Bafilomycins Produced in Culture by *Streptomyces* spp. Isolated from Marine Habitats Are Potent Inhibitors of Autophagy†

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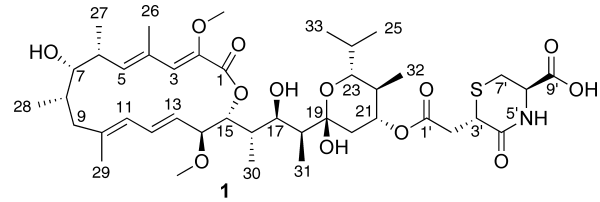
Five new bafilomycins, F (**1**) to J (**5**), have been isolated from laboratory cultures of two *Streptomyces* spp. obtained from marine sediments collected in British Columbia, and their structures have been elucidated by detailed analysis of spectroscopic data and the synthesis of model compounds. The new bafilomycins F (**1**), G (**2**), H (**3**), and J (**5**) along with several co-occurring known analogues showed potent inhibition of autophagy in microscopy and biochemical assays. The thiomorpholinone fragment present in bafilomycin F (**1**) has not previously been found in a natural product.

Autophagy is a catabolic process used by cells to degrade proteins and organelles present in the cytoplasm into low molecular weight products that are recycled.[1] Autophagy is upregulated during periods of nutrient starvation, and it is involved in mammalian developmental processes, innate and adaptive immunity, degradation of invading bacteria, and diseases such as neurodegeneration.[1b] Studies in yeast have identified over 30 autophagy-related (Atg) genes.[1a] There are currently no known direct inhibitors of any of the Atg proteins. In an effort to discover new chemical genetics tools for studying autophagy, a library of crude microbial extracts has been screened in an automated microscopy assay designed to detect modulators of autophagosome accumulation.[2,3] The crude extract library was prepared by extraction of solid agar laboratory cultures of microorganisms obtained from both cold temperate and tropical marine habitats. Two cultures that produced extracts with extremely potent induction of autophagosome accumulation were identified in the screen, and 16S RNA analysis identified both of the producing organisms as *Streptomyces* sp. Assay-guided fractionation of the extracts led to the isolation of the new compounds bafilomycin F (**1**), bafilomycin G (**2**), bafilomycin H (**3**), bafilomycin I (**4**), and bafilomycin J (**5**) along with the known compounds bafilomycin A_1 (**6**),[4] bafilomycin B_1 (**7**),[4] and bafilomycin D (**8**).[5] Details of the isolation, structure elucidation, and autophagy-inhibiting activities of the new and known bafilomycins are presented below.

Results and Discussion

The *Streptomyces* sp. strains RJA71 and RJA635 were obtained from marine sediments collected off the coast of British Columbia, and production cultures were grown as lawns on solid agar. The solid agar culture of strain RJA635 was extracted repeatedly with EtOAc, and the combined EtOAc extracts were dried *in vacuo* to give a brown gum, which was fractionated by sequential application of Si gel flash chromatography, reversed-phase flash chromatography, and reversed-phase HPLC to give the new macrolide bafilomycin F (**1**) along with the known compounds bafilomycin A_1 (**6**), bafilomycin B_1 (**7**), and bafilomycin D (**8**). The structures of the known compounds **6**, **7**, and **8** were confirmed by comparison of their spectroscopic data with literature values.[4,5]



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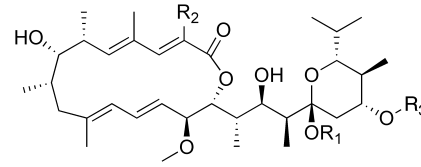
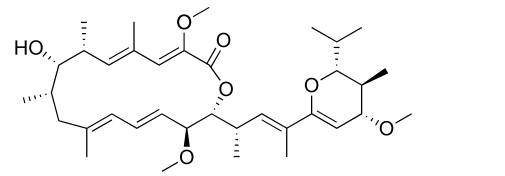


2 R_1 = H, R_2 = OCH_3, R_3 = CH_3
3 R_1 = CH_3, R_2 = OCH_3, R_3 = CH_3
5 R_1= H, R_2 = CH_3, R_3 = CH_3
6 R_1 = H, R_2 = OCH_3, R_3 = H
7 R_1 = H, R_2 = OCH_3, R_3 =



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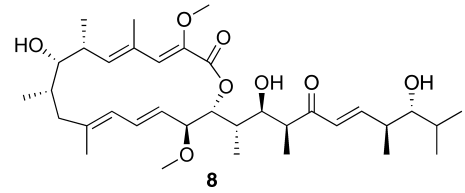

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The cells and media from solid agar culture of strain RJA71 were lyophilized together and then extracted with MeOH, which was dried *in vacuo* to give a brown gum. Partitioning the gum between EtOAc and H_2O gave an active EtOAc-soluble component that was fractionated by sequential application of Sephadex LH-20 chromatography and reversed-phase HPLC to give samples of bafilomycin G (**2**), bafilomycin H (**3**), bafilomycin I (**4**), and bafilomycin J (**5**). ESIHRMS and 1D ^{1}H NMR data showed that

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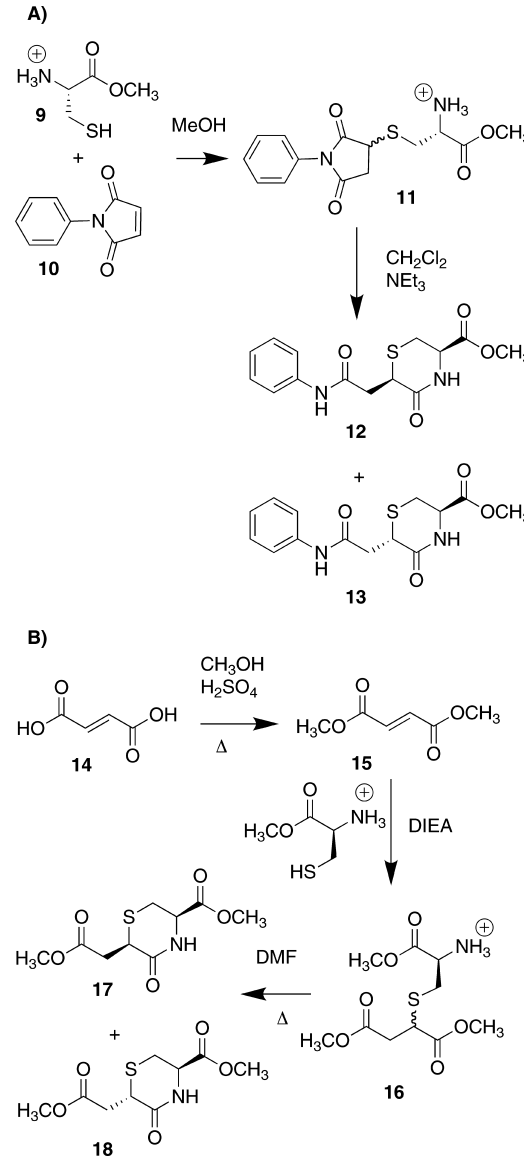
each of the bafilomycins G (**2**), H (**3**), and J (**5**) was contaminated with small amounts of the corresponding analogues that simply have a C-21 alcohol functionality in place of the C-21 methyl ether assigned to the named structures. Significant effort was put into attempts to separate the alcohol-containing contaminants from the methyl ethers, but this proved to be impossible in our hands. Therefore, only the major component methyl ethers **2**, **3**, and **5**, which are clearly defined in the NMR data (Supporting Information), are described here.

Bafilomycin F (**1**) gave a $[M - H]^-$ ion at *m/z* 822.4096 in the negative ion HRESIMS, consistent with a molecular formula of $C_{42}H_{65}NO_{13}S$, requiring 11 sites of unsaturation. Analysis of the 1D and 2D NMR data obtained for bafilomycin F showed that it contained the C-1 to C-33 macrolide substructure present in bafilomycin A_1 (**6**), which was acylated at the C-21 alcohol. The presence of the C-21 acyl substitution in **1** was identified by the downfield shift of H-21 to δ 4.95 (δ 3.69 in **6**[4]) and the observation of a HMBC correlation between the H-21 resonance and a carbonyl resonance at δ 171.5 (C-1′). Subtraction of the atoms present in the macrolide fragment ($C_{35}H_{57}O_9$) of **1** from the molecular formula ($C_{42}H_{65}NO_{13}S$) showed that the C-21 acyl substituent had to account for $C_7H_8NO_4S$ and four sites of unsaturation.

Two isolated spin systems that could be assigned to the C-21 acyl substituent were identified from the COSY data (CD_3OD). The first system consisted of a pair of geminal methylene proton resonances at δ 3.20 (H-2a′) and 3.01 (H-2b′) that were coupled to a methine resonance at δ 3.67 (H-3′), and the second consisted of a two-proton methylene resonance at δ 3.12 (H_2-7′) that was coupled to a methine resonance at δ 4.25 (H-6′). HMBC correlations observed between the H-2a′ (δ 3.20) and H-3′ (δ 3.67) resonances and a resonance at δ 171.5 (C-1′) and between the H2a′, H2b′ (δ 3.01), and H-3′ resonances and a carbon resonance at δ 175.1 (C-4′) indicated that the first spin system had carbonyl substituents on each of the methylene and methine carbons. The H-3′ resonance at δ 3.67 showed a HMBC correlation to a carbon resonance at δ 33.7 (C-7′), which was in turn correlated to the methylene resonance at δ 3.12 (H_2-7′) in the HSQC spectrum, and the H_2-7′ methylene resonance at δ 3.12 showed a HMBC correlation to a carbon resonance at δ 39.6 (C-3′), which was correlated in the HSQC spectrum to the H-3′ resonance (δ 3.67), demonstrating that C-3′ and C-7′ were linked via a nonprotonated atom. The chemical shifts of the carbons indicated a possible linkage via the sulfur atom. A HSQC correlation showed that the methine resonance at δ 4.25 (H-6′) in the second proton spin system was attached to a carbon with a chemical shift of δ 60.6 (C-6′), typical of an amino acid α methine carbon, and the possibility of a sulfur atom being attached to its vicinal methylene neighbor (C-7′) suggested the presence of a cysteine residue. The final carbonyl resonance observed at δ 178.1 in the ^{13}C NMR spectrum (CD_3OD) was assigned to C-9′, and a COSY spectrum of **1** recorded in DMSO-d_6 showed a correlation between the H-6′ resonance at δ 3.76 and an exchangeable resonance at δ 7.01, assigned to NH-5′, accounting for the remaining pieces of the cysteine fragment. All of the above data were in agreement with a biogenesis in which the fumaric acid residue found in bafilomycins B_1 (**7**) and C_1[4] had been elaborated by amide formation with a cysteine α-amino nitrogen followed by an intramolecular Michael addition of the sulfur atom to give a thiomorpholinone ring as shown in **1**.

In order to confirm the presence of the thiomorpholinone ring and to determine the relative configurations at C-3′ and C-6′, model compounds **12**, **13**, **17**, and **18** were synthesized as shown in Scheme 1.[6] The diastereomers **12** and **13** were separated by Si gel chromatography, and compound **12** was crystallized. Analysis of a crystal of **12** by X-ray diffraction analysis showed that the substituents on the thiomorpholinone ring were *cis* (Supporting Information).[7] We were not able to separate the mixture of stereoisomers **17** and **18** by HPLC, but it was possible to assign

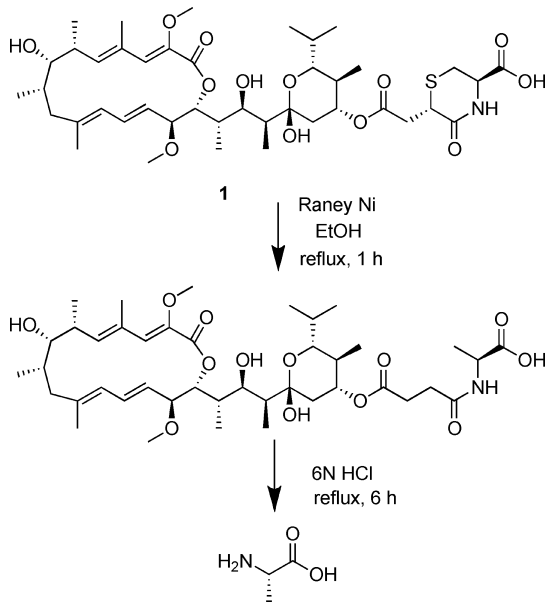
Scheme 1



resonances to each compound in the NMR data for the mixture. NOESY correlations were observed between H-3 and H-6 in both *cis* isomers **12** and **17** but not in the *trans* isomers **13** and **18**. The ^1H and ^{13}C NMR shifts assigned to the thiomorpholinone rings in the model compounds **12**, **13**, **17**, and **18** all showed good agreement with the shifts assigned to the corresponding atoms in **1** (Supporting Information), confirming the nature of the C-21 acyl substituent. The best fit between the chemical shifts for the thiomorpholinone ring in **1** and the model compounds was with the *trans* isomers **13** and **18** (Supporting Information), especially at H-3′ (**1**: δ 3.67; **12**: δ 4.09; **13**: δ 3.87; **17**: δ 4.00; **18**: δ 3.79), and, therefore, we tentatively assigned the relative configuration of the thiomorpholinone ring in **1** as *trans*. The lack of NOESY correlations between H-3′ and H-6′ in **1** was in agreement with the *trans* relative configuration. The absolute configuration in the thiomorpholinone ring of **1** was determined by chemical degradation to alanine (Scheme 2), which was shown by Marfey's analysis to be L. Therefore, the absolute configuration of the C-21 acyl substituent in **1** is assigned as 3′*S*,6′*R*. We have assumed that the remainder of bafilomycin F (**1**) has the same absolute configuration reported for bafilomycin A_1 (**6**),[8] as shown.

Bafilomycin G (**2**) gave a $[M + Na]^+$ ion at *m/z* at 659.4142 in the positive ion HRESIMS, consistent with a molecular formula of $C_{36}H_{60}O_9$, differing from the molecular formula of bafilomycin

Scheme 2



A_1 (**6**) simply by the addition of CH_2. Comparison of the 1D and 2D NMR data obtained for **2** (Table 1) with literature values and the data that we re-collected for bafilomycin A_1 (**6**) showed that **2** was closely related to **6**. A HMBC correlation observed between a methyl ether resonance at δ 3.25 (δ_C 56.3) and a carbon resonance at δ 80.2, assigned to C-21 in **2** by COSY and HSQC data, confirmed that the C-21 OH present in bafilomycin A_1 (**6**) had been replaced by a OMe in bafilomycin G (**2**).

Bafilomycin H (**3**) gave a $[M + Na]^+$ ion at *m/z* at 673.4301 in the positive ion HRESIMS, consistent with a molecular formula of $C_{37}H_{62}O_9$, differing from the molecular formula of bafilomycin G (**2**) simply by the addition of CH_2. The 1D and 2D NMR data obtained for **3** (Table 1) were nearly identical to the data obtained for bafilomycin G (**2**), indicating that the compounds were closely related. The major difference in the NMR data for **2** and **3** was that the OH-19 proton at δ 5.84 in **2** was replaced by a methyl singlet (19-OMe: δ_C 46.4; δ_H 3.12), suggesting that bafilomycin H (**3**) was the methyl ketal of bafilomycin G (**2**). A correlation observed between the methyl resonance at δ 3.12 and the C-19 ketal carbon (δ 103.8) in the HMBC spectrum obtained for **3** confirmed this assignment. The methyl ketal in **3** may be an isolation artifact formed during MeOH extraction of the culture.[9]

Bafilomycin I (**4**) gave a $[M + Na]^+$ ion at *m/z* at 623.3917 in the positive ion HRESIMS, consistent with a molecular formula of $C_{36}H_{56}O_7$, which differed from the molecular formula of bafilomycin A_1 (**6**) by the loss of $2 \times H_2O$. Comparison of the 1D and 2D NMR data obtained for **4** with that of **2** (Table 1) showed that these compounds were closely related. In particular, the NMR data showed that the C-1 to C-15 macrocyclic rings were identical in **2** and **4**. Similarly, the NMR data confirmed that the substituents at C-21 (OMe), C-22 (Me), and C-23 (isopropyl) were also the same in **2** and **4** (Table 1). However, unlike in **2**, the C-21 methine proton resonance at δ 3.55 in **4** showed a COSY correlation to a

Table 1. ^{1}H NMR Data for **1**−**5**

	1[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]
position	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)
2-(O)Me/Me	3.61, s	3.63, s	3.48, s	3.69, s	2.12, s
3	6.69, s	6.83, s	6.83, s	7.04, s	7.39, s
26	1.97, s	2.01, s	1.97, s	2.04, d (0.8)	1.63, s
5	5.88, d (8.9)	5.82, d (9.0)	5.83, d (8.9)	5.99, d (9.1)	5.87, d (8.8)
6	2.52, m	2.29, m	2.27, m	2.20, m	2.23, m
27	1.05, d (6.9)	0.88, d (7.0)	0.87, d (7.0)	0.89, d (7.0)	0.83, d (7.0)
7	3.24, m	2.84, t (5.8)	2.86, m	3.00, m	2.83, t (6.0)
7-OH		0.83, d (5.8)	0.87, m	0.86, d (6.0)	0.83, d (6.0)
8	1.84, m	1.74, m	1.74, m	1.66, m	1.76, m
28	0.92, d (6.9)	0.73, d (6.2)	0.73, d (5.6)	0.81, d (7.0)	0.72, d (6.1)
9a	2.02, m	1.97, m	1.95, m	2.25, dd (14.4, 10.9)	1.97, m
9b	2.02, m	1.72, m	1.74, m	1.84, d (14.4	1.75, m
29	1.89, s	1.87, s	1.87, s	1.66, s	1.88, s
11	5.76, d (10.8)	5.61, d (10.7)	5.65, d (10.7)	5.95, d (11.1)	5.72, d (10.7)
12	6.59, dd (14.9, 10.8)	6.47, dd (15.0, 10.7)	6.50, dd (15.0, 10.7)	6.69, dd (15.1, 11.1)	6.53, dd (14.9, 10.7)
13	5.10, dd (14.9, 8.9)	5.08, dd (15.0, 9.3)	5.16, dd (15.0, 9.2)	5.45, dd (15.1, 6.4)	5.22, dd (14.9, 9.3)
14	3.98, t (8.4)	3.90, t (9.2)	3.93, t (9.0)	3.94, t (5.4)	3.96, t (8.9)
14-OMe	3.24, s	3.07, s	3.08, s	3.18, s	3.11, s
15	5.03, d (7.7)	5.38, d (8.6)	5.58, d (8.5)	5.42, dd (8.2, 4.3)	5.45, d (7.7)
16	2.07, m	2.46, m	2.39, m	2.96, m	2.43, m
30	0.85, d (4.4)	0.86, d (6.8)	0.99, d (6.9)	1.16, d (6.7)	0.86, d (6.9)
17	4.14, dd (10.5, 1.4)	4.54, ddd (10.7, 3.9, 1.7)	3.96, dd (10.2, 4.4)	6.35, d (10.1)	4.54, d (10.6)
17-OH		5.20, d (3.7)	4.51, d (4.4)		5.45, m
18	1.79, m	1.91, m	2.29, m		1.90, m
31	0.98, d (7.2)	1.21, d (7.1)	1.32, d (6.8)	1.75, s	1.21, d (7.5)
19-OH(OMe)		5.84, m	3.12, s		5.97, m
20a	2.29, dd (12.0, 4.8)	2.69, m	2.92, m	5.12, d (2.6)	2.70, m
20b	1.30, m	1.21, m	1.82, m		1.22, m
21	4.95, dt (10.9, 4.8)	3.54, m	3.39, m	3.55, dd (7.8, 2.6)	3.55, m
21-OMe		3.25, s	3.20, s	3.20, s	3.25, s
22	1.61, m	1.72, m	1.65, m	2.02, m	1.73, m
32	0.86, d (4.1)	1.06, d (6.4)	1.00, d (12.6)	0.88, d (6.7)	1.06, d (6.5)
23	3.56, dd (10.4, 1.5)	3.85, d (10.4)	3.19, d (9.3)	3.37, dd (9.3, 3.4)	3.88, d (10.1)
24	1.91, m	1.92, m	1.85, m	1.89, m	1.95, m
33	0.95, d (7.2)	1.11, d (6.8)	1.10, d (6.9)	1.09, d (6.8)	1.22, d (7.1)
25	0.80, d (6.9)	0.94, d (6.8)	0.94, d (6.7)	0.92, d (6.8)	0.95, d (6.7)
2′a	3.20, m				
2′b	3.01, dd (14.8, 7.4)				
3′	3.67, dd (7.4, 2.5)				
6′	4.25, m				
7′	3.12, m				

[a] Spectra collected in MeOD at 600 MHz. [b] Spectra collected in C_6D_6 at 600 MHz.

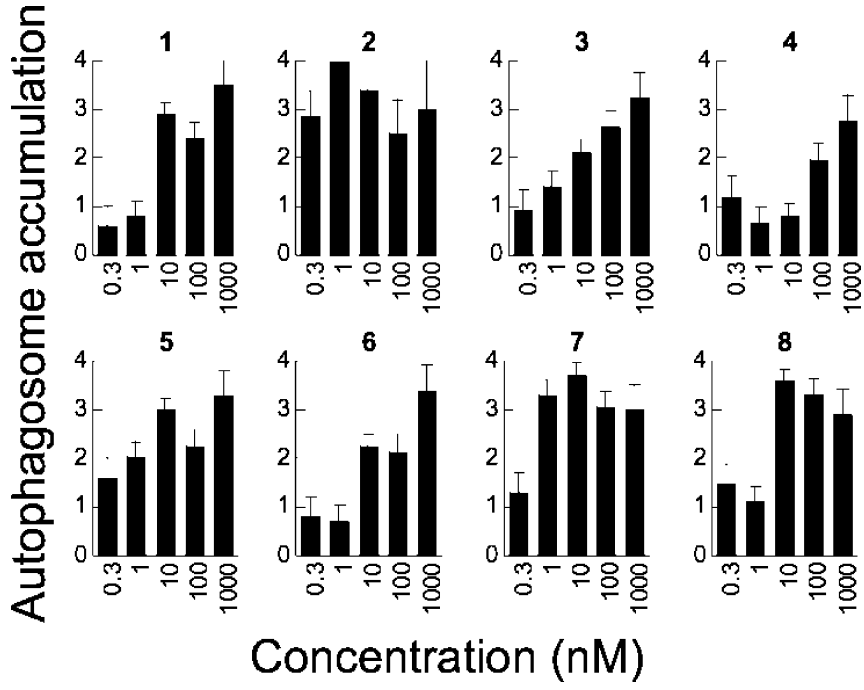


Figure 1. Stimulation of autophagosome accumulation by compounds **1**−**8**. MCF-7 cells expressing EGFP-LC3 were exposed to the compounds for 4 h. The formation of punctate EGFP-LC3 was determined quantitatively by automated microscopy and expressed relative to control, untreated cells. The bars represent means ± SD ($n = 3$).

vicinal olefinic methine resonance at δ 5.12 (H-20) and HMBC correlations to olefinic carbon resonances at δ 97.6 (C-20) and 154.3 (C-19), indicating the presence of a $\Delta^{19,20}$ alkene. The H-16 methine resonance at δ 2.96 in the ^{1}H NMR spectrum of **4** also showed a COSY correlation to a vicinal olefinic methine resonance at δ 6.35 (H-17) and HMBC correlations to olefinic carbon resonances at δ 129.3 (C-18) and 129.8 (C-18), suggesting the presence of a $\Delta^{17,18}$ alkene. An olefinic methyl resonance at δ 1.75, assigned to Me-31, showed HMBC correlations to the carbon resonances at δ 129.3 (C-17), 129.8 (C-18), and 154.3 (C-19), confirming that two alkenes were conjugated. The observation of a ROESY correlation between the Me-31 resonance at δ 1.75 and the H-16 methine resonance at δ 2.96 showed that the $\Delta^{17,18}$ alkene had the *E* configuration. We have assumed that the absolute configurations at C-21, C-22, and C-23 and at the stereogenic centers in the C-1 to C-15 macrocyclic ring in bafilomycin I (**4**) are identical to the corresponding absolute configurations in bafilomycin A_1 (**6**).[8]

Bafilomycin J (**5**) gave a $[M + Na]^+$ ion at *m*/*z* at 643.4168 in the positive ion HRESIMS, consistent with a molecular formula of $C_{36}H_{60}O_8$, which differed from the molecular formula of bafilomycin G (**2**) simply by loss of one oxygen atom. Detailed analysis of the 1D and 2D NMR data obtained for **5** showed that it was nearly identical to **2** (Table 1). The only difference between **2** and **5** was that the methyl ether at C-2 in bafilomycin G (**2**) (OMe-2: δ_C 59.7; δ_H 3.63) was replaced by a methyl group (δ_C 14.2; δ_H 2.12) in bafilomycin J (**5**), accounting for the one oxygen atom difference in their molecular formulas. Consistent with this assignment was the observation of a HMBC correlation between the olefinic methyl resonance at δ 2.12 and the macrolide carbonyl resonance at δ 172.5 (C-1). A methyl substituent at C-2 has previously been encountered in the related macrolide oxohygrolidin.[5]

The ability of bafilomycins **1** to **8** to induce autophagosome accumulation was measured using an automated microscopy screening assay.[2] In the presence of **1** to **8** the fluorescence intensity associated with autophagosomes increased, indicating that the compounds induced autophagosome accumulation either by stimulating the formation of autophagosomes or by inhibiting their degradation by preventing the maturation of autophagosomes into autolysosomes. The effect of various concentrations of **1** to **8** on autophagosomal content is shown in Figure 1. Compound **4** was the least potent, showing activity at 100 nM and above. Compounds **1**, **3**, and **8** showed good activity at 10 nM, and compounds **5**, **6**, and **7** were active at 1 nM and above. Bafilomycin G (**2**) was the most potent, being fully active at 0.3 nM, the lowest concentration tested in this assay. For a comparison, 4 h exposure to well-characterized autophagic stimuli such as amino acid and serum starvation or 20 nM rapamycin caused ~3-fold increase in autophagosomal content, while some recently identified autophagy modulators caused a 10−15-fold increase in this assay.[2a]

To determine whether the compounds induced autophagy or inhibited this process at a late stage, they were subjected to a biochemical test. Autophagy entails the recruitment of the protein LC3 to autophagosomes and its degradation by lysosomal hydrolases upon fusion of autophagosomes with lysosomes. In the cell line used, LC3 is tagged by fusion at its N-terminus with enhanced green fluorescent protein (EGFP). The EGFP moiety of the EGFP-LC3 fusion protein is much more resistant to degradation by lysosomal hydrolases than the LC3 portion. Compounds that stimulate autophagy, such as the mTOR inhibitor rapamycin, cause increased accumulation of the EGFP moiety (Figure 2A). Compounds **1**−**3** and **5**−**8** did not increase EGFP levels (Figure 2A), indicating that they do not stimulate autophagy. Notably, compound **4** clearly and reproducibly increased EGFP levels, suggesting that it induces autophagy. To determine whether the compounds inhibited EGFP-LC3 degradation by autophagy, cells were co-incubated with rapamycin to induce autophagy and with compounds **1**−**8**. Compounds **1**−**3** and **5**−**8** inhibited EGFP production, showing that they inhibit autophagic protein degradation. Compound **4** did not prevent EGFP formation by rapamycin, indicating that it does not inhibit autophagy. Bafilomycins A_1, B_1, and D are potent and selective inhibitors of the vacuolar type H^+-ATPase responsible for maintaining the acidic pH of lysosomes.[10] The bafilomycins thus block lysosomal acidification and the activity of the acid hydrolases responsible for autophagic degradation. The observation that **1**−**3** and **5**−**8** block autophagosomal degradation is consistent with their known mechanism of action. However, the observation

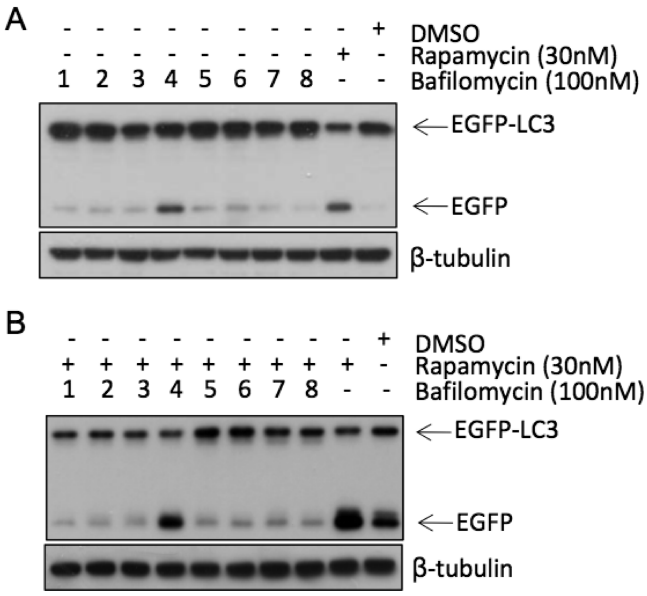


Figure 2. Effect of compounds **1**−**8** on EGFP-LC3 degradation. MCF-7 cells expressing EGFP-LC3 were exposed for 4 h to the different compounds and combinations. The formation of the EGFP band was monitored by Western blotting using antibodies to GFP. Protein loading was monitored with an antibody to β-tubulin.

that the closely related bafilomycin I (**4**) appears to stimulate autophagy instead of inhibiting it was very unexpected.

Bafilomycins F (**1**) to J (**5**) contain a number of structural variants not previously encountered in this family of macrolides. The most striking new variation is the thiomorpholinone fragment present in bafilomycin F (**1**), which is an unknown substructure in any natural product and does not appear to hinder activity. Also of note is the conjugated $\Delta^{17,18}$, $\Delta^{19,20}$ diene in bafilomycin I (**4**), which results in significant attenuation of the autophagy-inhibiting activity of the bafilomycin scaffold.

Experimental Section

General Experimental Procedures. Optical rotations were recorded with a JASCO P-1010 polarimeter equipped with a halogen lamp (589 nm) and a 10 mm microcell. UV spectra were recorded using a Waters 2487 dual λ absorbance detector. NMR spectra were recorded on a Bruker Avance 600 equipped with a cryoprobe at 600 MHz in C_6D_6 or MeOD and referenced to the solvent. ESIMS spectra were obtained with Micromass LCT and Bruker Esquire-LC mass spectrometers. A Waters 10 g silica Sep-pak and a Waters 2 g C_{18} Sep-pak were used for normal-phase and reversed-phase flash chromatrography, respectively. Sephadex LH-20 was used for column chromatography. A Waters 1500 Series pump system equipped with a Waters 2487 dual λ absorbance detector and a CSC-Inertsil 150A/ODS2 column or Alltech Apollo silica semipreparative column (10 × 250 mm, 5 μm) was used for HPLC. Single-crystal X-ray diffraction measurements were made on a Bruker X8 APEX II diffractometer with graphite-monochromated Mo Kα radiation. The data were collected at a temperature of -100 ± 0.1 °C to a maximum 2θ value of 55.0°. Data were collected in a series of ϕ and ω scans in 0.50° oscillations with 4.0 s exposures. The crystal to detector distance was 36.00 mm.

Microbial Isolates RJA71 and RJA635. Isolate RJA71 was obtained from a marine sediment collected in the Queen Charlotte Islands, British Columbia. It was identified as *Streptomyces* sp. YIM26 by 16S RNA analysis (GenBank accession number AF389343.1). Production cultures were grown as lawns on solid tryptic soy agar at 25 °C. Isolate RJA635 was obtained from a marine sediment collected in Indian Arm, British Columbia. It was identified as *Streptomyces halstedii* by 16S RNA analysis (GenBank accession number AB184831.1). Production cultures were grown as lawns on solid ISP2 media at 25 °C.

Isolation of Bafilomycins. The solid agar culture of strain RJA635 was extracted with EtOAc and dried *in vacuo* to give the crude extract. The crude extract was subjected to Si gel flash chromatography (step gradient: hexanes to EtOAc to MeOH). The fractions eluting with 3:1 EtOAc−hexanes were further purified by normal-phase HPLC (1:1 EtOAc−hexanes) and reversed-phase C_{18} flash chromatography (7:3 CH_3CN-H_2O) to give bafilomycin A_1 (**6**, 1.2 mg), bafilomycin B_1 (**7**, 3.8 mg), and bafilomycin D (**8**, 1.0 mg). The fractions eluting from the Si gel flash chromatography fractionation with 100% MeOH were subjected to reversed-phase C_{18} flash chromatography (step gradient: H_2O to MeOH). The fractions eluting with 3:1 MeOH−H_2O were purified by reversed-phase HPLC (3:1 MeOH−H_2O) to give bafilomycin F (**1**, 2.0 mg).

The solid agar culture of strain RJA71 was extracted twice with MeOH. Concentration of the 2 L of combined MeOH extracts *in vacuo* gave a gummy brown residue, which was partitioned between EtOAc (3 × 75 mL) and H_2O (300 mL). The combined EtOAc extract was evaporated to dryness and fractionated on Sephadex LH-20 using 4:1 MeOH−CH_2Cl_2 as eluent to generate an active fraction. Reversed-phase HPLC, using a CSC-Inertsil 150A/ODS2, 5 μm 25 × 0.94 cm column with 87:13 MeCN−H_2O as eluent, gave pure samples of bafilomycin G (**2**) (0.8 mg) and bafilomycin H (**3**) (4.4 mg) and a mixture of bafilomycin I (**4**) and bafilomycin J (**5**). Pure samples of bafilomycin I (**4**) (0.2 mg) and bafilomycin J (**5**) (0.4 mg) were obtained after an additional reversed-phase HPLC purification using 17:3 MeOH−H_2O as eluent.

Bafilomycin F (1): clear glass; $[\alpha]^{20}_D$ +10.0 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ϵ) 244 (4.4), 281 (4.0) nm; ^{1}H NMR and ^{13}C NMR, see Tables 1 and 2; HRESIMS(−) *m*/*z* 822.4096 (calcd for $C_{42}H_{64}NO_{13}S$, 822.4098).

Table 2. ^{13}C NMR (δ_C) Data for **1**−**5**

position	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]
1	168.1	167.6	167.0	164.1	172.5
2	142.3	142.1	142.3	142.7	122.8
2-(O)Me/Me	60.6	59.7	59.4	59.7	14.2
3	134.9	133.1	132.4	131.8	146.5
4	133.3	133.1	133.0	132.1	134.4
26	14.1	14.3	14.2	14.1	15.0
5	145.6	142.9	142.5	140.8	144.9
6	38.5	37.1	37.1	38.4	37.0
27	17.9	17.3	17.3	18.6	17.4
7	81.2	80.7	80.7	80.8	80.7
8	41.7	40.5	40.4	38.1	40.4
28	22.5	21.8	22.0	23.9	21.6
9	42.5	41.5	41.6	41.8	41.5
10	145.0	142.9	142.7	140.7	142.7
29	20.1	20.3	20.2	18.0	20.3
11	125.6	125.5	125.6	125.8	125.4
12	134.7	133.4	133.2	131.1	133.0
13	127.0	127.8	127.9	126.1	128.0
14	84.4	82.5	83.0	83.5	82.9
14-OMe	55.9	55.2	55.3	55.8	55.3
15	77.7	77.3	77.6	78.6	76.7
16	39.5	37.5	39.2	35.8	37.9
30	10.5	10.1	11.0	17.4	10.1
17	72.0	71.2	70.2	129.3	71.0
18	43.6	42.9	39.3	129.8	42.6
31	7.2	7.6	8.0	13.1	7.4
19	100.4	99.5	103.8	154.3	99.5
19-OMe			46.4		
20	40.6	39.6	35.2	97.6	39.4
21	76.4	80.2	79.8	79.4	80.2
22	39.4	40.0	38.9	34.4	40.0
32	12.6	12.6	12.6	14.3	12.6
23	77.3	76.5	77.9	83.6	76.4
24	29.2	28.5	28.8	28.6	28.5
33	21.8	21.7	21.0	20.4	21.4
25	14.6	14.7	14.4	15.3	14.7
1′	171.5	56.3	56.0	54.9	56.3
2′	42.6				
3′	39.6				
4′	175.1				
6′	60.6				
7′	33.7				
9′	178.1				

[a] Spectra collected in MeOD at 600 MHz. [b] Spectra collected in C_6D_6 at 600 MHz.

Bafilomycin G (2): clear glass; $[\alpha]^{20}_D$ −29 (*c* 0.4, MeOH); UV (87% MeCN−H_2O) λ_{max} (log ϵ) 245 (4.6), 286 (4.0) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; (+)-HRESIMS $[M + Na]^+$ *m/z* 659.4142 (calcd for $C_{36}H_{60}O_9Na$, 659.4135).

Bafilomycin H (3): clear glass; $[\alpha]^{20}_D$ −1.8 (*c* 2.1, MeOH); UV (87% MeCN−H_2O) λ_{max} (log ϵ) 245 (4.6), 286 (4.0) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; (+)-HRESIMS $[M + Na]^+$ *m/z* 673.4301 (calcd for $C_{37}H_{62}O_9Na$, 673.4291).

Bafilomycin I (4): clear glass; $[\alpha]^{20}_D$ +140 (*c* 0.1, MeOH); UV (85% MeOH−H_2O) λ_{max} (log ϵ) 245 (4.6), 283 (4.0) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; (+)-HRESIMS $[M + Na]^+$ *m/z* 623.3917 (calcd for $C_{36}H_{56}O_7Na$, 623.3923).

Bafilomycin J (5): clear glass; $[\alpha]^{20}_D$ +40 (*c* 0.2, MeOH); UV (85% MeOH−H_2O) λ_{max} (log ϵ) 245 (4.6), 283 (4.0) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; (+)-HRESIMS $[M + Na]^+$ *m/z* 643.4168 (calcd for $C_{36}H_{60}O_8Na$, 643.4186).

Synthesis of Model Compounds 12 and 13. Compounds **12** and **13** were synthesized according to a literature protocol.[6] A solution of cysteine methyl ester hydrochloride (**9**) (343 mg, 2.0 mmol) in MeOH was added to a solution of *N*-phenylmaleimide (**10**) (363 mg, 2.1 mmol) in CH_2Cl_2. The reaction was stirred at room temperature for 2 h. The mixture was concentrated *in vacuo* and purified by Si gel chromatography (step gradient elution: CH_2Cl_2 to MeOH) to give **11** (652 mg, 95%). To a solution of **11** (652 mg, 1.90 mmol) in MeOH was added diisopropylethylamine, and the reaction mixture was stirred at room temperature for 2 days. The reaction mixture was dried *in vacuo* and purified by Si gel chromatography (step gradient elution: CH_2Cl_2 to EtOAc) to give a pure **12** (85 mg) and pure **13** (26 mg) along with a mixture of **12** and **13** (320 mg, total yield = 74%). Compound **12** was recrystallized from MeOH.

Compound **12**: colorless needles (MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ 7.52 (2H, d, J = 7.8 Hz, H-12, H-16), 7.28 (2H, t, J = 7.8 Hz, H-13, H-15), 7.07 (1H, t, J = 7.8 Hz, H-14), 4.47 (1H, dd, J = 6.3, 4.2 Hz, H-6), 4.09 (1H, dd, J = 8.2, 5.7 Hz, H-3), 3.78 (3H, s, H-17), 3.28 (2H, m, H-2a, H-7a), 3.07 (1H, m, H-7b), 2.61 (1H, dd, J = 15.4, 8.2 Hz, H-2b); ^{13}C NMR (CD_3OD, 100 MHz) δ 171.8 (C, C-9), 171.4 (C, C-4), 170.8 (C, C-1), 139.8 (C, C-11), 129.8 (CH, C-13, C-15), 125.2 (CH, C-14), 121.3 (CH, C-12, C-16), 57.7 (CH, C-6), 53.4 (CH_3, C-17), 39.5 (CH_2, C-2), 39.4 (CH, C-3), 28.8 (CH_2, C-7); HRESIMS(+) *m/z* 331.0734 (calcd for $C_{14}H_{16}N_2O_4SNa$, 331.0728).

Compound **13**: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.52 (2H, d, J = 7.8 Hz, H-12, H-16), 7.28 (2H, t, J = 7.8 Hz, H-13, H-15), 7.07 (1H, t, J = 7.8 Hz, H-14), 4.50 (1H, m, H-6), 3.87 (1H, dd, J = 8.6, 4.8 Hz, H-3), 3.78 (3H, s, H-17), 3.26 (1H, m, H-7a), 3.07 (1H, m, H-7b), 3.04 (1H, m, H-2a) 2.85 (1H, dd, J = 15.3, 8.6 Hz, H-2b); ^{13}C NMR (CD_3OD, 100 MHz) δ 171.8 (C, C-9), 171.4 (C, C-4), 170.8 (C, C-1), 139.8 (C, C-11), 129.8 (CH, C-13, C-15), 125.2 (CH, C-14), 121.3 (CH, C-12, C-16), 58.1 (CH, C-6), 53.4 (CH_3, C-17), 40.9 (CH_2, C-2), 39.0 (CH, C-3), 27.0 (CH_2, C-7); HRESIMS(+) *m/z* 331.0732 (calcd for $C_{14}H_{16}N_2O_4SNa$, 331.0728).

Synthesis of Model Compounds 17 and 18. To fumaric acid (**14**) (1.45 g, 12.5 mmol) suspended in MeOH (20 mL) was added concentrated H_2SO_4 (0.5 mL), and the mixture was heated to reflux for 1 h. The mixture was then cooled in an ice bath and neutralized with 10% Na_2CO_3. CH_2Cl_2 was added, and the layers were separated. The organic layer was dried *in vacuo*, redissolved in CH_2Cl_2, and purified by Si gel chromatography (eluent: CH_2Cl_2) to give dimethylfumarate (**15**) (1.63 g, 91%).

To a solution of L-cysteine methyl ester hydrochloride (**9**) (858 mg, 5.0 mmol) in MeOH (20 mL) was added diisopropylethylamine (1.8 mL, 10.3 mmol), followed by a solution of dimethylfumarate (**15**) (734 mg, 5.1 mmol) in CH_2Cl_2. The reaction was stirred for 15 min at room temperature, at which point TLC analysis indicated that the reaction was complete. The reaction mixture was purified by Si gel chromatography (step gradient elution: CH_2Cl_2 to EtOAc) to give **16**. Compound **16** was dissolved in DMF and heated to 140 °C for 6 h. The reaction mixture was cooled, dried *in vacuo*, and purified by Si gel chromatography (step gradient elution: CH_2Cl_2 to EtOAc) to give a mixture of **17** and **18** that could not be separated by chromatography (191 mg, 15% over two steps).

Compound **17**: ^{1}H NMR (CD_3OD, 400 MHz) δ 4.49 (1H, dd, J = 6.5, 4.1 Hz, H-6), 4.00 (1H, t, J = 6.8 Hz, H-3), 3.79 (3H, s, H-11), 3.69 (3H, s, H-10), 3.31 (1H, m, H-7a), 3.08 (1H, m, H-7b), 2.95 (1H, m, H-2a), 2.60 (1H, dd, J = 16.5, 7.3 Hz, H-2b); ^{13}C NMR (CD_3OD, 100 MHz) δ 172.8 (C, C-4), 171.6 (C, C-9), 170.8 (C, C-1), 57.5 (CH, C-6), 53.4 (CH_3, C-11), 52.5 (CH_3, C-10), 38.9 (CH, C-3), 36.8 (CH_2, C-2), 28.8 (CH_2, C-7); HRESIMS(+) *m/z* 270.0413 (calcd for $C_9H_{13}NO_5SNa$, 270.0412).

Compound **18**: ^{1}H NMR (CD_3OD, 400 MHz) δ 4.53 (1H, t, J = 4.8 Hz, H-6), 3.79 (1H, m, H-3), 3.79 (3H, s, H-11), 3.69 (3H, s, H-10), 3.28 (1H, m, H-7a), 3.08 (1H, m, H-7b), 2.97 (1H, m, H-2a), 2.85 (1H, dd, J = 16.6, 7.8 Hz, H-2b); ^{13}C NMR (CD_3OD, 100 MHz) δ 172.8 (C, C-4), 172.0 (C, C-9), 170.6 (C, C-1), 58.0 (CH, C-6), 53.4 (CH_3, C-11), 52.4 (CH_3, C-10), 38.5 (CH, C-3), 38.4 (CH_2, C-2), 27.1 (CH_2, C-7); HRESIMS(+) *m/z* 270.0413 (calcd for $C_9H_{13}NO_5SNa$, 270.0412).

Biological Assays. The automated microscopy cell-based assay used to detect autophagosome accumulation and the biochemical EGFP-LC3 degradation assay have been described in detail.[2a]

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Supporting Information Available: NMR spectra for bafilomycins F (**1**) to J (**5**); experimental details for X-ray diffraction analysis of **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (a) Suzuki, K.; Ohsumi, Y. *FEBS Lett.* **2007**, *581*, 2156–2161. (b) Martinez-Vicente, M.; Cuervo, A. M. *Lancet Neurol.* **2007**, *6*, 352–361. (c) Yoshimori, T. *Cell* **2007**, *128*, 833–836. (d) Rubinstein, D. C.; Gestwicki, J. E.; Murphy, L. O.; Klionsky, D. J. *Nat. Rev. Drug Discovery* **2007**, *6*, 304–312. (e) Schmid, D.; Munz, C. *Immunity* **2007**, *27*, 11–21. (f) Xiao, G. *Cytokine Growth Factor Rev.* **2007**, *18*, 233–243. (g) Ferraro, E.; Cecconi, F. *Arch. Biochem. Biophys.* **2007**, *462*, 210–219. (h) Mizushima, N. *Proc. Jpn. Acad., Ser. B* **2007**, *83*, 39–46. (i) Yorimitsu, T.; Klionsky, D. J. *Trends Cell Biol.* **2007**, *17*, 279–285.

(2) (a) Balgi, A. D.; Fonseca, B. D.; Donohue, E.; Tsang, T. C. F.; Lajoie, P.; Proud, C. G.; Nabi, I. R.; Roberge, M. *PLoS ONE* **2009**, *4*, e7124. (b) Klionsky, D. J.; Cuerva, A. M.; Seglen, P. O. *Autophagy* **2007**, *3*, 181–206.

(3) Keyzers, R. A.; Daoust, J.; Davies-Coleman, M. T.; Van Soest, R.; Balgi, A.; Donohue, E.; Roberge, M.; Andersen, R. J. *Org. Lett.* **2008**, *10*, 2959–2962.

(4) Werner, G.; Hagenmaier, H.; Albert, K.; Kohlshorn, H.; Drautz, H. *Tetrahedron Lett.* **1983**, *24*, 5193–5196.

(5) Kretschmer, A.; Dorgerloh, M.; Deeg, M.; Hagenmaier, H. *Agric. Biol. Chem.* **1985**, *49*, 2509–2511.

(6) Shih, H. C.; Rankin, G. O. *J. Heterocycl. Chem.* **1988**, *25*, 589–590.

(7) Crystallographic data for **12** have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 757295). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

(8) Baker, G. H.; Brown, P. J.; Dorgan, R. J. J.; Everett, J. R.; Ley, S. V.; Slawin, A. M. Z.; Williams, D. J. *Tetrahedron Lett.* **1987**, *28*, 5565–5568.

(9) Hatfield, G. M.; Woodard, R. W.; Son, J.-K. *J. Nat. Prod.* **1992**, *55*, 753–759.

(10) Bowman, E. J.; Siebers, A.; Altendorf, K. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7972–7976.

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