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Chemopreventive Potential of Cyclic Diarylheptanoids

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Abstract—Eleven cyclic diarylheptanoids and seven related compounds were screened as potential antitumor promoters by using the in vitro short-term 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein–Barr virus early antigen (EBV-EA) activation assay. In addition, the cyclic diarylheptanoid myricanone (**2**) was examined for antitumor initiating activity in a two-stage carcinogenesis assay of mouse skin tumors induced by peroxynitrite as an initiator and TPA as a promoter. Myricanone (**2**) exhibited significant antitumor-initiating effect on mouse skin. These data suggest that cyclic, as well as linear, diarylheptanoids might be valuable chemopreventors.

Introduction

Diarylheptanoids belong to a class of natural products based on 1,7-diphenylheptane. Their structures are classified into linear and cyclic types, and the latter is divided further into biphenyl ([7.0]-metacyclophanes) and diphenyl ether (14-oxa-[7.1]-metaparacyclophanes) types. Each cyclic type may be formed from the corresponding linear type by phenolic oxidative coupling.[1]

Linear type diarylheptanoids exhibit a broad range of potent biological activities including anti-inflammatory, antihepatoxic, antifungal, antibacterial, and related effects.[2] For example, curcumin (**1**) (Fig. 1), a yellow component present in turmeric (*Curcuma longa* Linn) and that has been used as a coloring agent exerts anticarcinogenic or anti-mutagenic effects in diverse animal models and also in cultured cells.[3–9] It has also been found to have antioxidant and oxygen radical scavenging activities, including the inhibition of ROS (superoxide, peroxyl, and hydroxy radicals) and NO donor effect.[10,11] However, few studies have been reported on cancer chemopreventive activity of the cyclic diarylheptanoid class.

Recently, we described the significant inhibitory effect of the cyclic diarylheptanoid myricanone (**2**) (Fig. 1), derived from *Myrica rubra,* using the in vitro short-term 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein–Barr virus early antigen (EBV-EA) activation assay.[12] We have also found that **2** strongly inhibited tumor promotion induced by TPA following initiation with 7,12-dimethylbenz [α] anthracene (DMBA) in mouse skin.[12] In this study, we have extended our screening to clarify the structure–activity relationships for antitumor promotion activity in this compound class. In addition, we also assayed the antitumor-initiating activity of **2** in a two-stage mouse skin carcinogenesis test initiated by peroxynitrite. These results should help in understanding the physiological functions and biochemical mechanism of cancer chemopreventive activity of cyclic diarylheptanoids.

Chemistry

The chemical structures are shown in Figure 1. The isolation of test compounds, 12-dehydroporson (**3**), porson (**4**), myricanone oxime (**5**), myricamine (**6**), acerogenin K (**7**), isomyricanone (**8**), acerogenin C (**10**), acerogenin C oxime (**11**), galeon (**12**), (−)-centrolobol (**13**), (−)-di-*O*-methylcentrolobol (**14**), and (+)-rhododendrol (**16**), was described previously.[13,14] Compound **15** was synthesized by refluxing 5-bromovanillin with copper powder in anhydrous DMF. Compound **17** was purchased from Tokyo Kasei Kogyo Co. Compound **18** was synthesized by methylation of **17** with CH_3I/K_2CO_3 in refluxing dry acetone. The structures of compounds **15** and **18** were confirmed by NMR analysis and MS analysis, respectively. All compounds were recrystallized and were homogeneous by TLC.

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Results and Discussion

In vitro EBV-EA activation

The primary screening test was carried out utilizing a short-term in vitro synergistic assay on EBV-EA activation. Table 1 lists inhibitory effects of **1**–**18** on the EBV-EA activation induced by TPA and the associated viability of Raji cells. Comparison of the data in Table 1, including statistical discussion, suggests the following general trends.

At 100 mol ratio/TPA, all compounds tested in this study, except **6**, showed inhibitory effects on EBV-EA activation without cytotoxicity on Raji cells as shown in Table 1. Particularly, myricanone (**2**), 12-dehydroporson (**3**), and curcumin (**1**) showed strongly increased inhibitory effects with increasing concentration, as reflected by complete inhibition at 1000 mol ratio/TPA. Compounds **1**[20] and **2**[12] have been reported previously as potent antitumor promoters.

We previously reported that the extended quinol structure of **2** is important in maintaining the antitumor promoting activity of cyclic diarylheptanoids.[12] Therefore, compounds **3** and **4**, which are methylated at the 5-hydroxy group, should be less potent than curcumin (**1**). However, although **4** showed reduced activity as expected, **3** was essentially equally potent to **1**. Comparing the structures of **3** (α-diketo at C-11,12 positions), **4** (α-ketol), and monomethylmyricanone (11-ketone), which was also less active than **2** as reported previously (data not shown),[12] indicated that the α-diketo moiety in the heptane chain is important for activity.

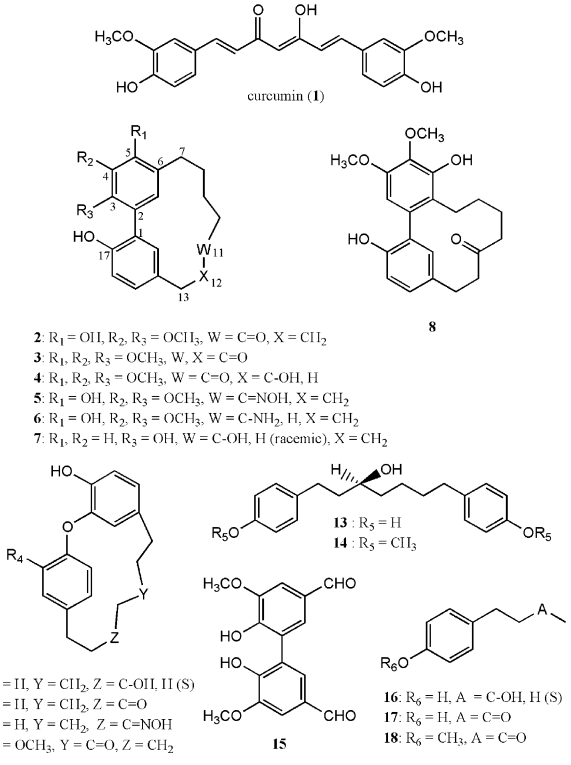

Figure 1. Structures of cyclic diarylheptanoids and derivatives.

In contrast, replacing the C-11 carbonyl group of **2** with other functional groups, including oxime (**5**) and amine (**6**), decreased the activity. In addition, **7**, which has a hydroxy rather than a carbonyl group at C-11, showed reduced activity. Also, isomerization of **2**, which gave the [7-*ortho*, *meta*-0] cyclophane structure **8**, decreased the activity. Thus, the 13-membered ring seems to be important for the antitumor-promoting activity, perhaps because of steric factors.

All tested diphenyl ether-type cyclic diarylheptanoids (**9**–**12**) were less active than the biphenyl-type compounds (**2**, **3**, and 13-oxomyricanol).[12] In addition, **13**, which is the linear biosynthetic precursor to **9**,[12] and **14** exhibited moderate activity. Thus, cyclization to the diphenyl ether-type cyclic diarylheptanoid diminished the antitumor promoting activity. We also tested **15**–**18**, which constitute partial structures found in **2**. These four compounds showed reduced activity, thus emphasizing the importance of the cyclic structure.

In summary, the following conclusions were drawn. (a) The presence of an α-diketo moiety in the side chain is important to activity. (b) Replacing the side-chain ketone with other substituents (amine, oxime, hydroxyl) reduced activity. (c) Cyclization as the *meta, meta*-bridged biphenyl structure, which produces a 13-membered ring, rather than the diphenyl ether-type structure is important for antitumor promotion activity.

In vivo two-stage carcinogenesis test on mouse skin papillomas initiated by peroxynitrite

On the basis of the above results, myricanone (**2**) was the most active antitumor promoting agent among the tested cyclic diarylheptanoids. To elucidate the biochemical mechanism of cancer chemopreventive activity of **2**, we tested **2** for its effect on in vivo mouse skin carcinogenesis, initiated by peroxynitrite and promoted by TPA. The incidence (%) of papilloma-bearing mice and the average numbers of papillomas per mouse are presented in Figure 2A and B, respectively.

Figure 2A shows the time course of tumor formation in the two groups. In group I (control, treated with peroxynitrite/TPA), the first tumor appeared after 6 weeks. In group II (treated with peroxynitrite and myricanone/TPA), the first tumor appeared after 8 weeks. The percentage of tumor-bearing mice in group I was 100% after 10 weeks, whereas that in group II was approximately 26% at that same time and 86% after 20 weeks of promotion. Figure 2B shows the average number of tumors per mouse. Group I produced approximately eight tumors per mouse after 20 weeks, whereas group II had only approximately five tumors per mouse. Therefore, treatment with 0.0025% of myricanone (group II) caused 38% reduction in the average number of tumors per mouse after 20 weeks of promotion.

From these results, myricanone (**2**) appears effective for the inhibition of peroxynitrite-induced carcinogenesis on mouse skin. Peroxynitrite, which is produced by the reaction of nitric oxide (NO) with superoxides, is a

potent oxidant as well as nitrating and hydroxylating agent. We have previously reported that peroxynitrite shows tumor initiation.[17] Together with results from our previous study,[12] these data show that **2** plays a useful physiological role, including both anti-initiating and anti-promoting activity on two-stage carcinogenesis. Curcumin (**1**) is known to have potent antioxidant activity, which suggests that it intercepts and neutralizes potent chemical carcinogens, such as ROS (superoxide, peroxyl, hydroxy radicals) and NO donors.[10,11] Correspondingly, possible antioxidative effects of the cyclized curcumin derivative myricanone (**2**) might account for the observed inhibition of peroxynitrite-induced carcinogenesis with this compound.

Conclusions

In summary, not only linear-type (**1**) but also cyclic-type (**2**) diarylheptanoids can have substantial chemopreventive properties, and **2** could be a valuable chemopreventor. Additional study is needed to elucidate the relationship of diarylheptanoid structure to chemopreventor effects. However, these derivatives offer new insights into possible derivatives that may hold promise for the development of potent new chemopreventive agents.

Experimental

Chemicals

Cell culture reagents, *n*-butyric acid, and other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). TPA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). EBA-EA positive serum from a patient with nasopharyngeal carcinoma (NPC), used for an immunofluorescence test, was a gift from Prof. H. Hattori, Department of Otorhinolaryngology, Kobe University. Peroxynitrite was purchased from Dojindo Laboratories Co (Kumamoto, Japan).

Cells

EBV genome carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured in RPMI-1640 medium (Nissui, Tokyo, Japan) under the conditions described previously.[15] Spontaneous activation of EBV-EA in our subline of Raji cells was less than 0.1%.

Animals

Specific pathogen-free (SPF) female SENCAR mice (6 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan), and maintained under SPF conditions in Kyoto Prefectural University of Medicine, Animal Center. The mice were housed five per polycarbonate cage, in a temperature-controlled room at $24\pm2\,^{\circ}C$, and given food (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and water ad libitum during the experiments.

In vitro EBV-EA activation experiments

The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type), an EBV genome-carrying human lymphoblastoid cell, which were cultivated in 10% fetal bovine serum (FBS) RPMI 1640 medium. The indicator cells (Raji, 1×10^6/mL) were incubated at 37 °C for 48 h in 1 mL of medium contain-

Table 1. Relative ratio[a] of EBV-EA activation with respect to positive control (100%) in presence of myricanone and its derivatives (**1**–**18**)

Compound	Concentration (mol ratio/TPA)[b]				
	1000	500	100	10	IC_{50}[c]
Curcumin (**1**)[d]	0.0±0.5 (60)	22.8±1.8 (>80)	81.7 +1.9 (>80)	100.0±0.2 (>80)	320
Myricanone (**2**)[d]	0.0±0.3 (60)	25.4±1.2 (>80)	76.4±2.2 (>80)	90.9±0.6 (>80)	260
3	0.0±0.6 (70)	28.5±1.2 (>80)	77.0±1.6 (>80)	96.8±0.5 (>80)	300
4	10.5±0.8 (60)	39.6±1.4 (>80)	75.4±1.7 (>80)	100.0±0.6 (>80)	450
5	21.8±1.1 (70)	51.7±1.9 (>80)	84.9±2.0 (>80)	100.0±0.3 (>80)	500
6	40.6±1.3 (70)	72.1±2.1 (>80)	93.7±1.9 (>80)	100.0±0.0 (>80)	820
7	7.8±1.2 (60)	37.4±1.1 (>80)	76.8±2.0 (>80)	100.0±0.3 (>80)	420
8	5.7±0.5 (60)	33.7±1.2 (>80)	75.9±2.0 (>80)	100.0±0.7 (>80)	350
9[d]	18.2±0.6 (60)	47.0±1.5 (>80)	83.5±2.7 (>80)	100.0±0.3 (>80)	490
10	20.3±0.6 (60)	60.1±1.3 (>80)	82.0±1.9 (>80)	100.0±0.2 (>80)	620
11	15.5±0.4 (60)	51.7±1.9 (>80)	75.0±2.2 (>80)	100.0+0.4 (>80)	450
12	18.6±0.3 (60)	54.0±1.2 (>80)	78.9±1.9 (>80)	100.0+0.2 (>80)	580
13	5.0±0.5 (60)	36.6±1.2 (>80)	74.0±2.0 (>80)	96.0±0.5 (>80)	320
14	8.3±0.5 (60)	39.7±1.1 (>80)	77.2±1.9 (>80)	100.0±0.6 (>80)	420
15	9.5±0.3 (60)	43.2±1.9 (>80)	72.4±2.1 (>80)	100.0±0.5 (>80)	340
16	8.7±0.5 (60)	39.8±1.2 (>80)	75.1±1.9 (>80)	100.0±0.3 (>80)	360
17	10.2±0.6 (60)	40.4±1.5 (>80)	75.7±1.8 (>80)	100.0±0.8 (>80)	400
18	14.8±0.9 (60)	43.0±1.9 (>80)	76.9±2.1 (>80)	100.0±0.4 (>80)	460

[a]Values represent relative percentage to the positive control value (100%). Data are expressed as mean±SD. Values in parentheses are viability percentages of Raji cells.
[b]Mol ratio/TPA (20 ng = 32 pmol/mL). The values of all compounds at 500 mol ratio/TPA and the values of **3** at 100 mol ratio/TPA were significantly different from the control value ($p<0.05$) without cytotoxicity.
[c]The concentration (Mol ratio/TPA) needed to inhibit 50% of positive control activated with TPA.
[d]Ishida et al. reported previously.[12]

ing *n*-butyric acid (4 mM, inducer), TPA (32 nM), and various amounts of the test compounds dissolved in 5 μL of DMSO. Smears were made from the cell suspension. The EBV-EA inducing cells were stained with high titer EBV-EA positive serum from NPC patients and detected by an indirect immunofluorescence technique.[16] In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. Triplicate assays were performed for each data point. The EBV-EA inhibitory activity of the test compound was expressed by comparison with that of the positive control experiment (100%), which was carried out with *n*-butyric acid (4 mM) plus TPA (32 nM). In the experiments, the EBV-EA induction was ordinarily around 35%, and this value was taken as the positive control (100%). *n*-Butyric acid (4 mM) alone induced 0.1% EA-positive cells. The viability of treated Raji cells was assayed by the trypan-blue staining method. Student's *t*-test was used for all statistical analyses.

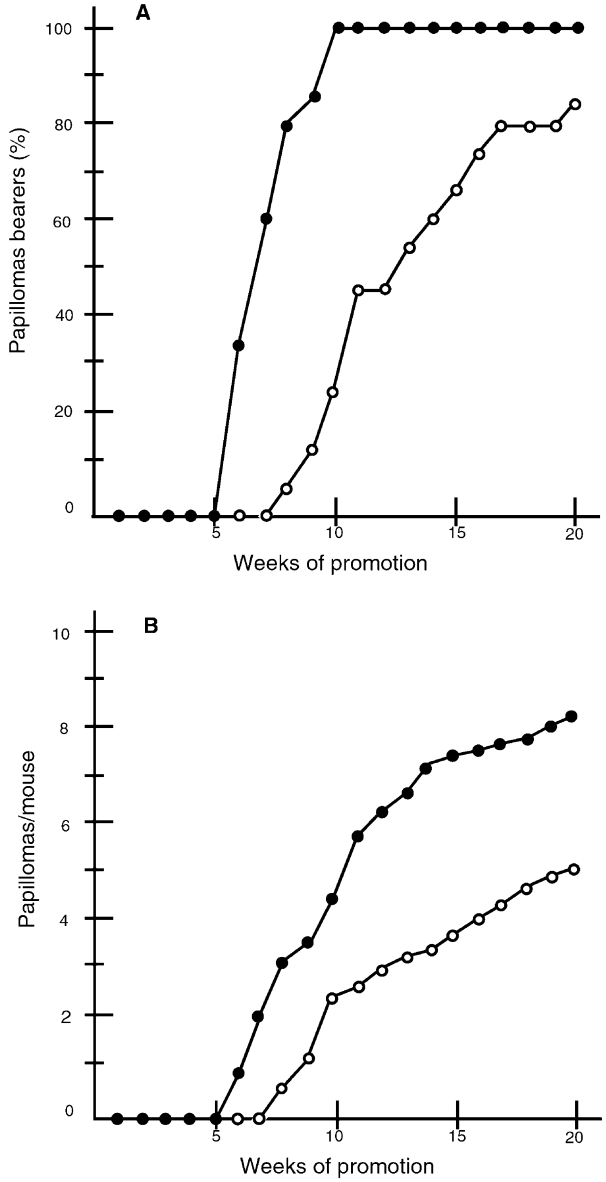

Figure 2. Inhibition of peroxynitrite/TPA-induced tumor promotion by multiple application of myricanone (**2**). (A) Percentage of mice bearing papillomas. (B) Average number of papillomas per mouse. ●, peroxynitrite (390 nmol) + TPA (1.7 nmol) (Group I); ○, peroxynitrite (390 nmol) + 0.0025% of **2** (2 weeks) + TPA (1.7 nmol) (Group II). At 20 weeks of promotion, the number of papillomas per mouse differed significantly ($p < 0.05$) between Group I and Group II. The number (standard deviations are shown in parentheses) of papillomas per mouse for each group was 8.1 (1.9) and 5.0 (2.0) for Groups I and II, respectively.

In vivo two-stage carcinogenesis test on mouse skin papillomas initiated by peroxynitrite[17]

The animals were divided into two experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were treated topically with peroxynitrite (390 nmol) in 1 mM NaOH (0.1 mL). One week after the initiation, papilloma formation was promoted by the twice-a-week application of TPA (1.7 nmol) in acetone (0.1 mL) on the skin (no papilloma formation was seen with topical application of the acetone solvent alone). Test samples (0.0025% myricanone) were orally fed with drinking water for only 2 weeks before the promotion treatment (1 week both before and after initiation). Two groups received initiation/promotion treatments as follows: (I) peroxynitrite/TPA, $n = 15$ (II) peroxynitrite and myricanone/TPA, $n = 15$. The incidence and numbers of papillomas were detected weekly for 20 weeks, as described.[18,19] Student's *t*-test was used for all statistical analyses.

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References and Notes

1. Henley-Smith, P.; Whiting, D. A.; Wood, A. F. *J. Chem. Soc., Perkin Trans. 1* **1980**, 614.
2. Keseru, G. M.; Nogradi, M. In *Studies in Natural Products Chemistry*; Atta-ur-Pahman, Ed.; Elsevier Science: New York, 1995; Vol. 17, p 357.
3. Soudamini, K. K.; Kuttan, R. *J. Ethnopharmacol.* **1989**, *27*, 227.
4. Nagabhushan, M.; Bhide, S. V. *J. Am. Coll. Nutr.* **1992**, *11*, 192.
5. Conney, A. H.; Lou, Y. R.; Xie, J. G.; Osawa, T.; Newmark, H. L.; Liu, Y.; Chang, R. L.; Huang, M. T. *Proc. Soc. Exp. Biol. Med.* **1997**, *216*, 234.
6. Rao, C. V.; Rivenson, A.; Simi, B.; Reddy, B. S. *Cancer Res.* **1995**, *55*, 259.
7. Abraham, S. K.; Sarma, L.; Kesavan, P. C. *Mutat. Res.* **1993**, *303*, 109.
8. Kuo, M. L.; Huang, J. T. S.; Lin, K. *Biochim. Biophys. Acta* **1996**, *1317*, 95.
9. Mehta, K.; Pantazis, P.; McQeen, T.; Aggarwal, B. B. *Anticancer Drugs* **1997**, *8*, 470.
10. Masuda, T.; Hidaka, K.; Shinohara, A.; Maekawa, T.; Takeda, Y.; Yamaguchi, H. *J. Agric. Food Chem.* **1999**, *47*, 71.
11. Onoda, M.; Inano, H. *Nitric Oxide* **2000**, *4*, 505.

12. Ishida, J.; Kozuka, M.; Wang, H. K.; Konoshima, T.; Tokuda, H.; Okuda, M.; Mou, X. Y.; Nishino, H.; Sakurai, N.; Lee, K. H.; Nagai, M. *Cancer Lett*. **2000**, *159*, 135.
13. Nagai, M.; Dohi, J.; Morihara, M.; Sakurai, N. *Chem. Pharm. Bull*. **1995**, *43*, 1674.
14. Inoue, T. *Yakugaku Zasshi* **1993**, *113*, 181.
15. Takasaki, M.; Konoshima, T.; Fujitani, K.; Yoshida, S.; Nishimura, H.; Tokuda, H.; Nishino, H.; Iwashima, A.; Kozuka, M. *Chem. Pharm. Bull*. **1990**, *38*, 2737.
16. Henle, G.; Henle, W. *J. Bacteriol*. **1966**, *91*, 1248.
17. (a) Tokuda, H.; Okuda, M.; Mou, X. Y.; Mukainaka, T.; Ichiishi, E.; Nishino, H.; Takasaki, M.; Konoshima, T. *Arch. Biochem. Biophys. Part B* **2000**, *3*, 256. (b) Tokuda, H.; Okuda, M.; Mou, X. Y.; Ichiishi, E.; Mukainaka, T.; Nishino, H.; Takasaki, M.; Konoshima, T. *Arch. Biochem. Biophys. Part B* **2000**, *3*, 284.
18. Weeks, C. W.; Slaga, T. J.; Hennings, H.; Gleason, G. L.; Bracken, W. M. *J. Natl. Cancer Inst*. **1979**, *63*, 401.
19. Tokuda, H.; Ohigashi, H.; Koshimizu, K.; Ito, Y. *Cancer Lett*. **1986**, *33*, 279.
20. Huang, M. T.; Ma, W.; Lu, Y. P.; Chang, R. L.; Fisher, C.; Manchand, P. S.; Newmark, H. L.; Conney, H. *Carcinogenesis* **1995**, *16*, 2493.