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Elucidation of Strict Structural Requirements of Brefeldin A as an Inducer of Differentiation and Apoptosis

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Abstract—Brefeldin A (BFA) can induce a wide variety of human cancer cells to differentiation and apoptosis and is in development as an anticancer agent. To elucidate structural requirements for cytotoxicity and induction of differentiation and apoptosis, BFA was structurally modified into various derivatives including 4-*epi*-BFA in this study. Their inducing activities of apoptosis were evaluated with their cytotoxicities, DNA fragmentation and morphological changes in human colon cancer cell HCT 116. The cytotoxicity of 4-*epi*-BFA (TX-1923) ($IC_{50}=60\,\mu M$) was 300 times lower than that of BFA ($IC_{50}=0.2\,\mu M$). Furthermore, 4-*epi*-BFA induced DNA fragmentation and apoptotic morphological changes at much higher concentrations (70 and 50 μM, respectively) than BFA (0.11 and 0.36 μM, respectively). These results indicated that the configuration of 4-hydroxyl group of brefeldin A plays a key role in the cytotoxicity and induction of apoptosis. On the other hand, 7-*O*-acetyl-BFA, 4-*O*-acetyl-BFA, and 4,7-di-*O*-acetyl-BFA exhibited potential activities in cytotoxicity and inducibility of apoptosis. We suggested that the structural determinants for BFA include the moiety of the Michael acceptor, the conformational rigidity of the 13-membered ring, and the configuration of 4-hydroxyl group.

Introduction

Brefeldin A (BFA) is a 16-membered macrolide antibiotic (Fig. 1) which was isolated from various fungal species such as *Penicillium decumbens*,[1] *Penicillium brefeldianum*,[2] *Penicillium cyaneum*,[3] and *Ascochyta imperfecta*.[4] There have been numerous synthetic studies on BFA[5–12] after its antiviral activity[13] and X-ray crystallographic structure[14] were established. BFA has been known as a disassembler of the Golgi apparatus, since BFA can block protein transport from the rough endoplasmic reticulum (ER) to the Golgi complex[15,16] and cause a redistribution of the *cis*-, *medial*-, and *trans*-Golgi proteins into the ER in mammalian cells.[17–19] Recently, our studies have revealed for the first time that BFA can induce cell differentiation and apoptosis in some human epithelial carcinoma cell lines.[20] When HCT 116 human colon cancer cell was treated with BFA, morphological changes indicating cell differentiation were observed. DNA-ladder, which is one of the biochemical criteria for apoptosis, was also observed following the morphological changes. In addition, ganglioside analysis of BFA-treated cells showed a remarkable increase in G_{M3} and decrease in neolactoseries ganglioside with longer carbohydrate moieties.[21] It is also reported that BFA-induced apoptotic cell death was independent of p53.[22] These results stimulated us to develop BFA into clinical use for cancer chemotherapy, including our design and synthesis of BFA analogues to seek the structural determinants[23] and others' modification to improve the aqueous solubility by preparing the prodrugs of BFA.[24]

Recently, induction of apoptosis has been considered as a new strategy for cancer chemotherapy.[25] BFA has been reported to exert its cytotoxic effects mainly by inducing differentiation and apoptosis in tumor cells, as mentioned above. Thus, BFA may be selected as a lead compound for developing a chemotherapeutic agent inducing apoptosis.

Keywords: brefeldin A; brefeldin A derivatives; structural requirements; apoptosis; cytotoxicity.

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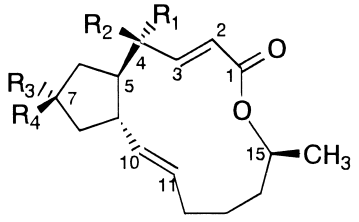
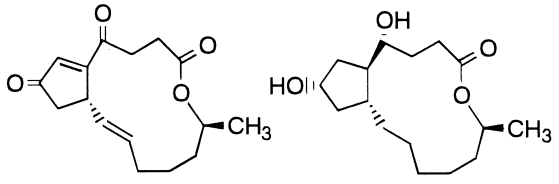
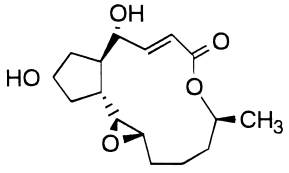
Here, we report the full account[23] of the design and synthesis of several BFA derivatives (Fig. 1) which were evaluated by cytotoxicities, DNA fragmentations, and morphological changes in HCT 116 cells. In the present study, we have made efforts to elucidate the structural requirements for BFA, especially the configurational importance of 4-hydroxyl group, in order to develop new inducing agents of differentiation and apoptois in cancer cells. We also discuss some structural requirements for BFA to induce apoptosis implicated in cytotoxic effects to tumor cells.



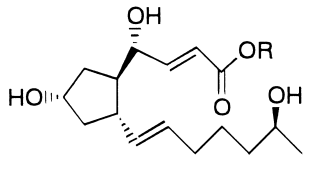
	R_1	R_2	R_3	R_4
brefeldin A (BFA)	OH	H	OH	H
TX-1923 (4-*epi*-BFA)	H	OH	OH	H
TX-1933	R_1, R_2 = O		OH	H
TX-1852	OH	H	R_3, R_4 = O	
TX-1856	OAc	H	OAc	H
TX-1872	OAc	H	OH	H
TX-1891	OH	H	OAc	H
TX-1870	OMe	H	OH	H
TX-1869	OMe	H	OMe	H



TX-1855 TX-1853



TX-1876



	R
TX-1875	H
TX-1873	CH_3
TX-1890	$C_6H_5CH_2$

Figure 1. Chemical structures of designed brefeldin A derivatives.

Results

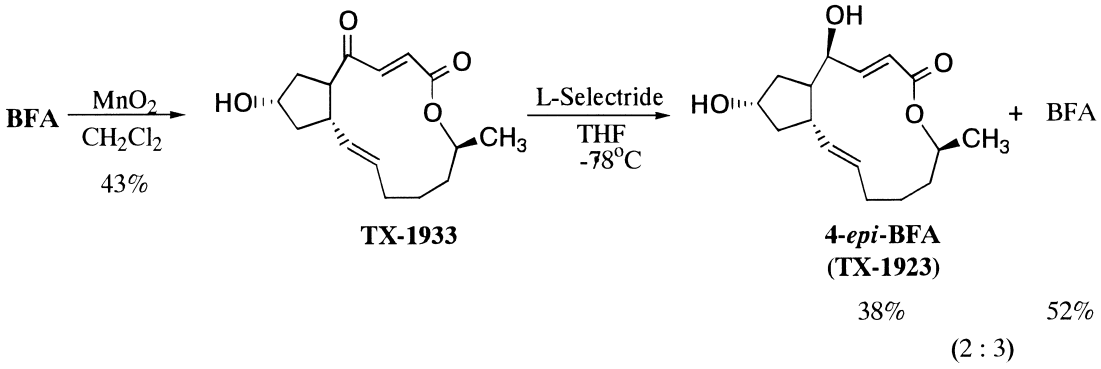
Design and synthesis of BFA derivatives

BFA possesses considerable structural complexity (Fig. 1), although it is a relatively small molecule (MW = 280). The structural characteristics of BFA include a transoid bicyclo[11.3.0]hexadecadienoate skeleton containing five stereocenters, two hydroxyl groups, two carbon–carbon double bonds, and one lactone moiety.

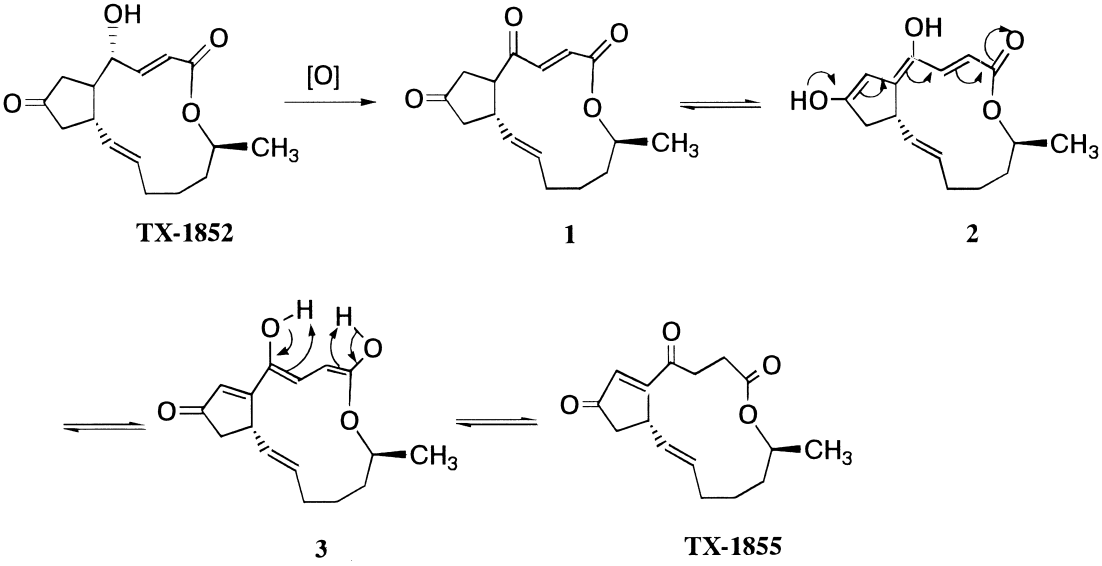
To understand the structural requirements for BFA, we designed and synthesized several BFA derivatives from BFA (Fig. 1). To synthesize 4-*epi*-BFA (TX-1923) for evaluating the stereospecificity of BFA, BFA was oxidized into 4-oxo-BFA followed by the reduction with sodium borohydride at −78 °C in methanol, as reported in Corey's paper in which 4-*epi*-BFA was mainly afforded (4-*epi*-BFA:BFA = 5:1).[26] The reaction, however, resulted in recovered BFA only. We tried to reduce the 4-carbonyl group with L-Selectride and obtained 4-*epi*-BFA in 38% yield and BFA in 52% yield (4-*epi*-BFA:BFA = 2:3) (Scheme 1). We here first reported the spectra data of 4-*epi*-BFA (see Experimental).

The hydroxyl groups were modified to estimate their contribution to apoptosis inducing activity. The oxidation of BFA with pyridinium chlorochromate at room temperature for 5 h afforded TX-1852 (7-oxo-BFA)[27] in 61% yield, while TX-1855 (5,6-dehydro-2,3-dihydro-4,7-dioxo-BFA) was produced in 41% yield by prolonging the reaction time from 5 h to 2 days. A mechanism for the formation of TX-1855 was proposed in Scheme 2. Following the oxidation of BFA to TX-1852, 7-hydroxyl group was successively oxidized to a keto intermediate **1**. Then, 4- and 7-ketones were enolated to form a long-ranged conjugated lactone **2**. To release the ring strain of the macrocycle, the three carbon–carbon double bonds migrated to give another dienol **3**, which was converted to keto form to give the product TX-1855. The acetylation of BFA with acetic anhydride in pyridine afforded di-*O*-acetyl-BFA (TX-1856), while by refluxing in acetic acid yielded 7-*O*-acetyl-BFA (TX-1891).[27] 4-*O*-Acetyl-BFA (TX-1872) could be obtained mainly by control of stoichiometry in the former condition. Besides, the methylation of BFA with iodomethane and silver oxide in DMF afforded 4-*O*-methyl-BFA (TX-1870) and di-*O*-methyl-BFA (TX-1869) simultaneously.

Furthermore, to identify whether the carbon–carbon double bonds are necessary for biological actions of BFA, tetrahydro-BFA (TX-1853)[27] was prepared under conventional hydrogenation condition in good yield. By epoxidation of BFA with 5% *m*-chloroperbenzoic acid in chloroform, only the isolated double bond was epoxidized whereas the conjugated double bond remained to give 10,11-epoxy-BFA (TX-1876)[3] (Scheme 3). To understand the effect of structural rigidity of BFA on its activity, the 13-membered ring of BFA was opened by hydrolysis to afford BFA seco-acid (TX-1875), whose NMR spectrum was identical with the reported data.[28] In addition, seco-BFA methyl ester (TX-1873) and seco-BFA benzyl ester (TX-1890) were synthesized since they have the similar functional groups with BFA (Scheme 3).



Scheme 1. Synthesis of 4-*epi*-BFA (TX-1923).



Scheme 2. A proposed mechanism for conversion of TX-1852 to TX-1855.

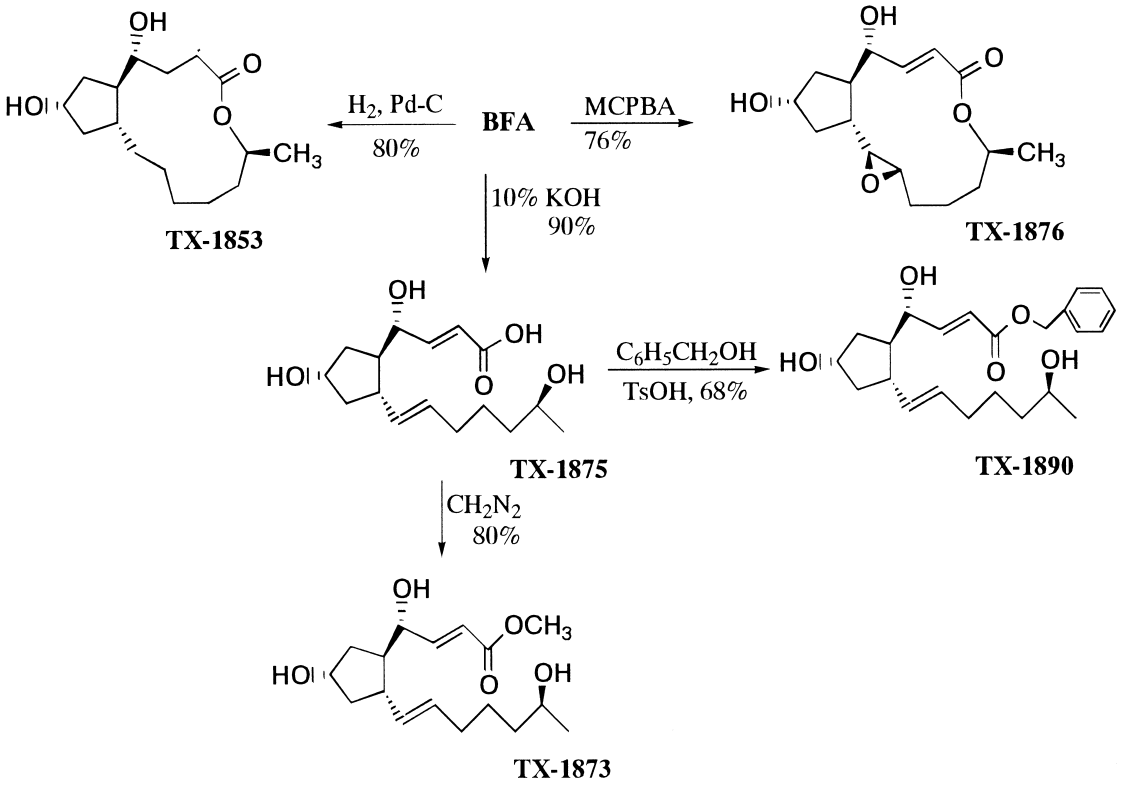
Induction of apoptosis and cytotoxicity in HCT 116 cells

The induction of apoptosis by 4-*epi*-BFA and other BFA derivatives was examined with apoptotic morphological changes and DNA ladder formation. After treatment of 50 μM of 4-*epi*-BFA, the cells showed membrane-bound apoptotic bodies, a hallmark of usual apoptotic morphological change, whereas the concentration of BFA for showing the similar morphological changes was 0.36 μM (data not shown). In addition, in the analysis of DNA fragmentation by agarose gel electrophoresis, the nucleosomal ladder formation (DNA ladder formation) was observed clearly with treatment of 70 μM of 4-*epi*-BFA, much higher concentration than BFA (0.11 μM) (Table 1). Some other derivatives, such as 4-*O*-acetyl-BFA (TX-1872), 7-*O*-acetyl-BFA (TX-1891), di-*O*-acetyl-BFA (TX-1856), and 10,11-epoxy-BFA (TX-1876), showed the morphological changes and DNA fragmentation at a minimum effective concentration (EC) (0.27, 0.16, 0.27, and 4.1 μM, respectively) (Table 1). Another derivative, such as TX-1852, TX-1855, TX-1869, TX-1870, TX-1873, TX-1875, and TX-1890, were also evaluated and were inactive at their individual concentrations (Table 1) which were much higher than that of BFA for showing the inducing activity.

The cytotoxicity of 4-*epi*-BFA and other BFA derivatives which had apoptosis inducing activity in the analysis of DNA ladder formation were evaluated in HCT 116 cells by MIT assay (Table 2). The cytotoxicity of 4-*epi*-BFA (TX-1923) was 300 times weaker ($IC_{50} = 60$ μM) than that of BFA ($IC_{50} = 0.2$ μM), indicating that only the configurational inversion of 4-hydroxyl group resulted in significant loss of cytotoxicity. On the other hand, 4-*O*-acetyl-BFA (TX-1872) ($IC_{50} = 0.1$ μM) and 7-*O*-acetyl-BFA (TX-1891) ($IC_{50} = 0.2$ μM) had higher cytotoxic effects than BFA, while 4,7-di-*O*-acetyl-BFA (TX-1856) ($IC_{50} = 2.0$ μM) and tetrahydro-BFA (TX-1853) ($IC_{50} = 3.0$ μM) were about 10–15 times weaker than that of BFA (Table 2).

Molecular orbital calculation of BFA and 4-*epi*-BFA

To understand the differences of BFA and 4-*epi*-BFA in biological activities, we calculated the molecular orbitals of the two compounds in water and in vacuum by using the PM3 method on MOPAC97 program (WinMOPAC



Scheme 3. Synthesis of derivatives with double bonds modification of lactone-ring opening.

ver. 2.0, Fujitsu, Japan). Figure 2 showed the lowest unoccupied molecular orbital (LUMO) energies and coefficients of BFA and 4-*epi*-BFA in water. The LUMO energy of BFA in water (0.074 eV) was lower than 4-*epi*-BFA (0.086 eV). Thus, BFA possesses higher electron affinity than 4-*epi*-BFA. Besides, comparing the highest occupied molecular orbitals (HOMO) of the two compounds made it clear that the coefficients of the double bond (C10–C11) were reversed in BFA and 4-*epi*-BFA (Fig. 3). C10 (HOMO coefficient = 0.391) appears to be more nucleophilic than C11 (HOMO coefficient = 0.389) of the double bond in BFA, while in 4-*epi*-BFA, the higher HOMO coefficient of C11 (0.385) indicates that C11 is more nucleophilic than C10 (0.377).

Hydrophobicity of drugs directly influences their pharmaceutical activities. The solvation free energy (dGW) is defined as free-energy changes for association in the vacuum (dGWv) and in the aqueous solution (dGWw). The value of dGW is considered one of the parameters for hydrophobicity and may be obtained by molecular orbital calculation as described.[29] Since the lower dGW value indicates the higher hydrophobicity, BFA (dGW = −73.0502 KJ) may be more hydrophobic than 4-*epi*-BFA (dGW = −70.7732 KJ) (Table 3) although the two compounds gave the same calculated partition coefficients (log $P = 1.61 \pm 0.40$), which are commonly used as hydrophobicity parameters in drug design and development. This fact suggested that dGW, in this case, is more reasonable than log P as a parameter of hydrophobicity, because dGW values reflected the steric effect of a molecule on its hydrophobicity.

Table 1. Induction of apoptosis in HCT 116 cells by BFA derivatives

Compound	DNA ladder EC (μM)[a]	Compound	DNA ladder EC (μM)[a]
4-*epi*-BFA (TX-1923)	70	TX-1870	ND (3.5)
		TX-1853	ND (3.5)
TX-1852	ND (3.6)[b]	TX-1876	4.1
TX-1855	ND (3.6)	TX-1873	ND (6.4)
TX-1856	0.27	TX-1875	ND (3.4)
TX-1872	0.27	TX-1890	ND (5.2)
TX-1891	0.16	BFA	0.11
TX-1869	ND (3.2)		

[a]The minimum effective concentration at which DNA ladder was identified.
[b]ND, not detected; the data indicated the maximum concentration examined.

Discussion

BFA has long been studied for its total synthesis and biological activities. Recent interests in this natural product focus on its potential as an anticancer drug. To gain insight into the structural requirements of BFA as

Table 2. Cytotoxicity of BFA derivatives on HCT 116 cells

Compound	IC_{50}[a] (μM)	Compound	IC_{50}[a] (μM)
4-*epi*-BFA (TX-1923)	60	TX-1853	3.0
		TX-1876	20
TX-1872	0.1	BFA	0.2
TX-1891	0.2		
TX-1856	2.0		

[a]Based on MIT assay described in Experimental.

a differentiation and apoptosis inducing agent and to develop a novel chemotherapeutic agent, we designed, synthesized, and evaluated BFA analogues. We observed that the activities of the synthetic compounds were highly dependent upon the precise structure of BFA.

4-*epi*-BFA (TX-1923), differing from BFA in configuration at only one stereocenter (C4), lost significant biological activity. The cytotoxicity of 4-*epi*-BFA ($IC_{50} = 60\,\mu M$) was much weaker than that of BFA ($IC_{50} = 0.2\,\mu M$) in HCT 116. Furthermore, BFA induced apoptosis of HCT 116 at 0.11 μM, while 4-*epi*-BFA, in contrast, showed such activity at as high as 70 μM. The results revealed that the configurational inversion of 4-hydroxyl group caused a remarkable decrease of the biological activities. Previously, 4-*epi*-BFA was reported to be inactive in inhibition of protein transportation from the ER to the Golgi complex.[30] Also, 4-*epi*-BFA has been found to disturb BFA activity in the mono-ADP-ribosylation assay.[31] The mechanism by which the 4-hydroxyl group influences the biological activities of BFA remains poorly understood. From the X-ray crystal structure of BFA,[14] we can draw a Newman projection formula for the conformation of C2–C5 moiety of BFA as shown in Figure 4(a). The conformation of 4-*epi*-BFA may be similar to that of BFA as shown in Figure 4(b) because the fact that the conformation of tetrahydro-BFA (TX-1853) derived from its X-ray crystal structure[23] was almost identical with that of BFA suggested that the 13-membered lactone ring is considerably rigid. The C2–C5 moieties consist of an α,β-unsaturated carbonyl and an allylic hydroxyl

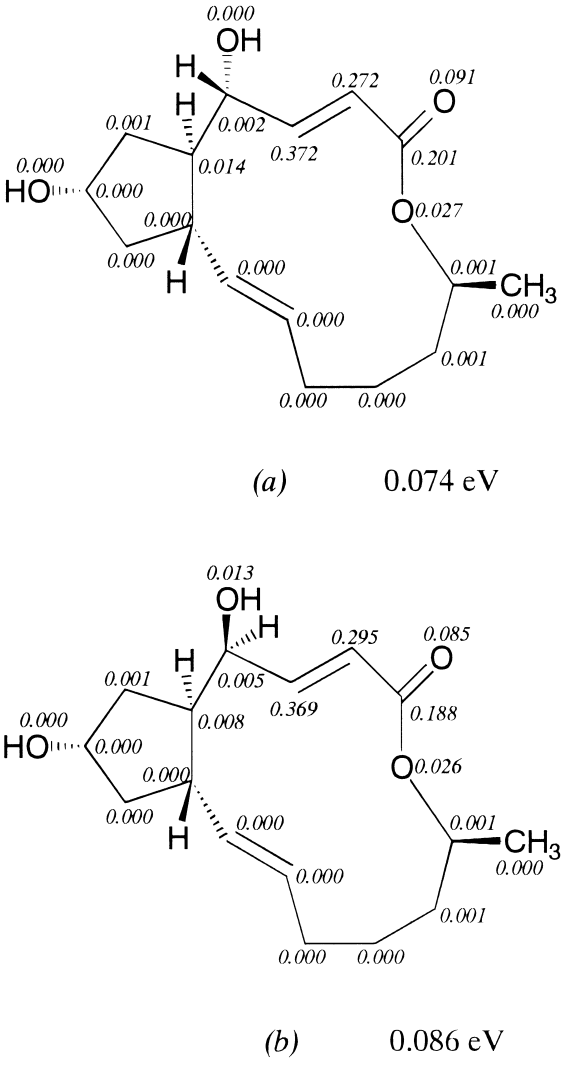


Figure 2. LUMO energies and coefficients of BFA (a) and 4-*epi*-BFA (TX-1923) (b) in water.

Table 3. Solvation free energies of BFA and 4-*epi*-BFA (TX-1923)[a]

Compound	DGWv (KJ)[b]	DGWw (KJ)[b]	DGW (KJ)[b]	Log *P*[c]
4-*epi*-BFA (TX-1923)	−681.5865	−752.3597	−70.7732	1.61 ± 0.40
BFA	−682.1024	−755.1526	−73.0502	1.61 ± 0.40

[a]The energies were defined by using free-energy changes for association in the aqueous solution and vacuum as described.[28]
[b]dGWv and dGWw indicate the energies in vacuum and water; dGW indicates the salvation free energies.
[c]The values of log *P* were calculated with ACD/log *P* version 1.0.

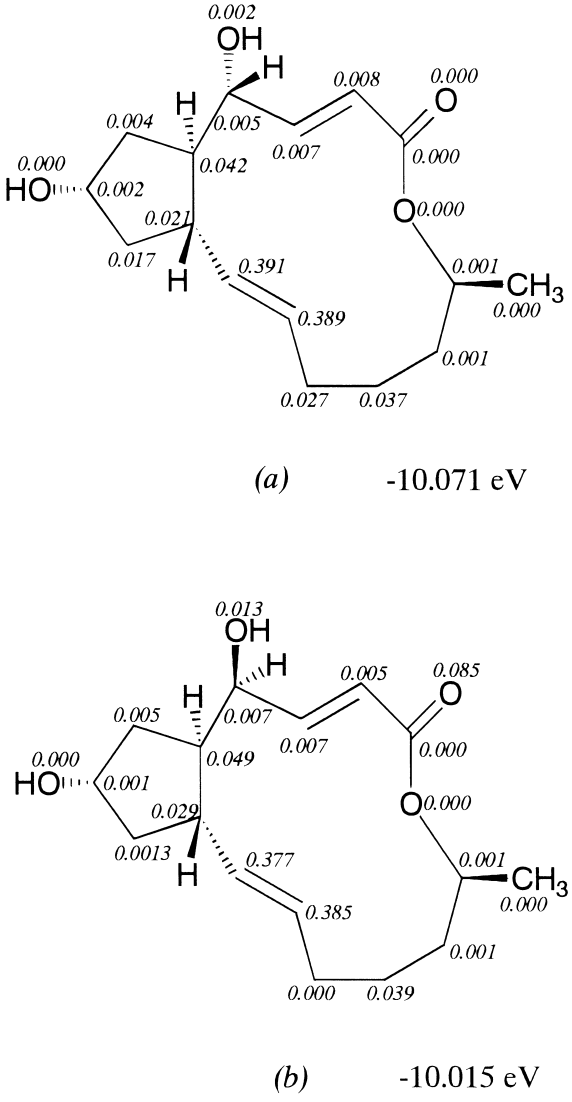


Figure 3. HOMO energies and coefficients of BFA (a) and 4-*epi*-BFA (TX-1923) (b) in water.

group. The structure–activity relationship of anticancer agents reveals that Michael acceptor reacting with a biological nucleophile plays an important role in cytotoxicity. So Michael acceptor has been generally considered one of the most useful pharmacophores in developing anticancer agents. It is meaningful to investigate what is different between BFA and 4-*epi*-BFA in Michael addition. Michael addition of various thiols to BFA was well documented[32] and the resulting major adduct was assigned the 3R configuration derived from the nucleophilic attack to C3 from the 'right' side for the 'left' side was hindered as shown in Figure 4(a). For a nucleophilic attack on π bond, the electronegative allylic substituent (e.g. 4-hydroxyl group) prefers to be *anti* conformation to the attacking agent because of electronic effect so that the withdrawal of electrons from the π system can be maximized.[32] In the case of BFA, however, the C–O bond and the C2–C3 double bond are in approximately eclipsed conformation and could not rotate to overlap σ^*-orbital of C–O bond to π^*-orbital in the transition state. So the direction of nucleophilic attack depends on the steric effect. That is, the nucleophile would attack on C3 of BFA from the side of OH and H to overlap with the LUMO of the Michael acceptor (Fig. 4(a)). On the other hand, for 4-*epi*-BFA, the nucleophilic attack from the same direction is hindered by the eclipsed hydroxyl group and the *anti* attack is also largely hindered by the steric downside cave (Fig. 4(b)), resulting in its reactivity of Michael addition becoming sluggish. MO calculation also supports that BFA is a more active Michael acceptor than 4-*epi*-BFA since LUMO energy of BFA is lower as depicted in Figure 3. This analysis may provide an explanation for the higher biological activities and cytotoxicity of BFA than 4-*epi*-BFA.

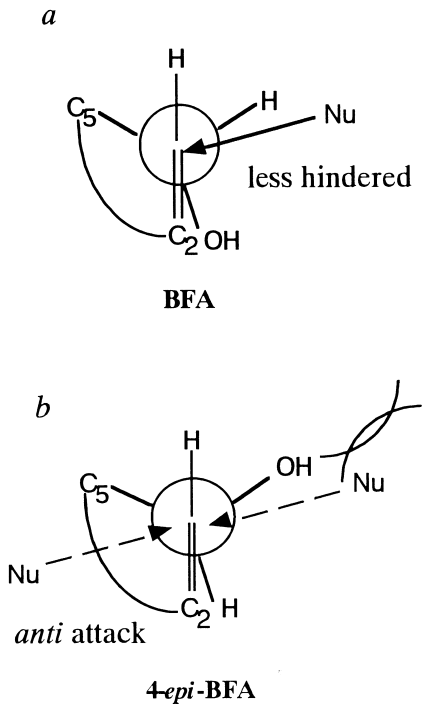


Figure 4. Different reactivity of C2–C5 moieties of BFA (a) and 4-*epi*-BFA (TX-1923) (b) in Michael addition.

The 4- and 7-hydroxyl groups were important to have cytotoxicity and apoptosis inducing effect, because methylation and oxidation of these hydroxyl groups (TX-1869, 1870, 1852, 1855) made inactive in DNA fragmentation as shown in Table 1. However, 4-*O*-acetyl-BFA (TX-1872), 7-*O*-acetyl-BFA (TX-1891), and 4,7-di-*O*-acetyl-BFA (TX-1856) showed potential cytotoxic and apoptosis inducing effects. These acetates may act after hydrolysis in cells as prodrugs. TX-1872 ($IC_{50} = 0.1\,\mu M$) and TX-1891 ($IC_{50} = 0.2\,\mu M$) were more active than BFA in cytotoxicity assay. In the DNA fragmentation analysis by agarose gel electrophoresis, the two analogues, TX-1872 (0.27 μM) and TX-1891 (0.16 μM) showed weaker activity than BFA (0.11 μM), revealing that the 4-hydroxyl group contributed more strongly than the 7-hydroxyl group in apoptosis inducing activity. Tetrahydro-BFA (TX-1853) did not show such effect regardless of its similar conformation with BFA. In addition, oxidized derivative, TX-1855, was also inactive. It has been reported that the Michael adducts of BFA with thiols were much less active than BFA in cytotoxicity.[24] These observations strongly suggest that the moiety from C1 to C4 position including Michael acceptor is a prerequisite for BFA.

The conformational rigidity was another important structural determinant. The ring-opened analogues, TX-1873, TX-1875, and TX-1890, lost the potency for induction of apoptosis, implying that the conformational rigidity of 13-membered lactone is a requisite also for activities of BFA.

In summary, we can draw the following conclusions from these studies with respect to structure–activity relationships. The γ-hydroxy-α,β-unsaturated carbonyl system as Michael acceptor is crucial for cytotoxicity and induction of apoptosis in HCT 116 cells. The apoptosis inducing ability of BFA appears to be rather sensitive to modification at its C4, especially to the configurational inversion of C4. Besides, the conformational requirements of 13-membered lactone are also stressed. This information could be valuable for developing an anticancer drug through inducing differentiation and apoptosis.

Experimental

Chemistry

All chemicals except BFA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Sigma-Aldrich Japan (Tokyo, Japan). BFA was supplied by Dr. B. Proksa (Sloyak Technical University). Solvents were generally distilled prior to use. Reactions were generally performed under a nitrogen atmosphere.

All reactions were monitored by TLC (thin-layer chromatography) which was performed on Merck $60F_{254}$ (0.25 mm) plates visualized with UV light and iodine vapor. Chromatographic separations were performed on silica gel columns (Kieselgel 60, 230–400 mesh, Merk). A Perkin–Elmer Model 1600 spectrophotometer

was used to record IR spectra (as KBr films). ^{1}H and ^{13}C NMR spectra were observed in the indicated deuterated solvents with a JEOL JNM-EX400 spectrometer. Chemical shifts are reported in ppm (δ) downfield from tetramethylsilane as internal standard, with peak multiplicities being indicated as follows: s, singlet; d, doublet; m, multiplet; dd, double of doublet. Mass spectra were obtained on a Shimazu GC–MS QP-1000 mass spectrometer (direct insertion probe). Melting points were obtained with Yanagimoto apparatus and were uncorrected.

TX-1852 (7-oxo-BFA), TX-1853 (tetrahydro-BFA), TX-1855 (5,6-dehydro-2,3-dihydro-4,7-dioxo-BFA), TX-1856 (4,7-di-*O*-acetyl-BFA), and TX-1891 (7-*O*-acetyl-BFA) were synthesized with the methods described previously.[27] The structures of these compounds were identified by ^{1}H and ^{13}C NMR spectra.

Synthesis of 4-oxo-BFA (TX-1933). To a solution of BFA (20 mg, 71.3 μmol) in dichloromethane (3 mL) was added manganese dioxide (195 mg, 2.2 mmol). The mixture was stirred for 2 h at rt, filtered on Celite with ethyl acetate. The solvent was removed by evaporation in vacuum and the residue was purified by chromatography on silica gel eluted with dichloromethane:methanol (30:1) to give less polar fraction 4-oxo-BFA (TX-1933),[26] as a colorless oil (8.6 mg, 43%) and polar one, unreacted BFA (8.2 mg, 41%). TX-1933, ^{1}H NMR ($CDCl_3$): δ 7.77 (d, 1H, J = 15.6 Hz), 6.45 (d, 1H, J = 16.0 Hz), 5.86 (m, 1H), 5.56 (dd, 1H, J = 9.2 and 15.6 Hz), 4.66 (m, 1H), 4.32 (m, 1H), 2.98 (dd, 1H, J = 8.8 and 18.0 Hz), 2.60 (m, 1H), 2.30–1.77 (m, 8H, including 1H of OH), 1.60 (m, 2H), 1.33 (d, 3H, J = 6.0 Hz), 1. 21 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 200.75, 166.18, 140.25, 135.90, 132.88, 128.29, 73.76, 72.09, 56.18, 45.46, 42.57, 35.55, 34.25, 32.21, 25.55, 20.26; FT-IR: ν_{max} 3432, 2926, 2855, 1718, 1702, 1258, 1064, 980 cm^{-1}; MS m/z 278 (M^+).

Synthesis of 4-*epi*-BFA (TX-1923). 4-oxo-BFA (5.5 mg, 20 μmol) in THF was cooled to −78 °C and then L-Selectride (1.0 M solution in tetrahydrofuran) 42 μL (42 μmol) was added. After stirring at −78 °C for 2.5 h, the reaction was quenched by the addition of acetone, warmed up to 0 °C, and then to rt. The solution was neutralized by aqueous hydrogen chloride followed by extraction with ethyl acetate. After evaporation, the residue was separated on preparative TLC in diethyl ether:acetone (1:5) to afford 4-*epi*-BFA (TX-1923, R_f 0.48, 2.1 mg, 38%) and BFA (R_f 0.35, 2.9 mg, 52%) (4-*epi*-BFA:BFA = 2:3). TX-1923, ^{1}H NMR ($CDCl_3$): δ 7.12 (dd, 1H, J = 8.8, 15.6 Hz), 5.73 (d, 1H, J = 15.6 Hz), 5.66 (m, 1H), 5.26 (dd, 1H, J = 9.7, 15.6 Hz), 4.88 (m, 1H), 4.32 (m, 1H), 4.19 (m, 1H), 2.76 (m, 1H), 2.06–1.70 (m, 10H, including 2H of OH), 1.35 (m, 2H), 1.24 (d, 3H, J = 6.3 Hz), 0.91 (m, 1H); FT-IR: ν_{max} 3367, 2928, 1712, 1644, 1449, 1292, 1257, 1111, 1078, 1002, 986, 804 cm^{-1}; MS m/z 280 (M^+).

Synthesis of 4-*O*-acetyl-BFA (TX-1872). The solution of BFA (50 mg, 0.178 mmol) and acetic anhydride (18 μL, 0.19 mmol) in pyridine (1.5 mL) was refluxed for 4 h. The reaction mixture was neutralized with aqueous hydrogen chloride followed by extraction with ethyl acetate. The organic layer was washed with brine and dried with anhydrous sodium sulfate. After the solvent was evaporated, the residue was purified by silica gel chromatography with 1% methanol in dichloromethane to afford two compounds, 4,7-di-*O*-acetyl BFA (TX-1856, 18 mg, 28%) and 4-*O*-acetyl BFA (TX-1872, 24 mg, 42%) (TX-1856:TX-1872 = 43:57). R_f in dichloromethane:methanol (9:1) were 0.76 (TX-1856) and 0.39 (TX-1872), respectively. TX-1872, mp 106–107 °C (white needle from methanol–hexane); ^{1}H NMR ($CDCl_3$): δ 7.25 (dd, 1H, J = 4.4, 16.4 Hz), 5.70 (m, 2H), 5.28 (m, 2H), 4.85 (m, 1H), 4.32 (m, 1H), 2.42 (m, 1H), 2.26–2.13 (m, 2H), 2.12 (s, 3H), 2.03–1.48 (m, 9H, including 1H of OH), 2.24 (d, 3H, J = 6.4 Hz), 0.93 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 170.06, 165.74, 147.30, 136.37, 130.61, 118.23, 76.26, 75.84, 72.11, 49.47, 44.31, 43.22, 40.95, 34.07, 31.80, 26.70, 26.52, 20.87; FT-IR: ν_{max} 3457, 2934, 1742, 1715, 1451, 1376, 1236, 1070, 1013, 977 cm^{-1}; MS m/z 322 (M^+).

Synthesis of di-*O*-methyl-BFA (TX-1869) and 4-*O*-methyl-BFA (TX-1870). To a solution of BFA (50 mg, 0.178 mmol) in DMF (1.0 mL) was added iodomethane (100 μL, 1.60 mmol) and silver oxide (248 mg, 1.07 mmol). The reaction mixture was stirred at rt overnight and then filtered. The filtrate was evaporated and the residue was separated by silica gel chromatography with ethyl acetate:hexane (1:1) to give di-*O*-methyl-BFA (TX-1869, 20 mg, 36%) and 4-*O*-methyl-BFA (TX-1870, 12 mg, 23%) (TX-1869:TX-1870 = 64:36). R_f in ethyl acetate:hexane (3:1) were 0.86 (TX-1869) and 0.64 (TX-1870), respectively. TX-1869, mp 64–65 °C (white amorphous from methanol–hexane); ^{1}H NMR (CD_3OD): δ 7.15 (dd, 1H, J = 3.6, 15.6 Hz), 5.79–5.67 (m, 2H), 5.17 (dd, 1H, J = 9.6, 15.2 Hz), 4.88 (m, 1H), 3.80 (m, 1H), 3.66 (m, 1H), 3.35 (s, 3H), 3.26 (s, 3H), 2.36 (m, 1H), 2.12 (m, 2H), 1.98 (m, 1H), 1.84–1.67 (m, 4H), 1.57 (m, 2H), 1.45 (m, 1H), 1.23 (d, 3H, J = 6.4 Hz), 0.90 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 166.33, 149.25, 130.10, 118.76, 115.29, 85.62, 80.96, 71.73, 57.71, 56.44, 50.44, 44.15, 40.33, 37.38, 34.24, 31.75, 26.44, 20.81; MS m/z 308 (M^+). TX-1870, mp 94–95 °C (white amorphous from methanol–hexane); ^{1}H NMR ($CDCl_3$): δ 7.08 (dd, 1H, J = 3.7, 15.9 Hz), 5.87 (dd, 1H, J = 1.5, 16.1 Hz), 5.63 (m, 1H), 5.24 (dd, 1H, J = 9.8, 15.1 Hz), 4.93 (m, 1H), 4.29 (m, 1H), 3.54 (m, 1H), 3.35 (s, 3H), 2.29 (m, 1H), 2.15 (m, 1H), 2.06–1.85 (m, 3H), 1.82–1.57 (m, 5H, including 1H of OH), 1.54–1.44 (m, 2H), 1.26 (d, 3H, J = 6.4 Hz), 0.90 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 166.24, 149.16, 136.36, 130.17, 118.87, 85.45, 72.46, 71.60, 57.76, 50.71, 44.35, 43.36, 41.35, 34.25, 31.84, 26.48, 20.89; MS m/z 294 (M^+).

Synthesis of BFA seco-acid (TX-1875). A solution of BFA (20 mg, 71.3 μmol) in 10% aqueous potassium hydroxide (1.0 mL) was refluxed for 1 h and then neutralized with iced 10% aqueous hydrogen chloride followed by extracttion with ethyl acetate. The organic layer was dried with anhydrous sodium sulfate and the solvent was evaporated to give a colorless oil, BFA seco-acid (TX-1875, 18 mg, 90%). TX-1875, ^{1}H NMR (CD_3OD): δ 6.94 (dd, 1H, J = 4.4, 15.6 Hz), 5.97 (dd,

1H, $J = 1.6, 15.6$ Hz), 5.47–5.38 (m, 2H), 4.20 (m, 2H), 3.71 (m, 1H), 2.43 (m, 1H), 2.15 (m, 1H), 2.02–1.95 (m, 3H), 1.81 (m, 1H), 1.57 (m, 1H), 1.46–1.22 (m, 5H), 1.13 (d, 3H, $J = 5.6$ Hz); FT-IR ($CHCl_3$): ν_{max} 3000–3700 (strongly wide wave), 1700, 1654, 1275, 1120, 1082, 981 cm^{-1}.

Synthesis of seco-BFA methyl ester (TX-1873). To a solution of TX-1875 (18 mg, 64 μmol) in methanol (1.0 mL) was added excessive diazomethane in ether at 0 °C. The reaction mixture was stirred at rt for 10 min and then evaporated. The residue was purified by silica gel chromatography with dichloromethane–methanol (15:1) to give a colorless oil, seco-BFA methyl ester (TX-1873, 15 mg, 80%). TX-1873, ^{1}H NMR ($CDCl_3$): δ 6.98 (dd, 1H, $J = 4.4$, 15.6 Hz), 6.06 (dd, 1H, $J = 2.0$, 15.6 Hz), 5.48–5.34 (m, 2H), 4.30 (m, 2H), 3.79 (m, 1H), 3.74 (s, 3H), 2.40 (m, 1H), 2.26–2.19 (m, 1H), 2.12–1.99 (m, 3H), 1.82–1.75 (m, 4H, including 3H of OH), 1.62 (m, 1H), 1.49–1.37 (m, 5H), 1.18 (d, 3H, $J = 5.8$ Hz); FT-IR ($CHCl_3$): ν_{max} 3394, 2928, 1723, 1706, 1653, 1437, 1290, 1174, 1088, 980 cm^{-1}; MS *m/z*: 312 (M^+).

Synthesis of 10,11-epoxy-BFA (TX-1876). BFA (10 mg, 35.7 μmol) was added to a solution of 5% *m*-chloroperoxybenzoic acid in chloroform (0.7 mL, 0.30 mmol). The suspension was stirred at rt overnight and then filtered and washed with methanol. The crude product was recrystallized with methanol to give 10,11-epoxy-BFA (TX-1876, 8 mg, 76%). TX-1876, mp 220 °C (decomposed, colorless needle from methanol); ^{1}H NMR (CD_3OD): δ 7.33 (dd, 1H, $J = 2.9$, 15.6 Hz), 5.98 (dd, 1H, $J = 2.0$, 15.6 Hz), 4.86 (m, 1H), 4.25 (m, 1H), 4.02 (m, 1H), 3.18 (m, 1H), 2.99 (m, 1H), 2.57 (m, 1H), 2.10–1.39 (m, 13H, including 2H of OH), 1.30 (d, 3H, $J = 5.6$ Hz); ^{13}C NMR (CD_3OD): δ 167.94, 154.30, 119.33, 75.27, 73.88, 73.15, 65.22, 61.78, 50.18, 43.53, 42.32, 40.78, 34.78, 31.44, 23.56, 21.60.

Synthesis of seco-BFA benzyl ester (TX-1890). A mixture of TX-1875 (50 mg, 0.168 mmol), benzyl alcohol (1.0 mL, 9.6 mmol), *p*-toluenesulfonic acid monohydrate (120 mg, 0.63 mmol), and molecular seives (4 Å) in benzene (5.0 mL) was refluxed for 4 h and then the solvent was removed by evaporation and the residue was chromatographed on silica gel and eluted by ethyl acetate: hexane (1:4) to afford a colorless oil, seco-BFA benzyl ester (TX-1890, 44 mg, 68%). TX-1890, ^{1}H NMR ($CDCl_3$): δ 7.35 (m, 5H), 7.01 (dd, 1H, $J = 4.4$, 15.6 Hz), 6.11 (dd, 1H, $J = 1.7$, 15.9 Hz), 5.48–5.30 (m, 2H), 5.18 (s, 2H), 4.32 (m, 2H), 3.78 (m, 1H), 2.41 (m, 1H), 2.37–2.19 (m, 1H), 2.10–1.98 (m, 3H), 1.84–1.74 (m, 2H), 1.63–1.25 (m, 8H, including 2H of OH), 1.18 (d, 3H, $J = 6.4$ Hz); FT-IR: ν_{max} 3381, 3034, 2930, 1716, 1706, 1654, 1458, 1377, 1278, 1168, 1120, 1084, 981, 743, 698 cm^{-1}; MS *m/z* 388 (M^+).

Biological assay

Cell culture. Human colon carcinoma HCT 116 cells were grown at 37 °C in the presence of 5% CO_2 in the cell culture medium DMEM supplemented with 2.5% fetal bovine serum, 25 μg/mL kanamycin sulfate, and 2.5 mg/mL $NaHCO_3$.

Detection of DNA fragmentation. The procedures were described in detail previously.[33] Briefly, cell suspension (1×10^6 cells) was centrifuged for 5 min at $400 \times g$, then the cell pellets were resuspended in 20 μL lysis buffer containing 50 mM Tris–HCl, pH 7.8, 10 mM EDTA, and 0.5% sodium-*N*-lauroylsarcosinate, followed by RNase. After incubation for 30 min at 50 °C, proteinase K was then added to the mixture and left to stand for 3 h at 50 °C. To 2% agarose gel (TBE buffer containing 89 mM Tris–HCl, 89 mM boric acid and 2 mM EDTA), 20 μL DNA sample was added. Electrophoresis was performed at 100 V/cm for 90 min. The DNA in the gel was visualized with ethidium bromide according to standard procedures.

Measurement of cytotoxicity using MTT. HCT 116 was seeded in 250 μL/well, incubated for 2 days in a 37 °C, 5% CO_2 humidified incubator. After BFA or its derivative was added, the cells were incubated for another 3 or 4 days. Then the medium was removed and MTT assay was carried out as described.[34] Briefly, 250 μL MTT/PBS (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS: phosphate buffer saline) solution (5 mg/mL) was added to each well. After the multiplate was incubated for 4 h, the supernatant was removed and 0.04 N HCl/isopropanol (400 μL) was added to each well to dissolve the formazan. 100 μL formazan solution was added to each well of 96-well Elisa Plate. The plate was read in a microtiter plate reader (Bio-Rad Model 450) using a 570 nm filter.

Determination of cell morphological changes. After drug treatment, the morphological assessment of the cells was performed under a phase microscope (Olympus INT-2 and CK-2, Olympus, Japan).

Calculation of molecular orbitals and solvation free energies

The semi-empirical molecular orbital (MO) calculations were performed with the PM3 method of Stewart[35] and MOPAC97 program (WinMOPAC V. 2.0, Fujitsu, Japan). The stable structures were initially built with the parameters including bond lengths, bond angles, and dihedral angles obtained from BFA's X-ray structure,[14] followed by the optimization with the eigenvector following (EF) method. The solvation free energy (dGW = dGWw − dGWv) was defined as the free-energy changes for association in water (dGWw) and in vacuum (dGWv). The dielectric constants (ϵ) of water and vacuum were 78.3 and 1.0, respectively.

Calculation of log *P*

The log *P* values of 4-*epi*-BFA and BFA were calculated using ACD/log*P* version 1.0 (Advanced Chemistry Development Inc., Toronto, Canada).

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