# Studies on Differentiation Inducers. V.[1] Steroid Glycosides from Periplocae Radicis Cortex

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**Six pregnane glycosides and three cardenolides were isolated as differentiation inducers using mouse myeloid leukemia (Ml) cells from Periplocae Radicis Cortex (bark of *Periploca sepium* BGE., Asclepiadaceae). The cardenolides showed much higher activities than the pregnane glycosides. Besides these nine compounds, commercially available cardenolides were tested for their differentiation inducing activities using Ml cells. Digitoxin and digoxin induced Ml cells into phagocytic cells, but others did not. In the presence of 1 nM of actinomycin-D, the activity of steroid glycosides was enhanced against Ml cells.**

**Key words** differentiation; *Periploca sepium*; Ml cell; cardenolide; pregnane glycoside

Some myeloid leukemia cell lines are known to differentiate into granulocytes or macrophage-like cells on treatment with various inducers. Differentiation inducers are expected to be a new type of antitumor agent, and we have reported the isolation and structural elucidation of several of these compounds such as triterpenes, flavones, lignans and pregnane glycosides.[1,2] In the course of our study, the methanolic extract of Periplocae Radicis Cortex (bark of *Periplocae sepium* BGE., Asclepiadaceae) was found to have differentiation inducing activity. The active components of the extract were investigated.

A suspension of the methanolic extract of Periplocae Radix in water was extracted with AcOEt. The water layer, showing differentiation inducing activity against mouse myeloid leukemia (Ml) cells, was passed through HP-20 and the absorbed materials were eluted with 50% aqueous methanol and 100% methanol, successively. The 100% methanol eluate was chromatographed on silica-gel and high performance liquid chromatography (HPLC) to afford active components **1**—**5**, **7**, **8** containing new pregnane glycosides named plocoside A (**2**) and B (**4**). Ml cells were also induced into phagocytic cells following

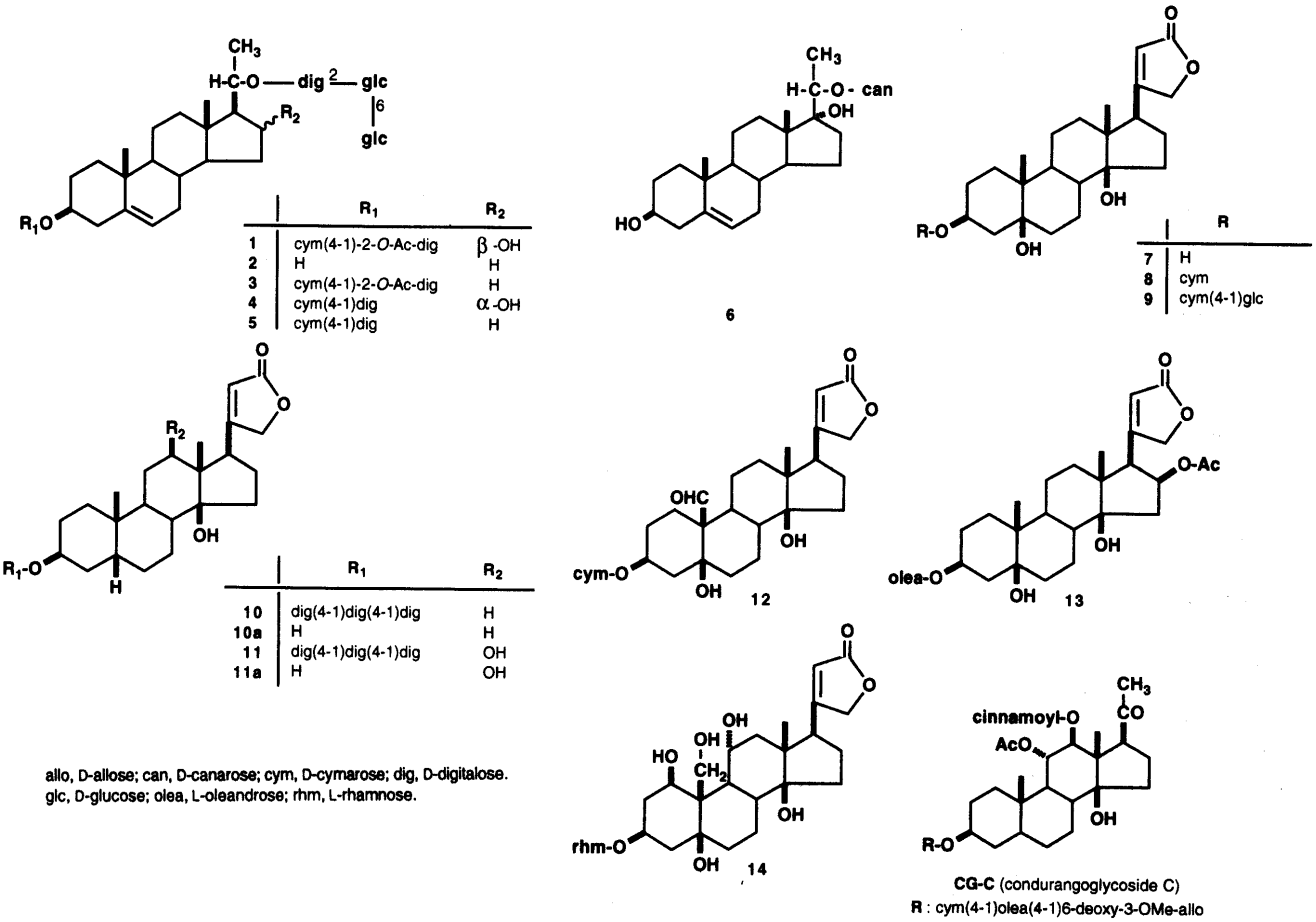


Chart 1

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treatment with the AcOEt layer, then the AcOEt layer was chromatographed on silica-gel followed by HPLC to give the active compounds **6** and **9**. The structures of seven known compounds were identified by comparison of various data with reported values as shown in Chart 1.[3)]

Plocoside A (**2**), $C_{40}H_{66}O_{16}$, $[\alpha]_D$ −23.5° was obtained as an amorphous powder. The $^1$H-NMR spectrum of **2** showed two singlet methyls ($\delta$ 0.64, 1.00), two doublet methyls ($\delta$ 1.47, 1.58), a methoxyl signal ($\delta$ 3.49) and three anomeric proton signals ($\delta$ 4.67, 5.25, 5.35). In the $^{13}$C-NMR spectrum, three anomeric carbon signals ($\delta$ 104.3, 104.5, 105.3), a methoxyl signal ($\delta$ 56.8) which was characteristic for D-digitalose and two olefinic carbon signals ($\delta$ 121.5, 141.8) were observed, and these data indicated the structure of **2** to be a pregnane glycoside having one D-digitalose and two D-glucose for the sugar moiety. Furthermore, this sugar chain was shown to be identical to that of S-10 isolated from the same plants by Itokawa *et al.*, and the $^{13}$C-NMR chemical shifts of C-3 ($\delta$ 71.3) showed the presence of no sugar chain at this position.[3a,b)] So, we determined the structure of **2** by comparing the spectral data with reported values as $\Delta^5$-pregnene-3$\beta$, 20(*S*)-diol 20-*O*-[$\beta$-D-glucopyranosyl (1-6)-$\beta$-D-glucopyranosyl(1-2)-$\beta$-D-digitalopyranoside] previously derived from S-5 previously isolated by Itokawa *et al.* from the same plants.[3b)]

Plocoside B (**4**), $C_{54}H_{90}O_{24}$, $[\alpha]_D$ 18.5° was obtained as an amorphous powder. $^1$H-NMR spectrum of **4** showed two singlet methyls ($\delta$ 0.62, 0.93), four doublet methyls ($\delta$ 1.51, 1.56 × 2, 1.65), three methoxyl signals ($\delta$ 3.54, 3.56, 3.57) and five anomeric proton signals ($\delta$ 4.70, 4.76, 5.26 × 2, 5.35). In the $^{13}$C-NMR spectrum of **4**, olefinic carbon signals ($\delta$ 122.3, 140.5), five anomeric carbon signals ($\delta$ 96.2, 104.0, 105.2, 105.3, 106.9) and three methoxyl groups ($\delta$ 58.7, 57.2, 57.3) were observed. These three methoxyl signals were characteristic for a D-cymarose and two D-digitaloses. The chemical shifts due to the sugar moiety were basically identical to that of S-5. Comparing the spectral data with reported values, the structure of **4** was concluded to be $\Delta^5$-pregnene-3$\beta$,16$\alpha$,20(*S*)-triol 3-*O*-[2-*O*-acetyl-$\beta$-D-digitalo pyranosyl(1-4)-$\beta$-D-cymaro pyranoside] 20-*O*-[$\beta$-D-glucopyranosyl (1-6)-$\beta$-D-glucopyranosyl(1-2)-$\beta$-D-digitalopyranoside] previously derived from glycoside $H_2$ isolated by Sakuma *et al.* from the same plants.[3d)]

The differentiation inducing activities of the steroid compounds (**1**—**9**) are indicated in Table 1. Pregnane glycosides (**1**—**6**) showed activities at a concentration of 50 $\mu$M. Although these compounds have one to five sugars in their structures, no relationship was observed between their numbers and their activities against Ml cells. Compounds **1**, **4** and **6**, having a hydroxyl group at the D-ring, exhibited higher differentiation inducing activities compared with other pregnane glycosides. Cardenolides (**7**—**9**) showed much higher activities than pregnane glycosides (**1**—**6**). More Ml cells became phagocytic cells by the treatment with 50 $\mu$M of cardenolides compared with the case of pregnane glycosides.

Much attention has been paid to the differentiation inducing activities of other cardenolides, which have been tested using Ml cells with commercially available compounds (Table 2). The tested compounds are listed in Chart 1, such as digitoxin (**10**), digitoxigenin (**10a**), digoxin (**11**), digoxigenin (**11a**), cymarin (**12**), oleandrin (**13**) and ouabain (**14**) in addition to the isolated cardenolides (**7**—**9**) from Periplocae Radicis Cortex. Digitoxin (**10**) and digoxin (**11**) were the most effective compounds among these ten, whereas their aglycones (**10a**, **11a**) showed poor activity. The differentiation inducing activities of cymarin

Table 1. Cell Growth and Phagocytic Activities of M1 Cells Treated with Steroid Glycosides from Periplocae Radix

| Compound | Conc. ($\mu$M) | G.R. (%) | Phagocytic[a)] activity |
|---|---|---|---|
| Cont. | | 100 | − |
| Dex. | 1 | 60 | +++ |
| **1** | 100 | 63 | ++ |
| | 50 | 61 | + |
| **2** | 100 | 78 | + |
| | 50 | 65 | + |
| **3** | 100 | 82 | + |
| | 50 | 83 | − |
| **4** | 100 | 72 | ++ |
| | 50 | 89 | + |
| **5** | 100 | 63 | + |
| | 50 | 64 | + |
| **6** | 100 | 78 | ++ |
| | 50 | 66 | ++ |
| **7** | 50 | 81 | ++ |
| | 10 | 75 | + |
| **8** | 50 | 46 | ++ |
| | 10 | 58 | + |
| **9** | 50 | 93 | ++ |
| | 10 | 88 | + |

Cont., control; Dex., dexamethasone. G.R., growth ratio. *a*) +, >10%; ++, >25%; +++, >50%.

Table 2. Cell Growth and Phagocytic Activities of M1 Cells Treated with Cardenolides

| Compound | Conc. ($\mu$M) | G.R. (%) | Phagocytic[a)] activity |
|---|---|---|---|
| Cont. | | 100 | − |
| Dex. | 1 | 62 | +++ |
| **7** | 50 | 95 | ++ |
| | 10 | 81 | + |
| **8** | 50 | 57 | ++ |
| | 10 | 71 | + |
| **9** | 50 | 82 | ++ |
| | 10 | 83 | + |
| **10** | 50 | 40 | ++ |
| | 10 | 69 | ++ |
| **10a** | 50 | 73 | + |
| | 10 | 85 | + |
| **11** | 50 | 73 | ++ |
| | 10 | 81 | + |
| **11a** | 50 | 79 | + |
| | 10 | 79 | − |
| **12** | 50 | 85 | + |
| | 10 | 100 | − |
| **13** | 50 | 30 | + |
| | 10 | 84 | − |
| **14** | 50 | 74 | − |
| | 10 | 96 | − |

Cont., control; Dex., dexamethasone. G.R., growth ratio. *a*) +, >10%; ++, >25%; +++, >50%.

Table 3. Differentiation Inducing Effect of Steroid Glycosides Employed with Differentiation Inducers

| | EtOH | Dex. 0.2 μM | CG-C 20 μM | 1 20 μM | 9 20 μM | 10 20 μM |
|---|---|---|---|---|---|---|
| EtOH | 100[a] | 64 | 44 | 83 | 79 | 76 |
| | —[b] | ++ | + | + | + | + |
| Ara-C | 84 | 68 | 46 | 91 | 70 | 59 |
| 10 nM | − | + | + | + | − | + |
| Act-D | 87 | 54 | 51 | 62 | 65 | 44 |
| 1 nM | − | +++ | ++ | ++ | − | ++ |
| dbc-AMP | 83 | 70 | 51 | 91 | 89 | 68 |
| 100 μM | − | ++ | + | − | + | − |

a) Growth ratio (%). b) Phagocytosis, +, >10%; ++, >25%; +++, >50%.

Table 4. $^1$H-NMR Spectral Data (δ in ppm)

| Proton No. | 2 | 4 |
|---|---|---|
| 6 H | 5.38 m | 5.33 m |
| 18 Me | 0.65 s | 0.64 s |
| 19 Me | 1.00 s | 0.93 s |
| 20 H | 3.73 br q ($J$=6) | 3.75 br q ($J$=6) |
| 21 Me | 1.58 d ($J$=6) | 1.57 d ($J$=6) |
| 3 cym | | |
| 1 H | | 5.26 dd ($J$=10, 2) |
| 6 Me | | 1.57 d ($J$=6) |
| OMe | | 3.54 s |
| dig | | |
| 1 H | | 4.72 d ($J$=8) |
| 6 Me | | 1.65 d ($J$=6) |
| OMe | | 3.55 s |
| 20 dig | | |
| 1 H | 4.67 d ($J$=7.5) | 4.75 d ($J$=7.5) |
| 2 H | 4.84 dd ($J$=10, 8) | 4.78 br |
| 6 Me | 1.47 d ($J$=6.5) | 1.52 d ($J$=6) |
| OMe | 3.49 s | 3.57 s |
| glc | | |
| 1 H | 5.25 d ($J$=7.5) | 5.10 d ($J$=8) |
| glc | | |
| 1 H | 5.36 d ($J$=8) | 5.35 d ($J$=8) |

Recorded at 270.00 or 500.0 MHz in $C_5D_5N$.

(**12**), a 19-oxygenated compound of peliprocymarin (**8**), was lower than **8**. Oleandrin (**13**) and ouabain (**14**), having highly oxygenated aglycones, scarcely showed any activity. In these cardenolide compounds, the hydrophobic property of their aglycone moieties might play an important role in inducing the differentiation of Ml cells.

In the previous paper, we reported the enhancing effect of dexamethasone on the differentiation inducing activities of flavones using Ml cells.[2b] The combined effect of steroid glycosides (**1**, **9**, **10**, condurangoglycoside C) with other known differentiation inducers is shown in Table 3. Arabinofuranosyl cytosine (ara-C), known as an inhibitor of DNA polymerase, actinomycin-D (act-D), known as inhibitor of RNA polymerase, and dibutyryl c-AMP (dbc-AMP), known as a c-AMP analog, were employed at concentrations which showed no effects on the cell growth or differentiation of Ml cells. 1 nM of act-D enhanced the activities of steroid compounds, but ara-C and dbc-AMP did not.

Zhang *et al.* reported the differentiation inducing activities of bufalin against human myeloid leukemia cells and the compatibility between the activities and their inhibitory effect on $Na^+/K^+$ ATPase activity.[4] Cardenolide glycosides were also known to have an inhibitory effect on $Na^+/K^+$ ATPase activity, and similar properties between cardenolides and bufalin will be informative in discussing the mechanism involved in cardenolide-mediated induction of the differentiation of Ml cells.

Table 5. $^{13}$C-NMR Spectral Data in $C_5D_5N$ (δ in ppm)

| Carbon No. | 2 | 4 | Carbon No. | 2 | 4 |
|---|---|---|---|---|---|
| Aglycone moiety | | | Sugar moiety | | |
| 1 | 37.9 | 37.4[a] | dig | | |
| 2 | 32.7 | 30.4 | 1 | | 106.9 |
| 3 | 71.3[a] | 78.2[b] | 2 | | 71.2[d] |
| 4 | 43.5 | 39.4 | 3 | | 84.9 |
| 5 | 141.8 | 140.5 | 4 | | 70.0 |
| 6 | 121.5 | 122.3 | 5 | | 71.3[d] |
| 7 | 32.3 | 32.1 | 6 | | 17.3[e] |
| 8 | 32.0 | 31.4 | OMe | | 57.2[f] |
| 9 | 50.5 | 49.9 | 20 dig | | |
| 10 | 37.0 | 36.9[a] | 1 | 104.3 | 104.0 |
| 11 | 21.2 | 20.9 | 2 | 76.3 | 75.9 |
| 12 | 39.3 | 39.4 | 3 | 85.4 | 85.7 |
| 13 | 42.7 | 42.9 | 4 | 68.4 | 69.5 |
| 14 | 58.2 | 53.5 | 5 | 72.2[b] | 71.5 |
| 15 | 27.1 | 35.1 | 6 | 17.5 | 17.4[e] |
| 16 | 24.6 | 77.2[c] | OMe | 56.8 | 57.3[f] |
| 17 | 56.8 | 69.1 | glc | | |
| 18 | 12.8 | 13.9 | 1 | 104.5 | 105.2 |
| 19 | 19.7 | 19.5 | 2 | 75.4[c] | 75.0 |
| 20 | 81.9 | 82.0 | 3 | 77.8 | 77.3[c] |
| 21 | 23.3 | 23.7 | 4 | 70.8[a] | 70.9 |
| Sugar moiety | | | 5 | 77.4[b] | 76.7 |
| 3 cym | | | 6 | 70.0 | 68.4 |
| 1 | | 96.2 | glc | | |
| 2 | | 37.1[a] | 1 | 105.3 | 105.3 |
| 3 | | 77.8 | 2 | 75.5[c] | 75.8 |
| 4 | | 83.6 | 3 | 78.2[d] | 78.3[b] |
| 5 | | 71.1[d] | 4 | 71.9 | 71.3[d] |
| 6 | | 18.9 | 5 | 78.3[d] | 78.5 |
| OMe | | 58.7 | 6 | 63.0 | 63.0 |

Recorded at 67.8 or 125.65 MHz in $C_5D_5N$. *a*—*f*) Assignment may be interchanged within each column.

## Experimental

**General Procedure** Optical rotations were measured on a JASCO DIP-360 digital polarimeter. Ultraviolet(UV) spectra were measured on a Hitachi U3410 spectrophotometer. Mass spectra(MS) were taken on a JEOL JMS-SX 102 mass spectrometer. $^1$H- and $^{13}$C- NMR spectra were recorded on a JEOL JNM-GSX 270 and JNM-GSX 500 spectrometer (270.05, 67.8 MHz, 500.00, 125.65 MHz, respectively) and chemical shifts are given in δ (ppm) with tetra-methylsilane (TMS) as an internal standard (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad). HPLC was carried out on a JASCO model 800 series using PRO-10 Zorbax and a D-ODS-7 YMC column.

**Isolation** Commercially available Periplocae Radicis Cortex (3 kg from Niiya in Shimizu) was extracted with hot MeOH under reflux. The extract was concentrated under reduced pressure and the extract was partitioned with AcOEt and water. The water layer was passed through a Diaion HP-20 column. After the content of the column was washed with water, the absorbed material was eluted with 50% MeOH and 100% MeOH successively to give a brown gum(water eluate 158 g, 50% MeOH eluate 33 g, 100% MeOH eluate 48 g). The 100% MeOH eluate was chromatographed repeatedly on a silica gel column with a $CHCl_3$–MeOH solvent system and HPLC to afford the active

components **1** (250 mg), **2** (300 mg), **3** (550 mg), **4** (800 mg), **5** (1.0 g), **7** (50 mg) and **9** (130 mg). From the AcOEt layer (55 g), the active compounds **6** (160 mg) and **8** (50 mg) were isolated using chromatography on silica gel and HPLC.

Plocoside A (**2**): Colorless amorphous powder, $[\alpha]_D$ −23.5° ($c$= 0.57, MeOH). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 238 (3.09), 256 (3.03). *Anal.* Calcd for $C_{40}H_{66}O_{16}\cdot 2H_2O$: C, 57.26; H, 8.41. Found: C, 57.02; H, 8.18. FAB-MS $m/z$: 803 $[M+H]^+$. $^1$H-NMR: Table 4 and $^{13}$C-NMR: Table 5.

Plocoside B (**4**): Colorless amorphous powder, $[\alpha]_D$ 18.5° ($c$=0.54, MeOH). UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 225 (3.14), 237 (2.90). *Anal.* Calcd for $C_{54}H_{90}O_{24}\cdot 3/2H_2O$: C, 56.38; H, 8.15. Found: C, 56.27; H, 7.98. FAB-MS $m/z$: 1146 $[M+Na]^+$. $^1$H-NMR: Table 4 and $^{13}$C-NMR: Table 5.

**Materials** Eagle's minimum essential medium (MEM), Eagle's MEM amino acids and vitamins medium were purchased from Nissui Pharmaceutical Co., Ltd. Calf serum (CS) was from Gibco. Dexamethasone was from Nakalai Chemicals, Ltd. 12-*O*-Tetradecanoylphorbal 13-acetate (TPA), oleandrin and ouabain were from Sigma Chemical Co. Polystyrene latex particles were from The Dow Chemical Company. Digitoxin and digoxin were from Tokyo Chemical Industry Co., Ltd. Cymarin was from Fluka Chemika-Biochemika.

**Cell Culture** Ml cells were grown in Eagle's MEM medium containing 10% heat-inactivated CS and were diluted when the cell density reached about $2\times10^6$ cells per ml in a 5% $CO_2$ humidified atmosphere at 37 °C.

**Measurement of Phagocytosis** Phagocytic activity was assayed as reported previously.[1)]

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