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RESEARCH ARTICLE

Cancer preventive agents 11. Novel analogs of dimethyl dicarboxylate biphenyl as potent cancer chemopreventive agents†

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Abstract

Context: Dimethyl dicarboxylate biphenyl (DDB) is a clinically used hepatoprotectant and has also been found to have chemopreventive activity.

Materials and methods: Sixteen novel analogs (**5-20**) were designed, synthesized, and evaluated for their cancer preventive activity. The 2,2′-bismethyl ester (**5-18**) and ether (**19**, **20**) DDB analogs were synthesized by insertion of various linear alkyl, short fatty acid, polar, and aromatic groups. All synthesized analogs were evaluated in an *in vitro* short-term 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein Barr virus early antigen (EBA-EA) activation assay. Three of the most potent compounds were also tested for inhibitory effects on skin tumor promotion in an *in vivo* two-stage mouse-skin carcinogenesis test using 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a promoter.

Results: Compound **19** with bisprenyl ethers had the most significant cancer preventive activity (100% inhibition of activation at 1×10^3 mol ratio/TPA, 78.4%, 49.7%, and 10.9% inhibition at 5×10^2, 1×10^2, 1×10 mol ratio/TPA, respectively) *in vitro*. Compound **19** also exhibited a remarkable inhibitory effect on skin tumor promotion in the *in vivo* two-stage mouse-skin carcinogenesis test.

Discussion and conclusions: Thus, DDB analog **19** could be a valuable candidate as a cancer preventive agent or as a lead for the development of new antitumor promoter drugs.

Keywords: Cancer chemopreventive agents, DDB analogs, antitumor promoters

Introduction

Carcinogenesis is a complex process involving initiation, promotion, and progression steps. The promotion step is a long and reversible process and has been widely studied (Itoigawa et al., 2002). Inhibition of this step, which is known as cancer prevention, should be an effective approach to control cancer, and various phytochemicals, such as carotenoids, green tea polyphenols, curcumin, glycyrrhizin and its related compounds, herbs and medicinal plants, have been reported to exhibit cancer preventive ability (Nishino et al., 2000). However, despite its recognized effectiveness at blocking the long process of cancer development, relatively limited numbers of studies have been reported on cancer prevention. Therefore, more efforts aimed at the discovery and development of cancer preventive agents are needed. To

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evaluate cancer preventive ability, a short-term *in vitro* assay can be applied for determining cancer preventive agents. Epstein–Barr virus (EBV) is known to be activated by tumor promoters to produce early antigens. Inhibition of EBV early antigen (EBV-EA) is used to evaluate antitumor promoting ability (Ito et al., 1981).

Dimethyl dicarboxylate biphenyl (DDB, **1**), a synthetic analog of schizandrin C (Figure 1) isolated from *Fructus Schizandrae chinensis*, is a hepatoprotective agent used to treat hepatitis B in China and to treat HBV and HCV in many Asian countries (Sun & Liu, 2005; Jin et al., 2007). In addition, DDB was shown to reverse multidrug resistant cancer cells, breast carcinoma MCF-7/Adr, KBv200, and Bel_{7402} *in vitro* and increase antitumor activity of vincristine to KBv200 xenografts *in vivo* (Jin et al., 2007). DDB also prevented the oncogenic transformation of WB-F344 rat liver epithelial cells induced by 3-methylcholanthrene and 12-*O*-tetradecanoyl phorbal 13-acetate (TPA) at the doses of 1, 2, and 4 μmol/L (Sun & Liu, 2005). In a soft-agar colony formation assay, colony numbers were reduced in transformed cells treated with DDB. Furthermore, DDB could inhibit TPA-induced down-regulation of the gap junctional intercellular communication (GJIC). These findings suggest that DDB has chemopreventive potential.

In our design of new DDB analogs, we found that known DDB analogs with different functional groups were previously synthesized and tested for cancer preventive ability (Xie et al., 1995). In addition, while many 2,2′-carboxylate ester derivatives have been covered in various patents and papers, the modification of a 2,2′-bismethylene alcohol DDB intermediate (**21**) appears to be a new avenue of exploration. A prenylated side chain, which has been found to be effective in cancer chemoprevention studies of other compound classes (Tatsuzaki et al., 2010), was a logical first choice. In addition, short unsaturated fatty acid chains, which have resulted in good chemopreventive activity in betulinic acid derivatives, were included in our modification scheme (Nakagawa-Goto et al., 2009). Water-solubility is an important factor for drug discovery, because it is always associated with important pharmaceutical drug indices. Thus, a hydrophilic carboxylic acid moiety was incorporated into new DDB analogs by coupling the 2,2′-bismethylene alcohol DDB intermediate with succinic and glutaric anhydrides. Accordingly, in this study, we will discuss the synthesis of new DDB analogs (Figure 2), structure–activity relationship findings, and EBV-EA inhibition ability. *In vivo* data of the most potent compounds are also described.

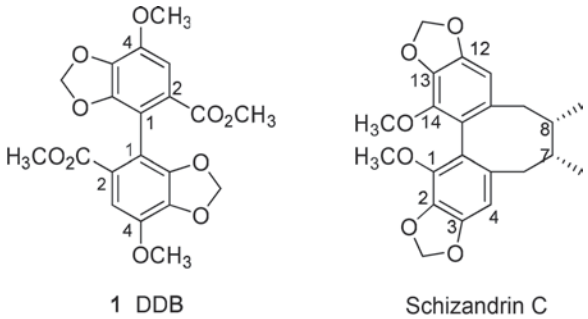


Figure 1. Structures of DDB and schizandrin C.

Materials and methods

Chemistry

^{1}H NMR (400 MHz) spectra were measured on a Varian Inova spectrometer with TMS as the internal standard. All chemical shifts are reported in ppm. Mass spectra were measured on a Shimadzu LCMS-2010 (ESI-MS). All reactions were monitored by thin-layer chromatography (TLC) on aluminum sheets (silica gel 60 F254 plate, 20 × 20, Merk). Melting points were recorded on a Fisher Johns melting apparatus without correction. Medium-pressure column chromatography was used in Biotage Flash and Isco companion systems with silica 40 μm columns from Grace Inc. All final compounds were > 95% pure based on high performance liquid chromatography (HPLC). Anhydrous solvents were purchased from commercial suppliers.

General procedure for compounds 5-7 and 9-14

To a solution of **21** in dichloromethane, the appropriate carboxylic acid (5 eq. mole), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (5 eq. mole) and 4-dimethylamino pyridine (1 eq. mole) were added and stirred overnight. The reaction mixture was subjected to preparative TLC (hexane-ethyl acetate) without work-up.

Compound **5**: Yield, 86%; Colorless prisms; mp: 109°C–110°C; ^{1}H NMR ($CDCl_3$) δ 5.94 (d, J = 1.2 Hz, 2H), 5.92 (d, J = 1.6 Hz, 2H), 4.97 (d, J = 1.2 Hz, 2H), 4.89 (d, J = 1.2 Hz, 2H), 4.06 (s, 6H), 2.18 (t, J = 7.6 Hz, 6H), 1.57 (sext, J = 7.6 Hz, 4H), 0.88 (t, J = 7.2 Hz, 6H); ESI-MS *m/z*: 678 $[M+18(H_2O)]^+$. Compound **6**: Yield, 75%; Colorless prisms; mp: 127°C–128°C; ^{1}H NMR ($CDCl_3$) δ 7.92 (d, J = 7.6 Hz, 4H), 7.46–7.52 (m, 2H), 7.36 (t, J = 7.6 Hz, 4H), 5.88 (s, 2H), 5.70 (s, 2H), 5.31 (d, J = 11.6 Hz, 2H), 5.12 (d, J = 11.6 Hz, 2H), 4.07 (s, 3H); ESI-MS *m/z*: 746 $[M+18(H_2O)]^+$. Compound **7**: Yield, 85%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.69 (s, 2H), 5.97 (s, 2H), 5.95 (s, 2H), 4.85 (s, 4H), 3.95 (s, 6H), 1.99 (s, 6H); ESI-MS *m/z*: 464 $[M+18(H_2O)]^+$. Compound **9**: Yield, 97%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 1H), 5.96 (d, J = 1.4 Hz, 2H), 5.95 (d, J = 1.4 Hz, 2H) 4.86 (s, 4H), 3.93 (s, 6H), 2.22 (t, J = 7.2 Hz, 4H), 1.59 (m, 4H), 0.90 (t, J = 7.2 Hz, 6H); ESI-MS *m/z*: 520 $[M+18(H_2O)]^+$. Compound **10**: Yield, 99%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.96 (d, J = 1.5 Hz, 2H), 5.94 (d, J = 1.5 Hz, 2H), 4.85 (s, 4H), 3.93 (s, 6H), 2.23 (t, J = 7.6 Hz, 4H), 1.54 (pent, J = 7.6 Hz, 4H), 1.34–1.25 (m, 4H), 0.88 (t, J = 7.6 Hz, 6H); ESI-MS *m/z*: 548 $[M+18(H_2O)]^+$. Compound **11**: Yield, 88%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.96 (d, J = 1.5 Hz, 2H), 5.94 (d, J = 1.5 Hz, 2H), 4.85 (s, 4H), 3.93 (s, 6H), 2.24–2.20 (m,

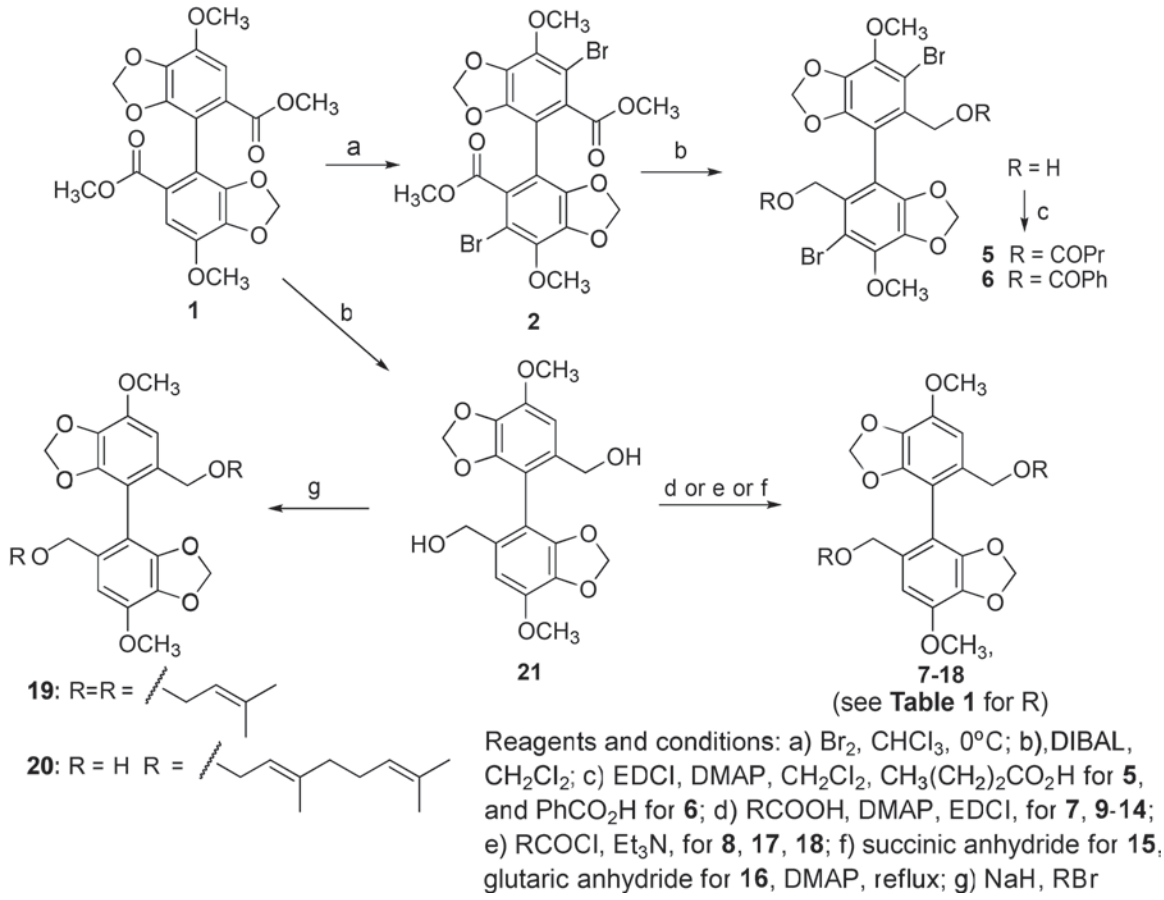


Figure 2. Syntheses of DDB analogs.

4H), 1.57–1.19 (m), 0.87 (t, J=6.8 Hz, 6H); ESI-MS m/z: 744 $[M+18(H_2O)]^+$. Compound **12**: Yield, 8%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.96–6.87 (m), 6.7 (s, 2H), 5.94 (s, 4H), 5.78 (d, J= 15.6 Hz, 2H), 4.94 (d, J=12.4 Hz, 2H), 4.85 (d, J=12.4 Hz, 2H), 3.93 (s, 6H), 1.85 (d, J=6.8 Hz, 6H); ESI-MS m/z: 544 $[M+46(HCOOH)]^+$. Compound **13**: Yield, 51%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.70 (s, 2H), 5.94 (d, J=1.5 Hz, 2H), 5.93 (d, J=1.5 Hz, 2H), 5.62 (s, 2H), 4.95 (d, J=12.5 Hz, 2H), 4.82 (d, J=12.5 Hz, 2H), 3.93 (s, 6H), 2.12 (s, 6H), 1.86 (s, 6H); ESI-MS m/z: 568 $[M+42(CH_3CN+H)]^+$. Compound **14**: Yield, 55%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.19 (d, J=9.8 Hz, 1H), 7.15 (d, J=9.8 Hz, 1H), 6.70 (s, 2H), 6.18–6.08 (m, 4H), 5.93 (d, J=1.5 Hz, 4H), 5.71 (s, 1H), 5.68 (s, 1H), 4.96 (d, J=12.3 Hz, 4H), 4.87 (d, J=12.3 Hz, 4H), 3.93 (s, 6H), 1.83 (d, J= 5.2 Hz, 6H); ESI-MS m/z: 516 $[M-34]^+$.

General procedure for compounds 8, 17, and 18

Compound **21** and triethylamine (5-10 eq. mole) were first added to anhydrous dichloromethane, and then the appropriate acyl chloride (2.2 eq. mole) was added at 0°C under nitrogen. The reaction was warmed gradually to room temperature and stirred for 1–3 h. After the reaction was completed, water and saturated sodium carbonate solution were added and the reaction mixture was extracted with dichloromethane, dried over sodium sulfate, and concentrated. Further purification was done by combiflash (hexane-ethyl acetate gradient).

Compound **8**: Yield, 99%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.95 (d, J=6.5 Hz, 4H), 4.85 (s, 4H), 3.93 (s, 6H), 2.26 (q, J=7.2 Hz, 4H), 1.08 (t, J=7.6 Hz, 6H); ESI-MS m/z: 492 $[M+18(H_2O)]^+$. Compound **17**: Yield, 85%; Orange amorphous solid; ^{1}H NMR ($CDCl_3$) δ 7.43 (s, 2H), 7.34 (d, J=15.6Hz, 2H), 6.73 (s, 2H), 6.58 (d, J=1.2 Hz, 2H), 6.44 (m, 2H), 6.22 (d, J=16 Hz, 2H), 5.95 (d, J=2.4 Hz, 4H), 5.01 (d, J=12.4 Hz, 2H), 4.95 (d, J=12.4 Hz, 2H), 3.93 (s, 6H); ESI-MS m/z: 620 $[M+18(H_2O)]^+$. Compound **18**: Yield, 85%; Colorless amorphous solid; ^{1}H NMR ($CDCl_3$) δ 7.53 (m, 2H), 7.34 (s, 2H), 6.77 (s, 2H), 6.75 (s, 2H), 5.99 (s, 4H), 5.93 (s, 2H), 5.88 (s, 2H), 5.10 (d, J=12.4 Hz, 2H), 5.02 (d, J=12.4 Hz, 2H), 3.93 (s, 6H); ESI-MS m/z: 676 $[M+18(H_2O)]^+$.

Compounds 15 and 16

Succinic anhydride (for **15**) or glutaric anhydride (for **16**) (1.2 eq. mole) and DMAP (5% w/w) was added to a flask containing **21** in anhydrous tetrahydrofuran. The solution was refluxed under nitrogen overnight. A solution of 1N hydrochloric acid was added to acidify the reaction mixture and ethyl acetate was used three times successively for extraction of the aqueous layer. Preparative TLC (dichloromethane–methanol) was applied to purify the desired compound from the combined aqueous extracts.

Compound **15**: Yield, 28%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.67 (s, 2H), 5.96 (d, J=16.8 Hz, 4H), 4.94 (d, J=12.6 Hz, 2H), 4.89 (d, J=12.6 Hz, 2H), 3.93 (s,

6H), 2.59–2.54 (m, 8H); ESI-MS *m/z*: 580 $[M+18(H_2O)]^+$. Compound **16**: Yield, 7%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.68 (s, 2H), 5.96 (d, J = 13.6 Hz, 4H), 4.90 (d, J = 12.5 Hz, 2H), 4.84 (d, J = 12.5 Hz, 2H), 2.38–2.31 (m, 8H), 1.93–1.84 (m, 4H); ESI-MS *m/z*: 608 $[M+18(H_2O)]^+$.

Compounds 19 and 20

Compound **21** in anhydrous tetrahydrofuran was added slowly to a flask with sodium hydride (5 eq. mole) in anhydrous tetrahydrofuran under nitrogen at 0°C. After 10 min, 3,3-dimethylallyl bromide (3 eq. mole for **19**) or geranyl bromide (3 eq. mole for **20**) was added. When the starting material disappeared, water was added to quench the reaction. The aqueous solution was partitioned with ethyl acetate. The organic layer was washed with sodium bicarbonate solution and then dried over sodium sulfate. Desired compounds were purified by preparative TLC with a hexane-ethyl acetate system.

Compound **19**: Yield, 43%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.78 (s, 2H), 5.92 (s, 4H), 5.23 (m, 2H), 4.23 (d, J = 12.1 Hz, 2H), 4.16 (d, J = 12.1 Hz, 2H), 3.94 (s, 6H), 3.82 (d, J = 6.9 Hz, 4H), 1.70 (s, 6H), 1.59 (s, 6H); ESI-MS *m/z*: 521 $[M+23(Na)]^+$. Compound **20**: Yield, 47%; Colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.76 (s, 1H), 6.69 (s, 1H), 5.69–5.93(m, 4H), 5.11–5.05 (m, 2H), 4.39–4.11 (m, 4H), 3.94 (s, 6H), 3.84 (m, 2H), 2.10–1.95 (m, 4H), 1.67 (s, 3H), 1.58 (s, 3H), 1.57 (s, 3H); ESI-MS *m/z*: 481 $[M-17(OH)]^+$.

In vitro EBV-EA activation experiment

Raji cells (10^6 cells/mL) were incubated at 37°C for 48 h in RPMI-1640 medium with 10% fetal calf serum (FCS), *n*-butyric acid (4 mmol), TPA (32 pmol), and test compounds. Smears were made from the cell suspension, and the EBV-EA inducing cells were stained by an indirect immunofluorescence technique. In each assay, at least 500 cells were counted and the number of stained cells (positive cells) was recorded. Each assay was repeated three times for one test compound. The EBV-EA-inhibiting activity of the test compound was estimated on the basis of the percentage of the number of positive cells compared with that of the control without the test compound. The viability of the cells was assayed by the Trypan Blue staining method. For the determination of cytotoxicity, the cell viability was required to be more than 60% (Iwase et al., 2000).

In vivo two-stage mouse skin carcinogenesis test

A total of 30 female imprinting control region (ICR) mice (6 weeks old, purchased from SLC Co. Ltd., Shizouka, Japan) were used. Two groups, with each group consisting of 15 animals, housed at five/cage, were painted with 390 nmol of 7,12-dimethylbenz[*a*]anthracene (DMBA) in acetone, 0.1 mL/mouse, on a shaved region of skin on the back. After 1 week, the mice were treated topically with 1.7 nmol of TPA in acetone (0.1 mol) twice a week for 20 weeks. One hour prior to TPA treatment, the animals in group I were treated with acetone (0.1 mL) alone, serving as a promotion-positive control. The animals in group II were treated with the test compound (85 nmol) in acetone (0.1 mL). The incidence of papilloma was observed weekly for 15 weeks. The differences in the occurrence of mouse skin papillomas between the control and treatment groups were analyzed by means of the Student's *t*-test after 15 weeks of promotion.

Results and discussion

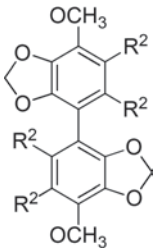
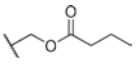
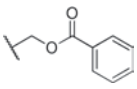
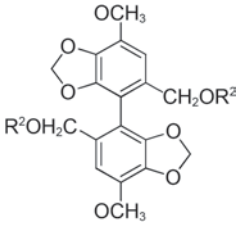
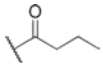
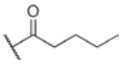
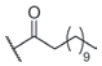
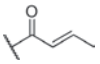
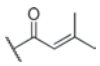
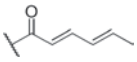
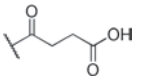
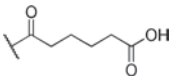
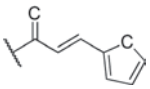
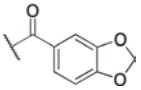
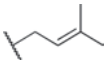
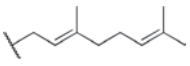
Chemistry

DDB analogs (Table 1) were synthesized following literature methods (Xie et al., 1995). Reduction of **1** and 3,3′-dibromo-DDB (**2**) with diisobutylaluminum hydride (DIBAL) resulted in the related 2,2′-methylene alcohols **21** and **3**, respectively (Figure 2). Diols **21** and **3** were then converted to various ester and ether analogs as shown in Figure 2. Esterifications of **21** and **3** were carried out in the presence of either excess carboxylic acid, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), and 4-dimethylaminopyridine (DMAP) or acyl chloride and triethylamine at 0°C. A hydrophilic group was introduced by coupling with succinic anhydride or glutaric anhydride. Bisprenyl ether **19** and mono-geranyl ether **20** were obtained by Williamson ether synthesis of **21** with prenylbromide and geranylbromide, respectively, in the presence of sodium hydride. It should be noted that the bisgeranyl ether was not stable and decomposed easily after purification. Therefore, only the mono-geranyl ether **20** was obtained.

In vitro EBV-EA inhibition of DDB analogs

All analogs were evaluated in a short-term *in vitro* EBV-EA inhibition assay to determine their cancer prevention potential, and the results are shown in Table 1. All tested compounds showed relatively potent inhibition of EBV activation. The analogs with unsaturated alkyl side chains and terminal carboxylic acids, such as **12–16, 19, and 20**, significantly inhibited EBV-EA activation, showing 95.9%–100% inhibition at the highest tested concentration, and showed greater inhibitory effects than the parent compound **1**. In particular, the most potent compound **19** displayed 100% inhibition at 1×10^3 mol ratio/TPA, and 78.4, 49.7, and 10.9% inhibition at 5×10^2, 1×10^2, 1×10 mol ratio/TPA, respectively, with an IC_{50} value of 252 mol ratio/TPA. At the higher concentrations of 1×10^3 and 5×10^2 mol ratio/TPA, the inhibition values with **19** were comparable to those of curcumin, which is a known potent cancer preventive agent. Moreover, even at low concentrations, **19** inhibited EBV-EA activation and the inhibitory effects of **19** were notably greater than those of curcumin at 1×10^2 and 1×10 mol ratio/TPA. The analogs with prenyl-like unsaturated alkyl groups, such as **12–14**, **19**, and **20** exhibited relatively high activity. This finding is consistent with other reports that a prenyl-like group tends to enhance the inhibitory effect on EBA activation (Tatsuzaki et al., 2010). The presence of an aromatic ring on the C-2,2′ side chain, as found in **6**, **17**,

Table 1. DDB analogs and their EBV-EA inhibition ability.

Cmpd No.		R^1	R^2	Percentage of EBV-EA positive cells concentration (mol ratio/TPA[b]) 1000	500	100	10	IC_{50}[d]
1		H		6.3 (70)[c]	32.8	67.8	97.3	341
2		Br		14.6 (60)	40.2	76.0	100	403
3		Br		8.4 (60)	36.0	71.2	100	358
4		Br		7.9 (60)	35.8	68.2	98.6	349
5		Br		7.9 (60)	35.8	68.2	98.6	349
6		Br		11.5 (60)	38.6	75.5	100	389
7				13.1 (60)	37.0	71.3	100	390
8				12.0 (60)	35.9	70.0	100	381
9				11.2 (60)	37.0	72.0	100	380
10				10.3 (60)	37.4	71.7	100	379
11				15.6 (60)	41.0	76.9	100	426
12				3.1 (70)	27.0	54.9	93.4	278
13				1.7 (70)	23.5	51.6	90.3	260
14				2.9 (70)	25.1	53.7	91.7	269
15				2.1 (60)	24.8	52.6	91.5	265
16				4.1 (60)	28.6	57.4	96.6	287
17				9.5 (60)	36.0	71.0	100	372
18				8.9 (60)	35.7	71.5	100	369
19				0 (70)	21.6	50.3	89.1	252
20[a]				1.9 (70)	24.6	52.9	91.6	263
curcumin				0 (60)	21.1	80.1	100	379

[a]In this structure, only one R^2 is the moiety shown above; the other R^2 is a hydrogen group.
[b]TPA concentration is 32 pmol/mL.
[c]Values in parentheses are viability percentages of Raji cells.
[d]IC_{50} in mol/ratio TPA.

and **18**, reduced the inhibitory effect on EBV-EA activation. The effect of bromide depended on the functional group at C-2 and -2′. With 2,2′-biscarbomethoxy substitution, the 3,3′-dibromo analog **2** showed lower potency than the parent compound **1**, while with 2,2′-bisbutyryloxymethyl substitution, the 3,3′-dibromo analog **5** showed higher potency than the related non-brominated compound **9**. The esters **7–10** with linear saturated fatty acids of varying lengths demonstrated almost similar potency, indicating that the length of the alkyl chain is not crucial for the activity; however, the activity decreased with a chain length of 12 carbons (analog **11**). Compounds **15** and **16** with terminal carboxylic acids on the 2,2′-fanctional groups exhibited better activity than the parent compound **1**. In a direct comparison, the succinate side chain (**15**) was better than glutarate side chain (**16**) in terms of potency.

In vivo mouse skin carcinogenesis inhibition

The *in vitro* inhibitory effects determined in the EBV-EA assay generally have been found to correlate well with *in vivo* inhibitory effects on tumor promotion as reported in many studies (Konoshima et al., 1994; Ishida et al., 2000, 2002; Sakurai et al., 2003; Wang et al., 2006). Therefore, based on the *in vitro* data, only three of the most potent compounds (**13**, **15**, and **19**) were examined in a two-stage *in vivo* skin carcinogenesis test evaluating mouse skin papilloma induced by DMBA as an initiator and TPA as a promoter (Table 2). The compounds' activities were determined by both the percentage of papilloma-bearing mice (Figure 3A) and the average number of papillomas/mouse (Figure 3B), compared with the positive control. All three compounds delayed the appearance of the first tumor for 2 weeks compared with the positive control.

Table 2. *In vivo* inhibitory effects of **13**, **15**, and **19** on two-stage mouse carcinogenesis.

	Papilloma (%)				Papillomas/mouse			
Week	Positive control[a]	**13**[b]	**15**[b]	**19**[b]	Positive control[a]	**13**[b]	**15**[b]	**19**[b]
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	6.6	0	0	0	0.4	0	0	0
7	20.0	0	0	0	0.9	0	0	0
8	40.0	6.6	13.3	6.6	1.8	0.7	0.9	0.6
9	73.3	13.3	26.6	13.3	2.4	1.6	2.0	1.4
10	86.6	26.6	33.3	26.6	3.5	2.0	2.2	1.8
11	100	33.3	40.0	33.3	3.9	2.4	2.6	2.0
12	100	40.0	53.3	40.0	4.3	2.6	2.9	2.4
13	100	53.3	66.6	53.3	5.2	3.3	3.6	3.1
14	100	66.6	73.3	66.6	5.9	4.3	4.5	4.0
15	100	73.3	73.3	66.6	6.3	4.6	5.1	4.4

[a]The positive control is DMBA (390 nmol) plus TPA (1.7 nmol).
[b]The concentration of compound is 85 nmol.

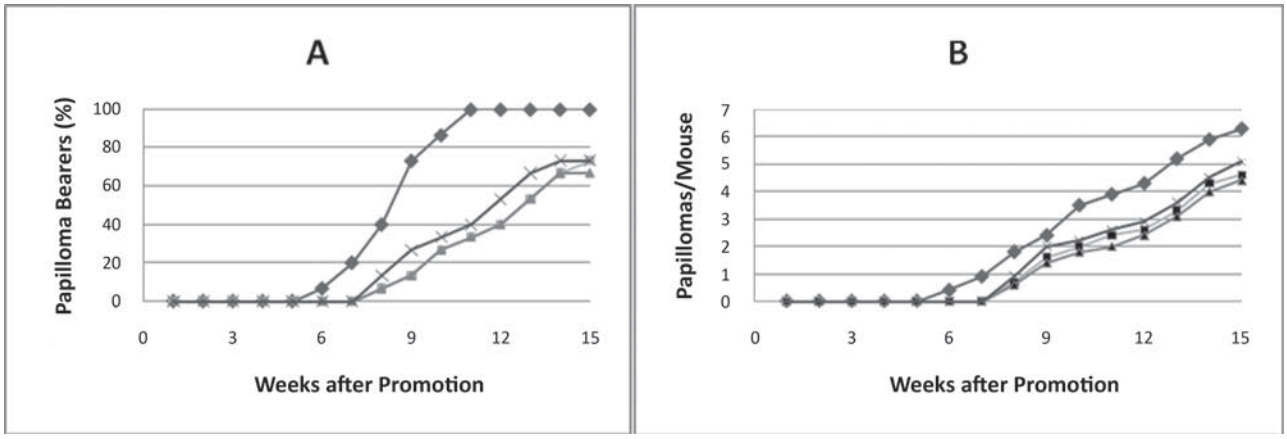


Figure 3. Inhibitory effects of compounds **13, 15,** and **19** on DMBA–TPA mouse skin carcinogenesis. Tumor formation in all mice was initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly beginning 1 week after initiation. (A) Papilloma percentage in mice. (B) Average number of papillomas/mouse. (◆) Control TPA alone; (■) TPA + compound **13** (85 nmol); (×) TPA+ compound **15** (85 nmol); (▲) TPA +compound **19** (85 nmol). After 15 weeks of promotion, a significant difference in the number of papillomas/mouse between the treated groups and the control group was evident (p <0.05). In Figure 3A, the trace for compound **19** is superimposed with that for compound **13**.

In the positive control group, 6.6, 40, and 100% of the mice bore papillomas after 6, 8, and 11 weeks of promotion, respectively, and 6.3 papillomas were formed/mouse after 15 weeks. However, in the groups treated with compounds **13** and **19**, 0, 7, and 33% of the mice bore papillomas at weeks 6, 8, and 11, respectively, and 4.4–5.1 papillomas/mouse were found with all three tested compounds, even after 15 weeks of promotion.

Conclusions

Several 2,2′-bismethyl ester and ether DDB analogs were designed and synthesized. All analogs showed potent EBV-EA inhibition *in vitro*. Among them, analogs **12–16**, **19**, and **20** with unsaturated side chains or terminal carboxylic acids significantly inhibited the EBV-EA activation. In particular, prenyl derivative **19** showed the highest inhibitory effects (100%, 78.4%, 49.7% and 10.9% inhibition at 1×10^3, 5×10^2, 1×10^2, 1×10 mol ratio/TPA, respectively), which were greater than those of curcumin at the low concentrations. In an *in vivo* assay, DDB analogs **13**, **15**, and **19** also delayed the formation of mouse skin papillomas after initiation and promotion by a cancer promoting substance. The DDB has been used clinically, which implies that DDB analogs have good probability to be further developed as potent cancer preventive agents for clinical use. Thus, DDB analog **19** could be a valuable candidate as a cancer preventive agent or as a lead for the development of new antitumor promoter drugs.

Declaration of interest

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