Structure−Activity Relationships of (1′*S*)-1′-Acetoxychavicol Acetate, a Major Constituent of a Southeast Asian Condiment Plant *Languas galanga*, on the Inhibition of Tumor-Promoter-Induced Epstein−Barr Virus Activation

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The structure−activity relationships of (1′*S*)-1′-acetoxychavicol acetate (ACA), a cancer chemopreventive agent of food origin, were investigated in an inhibitory test of tumor promoter teleocidin B-4-induced Epstein−Barr virus (EBV) activation in Raji cells. Through a test of 16 derivatives, the structural factors regulating activity were found to be as follows: (1) the absolute configuration at the 1′-position does not affect activity; (2) hydrogenation of the terminal methylene group abolishes activity; (3) both the phenolic and alcoholic hydroxyl groups are compulsorily acetylated, and it is necessary that the former is oriented only at the position para to the side chain; (4) an additional acetoxyl group is allowed to locate at the ortho or meta position; and (5) substitution of the hydrogen atom at the 1′-position by a methyl group reduces activity. Upon esterase blockade in Raji cells, (1′*R*,*S*)-ACA suppressed EBV activation, the extent of which was the same as tested in the control, suggesting that ACA bearing two acetoxyl groups is an intracellular structure prerequisite for activity exhibition. The present study suggests that nucleophilic attack to the 3′-position is important and involved in the interaction of ACA with an unidentified target molecule(s) participating in the process of EBV activation.

Keywords: *1′-Acetoxychavicol acetate; Epstein−Barr virus; structure−activity relationships; chemoprevention; teleocidin B-4*

INTRODUCTION

One of the biological effects of tumor promoters is the activation of Epstein−Barr virus (EBV), a herpesvirus causative for African Burkitt's lymphoma (Klein and Kelin, 1985), cervical carcinoma (Young and Sixbey, 1988), nasopharyngeal carcinoma (NPC) (Kumar and Mahanta, 1998), and gastric cancer in part (Leoneini et al., 1993). Some tumor promoters induce EBV activation partly through the activation of protein kinase C (PKC) (Natsukari et al., 1997), a major cellular receptor of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters and mitogen-activated protein kinase (Panousis et al., 1997). An EBV-infected lymphocyte is partly characterized by its high expression level of the oncogene c-*myc* (Nishikura et al., 1985). To date, we have been conducted inhibitory tests of tumor promoter-induced EBV activation for the estimation of the anti-tumor-promoting properties of edible plants as well as for the isolation of their active constituents [for reviews, see Murakami et al. (1996a, 1998)].

In 1993, we isolated (1′*S*)-1′-acetoxychavicol acetate [(1′*S*)-ACA) (**1**; Figure 1) from the rhizomes of a subtropical ginger, *Languas galanga* Stuntz (Zingiberaceae), as a potent inhibitor of EBV activation (Kondo et al., 1993). The rhizomes are ingested widely in Southeast Asia as a flavor or condiment and are occasionally used as a folk medicine for stomach health (Jacquat, 1990). Also, it is interesting to note that **1** was previously reported as an antiulcer (Mitsui, 1985) and antitumor agent (Itokawa, 1987). Thereafter, there have been a considerable number of reports indicating the effectiveness of ACA on the inhibition of chemically induced carcinogenesis or the expression of tumor markers in the initiation and postinitiation (promotion) phases in multistage carcinogenesis models (Murakami et al., 1996b; Ohnishi et al., 1996; Tanaka et al., 1997a,b; Kobayashi et al., 1998). To address the action mechanisms by which ACA suppresses oncogenic processes, it is important to note the biochemical and biological activities of ACA thus far found: (1) inhibition of xanthine oxidase activity (Noro et al., 1988); (2) suppression of superoxide (O_2^-) generation in leukocytes (Murakami et al., 1996b); (3) antilipid peroxidation (Murakami et al., 1996b); (4) inhibition of induction of proliferation markers such as silver-stained nucleolar organizer region's protein and 5-bromo-2′-deoxyuridine labeling (Ohnishi et al., 1996); (5) inhibition of polyamine synthesis (Ohnishi et al., 1996); (6) inhibition of ornithine decarboxylase activity (Ohnishi et al., 1996); and (7) induction of both glutathione *S*-transferase (GST) and quinone reductase (QR) activities (Tanaka et al., 1997b). The ability of xenobiotic enzymes to be induced may be important as the action mechanism in the initiation phase. On the other hand, we have recently

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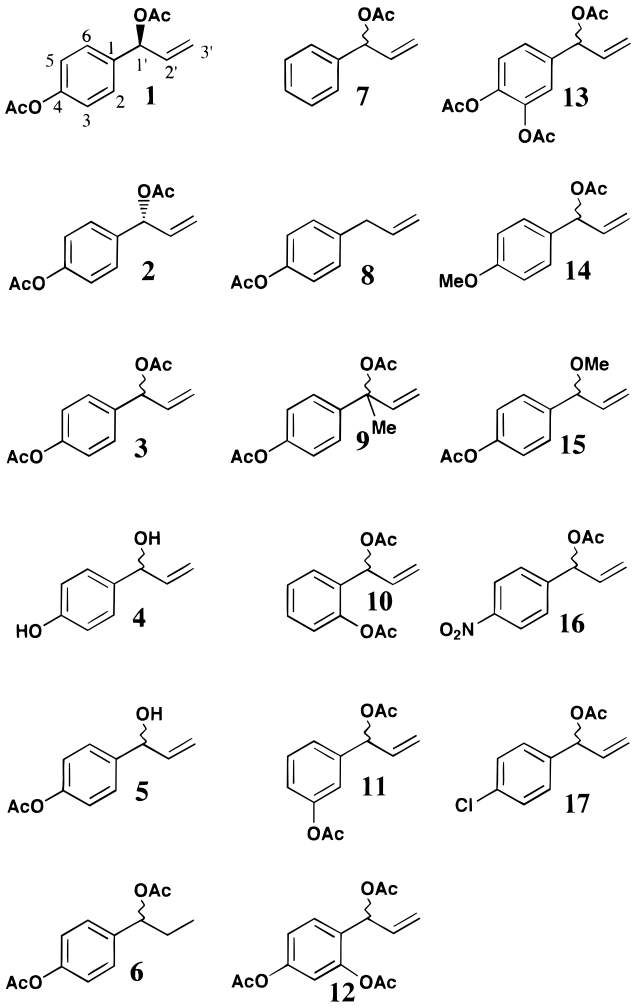

Figure 1. Structures of (1′*S*)-1′-acetoxychavicol acetate and its derivatives.

found that ACA inhibits inducible nitric oxide synthase expression by suppressing the degradation of Iκ-B in mouse macrophage RAW 264.7 cells (Ohata et al., 1998). Furthermore, ACA can suppress double-TPA application-induced hydrogen peroxide formation and other biochemical parameters related to inflammation in mouse skin (Nakamura et al., 1998). Because oxidative stress is closely associated with carcinogenesis (Kensler et al., 1989), we regard the inhibition of oxidative damage in inflammation by ACA as the most acceptable and major action mechanism in the postinitiation phase.

The intracellular target molecule(s) of ACA, with which it directly interacts, has (have) yet to be discovered. As one of the approaches to elucidate the action mechanism of ACA, we examined the structure−activity relationships of ACA on the inhibition of tumor-promoter-induced EBV activation using 16 ACA derivatives.

MATERIALS AND METHODS

Analytical Instruments. ^{1}H and ^{13}C NMR [Bruker DRX-300, tetramethylsilane (TMS) as an internal standard] and APCI-MS (MH-1200, Hitachi Co. Ltd., Tokyo, Japan) were recorded. HPLC (LC10AS and Chromatopac C-R6A, Shimadzu, Kyoto, Japan) was also used.

Chemicals. (1′*S*)-1′-Acetoxychavicol acetate (ACA) [(1′*S*)-ACA, **1**] and teleocidin B-4 were isolated from *Languas galanga* and *Streptoverticillium blastmyceticum* NA 34-17, respectively, as previously reported (Kondo et al., 1993; Irie et al., 1984). (1′*R*)-ACA (**2**) was a gift from Drs. Suzuki and Nozaki of Takasago International Corp. (1′*R*,*S*)-1′-Acetoxychavicol acetate (**3**), (1′*R*,*S*)-1′-hydroxychavicol (**4**), (1′*R*,*S*)-1′-hydroxychavicol acetate (**5**), (1′*R*,*S*)-2′,3′-dihydro-ACA (**6**), (1′*R*,*S*)-1-phenyl-2-propene-1-acetate (**7**), chavicol acetate (**8**), and *S*,*S*,*S*-tributyl phosphorotrithioate (DEF) were synthesized as previously reported (Kondo et al., 1993; Irie et al., 1985). Vinylmagnesium bromide was obtained from Aldrich (Milwaukee, WI). Raji cells and high-titer early antigen (EA)-positive sera from nasopharyngeal carcinoma patients (NPC) for the EBV assay were a kind gift from Dr. S. Imai of Hokkaido University. Fluorescence isothiocyanate (FITC)-labeled anti-human IgG was obtained from Dako Co. Ltd. (Glostrup, Denmark). Other chemicals were obtained from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan) and were of special grade.

Preparation of Derivatives. *(1′R,S)-1′-Acetoxy-1′-methylchavicol Acetate (**9**).* A solution of *p*-hydroxyacetophenone (108 mg) in 40 mL of dry THF was added to 1.0 M vinylmagnesium bromide in 100 mL of dry THF under N_2 atmosphere for 2 h. After evaporation of THF, the reaction mixture was partitioned between ethyl acetate (EtOAc) and NH_4Cl-saturated water to give 1′-hydroxy-1′-methylchavicol, which was then acetylated with 4-(dimethylamino)pyridine and acetic anhydride. The reaction mixture was purified by using preparative TLC (toluene/acetone, 9:1) and HPLC (μBondasphere C_{18} 19 mm × 15 cm column, Waters; mobile phase of 40% acetonitrile in water; flow rate = 7.0 mL; detection at 254 nm) to give **9** (24 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.87 (3H, s, 1′-Me), 2.06 (3H, s, 1′-OAc), 2.28 (3H, s, 4-OAc), 5.24 (1H, dd, J = 10.8, 0.5 Hz, H-3′a), 5.26 (1H, dd, J = 17.4, 0.5 Hz, H-3′b), 6.23 (1H, dd, J = 17.4, 10.8 Hz, H-2′), 7.05 (2H, d, J = 8.7 Hz, H-3,5), 7.38 (2H, d, J = 8.7 Hz, H-2,6); APCI-MS, *m*/*z* 249 [$(MH)^+$, $C_{14}H_{17}O_4$].

*(1′R,S)-1-(2-Acetoxyphenyl)-2-propene-1-acetate (**10**).* A solution of *p*-hydroxybenzaldehyde (1.5 g) in 5 mL of dry THF was added to 1.0 M vinylmagnesium bromide in 25 mL of dry THF under N_2 atmosphere for 3.5 h. After evaporation of THF, the reaction mixture was partitioned between EtOAc and NH_4Cl-saturated water to give 1-(2-hydroxyphenyl)-2-propen-1-ol, which was then acetylated with pyridine and acetic anhydride. The reaction mixture was purified by using preparative TLC (toluene/acetone, 9:1) and HPLC (μBondasphere C_{18} 3.9 mm × 15 cm column, Waters; mobile phase of 40% acetonitrile in water; flow rate = 1.0 mL; detection at 254 nm) to give **10** (800 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.03 (3H, s, 1′-OAc), 2.28 (3H, s, 4-OAc), 5.29 (1H, dd, J = 10.8, 1.0 Hz, H-3′a), 5.34 (1H, dd, J = 17.5, 1.0 Hz, H-3′b), 6.10 (1H, dd, J = 17.5, 10.8 Hz, H-2′), 6.55 (1H, br d, J = 8.9 Hz, H-1′), 7.39 (4H, m, H-2−5); APCI-MS, *m*/*z* 235 $[(MH)^+$, $C_{13}H_{15}O_4]$.

*(1′R,S)-1-(3-Acetoxyphenyl)-2-propene-1-acetate (**11**).* A solution of *m*-hydroxybenzaldehyde (1.5 g) in 5 mL of dry THF was added to 1.0 M vinylmagnesium bromide in 25 mL of dry THF under N_2 atmosphere for 3.5 h. After evaporation of THF, the reaction mixture was partitioned between EtOAc and NH_4Cl-saturated water to give 1-(3-hydroxyphenyl)-2-propen-1-ol, which was acetylated with pyridine and acetic anhydride. The reaction mixture was purified by using preparative TLC (toluene/acetone, 9:1) and HPLC (μBondasphere C_{18} 3.9 mm × 15 cm column, Waters; mobile phase of 40% acetonitrile in water; flow rate = 1.0 mL; detection at 254 nm) to give **11** (780 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.87 (3H, s, 1′-Me), 2.06 (3H, s, 1′-OAc), 2.28 (3H, s, 4-OAc), 5.24 (1H, dd, J = 10.4, 1.0 Hz, H-3′a), 5.26 (1H, dd, J = 17.5, 1.0 Hz, H-3′b), 5.93 (1H, ddd, J = 17.0, 10.0, 5.8 Hz, H-2′), 6.23 (1H, d, J = 5.8 Hz, H-1′), 7.05 (2H, d, J = 8.7 Hz, H-3,5), 7.38 (2H, d, J = 8.7 Hz, H-2,6); APCI-MS, *m*/*z* 235 $[(MH)^+$, $C_{13}H_{15}O_4]$.

*(1′R,S)-1-(2,4-Diacetoxyphenyl)-2-propene-1-acetate (**12**):* A solution of *o*,*p*-dihydroxybenzaldehyde (1.5 g) in 5 mL of dry THF was added to 1.0 M vinylmagnesium bromide in 25 mL of dry THF under N_2 atmosphere for 3.5 h. After evaporation of THF, the reaction mixture was partitioned between EtOAc and NH_4Cl-saturated water to give 1-(2,4-dihydroxyphenyl)-2-propen-1-ol, which was acetylated with pyridine and acetic anhydride. The reaction mixture was purified by using preparative TLC (toluene/acetone, 9:1) and HPLC (μBondasphere

C_{18} 3.9 mm × 15 cm column, Waters; mobile phase of 40% acetonitrile in water; flow rate = 1.0 mL; detection at 254 nm) to give **12** (500 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.10 (3H, s, 1′-OAc), 2.28 (6H, s, 2- and 3-OAc), 5.27 (1H, d, J = 10.6 Hz, H-3′a), 5.32 (1H, d, J = 17.6 Hz, H-3′b), 5.96 (1H, ddd, J = 17.6, 10.6, 5.8 Hz, H-2′), 6.26 (1H, d, J = 5.8 Hz, H-1′), 7.12 (1H, d, J = 2.1 Hz), 7.21 (2H, m); APCI-MS, m/z 293 [$(MH)^+$, $C_{15}H_{17}O_6$].

*(1′R,S)-1-(3,4-Diacetoxyphenyl)-2-propene-1-acetate (**13**).* A solution of *m*-hydroxybenzaldehyde (1.5 g) in 5 mL of dry THF was added to 1.0 M vinylmagnesium bromide in 25 mL of dry THF under N_2 atmosphere for 3.5 h. After evaporation of THF, the reaction mixture was partitioned between EtOAc and NH_4-Cl-saturated water to give 1-(3,4-dihydroxyphenyl)-2-propen-1-ol, which was acetylated with pyridine and acetic anhydride. The reaction mixture was purified by using preparative TLC (toluene/acetone, 9:1) and HPLC (μBondasphere C_{18} 3.9 mm × 15 cm column, Waters; mobile phase of 40% acetonitrile in water; flow rate = 1.0 mL; detection at 254 nm) to give **13** (380 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.05 (3H, s, 1′-OAc), 2.25 (3H, s, 2- or 3-OAc), 2.28 (3H, s, 2- or 3-OAc), 5.23 (1H, d, J = 10.4 Hz, H-3′a), 5.25 (1H, d, 17.2 Hz, H-3′b), 5.95 (1H, ddd, J = 17.2, 10.4, 5.8 Hz, H-2′), 6.40 (1H, d, J = 5.8 Hz, H-1′), 6.97 (1H, d, J = 2.2 Hz, H-2), 6.99 (2H, m, H-5,6); APCI-MS, m/z 293 [$(MH)^+$, $C_{15}H_{17}O_6$].

*(1′R,S)-1′-Acetoxychavicol Methyl Ether (**14**).* *p*-Methoxybenzaldehyde (1.2 mL) was added to 1.0 M vinylmagnesium bromide in 15 mL of dry THF under N_2 atmosphere for 2 h. After evaporation of THF, the reaction mixture was partitioned between EtOAc and NH_4Cl-saturated water to give 1′-hydroxychavicol methyl ether, which was acetylated with pyridine and acetic anhydride. The reaction mixture was purified by using preparative TLC (toluene/acetone, 10:1) and HPLC (μBondasphere C_{18} 19 mm × 15 cm column, Waters; mobile phase of 80% methanol in water; flow rate = 7.0 mL; detection at 254 nm) to give **14** (400 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.08 (3H, s, 1′-OAc), 3.79 (3H, s, 4-OMe), 5.22 (1H, dd, J = 10.8, 1.0 Hz, H-3′a), 5.26 (1H, dd, J = 17.5, 1.0 Hz, H-3′b), 6.00 (1H, ddd, J = 17.5, 10.8, 6.1 Hz, H-2′), 6.22 (1H, br d, J = 6.1 Hz, H-1′), 6.85 (2H, d, J = 8.9 Hz, H-3,5), 7.32 (2H, d, J = 8.9 Hz, H-2,6); APCI-MS, m/z 207 [$(M)^+$, $C_{12}H_{15}O_3$].

*(1′R,S)-1′-Methoxychavicol Acetate (**15**).* 3-Methoxybenzaldehyde (200 mg) in 2.5 mL of dry THF was added to 1.0 M vinylmagnesium bromide in 15 mL of dry THF under N_2 atmosphere for 3 h. After evaporation of THF, the reaction mixture was partitioned between EtOAc and NH_4Cl-saturated water to give (1*R,S*)-1-(3-methoxy)-2-propen-1-ol, which was acetylated with pyridine and acetic anhydride. The reaction mixture was purified by using preparative HPLC (μBondasphere C_{18} 19 mm × 15 cm column, Waters; mobile phase of 40% acetonitrile in water; flow rate = 7.0 mL; detection at 254 nm) to give **15** (72 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.29 (3H, s, 4-OAc), 3.78 (3H, s, 1′-OMe), 5.22 (1H, dd, J = 10.8, 1.0 Hz, H-3′a), 5.26 (1H, dd, J = 17.5, 1.0 Hz, H-3′b), 6.00 (1H, ddd, J = 17.5, 10.8, 6.1 Hz, H-2′), 6.22 (1H, d, J = 6.1 Hz, H-1′), 6.85 (2H, d, J = 8.9 Hz, H-3,5), 7.32 (2H, d, J = 8.9 Hz, H-2,4); APCI-MS, m/z 207 [$(MH)^+$, $C_{12}H_{15}O_3$].

*(1′R,S)-1-(4-Nitrophenyl)-2-propene-1-acetate (**16**).* *p*-Nitrobenzaldehyde (800 mg) in 2.5 mL of dry THF was added to 1.0 M vinylmagnesium bromide in 15 mL of dry THF under N_2 atmosphere for 3 h. After evaporation of THF, the reaction mixture was partitioned between EtOAc and NH_4Cl-saturated water to give (1*R,S*)-1-(4-nitrophenyl)-2-propen-1-ol, which was acetylated with pyridine and acetic anhydride. The reaction mixture was purified by using preparative HPLC (μBondasphere C_{18} 19 mm × 15 cm column, Waters; mobile phase of 40% acetonitrile in water; flow rate = 7.0 mL; detection at 254 nm) to give **16** (72 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.15 (3H, s, 1′-OAc), 5.34 (2H, m, H-3′ab), 5.96 (1H, ddd, J = 17.1, 10.4, 6.1 Hz, H-2′), 6.31 (1H, d, J = 6.1 Hz, H-1′), 7.52 (2H, d, J = 8.5 Hz, H-3,5), 8.22 (2H, d, J = 8.5 Hz, H-2,6); APCI-MS, m/z 222 [$(MH)^+$, $C_{11}H_{12}O_4N$].

*(1′R,S)-1-(4-Chlorophenyl)-2-propene-1-acetate (**17**).* *p*-Chlorobenzaldehyde (800 mg) in 2.5 mL of dry THF was added to 1.0 M vinylmagnesium bromide in 15 mL of dry THF under N_2 atmosphere for 2 h. After evaporation of THF, the reaction mixture was partitioned between EtOAc and NH_4Cl-saturated water to give (1*R,S*)-1-(4-chrolophenyl)-2-propen-1-ol, which was acetylated with pyridine and acetic anhydride. The reaction mixture was purified by using preparative HPLC (μBondasphere C_{18} 19 mm × 15 cm column, Waters; mobile phase of 60% acetonitrile in water; flow rate = 7.0 mL; detection at 254 nm) to give 17 (320 mg): ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.11 (3H, s, 1′-OAc), 5.27 (2H, dd, J = 10.1, 1.0 Hz, H-3′a), 5.30 (1H, dd, J = 17.1, 1.0 Hz, H-3′b), 5.98 (1H, ddd, J = 17.1, 10.1, 5.8 Hz, H-2′), 6.22 (1H, d, J = 5.8 Hz, H-1′), 6.94 (2H, d, J = 8.5 Hz, H-3,5), 7.80 (2H, d, J = 8.5 Hz, H-2,6); EIMS, m/z 211 [$(MH)^+$, $C_{11}H_{12}O_2Cl$].

EBV Activation Test. The inhibitory assay of EBV activation, using EBV genome-carrying human B lymphoblastoid Raji cells, was done basically in the same manner as reported previously (Kondo et al., 1993). Raji cells (5×10^5) were incubated in 1 mL of RPMI 1640 medium (supplemented with 10% fetal bovine serum, 200 units/mL penicillin, and 250 μg/mL streptomycin) containing sodium *n*-butyric acid (3 mM), teleocidin B-4 (50 nM), and the test compound dissolved in 5 μL of DMSO at 37 °C under 5% CO_2 atmosphere for 48 h. The cytotoxicity of the test compound was evaluated by cell viability, which was measured by staining the cells with trypan blue. After smears were made from the cell suspension, EA-positive cells were detected by a conventional indirect immunofluorescence technique with high-titer EA-positive sera from an NPC patient, followed by FITC-labeled IgG. The ordinary average percentage of EA-induced cells in the control experiment (with only *n*-butyrate, teleocidin B-4, and DMSO) was ~40%. Each experiment was done in triplicate, and the data were expressed as mean ± standard deviations ($m \pm$ SD).

Esterase Blockade. The influence of *S,S,S*-tributyl phosphorotrithioate (DEF) on the inhibition of ACA toward EBV activation was examined as previously reported (Irie et al., 1985) with some modifications. Raji cells (5×10^5 cells/mL) were preincubated with or without DEF (10 or 50 μM) at 37 °C under a 5% CO_2 atmosphere. After 24 h, extracellular DEF was removed by centrifugation at 2000*g* for 5 min followed by two washings with PBS, and the cells were suspended in a fresh medium in a density of 5×10^5 cells/mL. The cells were incubated with teleocidin B-4 (50 nM), *n*-butyrate (3 mM), and (1′*R,S*)-ACA (2.5 μM) at 37 °C for 48 h. The following procedure to measure the EBV activation was the same as described above.

RESULTS

Structure−Activity Relationships of ACA. We first examined the inhibitory activities of (1′*S*)-ACA (**1**) and its 16 derivatives toward teleocidin B-4-induced EBV activation in Raji cells. Teleocidin B-4 is an indole alkaloid-type of tumor promoter in mouse skin (Irie et al., 1984). EBV activation was measured by the induction rate of EBV early antigen (EA)-positive cells. The structures and activities of the test compounds are shown in Figure 1 and Table 1, respectively.

Naturally occurring **1** completely inhibited EBV activation at a concentration of 5 μM (IC_{50} = 1.0 μM) and exhibited a marked cytotoxicity (1% > cell viability) at ≥50 μM. No optical separation of the racemate (**3**) or other derivatives (**4**−**7**, **9**−**17**) was attempted because (1′*R*)-ACA (**2**) and (1′*R,S*)-ACA (**3**) showed activities (IC_{50} = 1.5 and 1.2 μM, respectively) very similar to that of **1**. Hydrogenated ACA was weakly active (**6**, IC_{50} = 29 μM). Replacement of the 1′-H by a methyl group resulted in a drastic activity decrease (**9**, IC_{50} = 24 μM). It is of great interest that two orientational isomers of ACA (**10**, **11**), in which a phenolic acetoxyl group is placed at the ortho and meta positions to the alkyl C_3 side chain, respectively, possessed no significant activity (IC_{50} = 57 and >100 μM, respectively). The derivatives

Table 1. Inhibitory Activities of (1′*S*)-ACA and Its Derivatives toward Tumor-Promoter-Induced EBV Activation in Raji Cells

	% inhibition (% cell viability) at a concentration of					
compound	100 μM	50 μM	5 μM	1.5 μM	0.5 μM	IC_{50} (μM)
1	ND[a] (1>)	ND[a] (1>)	100 (63)	60 ± 5 (>80)	36 ± 8 (>80)	1.0
2	ND[a] (1>)	ND[a] (1>)	100 (68)	50 ± 5 (>80)	32 ± 6 (>80)	1.5
3	ND[a] (1>)	ND[a] (1>)	100 (63)	55 ± 5 (>80)	35 ± 8 (>80)	1.2
4	48 ± 8 (>80)	32 ± 6 (>80)	0 (<80)	0 (>80)	0 (>80)	>100
5	8 ± 4 (>80)	0 (>80)	0 (>80)	0 (>80)	0 (>80)	>100
6	85 ± 6 (>80)	80 ± 1 (>80)	15 ± 4 (>80)	0 (>80)	0 (>80)	29
7	34 ± 3 (>80)	24 ± 8 (>80)	0 (>80)	0 (>80)	0 (>80)	>100
8	5 ± 3 (>80)	0 (>80)	0 (>80)	0 (>80)	0 (>80)	>100
9	85 ± (>80)	80 ± 1 (>80)	15 ± 5 (>80)	0 (>80)	0 (>80)	24
10	85 ± 2 (>80)	44 ± 2 (>80)	29 ± 9 (>80)	0 (>80)	0 (>80)	57
11	24 ± 3 (>80)	14 ± 8 (>80)	0 (>80)	0 (>80)	0 (>80)	>100
12	ND[a] (1>)	ND[a] (1>)	81 ± 11 (>80)	28 ± 6 (>80)	23 ± 4 (>80)	2.9
13	ND[a] (1>)	ND[a] (1>)	70 ± 4 (>80)	47 ± 12 (>80)	12 ± 6 (>80)	1.9
14	15 ± 5 (>80)	0 (>80)	0 (>80)	0 (>80)	0 (>80)	>100
15	0 (>80)	0 (>80)	0 (>80)	0 (>80)	0 (>80)	>100
16	35 ± 8 (>80)	17 ± 8 (>80)	0 (>80)	0 (>80)	0 (>80)	>100
17	15 ± 5 (>80)	0 (>80)	0 (>80)	0 (>80)	0 (>80)	>100

[a] Not detected due to cytotoxicity.

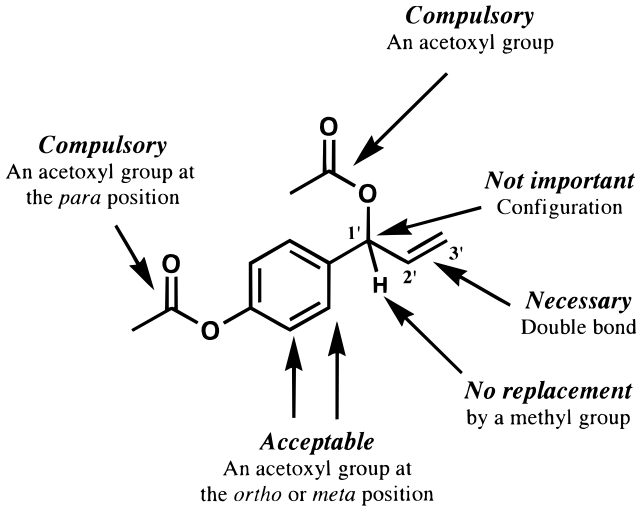

Figure 2. Deduced important structural factors of ACA for the inhibitory activity toward EBV activation.

lacking the acetoxyl group at the 4-position (**7**) or 1′-postion (**8**) in **1** were also inactive (IC_{50} > 100 μM each). Hydrolyzed derivatives of ACA (**4** and **5**, IC_{50} > 100 μM each) or those bearing a methoxyl group in place of an acetoxyl (**14** and **15**) were also inactive (IC_{50} > 100 and 66 μM, respectively). However, substitution of a phenolic acetoxyl group by the nitro (**16**) or choline (**17**) group caused a loss of activity. Importantly, the triacetoxyl derivatives **12** and **13** maintained an inhibitory potential comparable to that of **1** (IC_{50} = 2.9 and 1.9 μM, respectively). In summary, no derivatives in the present study showed an activity higher than that of **1**, and the activities of **2**, **3**, **12**, and **13** were all comparable to that of **1**.

Influence of Esterase Blockade. It is well-known that ester groups of organic compounds, in general, are hydrolyzed by intracellular esterase(s) to allow their hydrolysates to be trapped within cells. To confirm the importance of the presence of acetoxyl groups in (1′*S*)-ACA (**1**) for activity exhibition (see Figure 2), an esterase blockade was executed in the EBV assay. DEF, a potent potentiator of insecticide, was selected as an esterase inhibitor because of its low toxicity to Raji cells (Irie et al., 1985). As shown in Table 2, **1** at a concentration of 2.5 μM completely maintained its inhibitory activity toward EBV activation in Raji cells in which esterase(s) was blocked by DEF (10 or 50 μM) 24 h prior to ACA addition, as compared with the vehicle control.

Table 2. Influence of Esterase Inhibitor DEF on the Potency of (1′*R*,*S*)-ACA (3) in the Inhibition of EBV Activation

	% of EBV-EA-positive cells[a]		
pretreatment	control[b]	ACA (**3**)[c]	% inhibition
ethanol	53 ± 4	25 ± 5	52
DEF (10 μM)	57 ± 4	26 ± 3	54
DEF (50 μM)	57 ± 1	27 ± 6	52

[a] Mean ± SD (n = 3). [b] EBV was activated with teleocidin B-4 (50 nM) and *n*-butyrate (3 mM). [c] At a concentration of 2.5 μM.

DISCUSSION

The structural prerequisites of naturally occurring (1′*S*)-ACA (**1**) for EBV activation inhibition are remarkably strict. It should be noted that the activity of no derivatives, except for **2**, **3**, **12**, and **13** in the present study, was comparable to that of **1** and only four derivatives (**6**, **9**, **10**, and **15**) were weakly active ($^1/_{57}$–$^1/_{24}$ of **1**). Because both an enantiomer of **1** (**2**, IC_{50} = 1.5 μM) and a racemic mixture (**3**, IC_{50} = 1.2 μM) showed the same activity as **1**, the absolute configuration at the 1′-position does not affect inhibitory activity. The low activity of hydrogenated ACA (**6**, IC_{50} = 29 μM) indicates that the terminal methylene group is necessary for activity. The lack of activity from **5**, **8**, and **15** demonstrates that the functional group at the 1′-position is required to be acetylated. The phenolic hydroxyl group is also forced to be acetylated and yet is oriented only at the position para to the C_3 alkyl side chain because both the derivatives, with other functional groups (**7**, **14**, **16**, and **17**), and orientational isomers (**10**, **11**) had no notable activity (IC_{50} = 57–100 μM). An additional acetoxyl group to the ortho or meta position to the side chain did not diminish the activity (IC_{50} = 2.9 and 1.9 μM, respectively). The structural factors regulating the inhibitory activity of **1** are summarized as follows: (1) the absolute configuration at the 1′-position does not affect activity; (2) lack of a terminal methylene group results in marked activity reduction; (3) both phenolic and alcoholic hydroxyl groups are compulsorily acetylated, and the former should be oriented only at the

position para to the C_3 alkyl side chain; (4) an additional acetoxyl group is allowed at the ortho or meta position for the activity; and (5) substitution of the 1′-H by a methyl group drastically decreases the activity. Interestingly, the structure−activity relationships described here have some correlation with those on the antitumor activity of (1′*S*)-ACA derivatives (**1**, **4**, and **5**) against Sarcoma 180 ascites in mice, as reported by Itokawa et al. (1987).

It is important to note that the 3′-carbon is susceptible to nucleophiles and this may accompany double-bond migration and acetate elimination, that is, abnormal bimolecular displacement (S_N2'). A low activity of hydrogenated ACA (**6**) strongly supports the hypothesis that the high reactivity of the 3′-carbon with electrophiles, in part, is an increasing factor for activity and vice versa. Along a similar line, there is a premise for the properties of the functional group at C-1′, namely, the chemical trait of the leaving group. In this regard, it should be noted that the acetoxyl group was the best leaving group tested here as compared with the hydrogen atom (**7**), hydroxyl (**5**), or methoxyl (**15**) group. They were conversely inactive ($IC_{50} > 100\ \mu M$). Talalay et al. (1988) reported that the compounds which have an electrophilic terminal methylene, including Michael reaction acceptors, are capable of inducing phase II enzymes such as GST or QR by using a hepatocyte model. As expected, oral feeding of ACA significantly induced these xenobiotic enzymes in the liver and colon of rats (Tanaka et al., 1997b). Thus, the structure−activity relationships of ACA derivatives in their ability to induce xenobiotic enzymes remain to be examined in the future.

Despite the interesting data, there is no clear-cut explanation for the contrast in activity between (1′*R*,*S*)-ACA (**3**) ($IC_{50} = 1.2\ \mu M$) and the inactive derivatives **7**, **10**, **11**, **14**, **16**, and **17** ($IC_{50} = 57-100\ \mu M$), all of which are considered to be susceptible to both nucleophilic attack and acetate elimination. It is tempting, however, to speculate that the phenolic acetoxyl group only at the para position could appropriately be oriented for interaction with the specific target site(s). The hypothesis that ACA acts specifically toward biological systems may be indirectly supported by the data showing that ACA has no inhibition in some in vitro biological systems related to tumor promotion, for example, PKC activity, arachidonate release, prostaglandin E_2 synthesis, cyclooxygenase, and lipoxygenase activity (data not shown). If ACA undergoes nonspecific interactions with any nucleophiles, the above-mentioned specificity in biological activities may be impossible. In addition, ACA exhibited no detectable toxicity or marked body weight retardation in rodents by oral feeding (Ohnishi et al., 1996; Tanaka et al., 1997a,b). Such activity and toxicity profiles suggest that the mode of action of ACA is rather specific to biological systems rather than simple and nonspecific interactions with any nucleophilic groups of cellular components.

As shown in Table 1, neither hydrolyzed derivative (**4** or **5**) possessed any inhibitory activity ($IC_{50} > 100\ \mu M$). There are at least two explanations for this: (1) Although they are potentially active, no inhibitory activity was observed because of their lower permeability, as compared with ACA, to the cell membrane due to their relatively lower molecular hydrophorbicity; that is, the acetyl groups of **3** would function as a carrier group and would then be hydrolyzed by intracellular esterase(s), forming **4** and **5**, which are intracellular candidates interacting with target molecule(s). (2) Inversely, the activity of **3** drastically decreases when the two acetyl groups are hydrolyzed; namely, holding two acetoxyl groups is an active structure. Intracellular esterase(s) hydrolyzed two acetoxyl groups at some point after starting the EBV assay. It is extremely important to elucidate whether **3** exhibits activity before or after hydrolyzation of the acetoxyl groups. In the latter case, the activity potency of **3** should be reduced significantly by the esterase blockade. (1′*R*,*S*)-ACA, however, was postulated to exhibit activity before intracellular hydrolyzation, because **3** at 2.5 μM maintained a complete inhibitory activity in Raji cells preincubated with an esterase inhibitor, DEF (10 or 50 μM), for 24 h as compared with the control experiment (Table 2). Irie et al. (1985) reported that 10 μM DEF was appropriate for esterase blockade in Raji cells, and its preincubation time was optimized at 18−24 h. Thus, the results here, together with the validity of the experimental conditions, raise the possibility that both acetoxyl groups in **3** are necessary for an exhibition of activity.

In conclusion, the structure−activity relationships of ACA suggested that the nucleophilic attack to the 3′-position of ACA, accompanying acetate elimination, may be involved in the interaction of ACA with an unidentified target molecule(s) participating in the process of EBV activation. ACA is postulated to modify them by forming adducts. Furthermore, the phenolic acetoxyl group at the para position could play an important role in the interaction. The present findings may confirm the action mechanism(s) of ACA after the discovery of its target molecule(s).

ABBREVIATIONS USED

ACA, 1′-acetoxychavicol acetate; APCI-MS, aperture chemical ionization mass spectrum; DMSO, dimethyl sulfoxide; EBV, Epstein−Barr virus; EtOAc, ethyl acetate; GST, glutathione *S*-transferase; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PKC, protein kinase C; QR, quinone reductase; THF, tetrahydrofuran; TLC, thin-layer chromatography; TMS, tetramethylsilane; TPA, 12-*O*-tetradecanolyphorbol-13-acetate; NMR, nuclear magnetic resonance.

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