, 6.0, 6.0 Hz, H16a), 1.90 (1H, dddd, $J = 14.2$, 7.2, 7.2, 4.6 Hz, H16b), 1.72 (1H, dd, $J = 15.6$, 8.9 Hz, H14a), 1.68 (1H, dd, $J = 14.1$, 11.6 Hz, H11b), 1.46 (1H, dd, $J = 15.5$, 9.5, H14b), 0.84 (3H, d, $J = 6.9$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.9, 159.1, 153.6x (d, $^1J_{CF} = 246$ Hz), 144.7 ($J_{CF} = 12$ Hz), 134.1, 131.5 ($J_{CF} = 2$ Hz), 130.6, 130.2, 129.1, 128.3, 125.5 ($J_{CF} = 7$ Hz), 117.1 ($J_{CF} = 19$ Hz), 113.7, 96.8, 79.3, 72.8, 72.6, 66.3, 61.6 ($J_{CF} = 7$ Hz), 55.6, 55.3, 37.7, 37.3, 36.4, 35.5, 34.0, 13.4; ^{19}F NMR (470 MHz, $CDCl_3$) δ −133.0 (br ddq, $J = 11.7$, 4.5, 1.8 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{29}H_{37}FNaO_7$ ($M + Na^+$) 539.2421, found 539.2426.

(3*S*,5*R*,6*S*,8*E*)-3,4,5,6,7,10-Hexahydro-3-(2-hydroxyethyl)-13-fluoro-14-methoxy-5-(methoxymethoxy)-6-methyl-1*H*-2-benzoxacyclododecin-1-one (20). To a solution of **19** (296 mg, 0.57 mmol) and water (0.57 mL) in dichloromethane (11 mL), 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (182 mg, 0,80 mmol) was added at 0 °C. After being stirred at room temperature for 53 min, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ and saturated aqueous $Na_2S_2O_3$ at 0 °C, and extracted with ether. The organic layer was washed with saturated aqueous $Na_2S_2O_3$, water, and brine, dried over Na_2SO_4, filtrated, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (3:1 to 1:1 hexane/ethyl acetate) to afford **20** (220 mg, 0.55 mmol, 97%) as a pale bright-yellow oil (10:1 mixture of *E*:*Z* isomers at C9). $[\alpha]_D^{27}$ −37.8 (c 0.26, $CHCl_3$); $R_f = 0.29$ (1:1 hexane/ethyl acetate); IR (film) 3441, 2954, 1725, 1605, 1489, 1457, 1417, 1382, 1348, 1275, 1231, 1197, 1155, 1142, 1102, 1041, 975, 917, 822, 717 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.98 (1H, dd, $J = 11.5$, 8.5 Hz), 6.80 (1H, dd, $J = 8.4$, 4.5 Hz), 5.48 (1H, dddd, $J = 8.5$, 8.5, 4.7, 4.7 Hz, H15), 5.46 (1H, ddt, $J = 15.2$, 11.5, 2.6 Hz, H10), 5.30 (1H, ddt, $J = 15.2$, 9.6, 2.2 Hz, H9), 4.85 (1H, d, $J = 6.8$ Hz), 4.80 (1H, d, $J = 6.8$ Hz), 4.08 (1H, dd, $J = 9.4$, 3.4 Hz, H13), 3.92 (3H, d, $J = 2.0$ Hz), 3.82 (1H, ddd, $J = 11.6$, 7.7, 4.4 Hz, H17a), 3.75 (1H, ddd, $J = 11.2$, 6.0, 5.3 Hz, H17b), 3.63 (1H, dd, $J = 16.5$, 9.6 Hz, H8a), 3.43 (3H, s), 3.29 (1H, dddd, $J = 16.5$, 4.2, 2.4, 2.4 Hz, H8b), 2.29 (1H, ddd, $J = 14.4$, 5.6, 3.2 Hz, H11a), 2.18−2.07 (1H, m, H12), 1.94 (1H, dddd, $J = 14.5$, 9.5, 5.9, 4.4 Hz, H16a), 1.84 (1H, dddd, $J = 14.4$, 7.7, 4.7, 4.7 Hz, H16b), 1.75 (1H, dd, $J = 15.6$, 8.2 Hz, H14a), 1.69 (1H, ddd, $J = 14.3$, 11.6, 11.6 Hz, H11b), 1.46 (1H, dd, $J = 15.6$, 9.3 Hz, H14b), 0.85 (3H, d, $J = 6.7$ Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.1 (d, $J_{CF} = 2$ Hz), 153.5 (d, $^1J_{CF} = 247$ Hz), 144.3 (d, $J_{CF} = 12$ Hz), 134.1 (d, $J_{CF} = 3$ Hz), 131.6, 129.9, 128.2, 125.7 (d, $J_{CF} = 7$ Hz), 117.3 (d, $J_{CF} = 19$ Hz), 97.0, 73.5, 61.9 (d, $J_{CF} = 7$ Hz), 59.4, 55.6, 38.9, 37.6, 37.3, 35.5, 34.1, 13.4; ^{19}F NMR (470 MHz, $CDCl_3$) δ −132.8 (ddq, $J = 11.7$, 4.5, 1.8 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{21}H_{29}FNaO_6$ ($M + Na^+$) 419.1846, found 419.1861.

(3*S*,5*R*,6*S*,8*E*)-3,4,5,6,7,10-Hexahydro-13-fluoro-3-(3-iodo-2-propenyl)-14-methoxy-5-(methoxymethoxy)-6-methyl-1*H*-2-benzoxacyclododecin-1-one (21). To a solution of **20** (211 mg, 0.53 mmol) in dichloromethane (11 mL) Dess−Martin periodinane (339 mg, 0,80 mmol) was added at 0 °C. After being stirred at room temperature for 73 min, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ and saturated aqueous $Na_2S_2O_3$ at 0 °C, and extracted with ether. The organic layer was washed with saturated aqueous $Na_2S_2O_3$, water, and brine, dried over Na_2SO_4, filtrated, and concentrated under reduced pressure to afford aldehyde as a pale bright-yellow oil, which was used in the next reaction without further purification. Chromium chloride(II) (782 mg, 6.36 mmol) was dried by being heated with flame under reduced pressure for 15min. To a suspension of the chromium chloride(II) in THF (4.6 mL), a solution of the aldehyde and iodoform (835 mg, 2.12 mmol) in dioxane (27.6 mL) was added at 0 °C to give a reddish-brown suspension. After being stirred at room temperature for 26 h with light shielding, the reaction mixture was diluted with ether (32 mL), and the suspension was poured into a mixture of ether (12 mL) and water (36 mL) at 0 °C. The organic layer was separated, washed with saturated aqueous $Na_2S_2O_3$, saturated aqueous $NaHCO_3$, water, and brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (15:1 to 5:1 hexane/ethyl acetate) to afford **21** (221 mg, 0.43 mmol, 80%) as a pale-yellow oil (3.3:1 mixture of *E*:*Z* isomers at C17). $[\alpha]_D^{27}$ −84.5 (c 0.21, $CHCl_3$); $R_f = 0.49$ (3:1 hexane/ethyl acetate); IR (film) 2955, 2932, 1727, 1606, 1489, 1456, 1426, 1417, 1382, 1347, 1273, 1230, 1196, 1155, 1139, 1102, 1042, 975, 917, 821, 811 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.99 (1H, dd, $J = 11.5$, 8.5 Hz), 6.78 (1H, dd, $J = 8.5$, 4.4 Hz), 6.69 (1H, ddd, $J = 14.4$, 8.2, 6.2 Hz, H17), 6.15 (1H, d, $J = 14.5$ Hz, H18), 5.45 (1H, dddd, $J = 14.8$, 11.4, 2.7, 2.7 Hz, H10), 5.33 (1H, ddd, $J = 8.3$, 8.3, 4.6 Hz, H15), 5.28 (1H,dddd, $J = 15.2$, 9.6, 2.1, 2.1 Hz, H9), 4.85 (1H, d, $J = 6.7$ Hz), 4.78 (1H, d, $J = 6.7$ Hz), 4.05 (1H, dd, $J = 9.4$, 3.7 Hz, H13), 3.96 (3H, d, $J = 2.0$ Hz), 3.62 (1H, dd, $J = 16.3$, 9.5 Hz, H8a), 3.43 (3H, s), 3.28 (1H, dddd, $J = 16.4$, 3.9, 2.4, 2.4 Hz, H8b), 2.45 (1H, dddd, $J = 14.6$, 8.0, 6.3, 1.5 Hz, H16a), 2.33 (1H, dddd, $J = 14.9$, 8.2, 4.6, 0.9 Hz, H16b), 2.29 (1H, br d, $J = 14.2$ Hz, H11a), 2.17−2.07 (1H, m, H12), 1.68 (dd, $J = 15.1$, 8.6 Hz, H14a), 1.67 (1H, ddd, $J = 14.2$, 11.5, 11.5 Hz, H11b), 1.40 (1H, dd, $J = 15.4$, 9.5 Hz, H14b), 0.84 (3H, d, $J = 6.9$ Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.9 (d, $J_{CF} = 4$ Hz), 153.6 (d, $^1J_{CF} = 246$ Hz), 144.8 (d, $J_{CF} = 12$ Hz), 141.71, 136.9, 134.1 (d, $J_{CF} = 5$ Hz), 131.5, 128.3, 125.4 (d, $J_{CF} = 7$ Hz), 117.2 (d, $J_{CF} = 19$ Hz), 97.1, 79.4, 77.6, 73.8, 61.9 (d, $J_{CF} = 7$ Hz), 55.7, 42.4, 37.6, 37.2, 35.1, 34.1, 13.3; ^{19}F NMR (470 MHz, $CDCl_3$) δ −132.7 (ddq, $J = 11.3$, 4.5, 2.4 Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{22}H_{28}FINaO_5$ ($M + Na^+$) 541.0863, found 541.0881.

(3*S*,5*R*,6*S*,8*E*)-3,4,5,6,7,10-Hexahydro-13-fluoro-3-(3-iodo-2-propenyl)-14-hydroxy-5-(methoxymethoxy)-6-methyl-1*H*-2-benzoxacyclododecin-1-one (22). To a vigorously stirred solution of **21** (211 mg, 0.41 mmol) in dichloromethane (45 mL), a solution of tribromoborane in dichloromethane (1.0 M, 1.06 mL, 1.06 mmol) was added at −78 °C. After being stirred at −78 °C for 65 min, the reaction mixture was allowed to warm up to room temperature, then quenched with ice-cold water (70 mL) at −5 °C and extracted with dichloromethane. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (5:1 to 3:1 hexane/ethyl acetate) to afford **22** (138 mg, 0.30 mmol, 76%) as a colorless powder (4:1 mixture of *E*:*Z* isomers at C17). $[\alpha]_D^{27}$ +19.4 (c 0.69, $CHCl_3$); $R_f = 0.27$ (3:1 hexane/ethyl acetate, developed in twice); IR (film) 3469, 2956, 2359, 1703, 1612, 1491, 1425, 1356, 1291, 1274, 1168, 1141, 1028, 968, 820, 767, 669, 623 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.1 (1H, br s, -OH), 7.10 (1H, dd, $J = 8.5$ Hz), 6.63 (1H, dd, $J = 8.3$, 5.0 Hz), 6.57 (1H, ddd, $J = 14.3$, 7.4, 7.4 Hz, H17), 6.18 (1H, ddd, $J = 14.3$, 1.3, 1.3 Hz, H18), 5.52 (1H, dddd, $J = 10.6$, 5.9, 5.9, 1.0 Hz, H15), 5.40 (1H, ddd, $J = 15.5$, 4.6, 4.6 Hz, H9), 5.12 (1H, br ddd, $J = 15.2$, 9.3, 5.3 Hz, H10), 3.69 (1H, dd, $J = 8.7$, 3.4 Hz, H13), 3.59 (1H, br dd, $J = 16.7$, 5.1 Hz, H8a), 3.40 (1H, ddd, $J = 16.8$, 2.0, 2.0 Hz, H8b), 2.47−2.41 (2H, m, H16), 2.33 (1H, br dddd, $J = 13.5$, 6.7, 1.8, 1.8 Hz, H11a), 1.93 (1H, dd, $J = 15.0$, 10.6 Hz, H14a), 1.84−1.92 (2H, m, H12), 1.79 (1H, ddd, $J = 13.6$, 9.2, 9.2 Hz, H11b), 1.33 (1H, ddd, $J = 15.1$, 8.8, 1.0 Hz, H14b), 0.90 (3H, d, $J = 6.9$ Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.7, 150.5 (d, $^1J_{CF} = 241$ Hz), 149.4, 140.7, 136.8 (d, $J_{CF} = 5$ Hz), 135.9, 132.0, 127.4, 122.3 (d, $J_{CF} = 7$ Hz), 119.0 (d, $J_{CF} = 17$ Hz), 78.4, 73.7, 70.5, 41.4, 38.5, 38.2, 37.4, 35.2, 13.6; ^{19}F NMR (470 MHz, $CDCl_3$) δ −139.1 (br s); HRMS (ESI-TOF) *m/z* calcd for $C_{19}H_{22}FINaO_4$ ($M + Na^+$) 483.0445, found 483.0457.

Salicylihalamide A (1a) and B (1b). *N*,*N*′-dimethyl acetamide (DMA) used in this reaction was degassed by freeze−thaw method over 10 times. To the 20 mL Schlenk flask dried by heating with flame, under reduced pressure for 10 min Rb_2CO_3 (139 mg, 0.60 mmol) was added under argon atmosphere and dried by a drier under reduced pressure for 15 min and charged with argon gas after cooling to room temperature. To this flask, copper(I) thiophene

carboxylate (19 mg, 0.10 mmol), a solution of (2*Z*,4*Z*)-2,4-heptadienamide **8** (75 mg, 0.60 mmol) and 2,2′-bipyridine (20 mg, 0.12 mmol) in DMA (0.9 mL), which was dried over MS4A, and then the resulting mixture was immediately degassed until no bubbles were found. After being stirred for 30 min at room temperature with light shielding, a solution of **23** (44 mg, 0.10 mmol) in DMA (0.6 mL), which was dried over MS4A, was added and the resulting solution was immediately degassed until no bubbles were found. After being stirred for 1 h at 90 °C with light shielding, the reaction solution was diluted with ether at room temperatur, and was quenched with pH 7.0 phosphate buffer at 0 °C. The organic layer was separated, washed with pH 7.0 phosphate buffer, water and brine, and dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (hexane/ethyl acetate = 3/1 to 1/3) to afford the roughly purified substance (58.3 mg), which was purified by the preparative HPLC to afford salicylihalamide A (**1a**) (10.1 mg, 0.023 mol, 23%) and salicylihalamide B (**1b**) (11.4 mg, 0.026 mmol, 26%) as a colorless wax, respectively. HPLC conditions: column: COSMOSIL 5C18-MS-II (NACALAI TESQUE, INC.), 10 mm × 250 mm; detection: λ = 280 nm; flow rate: 2.0 mL/min; elution with a gradient solvent system: MeOH/H_2O = 7/3, 0−100 min: MeOH/H_2O = 7/3 → MeOH, 100−120 min: MeOH, 120−140 min; The t_R of salicylihalamide A (**1a**) and salicylihalamide B (**1b**) were 64.2 and 81.0 min, respectively.

1a. $[\alpha]_D^{19}$ = −32.6 (c 0.169, MeOH); R_f 0.25 (hexane/ethyl acetate = 1/1); IR (film) 3285, 2964, 1700, 1653, 1589, 1523, 1464, 1293, 1248, 1216, 1123, 1066, 971 cm^{-1}; ^1H NMR (500 MHz, C_6D_6) δ 11.5 (1H, br, C3-OH), 7.93 (1H, br dd, *J* = 11.4, 11.4 Hz, H22), 7.12 (1H, dd, *J* = 14.3, 10.9 Hz, H18), 6.99−6.94 (2H, m, H4 and H5), 6.62 (1H, dd, *J* = 11.6, 11.6 Hz, H21), 6.50 (1H, br d, −NH), 6.48 (1H, dd, *J* = 6.6, 2.3 Hz, H6), 5.62 (1H, ddddd, *J* = 10.8, 7.7, 7.7, 1.3, 1.3 Hz, H23), 5.54(1H, ddd, *J* = 10.0, 6.6, 6.6 Hz, H15), 5.28 (1H, ddd, *J* = 15.5, 4.3, 4.3 Hz, H9), 5.10 (1H, br d, *J* = 10.9 Hz, H20), 5.05 (1H, br ddd, *J* = 14.6, 6.9, 6.9 Hz, H10), 4.77 (1H, br ddd, *J* = 14.3, 7.2, 7.2 Hz, H17), 3.62 (1H, br dd, *J* = 16.5, 5.3 Hz, H8a), 3.44 (1H, dd, *J* = 8.3, 2.9 Hz, H13), 3.32 (1H, br d, *J* = 16.0 Hz, H8b), 2.17 (1H, ddd, *J* = 14.0, 7.2, 7.2 Hz, H16a), 2.12 (1H, ddd, *J* = 12.3, 5.7, 5.7 Hz, H11a), 2.05 (1H, ddd, *J* = 14.0, 6.9, 6.9 Hz, H16b), 1.97 (2H, tdd, *J* = 7.6, 7.6, 1.4 Hz, H24), 1.66 (1H, dd, *J* = 15.1, 10.8 Hz, H14a), 1.62 (1H, dd, *J* = 13.5, 8.9 Hz, H11b), 1.56−1.45 (1H, m, H12), 1.27 (1H, dd, *J* = 14.9, 8.6 Hz, H14b), 0.82 (3H, d, *J* = 6.9 Hz, H26), 0.77 (3H, t, *J* = 7.7 Hz, H25); ^{13}C NMR (125 MHz, C_6D_6) δ 171.2, 170.0, 163.0, 162.9, 142.6, 141.7, 136.9, 134.0, 132.7, 127.1, 126.0, 124.9, 123.6, 119.7, 117.1, 114.7, 107.0, 75.3, 70.4, 39.3, 38.6, 37.6, 36.4, 35.5, 20.8, 14.0, 13.9; HRMS (ESI-TOF) *m*/*z* calcd for $C_{26}H_{33}NNaO_5$ (M + Na^+) 462.2256, found 462.2253.

1b. $[\alpha]_D^{20}$ = −90.4 (c 0.025, MeOH); R_f 0.25 (hexane/ethyl acetate = 1/1); IR (film) 3286, 2960, 2924, 1695, 1653, 1587, 1507, 1464, 1294, 1247, 1211, 1119, 1032, 966 cm^{-1}; ^1H NMR (500 MHz, C_6D_6) δ 11.8 (1H, br, C3-OH), 7.97 (1H, br dd, *J* = 11.4, 11.4 Hz, H22), 7.57 (1H, br d, *J* = 9.2 Hz, -NH), 7.31 (1H, dd, *J* = 10.0, 10.0 Hz, H18), 6.98−6.94 (2H, m, H4 and H5), 6.62 (1H, ddd, *J* = 11.4, 11.4, 0.9 Hz, H21), 6.45 (1H, dd,6.3, 2.6 Hz, H6), 5.26−5.12 (2H, m, H15 and H10), 6.48 (1H, dd, *J* = 6.6, 2.3 Hz, H6), 5.62 (1H, ddddd, *J* = 10.8, 7.7, 7.7, 1.3, 1.3 Hz, H23), 5.44 (1H, d, *J* = 11.5 Hz, H20), 5.25−5.12 (2H, m, H15 and H10), 5.07 (1H, ddd, *J* = 15.3, 7.2, 7.2 Hz, H9), 4.48 (1H, ddd, *J* = 8.0, 8.0, 8.0 Hz, H17), 3.56 (1H, br dd, *J* = 16.5, 5.3 Hz, H8a), 3.24 (1H, br d, *J* = 16.9 Hz, H8b), 3.21 (1H, br d, *J* = 9.7 Hz, H13), 2.11−1.99 (1H, m, H11a), 2.04 (1H, ddd, *J* = 14.0, 7.4, 7.4 Hz, H16a), 1.94 (2H, tdd, *J* = 7.6, 7.6, 1.4 Hz, H24), 1.84 (1H, ddd, *J* = 14.3, 7.7, 7.7 Hz, H16b), 1.77−1.68 (1H, m, H11b), 1.72 (1H, dd, *J* = 14.7, 10.7 Hz, H14a), 1.45 (1H, br q, *J* = 6.8 Hz, H12), 1.19 (1H, dd, *J* = 15.2, 8.6 Hz, H14b), 0.82 (3H, d, *J* = 6.9 Hz, H26), 0.77 (3H, t, *J* = 7.4 Hz, H25); ^{13}C NMR (125 MHz, C_6D_6) δ 172.0, 162.9, 141.9, 137.3, 134.6, 132.8, 126.8, 125.4, 124.9, 123.7, 119.5, 117.2, 103.2, 76.1, 70.9, 39.4, 38.4, 38.0, 36.2, 31.4, 20.8, 14.0, 13.8; HRMS (ESI-TOF) *m*/*z* calcd for $C_{26}H_{33}NNaO_5$ (M + Na^+) 462.2256, found 462.2245.

4-^{19}F-Salicylihalamide A (6a) and B (6b). *N*,*N*′-dimethyl acetamide (DMA) used in this reaction was degassed by freeze−thaw method over 10 times. Rb_2CO_3 (139 mg, 0.60 mmol) in a Schlenk flask was dried by heating with a drier under reduced pressure for 15 min. After cooling to room temperature, copper(I) thiophenecarboxylate (19 mg, 0.10 mmol) was added in glove box. A solution of (2*Z*,4*Z*)-2,4-heptadienamide **8** (75 mg, 0.60 mmol) and 2,2′-bipyridine (20 mg, 0.12 mmol) in DMA (0.7 mL), which was dried over MS4A, was added and the resulting mixture was immediately degassed over and over again until no bubbles were found. After being stirred for 30 min at room temperature with light shielding, a solution of of **22** (46 mg, 0.10 mmol) in DMA (0.7 mL), which was dried over MS4A, was added and the resulting solution was immediately degassed over and over again until no bubbles were found. After being stirred for 1 h at 90 °C with light shielding, the reaction mixture was diluted with ether at room temperature and was quenched with pH 7.0 phosphate buffer at 0 °C. The organic layer was separated, washed with pH 7.0 phosphate buffer, water and brine, and dried over Na_2SO_4, filtrered, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (3:1 to 1:3 hexane/ethyl acetate) to afford the roughly purified substance (29 mg), which was purified by the preparative HPLC to afford 4-^{19}F-salicylihalamide A (**6a**) (7.1 mg, 0.015 mol, 15%) and 4-^{19}F-salicylihalamide B (**6b**) (10.1 mg, 0.022 mmol, 22%) as a colorless wax, respectively. HPLC conditions: column: COSMOSIL 5C_{18}-MSII, 10 mm × 250 mm; detection: 280 nm; flow rate: 2.0 mL/min, eluent: 70:30 MeOH/H_2O, 100 min, 70:30 → 100:0, 20 min, 100:0, 30 min. The retention times (t_R) of **6a** and **6b** were 63.4 and 73.4 min, respectively. **6a**: $[\alpha]_D^{28}$ −50.0 (c 0.213, MeOH); R_f = 0.34 (1:1 hexane/ethyl acetate); λ_{max} (MeOH) 282 nm (ε = 26200); IR (film) 3281, 2963, 2931, 1691, 1656, 1611, 1492, 1461, 1288, 1248, 1218, 1138, 1081, 1027, 971, 820, 678 cm^{-1}; ^1H NMR (500 MHz, C_6D_6) δ 10.5 (1H, br, C3-OH), 7.91 (1H, br dd, *J* = 11.5, 11.5 Hz, H22), 7.08 (1H, dd, *J* = 14.3, 10.6 Hz, H18), 6.72 (1H, dd, *J* = 9.7, 8.3 Hz, H5), 6.61 (1H, ddd, *J* = 11.5, 11.5, 0.9 Hz, H21), 6.56 (1H, br s, −NH), 6.19 (1H, dd, *J* = 8.3, 4.6 Hz, H6), 5.63 (1H, ddddd, *J* = 10.9, 7.7, 7.7, 1.3, 1.3 Hz, H23), 5.53 (1H, m, H15), 5.23 (1H, ddd, *J* = 15.5, 4.7, 4.7 Hz, H9), 5.10 (1H, br d, *J* = 11.5 Hz, H20), 5.13−5.03 (1H, m, H10), 5.05 (1H, br ddd, *J* = 14.6, 6.9, 6.9 Hz, H10), 4.85 (1H, ddd, *J* = 14.3, 7.2, 7.2 Hz, H17), 3.68 (1H, br d, *J* = 6.0 Hz, H13), 3.37 (2H, m, H8), 2.21 (1H, ddd, *J* = 14.3, 7.4, 7.4 Hz, H16a), 2.12 (1H, m, H11a), 2.08 (1H, ddd, *J* = 13.6, 6.0, 6.0 Hz, H16b), 1.97 (2H, tdd, *J* = 7.6, 7.6, 1.4 Hz, H24), 1.66 (1H, dd, *J* = 15.0, 9.9 Hz, H14a), 1.65−1.53 (2H, m, H11b and H12), 1.31 (1H, dd, *J* = 14.6, 9.2 Hz, H14b), 0.83 (3H, d, *J* = 6.9 Hz, H26), 0.77 (3H, t, *J* = 7.6 Hz, H25); ^{13}C NMR (125 MHz, C_6D_6) δ 169.7 164.2, 160.5, 151.5 (d, $^1J_{CF}$ = 240 Hz), 141.9, 137.2, 135.8, 130.8, 129.5, 126.0, 124.9, 121.7, 119.7, 117.5, 108.8, 76.0, 71.1, 38.3, 37.6, 36.7, 36.2, 20.8, 14.0, 13.7 (1 of the sp_2 carbons were obserded); ^{19}F NMR (470 MHz, C_6D_6) δ −138.6 (s); HRMS (ESI-TOF) *m*/*z* calculated for $C_{26}H_{33}FNO_5$ (M + H^+) 458.2343, found 458.2341. **6b**: $[\alpha]_D^{28}$ −71.0 (c 0.301, MeOH); R_f = 0.34 (hexane/ethyl acetate = 1/1); λ_{max} (MeOH) 282 nm (ε = 22,100); IR (film) 3357, 2964, 2931, 1707, 1654, 1590, 1491, 1274, 1248, 1207, 1170, 1137, 1032, 973, 678 cm^{-1}; ^1H NMR (500 MHz, C_6D_6) δ 7.90 (1H, dd, *J* = 11.5, 11.4 Hz, H22), 7.71 (1H, br s, −NH), 7.26 (1H, dd, *J* = 9.9 Hz, H18), 6.72 (1H, dd, *J* = 9.7, 9.7 Hz, H5), 6.67 (1H, dd, *J* = 11.7, 11.7 Hz, H21), 6.17 (1H, dd, *J* = 8.2, 4.6 Hz, H6), 5.63 (1H, br ddd, *J* = 10.9, 7.9, 7.9 Hz, H23), 5.51 (1H, d, *J* = 10.9 Hz, H20), 5.36 (1H, m, H15), 5.20−5.08 (2H, m, H9 and H10), 4.65 (1H, ddd, *J* = 8.3, 8.3, 8.0 Hz, H17), 3.60 (1H, m, H13), 3.34 (1H, br d, *J* = 15.7 Hz, H8a), 3.30 (1H, br d, *J* = 15.7 Hz, H8b), 2.17 (1H, ddd, *J* = 14.0, 7.2, 7.2 Hz, H16a), 2.11−2.01 (1H, m, H11a), 2.06 (1H, br d, *J* = 14.5 Hz, H16b), 1.97 (2H, tdd, *J* = 7.6, 7.6, 1.4 Hz, H24), 1.76 (1H, dd, *J* = 14.9, 10.0 Hz, H14a), 1.72−1.65 (1H, m, H11b), 1.65−1.55 (1H, m, H12), 1.30 (1H, dd, *J* = 14.9, 8.9 Hz, H14b), 0.85 (3H, d, *J* = 6.9 Hz, H26), 0.78 (3H, t, *J* = 7.7 Hz, H25); ^{13}C NMR (125 MHz, C_6D_6) δ 170.0, 164.0, 151.2 (d, $^1J_{CF}$ = 246 Hz), 142.0, 137.4, 136.7, 131.1, 128.9, 124.9 (d, J_{CF} = 12 Hz), 122.2 (d, J_{CF} = 5 Hz), 119.6,

118.3 (d, J_{CF} = 17 Hz), 105.2, 76.2, 71.3, 38.5, 38.3, 37.8, 36.3, 31.9, 20.8, 14.0, 13.6 (3 of the sp_2 carbons were obserded); ^{19}F NMR (470 MHz, C_6D_6) δ −138.4 (s); HRMS (ESI-TOF) *m/z* calcd for $C_{26}H_{32}FNNaO_5$ ($M + Na^+$) 480.2162, found 480.2162.

Biology. Materials. All the chemical used in this study were commercially available special grade ones without further treatment. Water was purified with a Millipore Simpli Laboratory system (Millipore Inc., Bedford, MA) and used within a few hours. The following reagents used in this protocol were as follows: SME buffer (0.3 M sucrose, 5 mM EDTA, 10 mM MOPS and pH 7.5 with NaOH), pepstatin A (10 mg/mL in ethanol), leupeptin (10 mg/mL in ethanol), reaction medium for proton pump assay (0.3 M sucrose, 40 mM KCl, 5 mM tricine, and pH 8.0 with NaOH), 9-amino-6-chloro-2-methoxyacridine (ACMA) (0.1 M in ethanol), valinomycin (1 mg/mL in ethanol), MgATP (0.1 M aq and pH 7.5 with NaOH), and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (1 mM in ethanol), Hanks' balanced salt solutions (HBSS) (137 mM NaCl, 5.3 mM KCl, 1.3 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 4.2 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 5.6 mM glucose, 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.4 with NaOH).

Assay of Synthesized Salicylihalamides Using Simian Kidney Cell. Simian (*Cercopithecus aethiops*) kidney COS-7 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum at 37 °C in 5% CO_2/95% air with a CO_2 incubator.[28] The cells were seeded on poly-L-lysine coated coverslips. After overnight culture, the synthesized salicylihalamide was added into the culture media to give a final concentration of 1 μM, and incubated at 37 °C for 5 h. Then, the cells were loaded with pH indicator LysoSensor Green DND-189 (Invitrogen, Molecular probes)[29] at a final concentration of 1 μM. After 1 min, an excess of the indicator was washed out with HBSS. The cells were observed using a fluorescence microscope, and images were acquired using a cooled CCD camera. For tryptan blue exclusion test, the intact or drug-treated cells were soaked in phosphate buffered saline containing 0.3% trypan blue. The cells were observed with bright-field and phase-contrast microscope.

Preparation of Chromaffin Granule Membranes (CGM) from Porcine Adrenal Glands. Preparation of chromaffin granule membranes (CGM) from porcine adrenal glands was operated according to refs 30, 31 in which the preparation of CGM from bovine adrenal glands was described. The following manipulation was carried out at 4 °C unless otherwise noted. Porcine adrenal glands are obtained from the local meat distributor (IKEDA meat-packing company Co., Ltd., Kyoto, Japan). Then 30−35 adrenal medullae were separated from the cortex by pinching the connecting tissue with pointed forceps. The isolated adrenal medullae were kept at 4 °C in 200 mL of freshly prepared SME buffer containing 5 μg/mL of pepstatin A and leupeptin. The medullae were briefly homogenized in a Waring blender and rehomogenized in the Polytron homogenizer on ice bath. The mixture was filtered through two layers of gauze, and the filtrate was kept on ice. The pink-colored filtrate was centrifuged at 2000*g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 10000*g* for 20 min. The obtained pellet was gently suspended in ca. 25 mL of SME containing the protease inhibitors. The suspension was applied on top of sucrose layers in polyallomer tubes. The bottom layer of 3.0 mL buffer contained 1.8 M sucrose, and the top layer of 3.6 mL buffer contained 1.2 M sucrose. The protease inhibitors were added to the each layer just before use. About 4.2 mL of the suspension was applied on the layers in six tubes and centrifuged at 72000*g* for 17 h. The supernatant was removed and the pale-pink pellet containing the chromaffin granules was suspended in a small amount of SME containing the protease inhibitors and homogenized with a glass homogenizer. The suspension was poured into 300 mL of the hypotonic solution (10 mM MOPS, pH 7.5 with NaOH, 1.25 mM Na_2ATP, and 1 μg/mL of the protease inhibitors). After vigorous stirring for 1 min, the solution was centrifuged at 3000*g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 200000*g* for 60 min. The chromaffin granule membranes were obtained as a brownish-pink pellet and diluted into 1.8 mL of the MOPS solution (20 mM MOPS, pH 7.5 with NaOH, 25% glycerol, 5 mM Na_2ATP, 5 mM monothioglycerol, and 10 μg/mL of the protease inhibitors). The resulting solution contained 4−8 mg of the membrane proteins including V-ATPase, judging from both the quantitative determination of the total protein concentration by the BCA assay, and the Western blotting with the antibody against subunit b on the V_1 domain of V-ATPase. The prepared CGM was suspended at the protein concentration of 5 mg/mL in the MOPS solution and kept frozen at −80 °C.

Assay of Synthetic Salicylihalamides in the Inhibitory Activity against Chromaffin Granule Membrane V-ATPase. The measurement of fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) as an indicator for proton uptake into the membrane vesicles was employed.[31] To a 2 mL of the reaction medium for proton pump assay, chromaffin granule membranes from porcine adrenal glands containing 50 μg of protein, 4.0 μL of 1.0 M ACMA, and 0.67 μL of valinomycin were added, and the resulting solution was incubated at 37 °C for 10 min. A solution of the synthesized salicylihalamide in DMSO (prepared to give 0.1% final concentration of DMSO) was added and incubated at 37 °C for 3 min, and then 10 μL of 0.1 M MgATP was added and incubated for 2 min. The proton gradients made by the V-ATPases in the membranes were canceled by addition of 3.4 μL of 1 mM FCCP. The fluorescence quenching in the presence of MgATP was monitored by a spectrofluorometer with an excitation wavelength of 412 nm and emission at 480 nm. The initial rate of quenching was estimated as the slope of the quenching trace performed on sets of 20 points (20 s of quenching trace) and is expressed as the rate of quenching/min. The proton transport activity is expressed as the percentage versus DMSO-matched controls with standard deviation from triplicate experiments. IC_{50} values were obtained graphically by plotting the percent activity of proton transport versus the log values of the inhibitor concentrations.

Acknowledgment. We thank Prof. Yoshinori Moriyama, Okayama University, for helpful advice on the isolation of chromaffin granule membranes. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (no. 16073211) from MEXT, Japan.

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JM801265E