A new cyclic diarylheptanoid, acerogenin M (1), has been isolated along with nine known diarylheptanoids, 2—10, and two known phenolic compounds, 11 and 12, from a MeOH extract of the stem bark of Acer nikoense MAXIM. The structure of 1 was determined on the basis of spectroscopic methods. Upon evaluation of the inhibitory effects on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation (1 µg/ear) in mice of nine of the compounds (2—6, 8, 10—12), six (2, 4—6, 8, 10) showed a marked anti-inflammatory effect with a 50% inhibitory dose (ID₅₀) of 0.26—0.81 mg per ear. In addition, upon an evaluation against the Epstein–Barr virus early antigen (EBV-EA) activation induced by TPA for all of the compounds, all exhibited moderate inhibitory effects against EBV-EA induction (IC₅₀ values of 356—534 mol ratio/32 pmol TPA).

Key words Acer nikoense; diarylheptanoid; anti-tumor-promoter; 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema; Epstein–Barr virus early antigen

The Aceraceae plant Acer nikoense MAXIM. is indigenous to Japan (Japanese name, Megusurino-ki) and its stem bark has been used as a folk medicine for the treatment of hepatic disorders and eye disease. The MeOH extract of its stem bark was reported to contain various diarylheptanoids and phenolic compounds, and some principal diarylheptanoid constituents have been reported to exhibit inhibitory effects on the release of β-hexosaminidase in RBL-2H3 cells and on the nitric oxide production in lipopolysaccharide-activated macrophages. In the course of our search for potential anti-tumor promoters from natural sources, we were especially interested to undertake the investigation of A. nikoense. In this paper, we present the isolation and structure elucidation of a new diarylheptanoid of the cyclic biphenyl ether type, 1, along with nine known diarylheptanoids, 2—10, and two known phenolic compounds, 11 and 12, from a MeOH extract of its stem bark, as well as their inhibitory effects on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice, and on Epstein–Barr virus early antigen (EBV-EA) activation induced by TPA, as primary screenings for anti-tumor promoters.

The n-hexane and MeOH extracts of A. nikoense stem bark and the EtOAc-, n-BuOH-, and H₂O-soluble fractions obtained from the MeOH extract were examined against TPA (1.7 pmol)-induced inflammation in mice and TPA (32 pmol)-induced EBV-EA activation in Raji cells. As shown in Table 1, the EtOAc-soluble fraction exhibited the most potent inhibitory activities in both assay systems (66% inhibition of inflammation in mice at a dose of 1.0 mg/ear; and 48.5% inhibition of TPA (32 pmol)-induced EBV-EA activation at a dose of 10 µg/ml). The EtOAc-soluble fraction was further investigated for the active constituents in this study. Chromatography of this fraction on normal-phase and reversed-phase silica gel column and reversed-phase prepara-

Table 1. Inhibitory Effects of the Extracts of Acer nikoense on TPA-Induced Inflammation in Mice and on the Induction of Epstein–Barr Virus Early Antigen

<table>
<thead>
<tr>
<th>Extract and fraction</th>
<th>Inhibition of inflammation</th>
<th>Percentage of EBV-EA induction&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±S.D.&lt;sup&gt;b&lt;/sup&gt; (×10⁻³ mm)</td>
<td>I.R.&lt;sup&gt;c&lt;/sup&gt; (%)</td>
</tr>
<tr>
<td>A (MeOH–CHCl₃–H₂O=2:1:1)</td>
<td>Control A</td>
<td>27.8±1.71</td>
</tr>
<tr>
<td>n-Hexane extract</td>
<td>12.3±1.50&lt;sup&gt;e&lt;/sup&gt;</td>
<td>34</td>
</tr>
<tr>
<td>MeOH extract</td>
<td>18.3±1.7&lt;sup&gt;f&lt;/sup&gt;</td>
<td>66</td>
</tr>
<tr>
<td>EtOAc-soluble fraction</td>
<td>9.5±1.2&lt;sup&gt;g&lt;/sup&gt;</td>
<td>19</td>
</tr>
<tr>
<td>Control B (MeOH–H₂O=1:1)</td>
<td>n-BuOH-soluble fraction</td>
<td>23.0±2.45&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>H₂O-soluble fraction</td>
<td>25.5±1.2&lt;sup&gt;i&lt;/sup&gt;</td>
<td>64</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ear thickness measured. <sup>b</sup>I.R.: inhibitory ratio at 1.0 mg/ear. <sup>c</sup> Each value represents the relative percentage to the positive control value. TPA (32 pmol, 20 ng/100 g body weight). <sup>d</sup> Values are expressed as mean±S.D. <sup>e</sup> Control group: 100% viability. <sup>f</sup> Values are expressed as mean±S.D. <sup>g</sup> Each value represents the relative percentage to the positive control value. TPA (32 pmol). <sup>h</sup> Values are expressed as mean±S.D. <sup>i</sup> Each value represents the relative percentage to the positive control value. TPA (32 pmol). <sup>j</sup> Values are expressed as mean±S.D. <sup>k</sup> Each value represents the relative percentage to the positive control value. TPA (32 pmol).
tive HPLC eventually yielded a new cyclic diarylheptanoid, 1, nine known diarylheptanoids, 2–10, and two known phenolic compounds, 11 and 12.

The high-resolution electron-ionization (HR-EI)-MS of compound 1 furnished a $M^+$ at $m/z$ 312.1363 according with the molecular formula $C_{19}H_{20}O_4$. The $^{13}$C-NMR spectrum of 1 (Table 2) exhibited 19 signals assignable to two benzene rings (five singlets and seven doublets), five methylenes, a carbonyl, and an oxymethine carbon. These findings, in combination with its IR spectrum which showed absorption bands at 3389, 1704, 1595, 1517, 1503, and 1053 cm$^{-1}$ assignable to hydroxyl, carbonyl, aromatic ring, and ether functions, and its UV spectrum which exhibited an absorption at 278 nm (log $\varepsilon$ 3.14), suggested that 1 is a biphenyl ether-type cyclic diarylheptanoid.\(^4\) Compound 1 was shown to possess a $\beta$-ketol system at C-7 ($C=O$) and C-9 (>CHOH) in the heptane chain by observation of $^{13}$C-$^1$H long-range couplings from H-4 to C-7, from H-6 to C-7, from H-8 to C-7, from H-10 to C-8, and from H-8, H-10 and H-11 to C-9, in the heteronuclear multiple-quantum coherence (HMBC) spectrum (Fig. 1). Furthermore, the presence of a hydroxyl group at C-2 was shown by $^{13}$C-$^1$H long-range couplings from H-3, H-4, and H-6 to C-2 observed in the HMBC spectrum of 1. The above evidence coupled with analysis of $^1$H-$^1$H correlation spectroscopy (COSY) (Fig. 1), $^1$H-detected multiple-quantum coherence (HMOC), and HMBC data indicated that 1 possesses a structure as formulated in Chart 1 which has been called acerogenin M.\(^10\) Stereochemistry at C-9 remains undetermined. Comparison of the $^{13}$C- and $^1$H-NMR data of 1 with those of the structurally related cyclic diarylheptanoids\(^4,5,7\) supported the proposed structure.

Identification of nine known compounds, aceroginin A (2),\(^1\) aceroside III (4),\(^12\) (R)-aceroside B (5),\(^7\) aceroside B\(_1\) (6),\(^7\) aceroside IV (8),\(^13\) acerogenin K (9),\(^9\) (−)-centrolobol (10),\(^14\) (+)-rhododendrol (11),\(^15\) and (+)-catechin (12),\(^16\) was made by spectral comparison with corresponding compounds in the literature. Two other known compounds, aceroside I (3)\(^11\) and acerogenin D (7),\(^9\) were identified.
them more inhibitory than quercetin (IC₅₀ 1.6 mg/ear). Three compounds, expressed as mean percentage of EBV-EA induction in mice, and the inhibitory effects were compared with a conventional numbering system of acerosides⁵) was adopted for the cyclic diarylheptanoids.

A conventional numbering system of acerosides⁵) was adopted for the cyclic diarylheptanoids.

Table 3. Inhibitory Effects of Compounds from *Acer nikoense* and Reference Compounds on TPA-Induced Inflammation in Mice and on the Induction of Epstein–Barr Virus Early Antigen

<table>
<thead>
<tr>
<th>Compound</th>
<th>ID₅₀⁽¹⁾ (mg/ear)</th>
<th>Concentration (mol ratio/32 pmol TPA)</th>
<th>Percentage of EBV-EA induction⁽²⁾</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td><strong>1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acerogenin M</td>
<td>13.3±0.4 (70)</td>
<td>52.4±1.5 (&gt;80)</td>
<td>76.4±2.1 (&gt;80)</td>
</tr>
<tr>
<td>Acerogenin A⁽⁶⁾</td>
<td>18.2±0.6 (60)</td>
<td>47.0±1.5 (&gt;80)</td>
<td>83.5±2.5 (&gt;80)</td>
</tr>
<tr>
<td>Aceroide I</td>
<td>&gt;1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aceroide III</td>
<td>15.4±0.5 (70)</td>
<td>55.5±1.5 (&gt;80)</td>
<td>76.3±2.0 (&gt;80)</td>
</tr>
<tr>
<td>5 (R)-Acerogenin B</td>
<td>0.81</td>
<td>57.1±1.6 (&gt;80)</td>
<td>79.0±2.3 (&gt;80)</td>
</tr>
<tr>
<td>Aceroide B₁</td>
<td>0.32</td>
<td>59.3±1.7 (&gt;80)</td>
<td>80.0±2.5 (&gt;80)</td>
</tr>
<tr>
<td>4 Aceroide D</td>
<td>13.5±0.4 (70)</td>
<td>45.1±1.5 (&gt;80)</td>
<td>81.3±2.3 (&gt;80)</td>
</tr>
<tr>
<td>Aceroide IV</td>
<td>0.49</td>
<td>61.5±1.7 (&gt;80)</td>
<td>82.7±2.8 (&gt;80)</td>
</tr>
<tr>
<td>Aceroine K⁽⁷⁾</td>
<td>7.8±1.2 (60)</td>
<td>37.4±1.1 (&gt;80)</td>
<td>76.8±2.0 (&gt;80)</td>
</tr>
<tr>
<td><strong>10</strong> (-)-Centrolobol⁽⁶⁾</td>
<td>0.66</td>
<td>36.6±1.2 (&gt;80)</td>
<td>74.0±2.0 (&gt;80)</td>
</tr>
<tr>
<td><strong>11</strong> (+)-Rhododendrol⁽⁸⁾</td>
<td>&gt;1.0</td>
<td>39.8±1.2 (&gt;80)</td>
<td>75.1±1.9 (&gt;80)</td>
</tr>
<tr>
<td><strong>12</strong> (+)-Catechin⁽⁹⁾</td>
<td>&gt;1.0</td>
<td>41.3±1.4 (&gt;80)</td>
<td>75.8±0.9 (&gt;80)</td>
</tr>
<tr>
<td>Reference compounds</td>
<td>Quercetin (mol ratio/32 pmol TPA)</td>
<td>21.6±1.1 (60)</td>
<td>55.7±1.8 (&gt;80)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.30</td>
<td>22.2±1.3 (60)</td>
<td>58.5±1.9 (&gt;80)</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>8.6±0.5 (70)</td>
<td>34.2±1.4 (&gt;80)</td>
<td>82.1±2.0 (&gt;80)</td>
</tr>
</tbody>
</table>

- ID₅₀: 50% inhibitory dose.  
- Values represent relative percentage to the positive control value (100%). Data are expressed as mean±S.D. Values in parentheses are the percentage viability of Raji cells.  
- The values of all compounds at 500 mol ratio/TPA were different from the control value (p<0.05) without cytotoxicity.  
- IC₅₀ represents the mol ratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol TPA.  
- EBV-EA induction data taken from literature.  
- a) EBV-EA induction data taken from literature.  

Based on the MS and NMR spectral analysis since the spectral data were unavailable in the literature.

Seven diarylheptanoids, 2—6, 8, and 10, and two phenolic compounds, 11 and 12, were evaluated with respect to their anti-inflammatory activity against TPA-induced inflammation in mice, and the inhibitory effects were compared with those of quercetin (3,3',4',5,7-pentahydroxylavone), a known inhibitor of TPA-induced inflammation in mice, and indomethacin, a commercially available anti-inflammatory drug, as shown in Table 3. All of the diarylheptanoids tested except for compound 3 inhibited the TPA-induced inflammation [ID₅₀ (50% inhibitory dose) 0.26—0.81 mg/ear], making them more inhibitory than quercetin (ID₅₀ 1.6 mg/ear). Three compounds, 2, 5, and 6, exhibited the strongest inhibitory effects (ID₅₀ 0.26—0.32 mg/ear) among those tested, which being comparable with that of indomethacin (ID₅₀ 0.30 mg/ear). The inhibitory effect against TPA-induced inflammation has been demonstrated to closely parallel that of the inhibition of tumor promotion in two-stage carcinogenesis initiated by 7,12-dimethylbenz[a]anthracene (DMBA) and then by TPA, a well-known promoter, in a mouse skin model.¹⁷ Thus, these diarylheptanoids can be expected to possess a high anti-tumor-promoting effect in the same animal model. Curcumin (diferuloylmethane),¹⁸—²² the major yellow pigment in turmeric (*Curcuma longa* L.), and other naturally occurring diarylheptanoids⁹—²¹,²³,²⁴ have also been demonstrated to possess marked anti-inflammatory¹⁸,¹⁹,²²,²³ and anti-tumor-promoting effects.²⁰,²¹,²⁴

The inhibitory effect on EBV-EA activation induced by TPA was further examined as a preliminary evaluation of the potential anti-tumor-promoting effects of the ten diarylheptanoids, 1—10, and the two phenolic compounds, 11 and 12. The results are shown in Table 3, together with comparable data for quercetin as well as β-carotene, a vitamin A precursor that has been intensively studied in cancer chemoprevention by using animal models.²⁵ All of the compounds tested showed inhibitory effects with IC₅₀ values of 356—534 mol ratio/32 pmol TPA, which were more inhibitory than quercetin (IC₅₀ 560 mol ratio/32 pmol TPA). Among them, three compounds, 9—11, exhibited potent inhibitory effects...
(IC$_{50}$ 356—392 mol ratio/32 pmol TPA) almost comparable with β-carotene (IC$_{50}$ 397 mol ratio/32 pmol TPA).

From the results of in vitro anti-inflammatory test and in vitro EBV-EA induction test, it may be suggested that diarylheptanoids from the EtOAc-soluble fraction of the MeOH extract of A. nikoense stem bark are useful agents that inhibit inflammation and chemical carcinogenesis.

**Experimental**

Optical rotations were measured on a JASCO P-1030 polarimeter in MeOH at 25°C. UV spectra on a Shimadzu UV-2200 spectrometer and IR spectra on a JASCO FTIR-300E spectrometer were recorded in KBr discs, respectively. NMR spectra were recorded with a JEOL ECA-600 spectrometer at 600 MHz (1H-NMR) and 150 MHz (13C-NMR) in CD$_3$OD with tetramethylsilane as internal standard. EI-MS (70 eV) and HR-EI-MS spectra were recorded on a JEOL JMS-BU20 spectrometer using a direct inlet system with tetramethylsilane as internal standard. EI-MS (70 eV) and HR-EI-MS with tetramethylsilane as internal standard. EI-MS (70 eV) and HR-EI-MS

Preparative HPLC [MeOH–H$_2$O–acetic acid (AcOH) (65 : 35 : 0.1, v/v/v)] was used for structure elucidation and to assign the 13C- and 1H-NMR spectra of these compounds.

Acerogenin M (1): Amorphous solid. [α]$_D$ +4.0° (c=0.10, MeOH). IR (KB) cm$^{-1}$: 3389 (OH), 1704 (C=C), 1696 (C=O), 1576 (N-H), 1453 (C-O-C), 1395 (C-N), 1244 (C-O), 1053 (C-O-C), 964 (C-N), 798 (C-O-C). UV A$_{max}$ (MeOH) nm (log ε): 213 (3.35), 231 (3.36), 278 (3.14), 303 (2.95). HR-ESI-MS m/z: 312.1363 [Caled for C$_{17}$H$_{20}$O$_2$ (M$^+$)]; 312.1361. ESI-MS m/z (%): 312 (M$^+$, 100), 294 (27), 284 (16), 269 (16), 256 (23), 241 (50), 225 (15), 212 (15), 198 (31), 152 (17), 121 (16), 117 (28), 107 (56). 13C- and 1H-NMR data, see Table 2.

Aceroside I (3): FAB-MS m/z: 483 (M+N$^+$). 13C- and 1H-NMR data, see Table 2.

**Animals**

The experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the College of Pharmacy, Nihon University, Chiba, Japan. Specific pathogen-free female ICR mice were obtained from Japan SLC (Shizuoka, Japan). The animals were housed, five per polycarbonate cage, in an air-conditioned specific pathogen-free room at 24±2°C. Food and water were available ad libitum.

**Assay of TPA-Induced Inflammation**

Ear edema in mice (TPA (1 µg, 1.7 nmol) dissolved in acetone (20 µl) was applied to the right ear of mice by a micropipette. A volume of 10 µl was delivered to both the inner and outer surfaces of the ear. The samples or their vehicles, MeOH–CHCl$_3$–H$_2$O (2:1:1, v/v/v), MeOH–H$_2$O–H$_2$O (1:1, v/v/v), or MeOH–H$_2$O (2:1, v/v), were applied topically about 30 min before TPA treatment. For ear thickness determinations, a pocket thickness gauge with a range of 0—9 mm, graduated at 0.1 mm intervals and measured the greatest surface area. The thickness was increased to reduce the tension, was applied to the tip of the ear. The ear thickness was measured before treatment (a), and 6 h after TPA treatment (b=TPA alone; b’=TPA plus sample). The following values were then calculated:

Edema A is induced by TPA alone (b—a). Edema B is induced by TPA plus sample (b’—a).

Intravenous inhibition ratio (%)=(Edema A—Edema B)/Edema A×100.

Each value was the mean of individual determinations from five mice. The 50% inhibitory dose (ID$_{50}$) values were determined by the method of probit-graphics interpolation for four dose levels. A statistical analysis was carried out by Student's t-test. Details of the in vivo anti-inflammatory test have been described previously.

**In Vivo EBV-EA Activation Experiment**

The inhibition of EBV-EA activation was assayed using Raji cells (EBV genome-carrying human lymphoblastoid cells; non-producer type), cultivated in 10% fetal bovine serum RPMI-1640 medium (Sigma, St. Louis, MO, U.S.A.). The indicator cells (Raji cells; 1×10$^5$ cells/ml) were incubated in 1 ml of the medium containing 4 mmol n-butyric acid as an inducer, 32 pmol of TPA (20 ng/ml in dimethyl sulfoxide), and a known amount (32, 16, 3.2, 0.32 nmol) of the test compound at 37°C in a CO$_2$ incubator. After 48 h, cell suspensions were centrifuged at 1000 rpm for 10 min, and the supernatant was removed. The activated cells were stained with high-titer EBV-EA-positive sera from naphosphgyral carcinoma patients, and the conventional indirect immunofluorescence technique was employed for detection. Each assay at least 500 cells were counted and the experiments were repeated three times. The average extent of EA induction was determined and compared with that on positive control experiments in which the cells were treated with n-butyric acid plus TPA where the extent of EA induction was ordinarily more than around 40%. The viability of treated Raji cells was assayed by the Trypan Blue staining method. Details of the in vitro assay of EBV-EA induction have been reported previously.

**References and Notes**


10) Whereas compound 1 can be referred to as 4,10-dihydroxy-2-oxatricyclo[13.2.2.1^{13}7]eicosa-3,5,7(20),15,17,18-hexaen-8-one when followed by the IUPAC nomenclature, a conventional numbering system of acerosides was adopted for 1 and the other diarylheptanoids of the cyclic biphenyl ether type in this study.


