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Solid-State NMR

Modification of Bafilomycin Structure to Efficiently Synthesize Solid-State NMR Probes that Selectively Bind to Vacuolar-Type ATPase

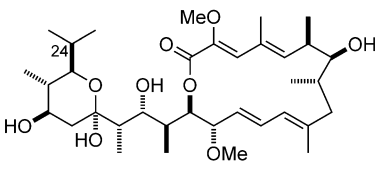
Hajime Shibata,[a, b] Hiroshi Tsuchikawa,[a] Tatsuru Hayashi,[a] Nobuaki Matsumori,[a] Michio Murata,*[a, b] and Takeo Usui[c]

Abstract: Bafilomycin (Baf) is one of the most potent inhibitors of vacuolar-type ATPase, which is strongly implicated in age-related diseases. However, the binding site of the antibiotic on the protein remains unclear because of the complexity of the structure of Baf bound to the target subunit in the transmembrane region. For conducting structural studies by applying solid-state NMR, which is one of the most promising methodologies available for structural analysis in membrane system, preparing bioactive fluorinated Baf analogues is essential. In this study two Baf analogues were carefully designed and efficiently synthesized through the convergent coupling of three segments. Biological evaluation revealed that the activity of 24-F-Baf was comparable to that of Baf, indicating its utility as a potential probe for solid-state NMR analysis. By contrast, desmethylated 24-F-Baf exhibited markedly diminished activity. The absence of two methyl groups caused a critical conformational change in the macrocyclic core structure necessary for binding to the target protein.

Introduction

Eukaryote cells contain acidic organelles such as lysosomes and endosomes, which possess unique structures and functions. The acidic nature of the organelles is considered to be critical for various physiological processes such as protein degradation and endocytosis. A membrane-bound protein, vacuolar-type ATPase(V-ATPase), is the main molecule that generates this acidic environment by actively transporting protons against the concentration gradient by using ATP. Since the discovery of V-ATPase in the vacuole of yeast,[1] it has been found not only in the endomembrane system of all eukaryotic cells but also in the plasma membranes of specialized cells such as osteoclasts and certain tumor cells.[2–4] Moreover, because V-ATPases have been implicated in numerous diseases such as osteoporosis, cancer, and diabetes, and have attracted considerable biomedical research scrutiny as a promising drug target, V-ATPase have been extensively investigated in terms of the pathology of these diseases and drug development.[5–7]

Bafilomycin A_1 (Baf, **1**), which was first isolated in 1983 from *Streptomyces griseus* ssp. *sulphurus*,[8] is a representative, highly potent V-ATPase inhibitor.[9] Baf is composed of two main parts, a 16-membered macrolactone and a tetrahydropyrane (THP) structure, tethered by a 4-carbon acyclic chain, encompassing 9 stereogenic centers (Figure 1). Early investigation of its mode of action implied that the Baf-binding target is the transmembrane Vo domain of V-ATPase.[10,11] Subsequent point-mutation[12] and photoaffinity-labeling[13] studies identified the putative binding site of Baf to be subunit c (and subunit a) in the Vo domain. However, despite the intensive use of Baf as an inhibitor in physiological studies on V-ATPase, the detailed interaction of Baf with V-ATPase at the atomistic level remains unclear. The most challenging problem is the structural



Bafilomycin A_1, Baf (**1**)

Figure 1. Structure of bafilomycin A_1.

[a] *Dr. H. Shibata, Dr. H. Tsuchikawa, T. Hayashi, Prof. Dr. N. Matsumori,[+] Prof. Dr. M. Murata*
Graduate School of Science
Osaka University
1-1 Machikaneyama, Toyonaka, Osaka 560-0043 (Japan)
Fax: (+81) 6-6850-5774
E-mail: murata@chem.sci.osaka-u.ac.jp

[b] *Dr. H. Shibata, Prof. Dr. M. Murata*
ERATO, Lipid Active Structure Project
Japan Science and Technology Agency
1-1 Machikaneyama, Toyonaka, Osaka 560-0043 (Japan)

[c] *Prof. Dr. T. Usui*
Faculty of Life and Environmental Sciences
University of Tsukuba
Tennodai, Tsukuba, Ibaraki 305-8572 (Japan)

[+] *Present address:*
Graduate School of Sciences
Kyushu University
6-10-1 Hakozaki, Higashi-ku, Fukuoka, 812-8581 (Japan)

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analysis of V-ATPase because it forms a macromolecular complex that exhibits high heterogeneity, and this prevents the application of X-ray crystallography. Although the entire structure of a yeast V-ATPase has been determined using cryo-electron microscopy and certain X-ray structures of its subunits were recently reported,[14,15] detailed structural information about the transmembrane Vo domain has remained unavailable thus far. Moreover, subunit c, the probable binding site of Baf, forms hetero-oligomers with its isoforms (subunit c′ and subunit c″) in yeast V-ATPases, which makes the structural analysis even more challenging.

One of the useful methods for the structural analysis of non-crystal, heterogeneous, and hardly solubilized entities is solid-state NMR; thus, this technique has attracted considerable attention over the last few decades.[16] Among the solid-state NMR techniques, rotational echo double resonance (REDOR)[17] has enabled us to obtain interatomic distance information. REDOR has been successfully applied in the structure elucidation of non-solubilized protein–ligand complexes[18,19] and drug–lipid assembles.[20] Therefore, solid-state NMR measurement appears to be the optimal approach for analyzing the structure of the highly complicated Baf–ATPase complex. However, certain key challenges must be overcome. First, the preparation of labeled compounds featuring an NMR-sensitive nucleus is essential. Specifically, ^{19}F is the most appropriate nucleus because it exhibits a high gyromagnetic ratio and low background signals in biological samples favorable for long and precise distance measurements.[21] However, the introduction of ^{19}F atoms into small compounds often causes large perturbations in biological activity.[22] Second, in NMR experiments conducted with labeled ligands in complex with receptors, most of the ligand molecules must bind to the original binding site while a negligible amount of the ligand stays unbound; this is because NMR signals are observed from both the bound and the unbound ligands and occasionally these overlap substantially, which seriously hampers the measurement of interatomic distances.

Recently, in a brief communication, we reported the synthesis of a 24-fluorinated Baf analogue (24-F-Baf **2**).[23] Here, we describe the underlying concept in designing NMR probes and synthetic schemes in greater detail. More importantly, in this study, we synthesized a new ^{19}F-labeled analogue, a 6,8-didesmethylated derivative (desmethyl-24-F-Baf **3**), which was designed in order to reduce the number of synthetic steps. The first analogue **2** was found to exhibit potent inhibitory activity toward V-ATPase, and thus it could serve as a useful probe for elucidating its protein-bound structure by using solid-state NMR. Conversely, the second analogue **3** unexpectedly showed considerably reduced activity, which indicated that the two methyl groups located at C-6 and C-8 are crucial for Baf activity. By performing NMR-based conformational analysis of them, we have revealed for the first time that critical conformational alteration of the macrolactone ring, including the direction of the C-7 hydroxy group, markedly influences the biological activity of Baf.

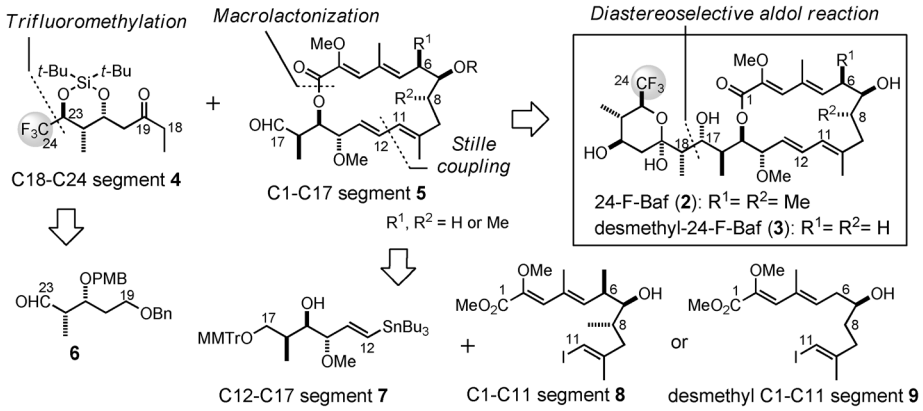
Results and Discussion

Design and synthetic plan for fluorinated bafilomycin analogues

In designing the fluorinated Baf analogues, our first consideration was that ^{19}F labeling must be introduced into the molecular framework in order to facilitate precise distance measurement. Next, we attempted to design a target compound based on considering mainly these three points: retention of biological activity, efficient synthesis on a multi-milligram scale, and chemical stability during several days of NMR measurement.

Initially, we aimed at substituting a hydroxy group with a fluorine atom because of their chemical compatibility. However, this could not be applied to all four hydroxy groups because replacing the 19- or 21-hydroxy group with fluorine would destabilize the product and because the groups at C7 and C17 were reported to be critical for Baf activity.[24] Next, we focused on the C-23 position in the THP ring because alteration of its substituent to larger or smaller groups such as pentadienyl or ethyl moieties did not compromise the activity.[25] Moreover, we envisioned that a CF_3 group could be introduced more readily than a fluorine atom and would be compatible with the *i*Pr group with respect to bulkiness.[26] Consequently, we designed 24,24-didesmethyl-24-F_3-Baf (24-F-Baf, **2**) as a synthetic target. As described in the synthetic part, desmethyl analogue **3** of the macrolide moiety was also designed as a target to facilitate the supply of an NMR probe in tens of milligrams.

Two fluorinated Baf analogues, **2** and **3**, could be synthesized using a common synthetic strategy (Scheme 1). Based on considering previous synthetic studies of **1**,[27] we concluded that C–C bond formation at C17 and C18 of ethyl ketone **4** and macrolactone **5** through a diastereoselective aldol reaction[27b] would be favorable because the CF_3 group appeared

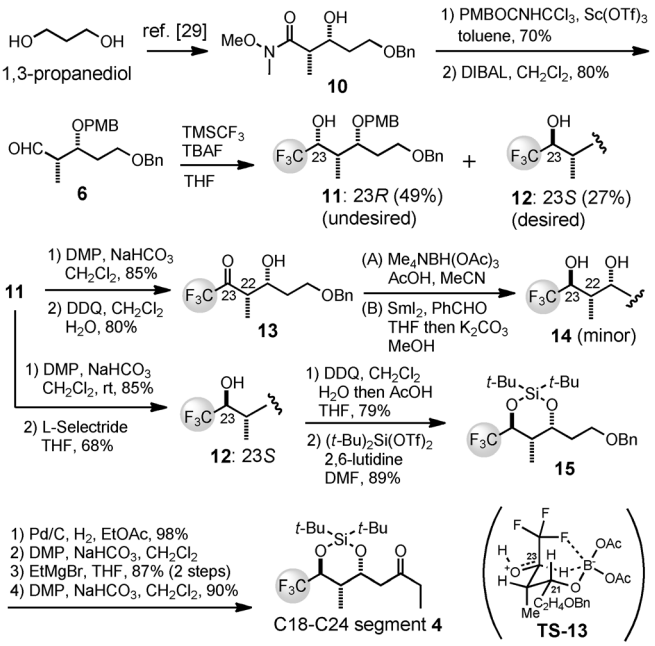


Scheme 1. Structure of fluorinated bafilomycin analogues and their retrosynthetic analysis.

to exert little effect on the stereoselectivity.[27a] A novel CF_3-labeled C18–C24 segment **4** could be synthesized through a trifluoromethylation and two-carbon elongation of aldehyde **6**, with the construction of the stereogenic center at C23. The C1–C17 segment **5** could be obtained via Stille coupling between C11 and C12,[27b,c,f] followed by macrolactonization from the known C12–C17 segment **7**[28] and C1–C11 segment **8**.[27c,f] Synthesis of segments **7** and **8** could be achieved by adopting the strategy reported for **1**, with certain modifications, and the desmethyl C1–C11 segment **9** could be synthesized in short steps even more easily than **8**. Notably, in order to synthesize fluorinated analogues efficiently, difficulties caused by fluorine, such as high electronegativity and unpredictable chemical reactivity, would have to be controlled.

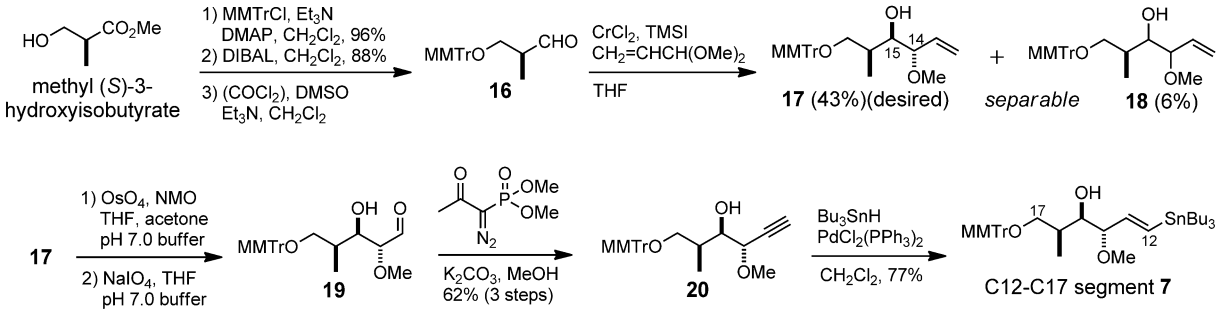
Synthesis of fluorinated bafilomycin analogues

The synthesis of the C18–C24 segment **4** commenced with the conversion of 1,3-propanediol into the chiral Weinreb amide **10** in 4 steps,[29] followed by *p*-methoxybenzyl (PMB) protection of the hydroxy group and diisobutylalminum hydride (DIBAL) reduction of amide to generate aldehyde **6** (Scheme 2). Next, stereoselective trifluoromethylation of **6** was attempted using trifluoromethyltrimethylsilane ($TMSCF_3$) treatment with tetrabutylammonium fluoride (TBAF),[30] which unexpectedly led to the preferential formation of the undesired 23*R* alcohol **11**. Thus, we sought to invert the configuration at C23 of the readily separable undesired 23*R*-isomer **11** through an oxidation–reduction sequence. After Dess–Martin periodinane (DMP) oxidation of alcohol **11** and PMB removal, 1,3-*anti*-selective reduction of **13** was attempted using the β-hydroxy group. First, we used triacetoxyborohydride[31] as a reducing agent, but the desired diol **14** was obtained as a minor product. We suspected that this unexpected result was obtained because of the undesired chelation of the axial CF_3 group to the reagent as shown in **TS-13**. Second, we conducted the hydride transfer of benzaldehyde in the presence of samarium iodide;[32] however, this resulted in simultaneous epimerization at the C22 position because of the high electron-withdrawing property of the CF_3 group. Finally, after DMP oxidation and subsequent reduction of ketone with L-Selectride, a non-chelating and bulky borohydride, we found that the desired 23*S* alcohol **12** was preferentially produced in a 4.2:1 ratio and 68% separated yield.[33] Next, the PMB group was removed, and the resulting diol was protected as a cyclic silyl ether to generate compound **15**. The deprotection of the primary alcohol followed by DMP oxidation furnished the aldehyde, and then the addition of ethylmagnesium bromide and subsequent oxidation of the alcohol generated the C18–C24 segment **4** in 80% yield in 4 steps.

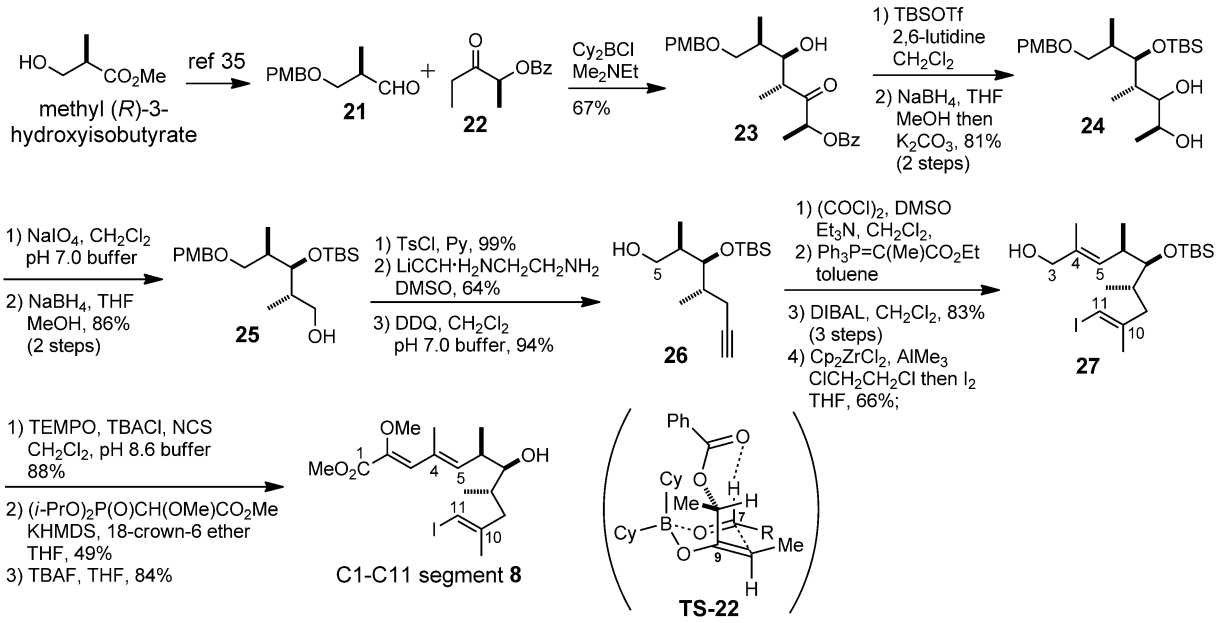


Scheme 2. Synthesis of the C18–C24 segment **4**. DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

Having obtained the common CF_3-labeled segment **4**, the synthesis of 24-F-Baf (**2**) was next examined. First, the known C12–C17 segment **7**[28] was prepared by modifying Toshima's protocol[27b] (Scheme 3). Methyl (*S*)-3-hydroxyisobutyrate was protected using the 4-monomethoxytrityl (MMTr) group, and then DIBAL reduction and Swern oxidation were performed to produce aldehyde **16**. Next, diastereoselective methoxyallylation of aldehyde **16** by using chromium(II) chloride and acrolein dimethyl acetal in the presence of trimethylsilyl iodide (TMSI)[34] generated the desired alcohol **17** as the main isomer. The terminal olefin of purified **17** was oxidatively cleaved to an aldehyde, and subsequent alkynation and hydrostannation generated the desired C12–C17 segment **7** in 48% yield in 4 steps.



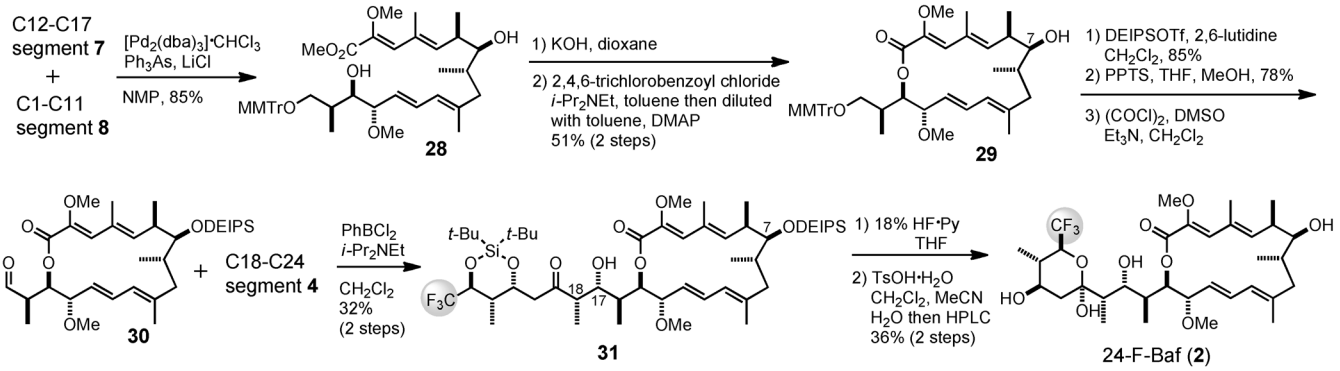
Scheme 3. Synthesis of the C12–C17 segment **7**. DMAP = 4-dimethylaminopyridine, NMO = *N*-methylmorpholine *N*-oxide.



Scheme 4. Synthesis of the C1–C11 segment **8**. TBACl = tetrabutylammonium chloride, NCS = *N*-chlorosuccinimide, KHMDS = potassium hexamethyldisilazane.

Next, the synthesis of the C1–C11 segment **8** was initiated with the preparation of aldehyde **21** from methyl (*R*)-3-hydroxyisobutyrate in 3 steps[35] (Scheme 4). Under the conditions reported by Paterson,[36] the (*E*)-boron enolate generated from ketone **22** reacted with aldehyde **21** to produce the desired *anti*-aldol adduct **23** as the main diastereomer (d.r. = 16:1). In this reaction, favorable selectivity was achieved through the transition state **TS-22**, in which the benzoyl moiety of ketone **22** formed hydrogen bonds with the aldehyde hydrogen to assist the reaction on the *Si*-face of aldehyde **21**. After *tert*-butyldimethylsilyl (TBS) protection of the secondary alcohol, the reduction of the ketone and removal of the benzoyl group was performed in a one-pot reaction to generate diol **24** in 79% yield in 2 steps. The subsequent oxidative cleavage of the diol and $NaBH_4$ reduction furnished the primary alcohol **25**, which was further converted into the known alkyne **26**[27c,f] via two-carbon elongation with lithium acetylide and removal of the PMB group. Lastly, following primarily a report by Marshall,[27f] the C1–C4 part and the vinyl iodide moiety at C10–C11 were constructed. On the primary alcohol of **26**, Swern oxidation and two-carbon elongation through Wittig reaction were performed, and then DIBAL reduction afforded an allyl alcohol, which was exposed to Negishi's condition[37] to produce the vinyl iodide **27** without any problem. Finally, the primary alcohol of **27** was oxidized using 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and the subsequent Horner–Wadsworth–Emmons reaction proceeded in a Z-selective manner in moderate yield, and this was followed by TBS removal to generate the desired C1–C11 segment **8**.

Having prepared the segments **4**, **7**, and **8**, we focused on their coupling and the completion of the synthesis of 24-F-Baf (**2**) (Scheme 5). Initially, the Stille coupling between segments **7** and **8** performed using Marshall's condition[27f] proceeded effectively to generate diene **28** in high yield. After the hydrolysis of the methyl ester,[27b] the obtained seco acid was exposed to the Yamaguchi condition[38] by closely following Carreira's protocol[27e] to afford macrolactone **29** in 51% yield in 2 steps. In this reaction, the free 7-hydroxy group was critical for suc-



Scheme 5. Synthesis of 24-F-Baf (**2**).

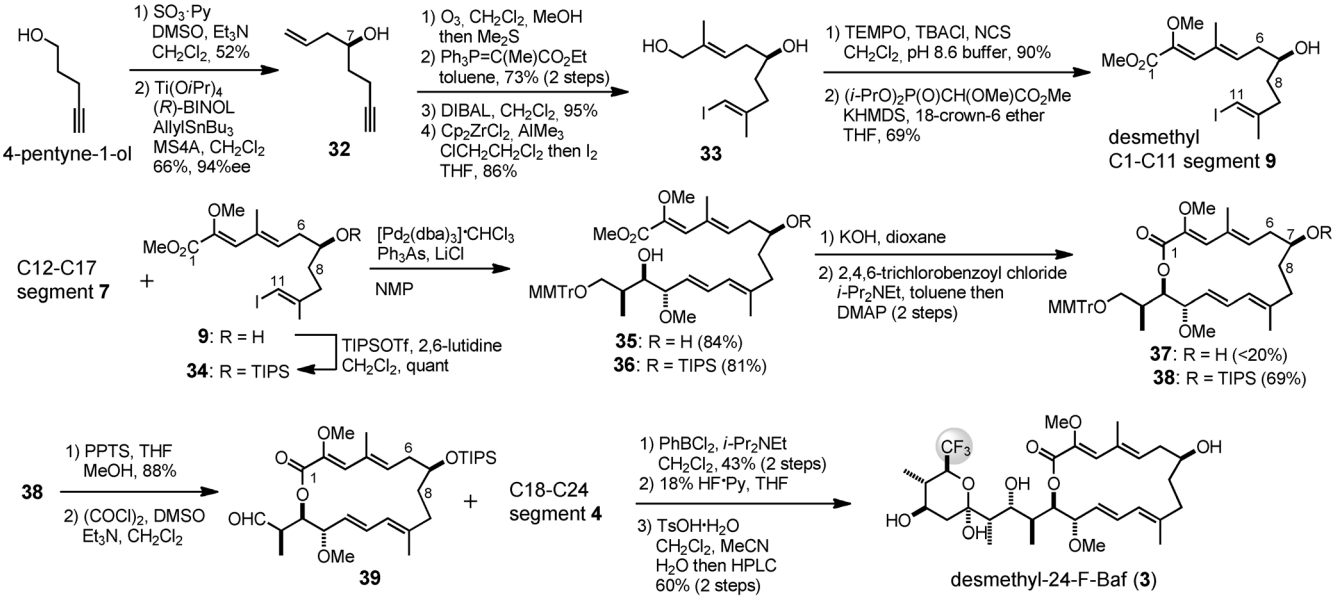
cessful cyclization as previously reported.[27c] Next, the secondary alcohol of **29** was protected using the diethylisopropylsilyl (DEIPS) group,[39] followed by the removal of the MMTr group by pyridinium-*p*-toluene sulfonate (PPTS) treatment; the resulting alcohol was oxidized to aldehyde **30**, which was used as the crude material in the next coupling reaction performed with segment **4**. Referring to Evans's method,[27a,b] we examined the aldol reaction between the crude aldehyde **30** and (*E*)-boron enolate formed by treating segment **4** with dichlorophenylborane and diisopropylethylamine. The reaction proceeded smoothly, and the desired β-hydroxyketone **31** was obtained in acceptable yield over 2 steps with high diastereoselectivity (d.r. = 20:1).[40] As we had aimed, the stereoselective aldol reaction at C17 and C18 was not influenced by the characteristic property of the CF_3 group. Lastly, the cyclic silyl and DEIPS groups were removed in a stepwise manner to afford 24-F-Baf (**2**) in 36% yield after HPLC purification.

As described later, 24-F_3-Baf **2** was found to retain the original biological activity of Baf. However, the synthesis of **2** was as laborious as that of the natural product. Thus, we attempted to enhance the synthetic efficiency by reducing the number of asymmetric centers. We planned to remove the methyl groups on the macrolactone, based on our hypothesis that the macrocyclic conformation is critical for the biological activity of Baf and based on Macromodel calculations, which showed that removing the two methyl groups at C-6 and C-8 did not appear to substantially alter the conformation of the 16-membered ring.[41] Other stereogenic substituents on the macrolides, such as 7-OH and 14-OCH_3, could not be removed: 7-OH is reported to be necessary[24b] and 14-OCH_3 is the common functionality in the plecomacrolide family. These considerations led us to design 6,8-24,24-tetradesmethyl-24-F_3-Baf (desmethyl-24-F-Baf, **3**) as the second synthetic target.

The synthesis of desmethyl-24-F-Baf (**3**) commenced with the construction of the desmethyl intermediate **9** (Scheme 6). The facile preparation of desmethyl C1–C11 segment **9** was first examined starting from 4-pentyne-1-ol. After the oxidation of alcohol to aldehyde, the single stereogenic center at C7 was constructed through Keck asymmetric allylation[42] to generate the known alcohol **32** in 66% yield and 94% *ee* (determined using a modified Mosher's method[43]). Next, the terminal olefin was converted into an aldehyde through ozonolysis, which was followed by the same transformation as in the case of segment **8** to afford the desired desmethyl C1–C11 segment **9** in 5 steps. Moreover, the established procedure was applied to the next 3 steps, that is, Stille coupling between segments **9** and **7**, saponification, and Yamaguchi macrolactonization. Unexpectedly, the cyclization reaction was not successful (the yield of **37** was $<20\%$), and scrutinizing its byproducts revealed the isomerization of the 2,4-diene moiety and, subsequently, the cyclization of the 7-hydroxy group to the C1 carbonyl carbon was found to occur, probably triggered by proton abstraction from the non-methylated C6 position by a base. Therefore, we speculated that a bulky protective group at the 7-hydroxy group could prevent this undesired side reaction in contrast to the case of the dimethylated compound **29**. After the triisopropylsilyl (TIPS) ether **34** was synthesized from segment **9**, the corresponding treatment in 3 steps resulted in the successful formation of the macrolactone **38** in 69% yield (in 2 steps), as expected. The subsequent conversion of **38** into aldehyde **39** and the following aldol reaction with segment **4** proceeded smoothly,[44] and the final stepwise deprotection generated smoothly the desired desmethyl-24-F-Baf (**3**).

Biological evaluation of fluorinated bafilomycin analogues

We evaluated the inhibitory effects of the two fluorinated Baf analogues on V-ATPase using two methods.[45] First, we examined the effects of the analogues on the acidification of intracellular acidic organelles by V-ATPase in rat 3Y1 fibroblasts;



Scheme 6. Synthesis of desmethyl-24-F-Baf (**3**). BINOL = 1,1′-bi-2-naphthol.

3Y1 cells were treated with Baf (**1**), 24-F-Baf (**2**), or desmethyl-24-F-Baf (**3**) for 2 h and then stained with 5 μM acridine orange for 15 min (Figure 2). In contrast to treatment with DMSO (negative control), in which normal acidic cells were observed as

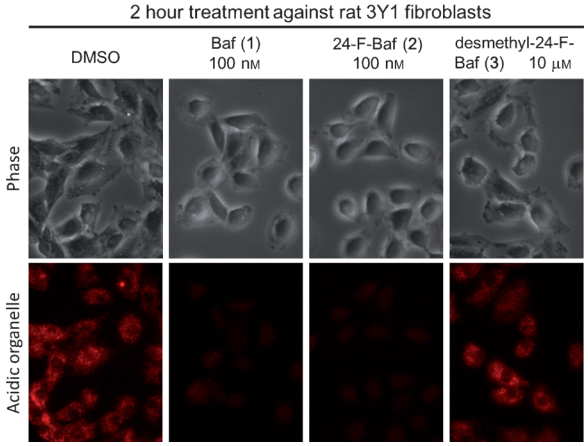


Figure 2. Effect of fluorinated Baf analogues on acridine orange stain of 3Y1 cells.

a red fluorescence of the acridine orange stain, treatment with 100 nM 24-F-Baf (**2**) caused complete disappearance of fluorescence and was as effective as treatment with 100 nM Baf (**1**) (positive control). This indicated that 24-F-Baf (**2**) strongly inhibited V-ATPase in the cells. Conversely, after treatment with 10 μM desmethyl-24-F-Baf (**3**), fluorescence was clearly observed, indicating a loss of the inhibitory activity. Next, we quantitatively assayed V-ATPase inhibition: we used V-ATPase in the purified vacuole membrane of budding yeast and measured the inorganic phosphate liberated from ATP (Table 1).[46]

Table 1. Yeast V-ATPase inhibition.

	IC_{50} [nM]
Baf (**1**)	2.3
24-F-Baf (**2**)	2.5
Desmethyl-24-F-Baf (**3**)	>10000

The results clearly showed that the inhibitory activity of 24-F-Baf (**2**) was similar to that of natural Baf (**1**) (IC_{50} = 2.5 and 2.3 nM, respectively), and that desmethyl-24-F-Baf (**3**) exerted no noticeable effect even at 10 μM. These results suggested that 24-F-Baf (**2**) could be useful for structural analysis of the Baf–V-ATPase complex by solid-state NMR, but that 24-desmethyl-F-Baf (**3**), which was designed for simplifying the synthetic process, was not suitable for such studies.

To further examine whether **3** completely lacked biological activity, cytotoxicity assays were performed using HL-60 and K562 cell lines (Supporting Information, Figure S1). Unexpectedly, desmethyl analogue **3** inhibited cell-growth at moderate level (ED_{50} was around 3 μM in the case of both cell lines); this was 100 times weaker than the cytotoxicity of **1**, but the effect of **3** here was not as weak as what was observed in the V-ATP assay.

Bioactivity based on conformational analysis

Based on the previous structure–activity relationship (SAR) obtained for Baf,[24b] the entire characteristic macrolactone structure was considered to be a prerequisite for Baf activity; however, this conclusion might not be warranted because one-site modification of the substituents in the macrolactone part was not performed except in the case of the 7-hydroxy group. Thus, our result showing that removing the two methyl groups at C6 and C8 critically influenced the activity provides new knowledge in terms of the SAR of synthetic derivatives. Therefore, we were motivated to investigate in detail the effect of the methyl groups on the conformation of the macrolactone ring by means of NMR analysis.

Initially, the ^{1}H chemical shifts and NOE correlation of the fluorinated analogues were compared with those of natural Baf (**1**) (Figure 3).[47] No large difference existed between 24-F-Baf

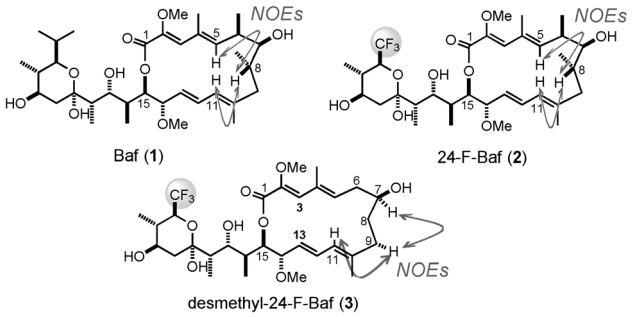


Figure 3. Comparison of NMR data at the C1–C15 position between Baf and fluorinated Baf analogues.

(**2**) and Baf (**1**) around the C1–C15 position ($\Delta\delta(^1H)$ < 0.05 ppm, NOE between H5 and H8 as well as H8 and H11 was observed). By contrast, analysis of desmethyl-24-F-Baf (**3**) revealed large differences in the chemical shift at H3 (**1**/**3** = 6.68/6.41, $\Delta\delta$ = 0.27) and H13 (**1**/**3** = 5.16/5.24, $\Delta\delta$ = 0.08) in addition to that in the C6–C9 region, which was assumed to change due to the lack of methyl groups. Moreover, distinct NOE correlation between H7 and H9 and H9 and H11 was observed instead of H5/H8 and H8/H11 correlations. These results suggested that conformational change occurred not only at the C6-C9 moiety but also over a wider range of the macrolactone because of the absence of C6/C8 methyl groups.

To perform conformational analysis in additional detail, an energy calculation by Macromodel was conducted using the Truncated Newton Conjugate Gradient (TNCG) algorithm constrained by experimentally obtained NOEs and coupling constants ($^3J_{HH}$) (Figure 4). Although our initial calculation of the lowest-energy conformation of natural Baf and 6,8-didesmethyl Baf without constraints revealed that the shape of the macrocyclic core of desmethyl derivative was almost the same as that of natural Baf (Supporting Information, Figure S2), the calculations performed under the NMR constraints revealed an

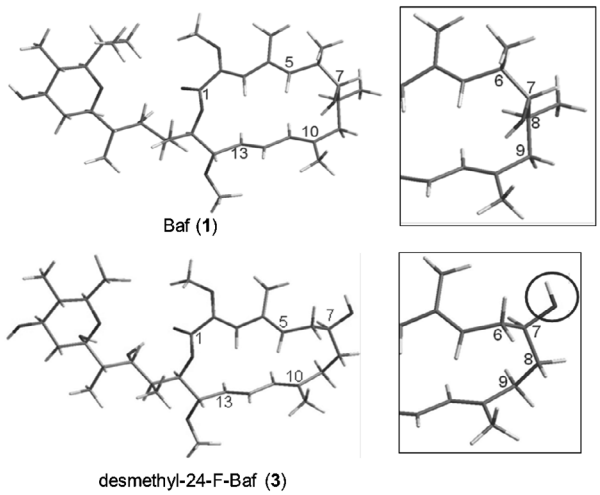


Figure 4. The lowest-energy conformation of Baf and desmethyl-24-F-Baf calculated using Macromodel with constraints from NOEs and $^3J_{HH}$ data.

unexpected conformational change of the macrolactone. The conformation of the C6–C9 position, including the direction of the critical 7-hydroxy group, was altered substantially and moreover, the entire structure of the macrocyclic core was considerably distorted and had become more compact than that of natural Baf **1**. As a result of this deformation, the relative position between the dienoate (C1–C5) and the diene (C10–C13) was changed, which possibly led to the chemical shift change at C3 and C13 positions.

Based on these results, we suggest that removal of the two methyl groups in Baf caused a substantial conformational change in the macrocyclic core and this led to the drastic drop in the V-ATPase inhibitory activity. Although it remains unclear to what extent the directional change of the 7-hydroxy group and the conformational alteration of the entire macrocyclic structure contribute to the biological function of Baf, we directly confirmed that these perturbations critically affect it. By contrast, desmethyl-24-F-Baf **3**, which completely lacked V-ATPase inhibitory activity, exhibited moderate cytotoxicity toward leukemia cell lines, which raises the possibility that **3** exerted its activity through target proteins other than V-ATPase; for example, Shacka et al.[48] have reported that **1** inhibits autophagy through a mechanism other than V-ATPase inhibition. Thus, the synthesis of slightly modified analogues can help elucidate hidden mechanisms of action of plecomacrolide family members, which might otherwise be masked by their powerful inhibition of V-ATPases.

Conclusions

Two fluorine-labeled Baf analogues, 24-F-Baf (**2**) and desmethyl-24-F-Baf (**3**), were designed and convergently synthesized from two common segments, C18–C24 segment **4** and C12–C17 segment **7**, and one respective segment, C1–C11 segment **8** or desmethyl C1–C11 segment **9**, through Stille coupling, macrolactonization, and diastereoselective aldol reaction. In this synthesis, the adverse effect of trifluoromethyl substitution was minimized by using appropriate synthetic route. The V-ATPase inhibitory activity of 24-F-Baf (**2**) was comparable to that of the natural product, indicating the utility of **2** as a potential molecular probe for investigating the inhibitory mechanism of Baf by using solid-state NMR. Moreover, although the V-ATPase inhibitory activity of desmethyl-24-F-Baf (**3**) was unexpectedly lost, detailed conformational analysis of its macrolactone moiety provided the new insight that the presence of C6 and C8 methyl groups is crucial for maintaining the proper architecture required for exhibiting a potent biological activity.

Experimental Section

C18–C24 segment 4

To a solution of alcohol **S15–3** (46.0 mg, 124.1 μmol) in CH_2Cl_2 (2.4 mL) was added $NaHCO_3$ (156 mg, 1.86 mmol) and DMP (158 mg, 372 μmol) at 0 °C. After being stirred at room temperature for 9 h, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ and $Na_2S_2O_3$. The aqueous layer was extracted with CH_2Cl_2, and the organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (hexane/EtOAc = 50:1 to 10:1) afforded ketone **4** (41.4 mg, 112 μmol, 90%) as a colorless oil.

$R_f = 0.70$ (hexane/EtOAc = 3:1); $[\alpha]_D^{24} = +50.2$ ($c = 1.91$, $CHCl_3$); IR (film): $\tilde{\nu} = 2971$, 2895, 1721, 1469, 1383, 1365, 1218, 1096, 1011, 961, 916, 826, 651 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 4.73$ (1H, dt, $J = 10.0$, 4.0 Hz), 4.10 (1H, quin, $J = 7.0$ Hz), 2.72 (1H, dd, $J = 15.0$, 10.0 Hz), 2.53 (1H, q, $J = 7.5$ Hz), 2.40–2.31 (2H, m), 1.07 (3H, t, $J = 7.5$ Hz), 1.03–0.99 ppm (21H, m); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta =$ 209.1, 124.8 (q, $J = 282$ Hz), 74.2 (q, $J = 31.2$ Hz), 71.1, 45.5, 36.8, 36.1, 27,2, 27.1, 21.4, 21.2, 13.4, 7.5 ppm; ^{19}F NMR (470 MHz, $CDCl_3$): $\delta = -78.15$ ppm (d, $J = 7.5$ Hz); HRMS (ESI-TOF) *m/z* calcd for $C_{17}H_{31}F_3O_3SiNa$ [$M+Na^+$] 391.1887, found: 391.1890.

24-F-Baf (2)

To a solution of alcohol **31** in THF (0.30 mL) was added 18% HF·Py (10.3 μL, 102 μmol) at 0 °C. After being stirred at room temperature for 1 h, the reaction mixture was poured into saturated aqueous $NaHCO_3$. The aqueous layer was extracted with ether, the organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Rough purification by silica gel column chromatography (hexane/EtOAc = 7:1 to 2:1) afforded 7-protected 24-F-Baf (5.3 mg). To a solution of 7-protected 24-F-Baf in CH_2Cl_2 (38 μL), MeCN (380 μL), H_2O (95 μL) was added $TsOH·H_2O$ (14.6 mg, 76.8 μmol) at 0 °C. After being stirred at room temperature for 3 h, $TsOH·H_2O$ (7.3 mg, 38.4 μmol) was added. The resulting mixture was stirred for 12 h and quenched with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2, the organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (hexane/EtOAc = 5:1 to 1:1), then by HPLC (COSMOSIL 5SL-II 10×250 mm, eluent: hexane/EtOAc = 1:1, flow rate: 2.0 mL min^{-1}, tR = 15.7 min) to afford 24-F-Baf (**2**) (1.2 mg, 1.87 μmol, 36% over 2 steps) as a white solid: $R_f = 0.38$ (hexane/EtOAc = 1:1); $[\alpha]_D^{22} = +10.3$ ($c = 0.0318$, $CHCl_3$); IR (film): $\tilde{\nu} = 3404$, 2959, 2924, 2850, 1685, 1461, 1259, 1099, 799 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 6.63$ (1H, s), 6.51 (1H, d, $J = 15.0$, 10.5 Hz), 6.17 (1H, d, $J = 2.0$ Hz), 5.81 (1H, d, $J = 10.5$ Hz), 5.75 (1H, d, $J = 9.0$ Hz), 5.15 (1H, dd, $J = 15.5$, 9.0 Hz), 4.94 (1H, dd, $J = 9.0$,

1.0 Hz), 4.87 (1H, d, J=4.0 Hz), 4.09–4.02 (1H, m), 4.04 (1H, qd, J=11.0, 6.5 Hz), 3.90 (1H, d, J=9.5 Hz), 3.75 (1H, ddd, J=11.0, 11.0, 5.0 Hz), 3.63 (3H, s), 3.33–3.26 (1H, m), 3.25 (3H, s), 2.56–2.49 (1H, m), 2.34 (1H, dd, J=12.0, 5.0 Hz), 2.18–2.10 (1H, m), 2.15 (1H, ddq, J=10.5, 7.0, 1.0 Hz), 2.00–1.92 (1H, m), 1.95–1.86 (1H, m), 1.87–1.79 (1H, m), 1.98 (3H, s), 1.94 (3H, s), 1.65–1.55 (1H, m), 1.31–1.20 (1H, m), 1.12 (3H, d, J=6.5 Hz), 1.07 (3H, d, J=7.0 Hz), 1.06 (3H, d, J=7.0 Hz), 0.93 (3H, d, J=6.0 Hz), 0.83 ppm (3H, d, J=7.0 Hz); ^{13}C NMR (151 MHz, $CDCl_3$): δ=167.4 143.3 142.6 141.4, 133.2, 133.2 133.1, 127.1 125.3 100.6, 81.9, 81.2, 76.5, 71.9 (q, J=30.0 Hz), 70.4 69.7 59.6, 55.5, 42.9 41.3, 41.0, 40.2 38.9 37.1 36.7 21.7 20.2 17.2 14.0, 11.9, 9.6 7.0 ppm; ^{19}F NMR (470 MHz, $CDCl_3$): δ=−73.36 ppm (d, J=7.0 Hz); HRMS (ESI-TOF) m/z calcd for $C_{33}H_{51}F_3O_9Na$ [M+Na$^+$] 671.3377, found: 671.3385.

Desmethyl C1-C11 segment 9

To a solution of iodoolefin **33** (974 g, 3.14 mmol) in CH_2Cl_2 (31 mL) and pH8.6 buffer (31 mL) was added TEMPO (73 mg, 0.47 mmol), TBACl (86 mg, 0.31 mmol) and NCS (629 mg, 4.71 mmol) at 0 °C. After being stirred at room temperature for 2 h, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ and $Na_2S_2O_3$. The aqueous layer was extracted with CH_2Cl_2, and the organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was passed through a silica gel (hexane/EtOAc=10:1 to 1:1) to afford aldehyde **S33**, which was used in the next step without further purification. To a solution of (iPrO)$_2$P(O)CH(OMe)CO$_2$Me (2.34 g, 8.74 mmol) in THF (87 mL) was added 18-crown-6 ether (2.31 g, 8.74 mmol) and KHMDS (0.5 M in THF, 15.9 mL, 7.95 mmol) at 0 °C. After being stirred at 0 °C for 30 min, a solution of aldehyde **S33** in THF (5.3 mL) was added at −15 °C. The resulting mixture was stirred at −15 °C for 12 h and quenched with saturated aqueous NH_4Cl. The aqueous layer was extracted with ether, and the organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (hexane/EtOAc=7:1 to 2:1) afforded segment **9** (754 mg, 1.91 mmol, 61% over 2 steps) as a colorless oil: R_f=0.58 (hexane/EtOAc=1/1); $[\alpha]_D^{24}$=+1.8 (c=1.06, $CHCl_3$); IR (film): $\tilde{\nu}$=3473, 2949, 1721, 1436, 1267, 1021, 776, 668 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=6.61 (1H, s), 5.93 (1H, q, J=1.0 Hz), 5.84 (1H, t, J=7.5 Hz), 3.79 (3H, s), 3.72–3.62 (1H, m), 3.66 (3H, s), 2.44–2.25 (4H, m), 1.99 (3H, d, J=1.0 Hz), 1.84 (3H, d, J=1.0 Hz), 1.69–1.50 ppm (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$): δ=165.4, 147.7, 143.4, 134.3, 133.8, 129.0, 75.3, 70.8, 60.4, 52.2, 36.7, 35.9, 35.1, 24.0, 15.1 ppm; HRMS (ESI-TOF) m/z calcd for $C_{15}H_{23}IO_4Na$ [M+Na$^+$] 417.0539, found: 417.0538.

Desmethyl-24-F-Baf (3)

To a solution of alcohol **S39** (38.9 mg, 42.5 μmol) in THF (4.3 mL) was added 18% HF·Py (42.9 μL, 425 μmol) at 0 °C. After being stirred at room temperature for 1 h, the reaction mixture was poured into saturated aqueous $NaHCO_3$. The aqueous layer was extracted with ether, the organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Rough purification by silica gel column chromatography (hexane/EtOAc=7:1 to 2:1) afforded 7-protected desmethyl-24-F-Baf (33.0 mg). To a solution of 7-protected desmethyl-24-F-Baf in CH_2Cl_2 (0.63 mL), MeCN (3.1 mL), H_2O (0.79 mL) was added TsOH·H_2O (123 mg, 650 μmol) at 0 °C. After being stirred at room temperature for 3 h, TsOH·H_2O (123 mg, 650 μmol) was added. The resulting mixture was stirred for 3 h and quenched with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2, the organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (hexane/EtOAc=3:1 to 1:2), then by HPLC (COSMOSIL 5SL-II 10×250 mm, eluent: hexane/EtOAc=1:1, flow rate: 2.0 mL min^{-1}, t_R=19.6 min) to afford desmethyl-24-F-Baf (**3**) (15.8 mg, 25.4 μmol, 60% over 2 steps) as a white solid: R_f=0.27 (hexane/EtOAc=1:1); $[\alpha]_D^{23}$=+92.6 (c=0.0343, $CHCl_3$); IR (film): $\tilde{\nu}$=3413, 2918, 2850, 2363, 2339, 1641, 1631, 1219, 1102, 772 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ=6.53 (1H, dd, J=15.0, 11.0 Hz), 6.41 (1H, s), 6.13 (1H, d, J=2.0 Hz), 5.82 (1H, d, J=10.5 Hz), 5.57 (1H, dd, J=10.0, 5.0 Hz), 5.24 (1H, dd, J=15.0, 9.5 Hz), 4.91 (1H, dd, J=9.0, 1.5 Hz), 4.83 (1H, dd, J=4.0, 1.0 Hz), 4.10–4.04 (1H, m), 4.09–3.99 (1H, m), 3.90 (1H, t, J=9.0 Hz), 3.74 (1H, ddd, J=10.0, 10.0, 5.0 Hz), 3.60 (3H, s), 3.55–3.48 (1H, m), 3.27 (3H, s), 2.65 (1H, ddd, J=13.0, 10.0, 4.0 Hz), 2.52–2.43 (1H, m), 2.34 (1H, dd, J=12.0, 5.0 Hz), 2.20 (1H, ddq, J=10.0, 7.0, 1.5 Hz), 2.15–2.08 (1H, m), 2.13–2.07 (1H, m), 1.96 (3H, s), 1.96–1.91 (1H, m), 1.84 (3H, s), 1.84–1.82 (1H, m), 1.64–1.55 (1H, m), 1.26–1.23 (1H, m), 1.12 (3H, d, J=5.5 Hz), 1.06 (3H, d, J=7.0 Hz), 0.84 ppm (3H, d, J=7.0 Hz); ^{13}C NMR (151 MHz, $CDCl_3$): δ=167.2, 142.2, 141.0, 135.4, 133.4, 133.0, 131.6, 129.0, 125.5, 125.0 (q, J=279 Hz), 100.7, 81.9, 76.1, 74.5, 72.0 (q, J=30 Hz,), 70.4, 69.6, 59.3, 56.6, 42.8, 41.1, 38.8, 38.2, 37.4, 36.6, 36.5, 16.3, 14.0, 11.9, 9.5, 7.0 ppm; ^{19}F NMR (470 MHz, $CDCl_3$): δ=−73.36 ppm (d, J=7.0 Hz); HRMS (ESI-TOF) m/z calcd for $C_{31}H_{47}F_3O_9Na$ [M+Na$^+$] 643.3064, found: 643.3070.

Staining of intracellular acidic organellae

Cells were stained with acridine orange as described previously.[49,50] 3Y1 cells were seeded onto coverslips and incubated at 37 °C for 2 h with 100 nM Baf(**1**) or 100 nM 24-F-Baf (**2**) or 10 μM desmethyl-24-F-Baf (**3**). They were then incubated for 15 min with 5 μM acridine orange. After three washes with phosphate buffered saline, the coverslips were photographed using a cooled charge-coupled device camera.

Yeast V-ATPase inhibition assay

V-ATPase inhibition activities of Baf(**1**) or 24-F-Baf (**2**) or desmethyl-24-F-Baf (**3**) were assayed against V-ATPases obtained from purified vacuole membrane of budding yeast.[46] The reaction mixture (135 μL) containing 5 μg of vacuolar membrane vesicles, 5 mM $MgCl_2$, 10 mM NH_4Cl, 5 mM NaN_3, 0.1 mM Na_3VO_4 and 25 mM Mes-Tris (pH 6.9) was incubated with or without inhibitors as DMSO solution at the indicated concentration for 10 min on ice. Then, the reaction was started by adding 15 μL of 50 mM of Na_2ATP. Activity assays were run for 60 min at 37 °C. 150 μL of 0.6 M perchloric acid was added to stop the reaction and the liberated inorganic phosphate was measured by malachite green method.[45]

Ab initio calculation of the lowest-energy conformation of Baf (1) and desmethyl-24-F-Baf (3) with constraints in the macrolactone ring

Conformations were calculated using the MacroModel software version 9.9. Initial atomic coordinates and structure files were generated from the crystal data of Baf.[51] The lowest-energy structure was obtained by the molecular mechanics simulation using a 7000 steps of Monte Carlo conformation search and TNCG energy minimization with MMFFs in vacuum. In order to emphatically observe the difference of conformation of the macrolactone ring, the conformation of the other part was preliminarily fixed by using two distance constraints for both compounds (2.5±1 Å between C1-carbonyl oxygen and C17-OH hydrogen atoms and C17-

OH oxygen and C19-OH hydrogen atoms), which were derived from the hydrogen bonds data in Baf crystal structure.[51] Three dihedral angle constraints from NOE correlations and $^3J_{H,H}$ coupling constants ($180^\circ \pm 35^\circ$ for O1-C1-C2-C3, C2-C3-C4-C5, and H14-C14-C15-H15) were used in the case of Baf (**1**). Two distance constraints from NOE correlations (2.5 ± 1 Å between C7-hydrogen and C9-α-hydrogen atoms and C9-β-hydrogen and C11-hydrogen atoms) and four dihedral angle constraints from NOE correlations and $^3J_{H,H}$ coupling constants ($180^\circ \pm 35^\circ$ for O1-C1-C2-C3, C2-C3-C4-C5, H6α-C6-C7-H7, H14-C14-C15-H15) were used in the case of desmethyl-24-F-Baf (**3**).

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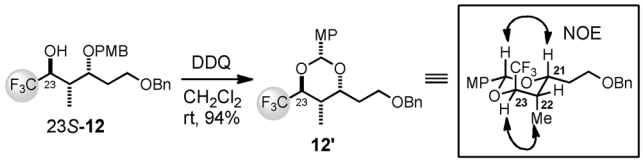

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