

## Purification and Structural Determination of Hematopoietic Stem Cell-Stimulating Monoacyldiglycerides from *Cervus nippon* (Deer Antler)

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A mixture of monoacyldiglycerides was newly isolated from the chloroform extract of antlers of *Cervus nippon*, guided by the hematopoietic stimulation of stem cells. The structures of monoacyldiglycerides were determined by various spectroscopic methods: FAB MS, CID tandem MS, and 1D and 2D NMR. A mixture of at least nine inseparable *sn*-3-monoacyldiglycerides was identified: 1 [ $C_{39}H_{72}O_6$  (C16:0/C18:1)], 2 [ $C_{39}H_{72}O_6$  (C18:1/C16:0)], 3 [ $C_{39}H_{70}O_6$  (C16:0/C18:2)], 4 [ $C_{39}H_{70}O_6$  (C18:2/C16:0)], 5 [ $C_{41}H_{74}O_6$  (C18:0/C18:2)], 6 [ $C_{41}H_{74}O_6$  (C18:2/C18:0)], 7 [ $C_{41}H_{74}O_6$  (C18:1/C18:1)], 8 [ $C_{43}H_{74}O_6$  (C18:0/C20:4)], and 9 [ $C_{43}H_{74}O_6$  (C20:4/C18:0)]. Among these nine monoacyldiglycerides in deer antlers, compound 3 was one of the major compounds and was efficiently synthesized from glycerol. Spectral data of synthetic monoacyldiglyceride 3 were compared with the corresponding data for the mixture of natural monoacyldiglycerides. The mixture of natural monoacyldiglycerides from deer antlers showed potent activity on the hematopoiesis (stimulation index =  $1.40 \pm 0.05$ ,  $p < 0.02$  at  $1 \mu\text{g/ml}$ ), and synthetic monoacyldiglyceride 3 showed even better activity (stimulation index =  $1.54 \pm 0.12$ ,  $p < 0.001$ , at  $1 \mu\text{g/ml}$ ).

**Key words** *Cervus nippon*; monoacyldiglyceride; hematopoietic stem-cell-stimulation activity; deer antler

Antlers from *Cervus nippon* TEMMINCK (Cervidae), have been claimed to exert growth, developmental, and promotion effects on the hematopoietic system, powerful general systemic activities including improvement of cardiac function, enhancement of systemic resistance to various diseases, as well as anti-aging and fatigue-recovery effects.<sup>1–4</sup> Many substances from *C. nippon* have been identified and claimed to contain active components, including free amino acids, polypeptides, trace elements, carbohydrates, hexosamines, mucopolysaccharides, uronic acids, sialic acids, prostaglandins, glycolipids, phospholipids, gangliosides, nucleic acids, hypoxanthine, cholesterol, cholest-5-ene-3,7 $\alpha$ -diol, and cholesterol esters.<sup>5–11</sup>

There is a considerable need for hematopoietic- and immune-stimulating agents for use in the protection and/or restoration of hematopoietic and immune systems during cytotoxic chemotherapy and radiotherapy. Examples of the known growth factors in the hematopoiesis system include granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and erythropoietin.<sup>12–14</sup> These hematopoietic cytokines, however, are effective only when injected and only for a very short period of time. Moreover, their effects are very short-lived, they are associated with undesirable side reactions, and they are very expensive. Therefore, there is a strong need for less expensive drugs that can be administered orally. We therefore investigated the bone-marrow stem-cell-stimulation effects of *C. nippon* extracts that showed strong activities in our screening studies using colony-forming units in culture assay. Monoacyldiglycerides were purified as active ingredients from *C. nippon* fractions by silica-gel-column chromatography. Here we report on the isolation and structural determination of monoacyldiglycerides from deer-antler extracts, guided by hematopoietic stem-cell-stimulation activity.

Monoacyldiglycerides occur in diverse seed oils,<sup>15</sup> and are present in bovine udder and milk fat.<sup>16,17</sup> The structures of several monoacyldiglycerides have been confirmed by gas chromatography (GC), GC-mass spectroscopy (MS), high-performance liquid chromatography (HPLC), liquid chromatography (LC)-MS, nuclear magnetic resonance (NMR), infrared (IR), and single-crystal X-ray analysis.<sup>16–19</sup> Monoacyldiglycerides were previously synthesized from corresponding phosphatidylethanolamines, phosphatidylcholines, phosphatidylglycerols, and diacylglycerols by acetylation and enzymolysis followed by acetylation or esterification.<sup>20–22</sup>

The water extract of deer antler exhibited interesting growth-promotion effects on the hematopoietic system in our preliminary *in vitro* experiment, and hence several fractionation and purification steps were utilized to search for the underlying active components. We focused on identifying substances having higher specific activities rather than on compounds present in higher amounts with low specific activities. The activity was expressed as a stimulation index (SI) at  $1 \mu\text{g/ml}$  in a colony-forming unit in culture assay. The highest specific activity was obtained with the chloroform extract (SI =  $1.11 \pm 0.02$ ) as compared to the other solvents and hexane and ethanol extracts. The bioactive fraction, Fr. 2E (SI =  $1.30 \pm 0.05$ ), was obtained from the chloroform extract by repeated silica-gel chromatography. Fr. 2E (70.0 mg) was further purified by silica-gel chromatography to afford 40.0 mg of Fr. 2E-a as a colorless oil (hexane–diethyl ether, 8:1; *R<sub>f</sub>* 0.15), which showed strong activity (SI =  $1.40 \pm 0.05$ ). Therefore, it was confirmed that more-purified fractions showed higher specific activities on the hematopoietic stimulation *in vitro*. From the hexane extract, 10.0 mg of the same Fr. 2E-a fraction were isolated using the same method as described above.

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The purified bioactive fraction, Fr. 2E-a, was characterized as *sn*-3-monoacyldiglycerides by fast atom bombardment-mass (FAB-MS) and NMR spectroscopy, as shown in Fig. 1.

For positive-ion-mode FAB-MS analysis, the sample was dissolved in chloroform-methanol and the solution was mixed with 3-nitrobenzyl alcohol as the matrix. The positive-ion FAB-MS spectrum of the newly isolated mixture of monoacyldiglycerides from deer antlers with NaI produced several prominent sodium-adducted molecules ( $[M+Na]^+$ ,  $m/z$ ), as shown in Fig. 2.

For further characterization, high-energy collision-induced dissociation (CID) tandem mass spectroscopy (MS/MS) coupled with FAB was successfully applied without prior separation and derivatization of the natural products (Fig. 3). The MS/MS spectra could be obtained with compounds at  $m/z=657$ , 659, 685, and 709 in the mixture. The rest of molecular ions exhibited insufficient molecular intensity in FAB-MS spectral data to undergo the tandem MS experiment and thus being ignored. The presence of sodiated diglyceride ions ( $[M+Na-R_n^1COOH]^+$ ) confirmed the composition of two long-chain acyl groups and one acetyl group. Each CID spectrum exhibited common fragment ions at  $m/z=123$ , 181, and 195, confirming the presence of the *sn*-3 acetyl group.

To identify possible regioisomers, CID MS/MS of monoacyldiglycerides isolated from deer antlers was performed. This could cause systematic fragmentation *via* charge-remote cleavage to provide additional information about the compositions of fatty acyl groups at *sn*-1 and *sn*-2 positions. For instance, molecular ions at  $m/z=319$  and 329 of compound **3** corresponded to sodiated propenyl ester ion with an *sn*-1 acyl group and vinyl ester ion with an *sn*-2 acyl group, respectively (Fig. 3B).

In general, the *sn*-1 and *sn*-3 fatty acyl groups are more prone to be alienated from the glycerol backbone than the *sn*-2 fatty acyl group, mainly due to a McLafferty-type rearrangement in the fragmentation pathway. Based upon our previous investigation of the regiospecificity of fatty acyl linkages and the double-bond positions in the polyunsaturated acid chain using CID MS/MS, the intensity ratio of

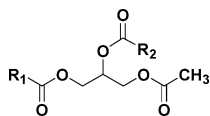


Fig. 1. General Structure of *sn*-3-Monoacyldiglycerides

both  $G_2$  [ $M+Na-R_2COOH$ ] and  $G_1$  [ $M+Na-R_1COOH$ ] ions can be utilized to determine the relative positions of the two fatty acyl groups in the glycerol backbone.<sup>23</sup> Our previous comparisons of the CID MS/MS data of synthetic compound **1** (C16:0/C18:1) with its synthetic regioisomer **2** (C18:1/C16:0), and compound **3** (C16:0/C18:2) with its regioisomer **4** (C18:2/C16:0) indicated that the  $G_2/G_1$  intensity ratio of a pure regioisomer of any monoacyldiglyceride must be close to 2.0 or higher.<sup>23</sup> However, as shown in Fig. 2, the values of  $G_2/G_1$  of the isolated monoacyldiglycerides in deer antlers are much less than 2.0:  $m/z=377/403$  (**1** and **2**, Fig. 3D), 377/401 (**3** and **4**, Fig. 3B), 405/401 (**5** and **6**, Fig. 3C), and 405/425 (**8** and **9**, Fig. 3A). These findings thus clearly indicate co-occurrence of *sn*-1/*sn*-2 regioisomers in all of the monoacyldiglycerides isolated in the present study. In addition, the fragment ion at  $m/z=403$  in the MS/MS spectrum is responsible for the  $G_1^*$  and  $G_2^*$  values of compound **7**, and thus supports the presence of this compound (Fig. 3C). It is also apparent that the major regioisomers are **1**, **3**, **5**, and **8**, while the minor regioisomers are **2**, **4**, **6**, and **9** within the four sets of monoacyldiglycerides **1** and **2** (Fig. 3D), **3** and **4** (Fig. 3B), **5** and **6** (Fig. 3C), and **8** and **9** (Fig. 3A) because the  $G_2/G_1$  ratios are all higher than 1.0.

Information on the location and number of double bonds in the fatty acid chains of the monoacyldiglycerides was directly acquired by examining high-mass-product ions generated by the loss of  $C_nH_{2n+2}$ . The tandem mass fragmentation pattern was typical for each ion being separated by 14 amu for saturated hydrocarbon and 12 amu for unsaturated hydrocarbon of fatty acid chain. Thus, the position of each C-C double bond and the degree of unsaturation of each fatty acyl chain on the glycerol backbone of natural monoacyldiglycerides were determined and confirmed by MS/MS.

From the FAB-MS and CID MS/MS data, we conclude that there are at least nine molecules in naturally occurring monoacyldiglycerides (compounds **1**–**9**), each containing a different diacylglycerol moiety: at  $m/z=657$  (**3**, C16:0/C18:2; and **4**, C18:2/C16:0), 659 (**1**, C16:0/C18:1; and **2**, C18:1/C16:0), 685 (**5**, C18:0/C18:2; **6**, C18:2/C18:0; and **7**, C18:1/C18:1), and 709 (**8**, C18:0/C20:4; and **9**, C20:4/C18:0). It is also observed that **1/2** (C16:0/C18:1 and *vice versa*) and **3/4** (C16:0/C18:2 and *vice versa*) are predominant in FAB-MS. Table 1 presents the molecular mass and fatty acid composition of nine monoacyldiglycerides from *C. nippon* obtained by FAB-MS and CID

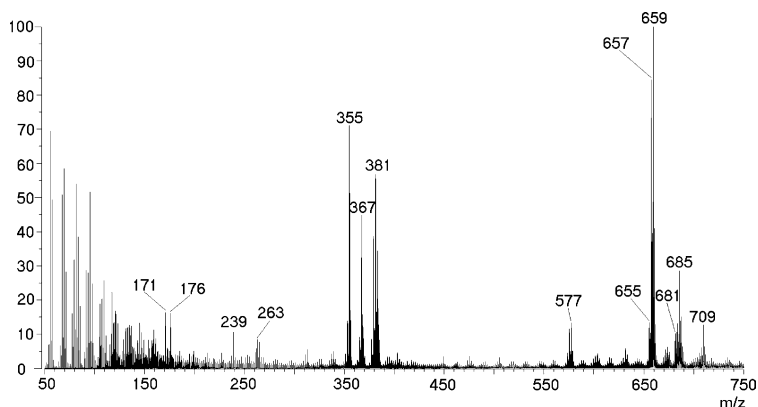


Fig. 2. Positive-Ion FAB-MS of Purified Monoacyldiglycerides from *Cervus nippon* (with NaI)

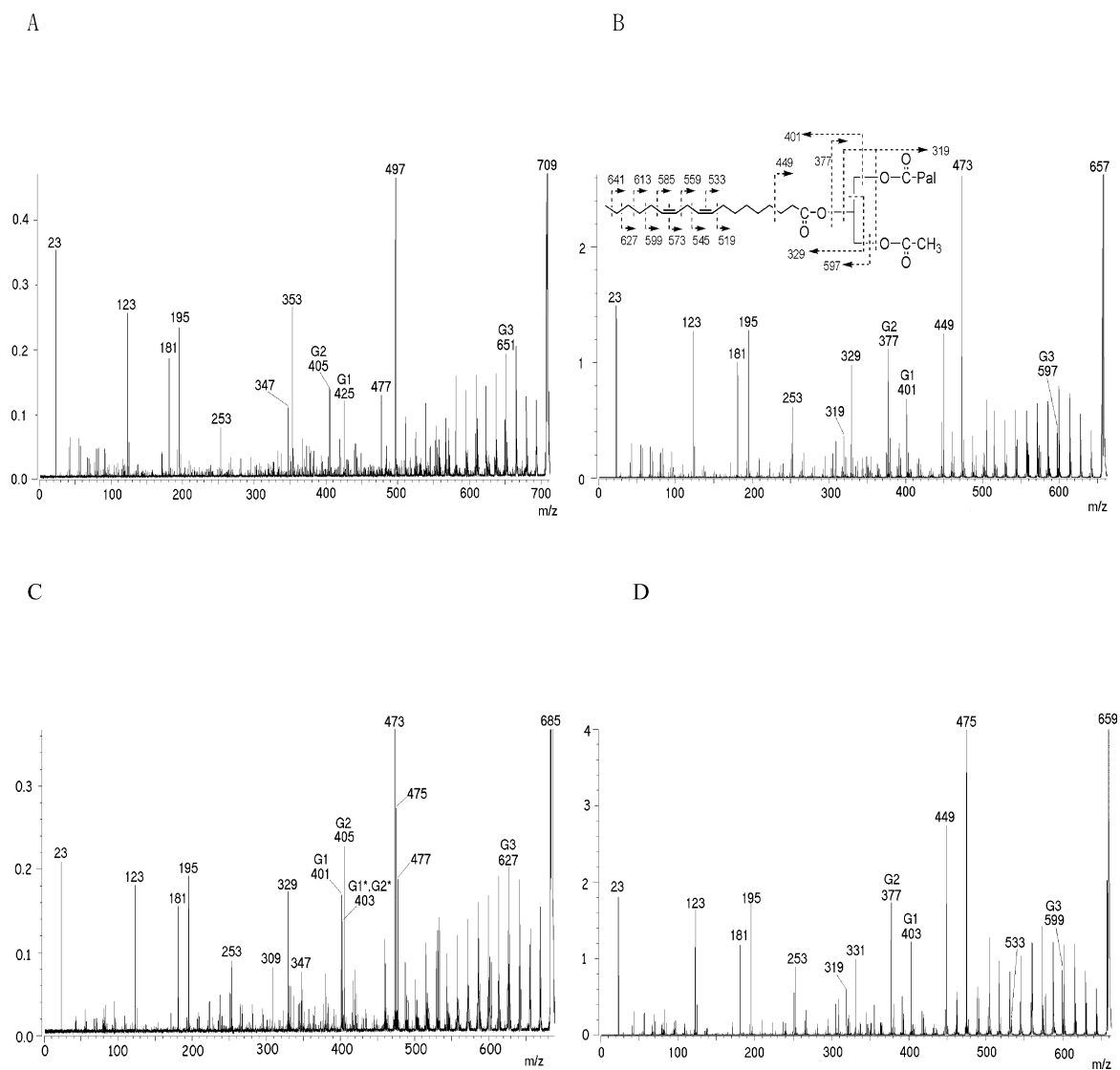


Fig. 3. CID MS/MS Spectra of Purified Monoacyldiglycerides

(A) Regioisomeric mixture of compounds **8** and **9** at  $m/z=709$ ,  $[M+Na]^+$ ; (B) regioisomeric mixture of compounds **3** and **4** at  $m/z=657$ ,  $[M+Na]^+$ ; (C) regioisomeric mixture of compounds **5** and **6** as well as compound **7** at  $m/z=685$   $[M+Na]^+$ ; and (D) regioisomeric mixture of compounds **1** and **2** at  $m/z$  659. In C,  $G_1$  and  $G_2$  are involved in compound **5** and *vice versa* in **6**, and  $G1^*$  and  $G2^*$  are in compound **7**.

Table 1. Accurate Masses of the Naturally Occurring Monoacyldiglycerides from *Cervus nippon* as Obtained by Positive-Ion FAB-MS and MS/MS

Compound	Elemental formula	$[M+Na]^+$ , $m/z$		Fatty acid composition <sup>a)</sup>	$G1^b$	$G2$	$G3$
		Calculated	Observed				
<b>1</b>	$C_{39}H_{72}O_6$	659.5	659.5	C16:0/C18:1	403	377	599
<b>2</b>	$C_{39}H_{72}O_6$	659.5	659.5	C18:1/C16:0	377	403	599
<b>3</b>	$C_{39}H_{70}O_6$	657.5	657.5	C16:0/C18:2	401	377	597
<b>4</b>	$C_{39}H_{70}O_6$	657.5	657.5	C18:2/C16:0	377	401	597
<b>5</b>	$C_{41}H_{74}O_6$	685.5	685.6	C18:0/C18:2	401	405	627
<b>6</b>	$C_{41}H_{74}O_6$	685.5	685.6	C18:2/C18:0	405	401	627
<b>7</b>	$C_{41}H_{74}O_6$	685.5	685.6	C18:1/C18:1	403	403	627
<b>8</b>	$C_{43}H_{74}O_6$	709.5	709.5	C18:0/C20:4	425	405	651
<b>9</b>	$C_{43}H_{74}O_6$	709.5	709.5	C20:4/C18:0	405	425	651

<sup>a)</sup> Individual molecular species are designated with the acyl groups at *sn*-1 and *sn*-2 positions listed in order (*i.e.*, C16:0/C18:1 = *sn*-1/*sn*-2). The fatty acyl groups are symbolized using the convention carbon number:double-bond number (C16:0, palmitoyl; C18:0, stearyl; C18:1, oleoyl; C18:2, linoleoyl; C18:3, linolenoyl; C20:4, arachdonoyl).

<sup>b)</sup>  $G_1$ ,  $G_2$ , and  $G_3$  correspond to  $[M+Na-R_1COOH]^+$ ,  $[M+Na-R_2COOH]^+$ , and  $[M+Na-R_3COOH]^+$ , respectively.

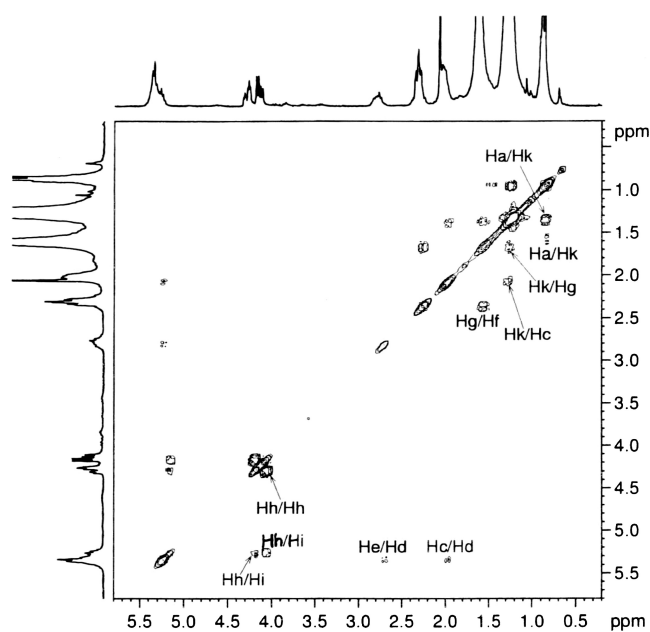


Fig. 4. The  $^1\text{H}$ - $^1\text{H}$  COSY NMR Spectrum of Purified Monoacyldiglycerides in  $\text{CDCl}_3$  at  $30^\circ\text{C}$  and at 250 MHz in Magnitude Mode

Chemical shifts are expressed relative to the  $\text{CDCl}_3$  signal at 7.24 ppm. The assignment for characteristic connectivities of cross peaks are indicated as Ha-k/Ha-k.

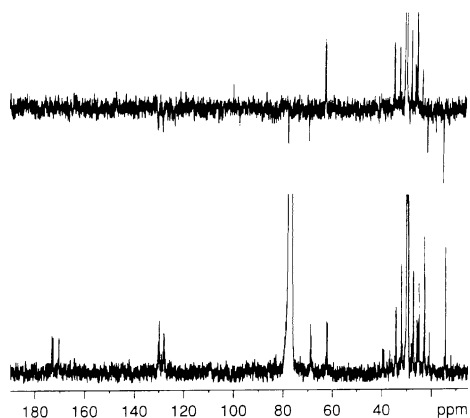


Fig. 5. The DEPT135 and  $^{13}\text{C}$ -NMR Spectra of Purified Monoacyldiglycerides in  $\text{CDCl}_3$  at  $30^\circ\text{C}$ , and at 62.5 MHz in Magnitude Mode

MS/MS. Three product ions corresponding to the sodiated diglyceride ions  $[\text{M}+\text{Na}-\text{R}_1^1\text{COOH}]^+$ ,  $[\text{M}+\text{Na}-\text{R}_2^1\text{COOH}]^+$ , and  $[\text{M}+\text{Na}-\text{R}_3^1\text{COOH}]^+$  are denoted as  $\text{G}_1$ ,  $\text{G}_2$ , and  $\text{G}_3$ , respectively.

To support the structural assignment of the isolated components clearly elucidated by mass spectrometry as *sn*-3-monoacyldiglycerides, we turned our attention to one- and two-dimensional FT-NMR spectroscopy. A series of 1D ( $^1\text{H}$ ,  $^{13}\text{C}$ , DEPT 135) and 2D  $^1\text{H}$ - $^1\text{H}$  correlation spectrometry (COSY) NMR analyses were performed on the mixture of isolated monoacyldiglycerides (Figs. 4, 5). Table 2 shows the assignments of the chemical shifts of all protons and carbons of the major monoacyldiglyceride, compound **3**, of the monoacyldiglyceride mixture isolated from deer antlers by 1D and 2D FT-NMR spectroscopic analyses.

The  $^1\text{H}$  and  $^1\text{H}$ - $^1\text{H}$  COSY NMR spectra provided a rough indication of the monoacyldiglyceride skeleton (Fig. 4).

Table 2. Chemical Shifts of Protons and Carbons of the Major Monoacyldiglyceride (Compound **3**) in  $\text{CDCl}_3$  Relative to MS/MS at  $30^\circ\text{C}$

Assignment	Proton $\delta$ (ppm)	Carbon $\delta$ (ppm)
A	0.86	14.12
B	2.05	20.68
C	2.01	27.19
D	5.32	127.5—130.5
E	2.75	25.60
F	2.30	34.17
G	1.60	24.85
H	4.19	62.34, 62.04
I	5.25	68.75
J	—	170.5, 172.9, 173.3
K	1.05—1.45	28.83—31.97

The triplet with a chemical shift of  $\delta$  0.86 is characteristic of terminal methyl groups of long alkyl chains (A). The broad and intense peak at  $\delta$  1.05—1.45 is typical of methylene protons in long-chain fatty acids (K). The peaks at  $\delta$  1.60 and 2.01 are attributable to beta protons in the acyl group (G) and alpha protons in the double bond (C) respectively. The sharp singlet at  $\delta$  2.05 can be assigned to methyl protons of the acetyl group (B). The peaks appearing at  $\delta$  2.30, 2.75, and 5.32 can be attributed to alpha protons in the acyl group (F), methylene protons between two double bonds (E), and vinyl protons of the unsaturated fatty acyl moiety (D), respectively. The vinyl protons in turn showed characteristic correlation with allylic protons ( $\text{H}_d/\text{H}_c$ ,  $\text{H}_d/\text{H}_e$ ) in the  $^1\text{H}$ - $^1\text{H}$  COSY NMR spectrum. Protons on the glycerol backbone gave rise to typical splitting patterns at  $\delta$  4.19 (H) and 5.25 (I) that were correlated with each other ( $\text{H}_i/\text{H}_h$ ). The spectral features associated with the  $\alpha$ - and  $\beta$ -protons of the acyl group were also correlated ( $\text{H}_f/\text{H}_g$ ).

The  $^{13}\text{C}$ -NMR spectra combined with DEPT 135 strongly indicated the presence of the monoacyldiglyceride skeleton, as shown in Fig. 5. In these spectra, carbon peaks at  $\delta$  20.7 and 170.5 are attributable to the methyl and carbonyl moieties of *sn*-3 acetyl groups in glycerol skeletons, respectively (B, J). Other carbonyl signals at  $\delta$  173.3 and 172.9 can be attributed to the carbonyl carbon resonances of long-chain fatty acyl groups attached at the *sn*-1 and *sn*-2 positions of glycerol (J). The signals at  $\delta$  62.3, 62.0, and 68.8 can be attributed to *sn*-3, *sn*-1, and *sn*-2 carbons of the glycerol moiety (H), respectively. Signals corresponding to the terminal methyl groups in fatty acyl chains appear at  $\delta$  14.1 (A). Peaks at  $\delta$  24.8, 25.6, 27.2, 28.9—32.0, and 34.2 are attributable to the  $\beta$ -carbons in the acyl groups (G), the methylene carbon between the two unsaturated carbons (E), the allyl carbon (C), several methylene carbons in the long-chain fatty acyl groups (K), and the  $\beta$ -carbons in the acyl groups (F), respectively. The chemical shifts of the signals corresponding to vinyl carbons ( $\delta$  127.5—130.5) confirmed the presence of several combinations of unsaturated fatty acid moieties in monoacyldiglycerides (D).

Thus, based on 1D and 2D NMR spectroscopy along with FAB-MS and MS/MS, the newly purified components responsible for the hematopoietic stem-cell-stimulation activity from *C. nippon* were identified as a mixture of seven *sn*-3-monoacylated diglycerides.

Compound **3** was one of the major compounds of the purified monoacyldiglycerides from deer antler, and hence it

was synthesized from glycerol and the appropriate fatty acids to confirm its structure and to provide larger quantities for further studies on the effect of hematopoietic stem cell stimulation *in vivo* and its underlying mechanism. All reactions were monitored by thin-layer chromatography (TLC) in order to obtain the target monoacyldiglyceride with high regioselectivity. The purity of all intermediates and the target molecule was verified by  $^1\text{H}$ ,  $^{13}\text{C}$ , and 2D COSY NMR, and high-resolution FAB-MS. As expected, the synthesized monoacyldiglyceride **3** showed potent activity on hematopoietic stem cell stimulation *in vitro* ( $\text{SI} = 1.54 \pm 0.12$  and  $1.46 \pm 0.11$ ,  $p < 0.001$ , at 1 and  $0.1 \mu\text{g/ml}$ , respectively), in a dose-dependent fashion.

## Experimental

**General Experimental Procedures** Melting points were measured on a Fisher-Johns melting-point apparatus and are presented here uncorrected. Infrared spectra were recorded on a Bio-Rad FTS 165 spectrometer. NMR spectra were obtained on a Bruker DPX 250 FT NMR spectrometer, determined in  $\text{CDCl}_3$ , and chemical shifts are reported in ppm ( $\delta$ ) and referenced to  $\text{CHCl}_3$  at 7.24 ppm. Peak assignments were made by  $^1\text{H}$ ,  $^{13}\text{C}$ , DEPT,  $^1\text{H}$ - $^1\text{H}$  and  $^1\text{H}$ - $^{13}\text{C}$  correlation experiments. The following abbreviations are used: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad; and dd, doublet of doublets. Positive-ion FAB mass spectra were obtained with a JEOL JMS-HX110/110A tandem mass four-sector spectrometer. Ions were produced by FAB using a cesium-ion gun operated at 22 kV and with a matrix of 3-nitrobenzyl alcohol.

All solvents and reagents used were purchased at the highest available commercial grade. Aqueous solutions were prepared using deionized and distilled water. TLC, high-performance TLC (HPTLC), and silica-gel-column chromatography were performed using Merck Kieselgel 60  $\text{F}_{254}$  (silica-gel) TLC plates and a Kieselgel 60, 230–400 mesh ASTM (silica gel).

**Animal Materials** Antlers from *C. nippon* TEMMINCK were purchased at an Asian drug market during April 1998 and April 1999. The sample was identified by Dae-Suk Han, emeritus professor, Department of Pharmacognosy, College of Pharmacy, Seoul National University. A voucher has been deposited at the Asan Institute for Life Sciences, University of Ulsan, Seoul, South Korea (no. AS-1999-153).

**Structure and Isolation** Samples of *C. nippon* (1.75 kg) were ground and refluxed with 7 l of hexane, with the residue dried, and subsequently refluxed with 7 l of  $\text{CHCl}_3$ . The residue was dried and then refluxed with 7 l of 70% ethanol. The resulting yields of dried hexane, chloroform, and ethanol extracts were 24.6, 14.0 and 91.0 g, respectively. The chloroform extract was loaded onto a silica-gel column (350 g,  $5.0 \times 50 \text{ cm}$ ) and eluted with chloroform-methanol (500 : 1 v/v) and was pooled according to the major spots on HPTLC (chloroform-methanol, 300 : 1 v/v) detected with UV (250 nm) or  $\text{I}_2$  vapor absorption to give seven fractions. Each fraction was tested for growth-stimulating effects on hematopoietic cells. Of fractions 1–7, Fr. 2 (380.5 mg)—the significantly active fraction—was rechromatographed on a silica-gel column (20.2 g,  $1.6 \times 20 \text{ cm}$ ) with chloroform-methanol (300 : 1 v/v) to give six fractions: Fr. 2A–F. Of the six fractions, Fr. 2E (70.3 mg)—exhibiting excellent bioactivity—was purified on a silica gel column (3.5 g,  $0.8 \times 15 \text{ cm}$ ) with hexane-ethyl acetate-acetic acid (30 : 1 : 0.5) to afford 40.0 mg of Fr. 2E-a (monoacyldiglycerides, a mixture of eight compounds, 1–8) as a colorless oil (hexane-diethyl ether, 8 : 1;  $R_f$  0.15). From the hexane extract, 10.0 mg of the same monoacyldiglycerides were extracted using the same method as described above.

**Synthesis of Compound 3** To a stirred solution of glycerol in acetone, palmitic acid, dicyclohexyl carbodiimide (DCC), and dimethyl aminopyridine (DMAP) were added and the reaction mixture was stirred overnight at  $0^\circ\text{C}$ . Purification by silica-gel-column chromatography afforded C1-monopalmitylated glycerol. The monoacylated glycerol was then treated with acetic anhydride in the presence of DMAP at  $-78^\circ\text{C}$  for 30 min to provide the desired C3-monoacylated product. The final C2-acylation of the column-purified C3-acetyl monoglyceride was then carried out with linoleic acid, DCC, and DMAP in dichloromethane for 4 h at  $0^\circ\text{C}$ . Silica-gel flash-column purification of the crude product afforded the desired target compound **3**. The overall yield of compound **3** synthesized in three steps from glycerol was 10%.

**Colony-Forming Unit in Culture Assay** Bone marrow cells were obtained from mice femora and tibiae. Bone marrow stem-cell-rich fraction was prepared by discontinuous Percoll-Hypaque gradient centrifugation. The cells at the interface between densities of 1.063 and 1.075 were collected. Bone marrow stem cells were cultured in 1.1% methylcellulose petri dishes. The 35-mm petri culture dishes contained the following components: the bone marrow stem cells under test ( $3 \times 10^5/\text{dish}$ ) with 1.1% methylcellulose, 20% fetal calf serum, and 1% penicillin-streptomycin antibiotics. Samples were dissolved in 0.1% ethanol, prepared at concentrations of 0.1 and  $1 \mu\text{g/ml}$ , and cultured in a  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  incubator. After 1 week, the number of colonies was counted under an inverted microscope and compared with control dishes. The colony-forming efficiency was expressed as the stimulation index (SI) according to the following equation:

$$\text{SI} = \frac{\text{total no. of colonies in sample group}}{\text{total no. of colonies in control group}}$$

**Statistical Analysis** All numerical data are expressed as mean  $\pm$  S.E.M. The statistical significance of the difference was determined by an unpaired Student's *t*-test.

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