# 28-Deacetylbelamcandal, a Tumor-Promoting Triterpenoid from *Iris tectorum*

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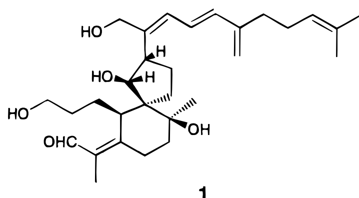
A spiroiridal-type triterpenoid, 28-deacetylbelamcandal (**1**), was isolated from the rhizomes of *Iris tectorum* as an active principle that stimulated differentiation of human promyelocytic leukemia (HL-60) cells, a short-term screening method for 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-type tumor promoters. In the same manner as TPA, compound **1** bound to protein kinase C (PKC) and activated PKC, and induced tumor necrosis factor-α release from HL-60 cells. In an in vivo study, groups treated with 100 μg 7, 12-dimethylbenz[*a*]anthracene plus 400 nmol of **1** showed 64.3% tumor incidence by week 20. It has thus been demonstrated that **1** represents a new structural class of mouse skin-tumor promoters.

Phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and compounds of other structural types, such as teleocidins and aplysiatoxins, are well-known potent tumor promoters in two-stage carcinogenesis experiments on mouse skin.[1] These classes of promoters activate calcium and phospholipid-dependent protein kinase C (PKC),[2,3] induce tumor necrosis factor-α (TNF-α) release from target cells,[4,5] and differentiate promyelocytic leukemia (HL-60) cells to macrophages.[6,7]

In a previous study,[8] methanolic extracts of various plants and crude drugs representing more than 180 species were tested for their activity to induce HL-60 cell adhesion, a short-term screening method for TPA-type tumor promoters.[9] Among these, methanolic extracts of several plants in the Iridaceae showed such activity,[8] suggesting potential tumor-promoting activity. Further fractionation of the methanolic extract of *Iris tectorum* Maxim. (Iridaceae) guided by the above-mentioned assay afforded a spiroiridal triterpenoid, 28-deacetylbelamcandal (**1**), as an active principle. This paper describes the isolation and biological evaluation of **1**, with regard to binding to PKC, activation of PKC activity, induction of TNF-α release from HL-60 cells, and tumor-promoting activity on mouse skin.




## Results and Discussion

The dried rhizomes of *I. tectorum* were extracted with MeOH at room temperature for 3 days, and the solvent was evaporated. The resultant MeOH extract was suspended in $H_2O$ followed by extraction with $CHCl_3$ to give a $CHCl_3$-soluble portion. Of these two extracts, the $CHCl_3$ extract caused a morphological change of HL-60 cells involving adhesion to culture plates. The $CHCl_3$ extract was subjected to column chromatography over Si gel using $CHCl_3$, EtOAc, and MeOH as eluents. A EtOAc-soluble fraction

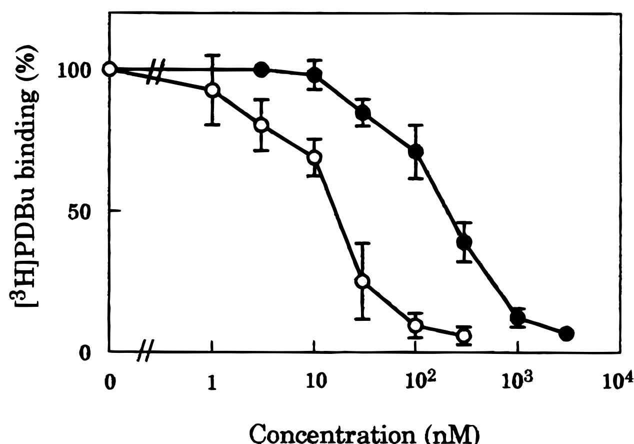



**Figure 1.** Inhibition of specific [$^3$H]PDBu binding to protein kinase C by **1** and TPA. [28-deacetylbelamcandal (**1**) (●), TPA (○)]. (Values are expressed as the mean ± S.E. of three experiments with duplicate assays.)

with biological activity was purified by a combination of reversed-phase chromatographic methods to yield compound **1** as an active component. Compound **1** was obtained as a white glassy solid and had a $[M^+ - H_2O]^+$ peak at *m*/*z* 468 in the EIMS. Detailed analysis of the 2D NMR spectra of **1**, including the $^{13}C-^1H$ COSY and HMBC spectra, revealed the compound to be a known bicyclic spiroiridal, 28-deacetylbelamcandal, which has been isolated previously from *Belamcanda chinensis* (Iridaceae).[10] The minimum concentration required to cause 100% adhesion of HL-60 cells was 500 nM for **1** and 3 nM for TPA (Table 1).

TPA-type tumor promoters bind to PKC in the presence of phospholipid and result in activation.[3,11] Also, it is well known that TPA promotes chemical carcinogenesis mediated through the activation of PKC.[2,3] An assay was carried out by examining the inhibitory effect of **1** on the binding of [$^3$H]phorbol 12,13-dibutylate (PDBu) to partially purified PKC.[12] Compound **1** inhibited the specific [$^3$H]PDBu binding to PKC, similar to TPA in a dose-dependent fashion (Figure 1). The $IC_{50}$ value (the concentration of compound that inhibited 50% of the [$^3$H]PDBu binding) of **1** was 200 nM. Figure 2 shows the result of examination for PKC activation of **1** by measuring the incorporation of $^{32}P$ into histone III−S from [$\gamma$-$^{32}$P]ATP.[13] Compound **1** directly enhanced PKC activity. In both tests, the potency of **1** was approximately 1/10−1/20 of that of TPA, and the activation

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**Table 1.** Biochemical and Tumor-Promoting Activities of 28-Deacetylbelamcandal (1) and TPA

| compound | HL-60 cell adhesion activity 100% adhesion (nM)[a] | inhibition of [$^3$H]PDBu binding $IC_{50}$ | PKC activity $ED_{50}$ (nM) | induction of TNF-α release $ED_{100}$ (nM)[b] | tumor-promoting activity: nmol/ application | tumor-promoting activity: tumor incidence mice (%) | tumor-promoting activity: average no. of tumors/mouse |
|---|---|---|---|---|---|---|---|
| 1 | 500 | 200 | 300 | 3000 | 400 | 64.3 | 4.0 |
| TPA | 3 | 15 | 15 | 6 | 4 | 93.3 | 7.3 |

[a] The concentration for 100% adhesion of all live HL-60 cells. [b] The concentration for 100 pg/mL TNF-α release from HL-60 cells.

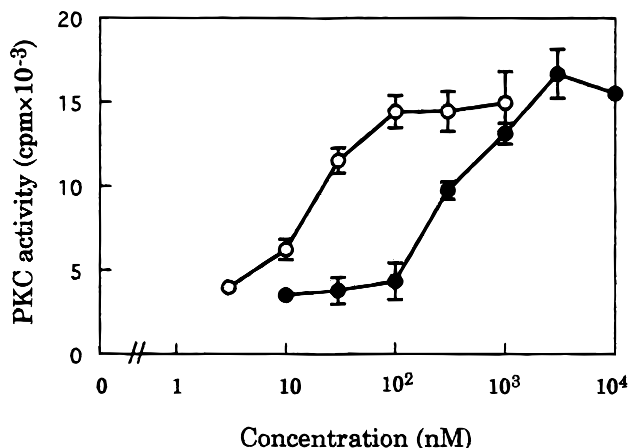


**Figure 2.** Activation of protein kinase C by **1** and TPA. [28-deacetylbelamcandal (**1**) (●), TPA (○)]. (Values are expressed as the mean ± S.E. of three experiments with duplicate assays.)

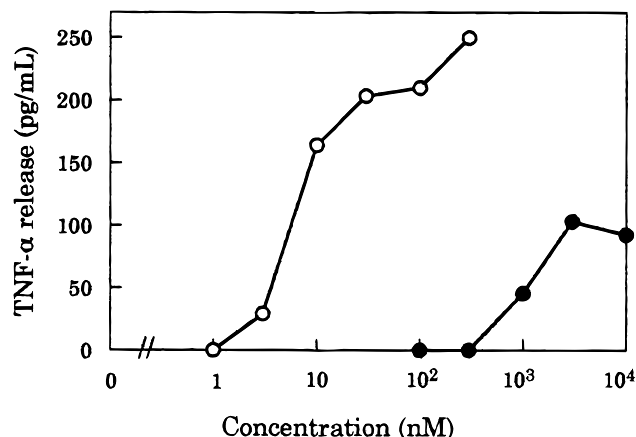


**Figure 3.** Induction of TNF-α release from HL-60 cells treated with **1** and TPA. [28-deacetylbelamcandal (**1**) (●), TPA (○)].

of PKC correlated well to that of binding to PKC (Table 1, Figures 1 and 2).

Recently, it has been reported that TPA-type and non-TPA-type tumor promoters both induce TNF-α mRNA expression in their target tissues and TNF-α release from the cell. However, their mechanisms of action are different, that is, activation of PKC and inhibition of protein phosphatases 1 and 2A, respectively.[5] Because TNF-α acts as an endogenous tumor promoter, evaluation of TNF-α release is a practical method to determine the potential tumor-promoting activity of an agent. When induction of TNF-α release from HL-60 cells was measured, **1** was found to be an active inducer of this type of activity. The $ED_{100}$ values (the concentration required to release 100 pg/mL TNF-α into the medium) of **1** and TPA were 3000 nM and 6 nM, respectively (Figure 3 and Table 1). These results suggested that **1** has potential as a tumor promoter, which acts in a manner similar to TPA.

Accordingly, the tumor-promoting activity of **1** was examined in a two-stage carcinogenesis experiment on mouse skin. Based on the results of these biochemical activities, the dose of **1** for the experiment was estimated to be about 100-fold the amount of that of TPA. Consequently, 400 nmol (200 μg)/application of **1** and 4 nmol/application of TPA were administered after initiation by 7,12-dimethylbenz[*a*]anthracene (DMBA). In the groups treated with DMBA plus **1** and with DMBA plus TPA the percentages of tumor incidence were 64.3% and 93.3%, and the average number of tumors per mouse were 4.0 and 7.3, in week 20, respectively (Figure 4 and Table 1). DMBA alone produced only one tumor on one mouse in week 20. The groups treated with **1** alone and TPA alone showed no tumors. 28-Deacetylbelamcandal (**1**), a bicyclic spiroiridal-type triterpenoid, has thus been found to be a new class of mouse skin-tumor promoter with a structure quite different from those of the phorbol ester, teleocidins, and aplysiatoxins.

Iridal-type triterpenoids are characteristic constituents of plants in the genera *Iris* and *Belamcanda*. Iridal

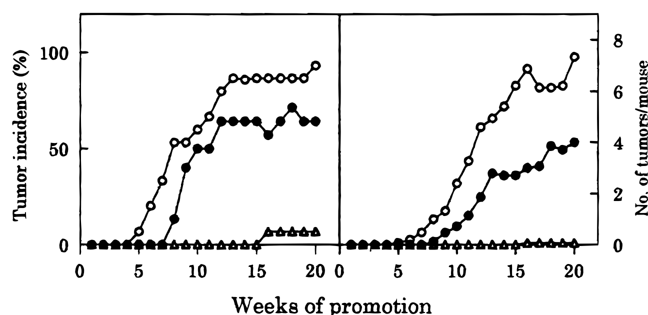


**Figure 4.** Tumor-promoting activity of 28-deacetylbelamcandal (**1**) and TPA in a two-stage carcinogenesis experiment on mouse skin. The groups were treated with DMBA plus 28-deacetylbelamcandal (**1**) (●), with DMBA plus TPA (○) and DMBA alone (△).

compounds have been reported to exhibit antiulcer activity[14] and piscicidal activity.[15] The present results suggest that other congeners of **1** may be potential mouse skin-tumor promoters.

## Experimental Section

**General Experimental Procedures.** The optical rotation was measured on a JASCO DIP-370 digital polarimeter. UV and IR spectra were recorded on a Shimadzu UV-2200 and a Shimadzu FTIR-8100A spectrometer, respectively. $^1$H and $^{13}$C NMR spectra were recorded in $CDCl_3$ on a JEOL JNM EX-400 FTNMR spectrometer (400 MHz) with TMS as internal standard. EIMS was recorded on a JEOL JMS D-300 spectrometer.

**Materials.** DEAE cellulose (DE−52), phenyl−Sepharose CL-4B, [$\gamma$-$^{32}$P]ATP, and the TNF-α ELISA kit were purchased from Amersham Pharmacia Biotech. UK, Ltd. (Amersham, Bucks, UK). Leupeptin was obtained from Chemicon International Inc. (Temecula, CA). [$^3$H]PDBu was purchased from NEN Life Science Products Inc. (Boston, MA). Phosphatidylserine was obtained from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). ATP, histone III−S, PDBu, and polyethylenimine were purchased from Sigma Chemical Co. (St. Louis, MO). TPA was obtained from Chemsyn Science Lab. (Lenexa, KS) and Sigma Chemical Co. Wakogel C-200 (Wako Pure Chemical Industry, Osaka, Japan) was used for column chromatography.

**Plant Material.** The rhizomes of *I. tectorum* were purchased from Nakajima Pharmaceutical Co. (Ohmiya, Japan) in November 1990. A voucher specimen has been deposited at the Department of Food Chemistry, Saitama Prefectural Institute of Public Health.

**Extraction and Isolation.** The MeOH extract (75.4 g) of the dried rhizomes of *I. tectorum* Maxim. (500 g) was partitioned between $CHCl_3$ and $H_2O$. The $CHCl_3$ layer was evaporated and subjected to column chromatography on Si gel and eluted with $CHCl_3$, EtOAc, and MeOH, in a stepwise fashion. The EtOAc-soluble fraction was further separated on liquid chromatography with the following columns in turn: LiChroprep RP-18 (Merck, Darmstadt, Germany; 25 mm i.d. × 310 mm, MeOH–$H_2O$, 85:15) and LiChrospher RP-18 (e) (Merck; 10 mm i.d. × 250 mm; MeCN–$H_2O$, 70:30), to yield compound **1** (50 mg).

**28-Deacetylbelamcandal (1):** white glassy substance; $[\alpha]_D$ +120° (*c* 0.06, $CHCl_3$) [lit.[10] $[\alpha]_D$ + 96.0° (*c* 0.4, MeOH)]; UV (EtOH) $\lambda_{max}$ (log $\epsilon$) 257 infl. (4.40), 268 (4.47), 278 (4.49), 287sh (4.38); IR $\nu_{max}$ ($CHCl_3$) 3360, 2920, 2875, 1650, 1610, 1470, 1350, 1270, 1230, 1190, 1050, 1000, 960; EIMS *m*/*z* 468 ($M^+$ − $H_2O$); $^1H$ NMR and $^{13}C$ NMR spectral data consistent with those of 28-deacetylbelamcandal (**1**) in the literature.[10]

**Induction of HL-60 Cells Adhesion.** Induction of HL-60 cell adhesion was examined as described previously.[8] HL-60 cells were cultivated in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum. The cells (3.2 × $10^4$ cells, 0.1 mL) were incubated with test compound for 48 h. Adherent cells accompanied with macrophage-like differentiation were observed by a microscope (×100).

**Partial Purification of Protein Kinase C.** PKC was partially purified by DE–52 and phenyl–Sepharose CL-4B chromatography by modifying the methods of Inagaki et al.[16] and Kitano et al.,[17] in the following manner. Five rabbit brains were homogenized with 5 volumes of 25 mM Tris HCl (pH 7.5) containing 2 mM EGTA, 5 mM DTT, 0.05% leupeptin, and 0.25 M sucrose. The homogenate was centrifuged for 60 min at 100 000*g*. The supernatant was applied to a DE–52 column (3 × 19 cm) equilibrated with 25 mM Tris HCl (pH 7.5) containing 2 mM EGTA, 5 mM DTT, and 0.001% leupeptin (Buffer A). After washing the column with both Buffer A and Buffer A containing 20 mM NaCl, the enzyme was eluted with a 800-mL linear concentration gradient of NaCl (0.02–0.4 M) in Buffer A. The active fraction was dialyzed against Buffer A containing 1 M NaCl and applied to phenyl–Sepharose CL-4B column (0.85 × 3 cm) equilibrated with Buffer A containing 1 M NaCl. PKC was eluted with a 40-mL linear concentration gradient of NaCl (1.0–0 M) in Buffer A. The specific activity of partially purified PKC was 70–80 nmol/min/mg. Protein was determined by using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard.

**Inhibition of [$^3$H]PDBu Binding to Protein Kinase C.** [$^3$H]PDBu binding to PKC was examined as described previously.[12] Binding was carried out in the standard reaction mixture (0.2 mL) in 20 mM Tris malate (pH 6.8), 100 mM KCl, 0.2 mM $CaCl_2$, 0.1 mg/mL phosphatidylserine, 30 nM [$^3$H]-PDBu (370 GBq/mmol), 0.5% DMSO, and 10 $\mu$L PKC. After incubation for 3 h in ice-cold $H_2O$, 4 mL of ice-cold 0.5% DMSO was added. The reaction mixture was poured onto a GF/B filter (Whatman, Maidstone, UK), which had been soaked in fresh 0.3% polyethylenimine solution for at least 1 h before use. The bound radioactivity was determined using a liquid scintillation counter.

**Activation of Protein Kinase C.** PKC activity was determined by measuring the incorporation of $^{32}$P into histone III–S from [$\gamma$-$^{32}$P]ATP, a modified procedure of Castagna *et al.*[13] The standard reaction mixture used (0.1 mL) contained 40 mM Tris HCl (pH 7.5), 10 $\mu$M [$\gamma$-$^{32}$P]ATP (200 dpm/pmol), 10 mM $MgCl_2$, 0.5 mg/mL histone III–S, 2.5 $\mu$g phosphatidylserine, 20 $\mu$M $CaCl_2$, and 5 $\mu$L PKC. After incubation for 10 min at 30 °C, the reaction was stopped by adding ice-cold 25% trichloroacetic acid, and acid-perceptible materials were collected on a GF/C filter (Whatman). The radioactivity of $^{32}$P was quantified by Cerenkov counting.

**Induction of TNF-α Release from HL-60 Cells.** HL-60 cells (5 × $10^5$ cells/mL) were incubated in RPMI-1640 medium containing 10% fetal bovine serum with **1** or TPA for 24 h. Cells were removed by centrifugation. TNF-α in medium released from cells was measured by an ELISA kit.[5]

**Tumor Promotion in Mouse Skin.** Female CD-1 mice were obtained from Charles River Japan, Inc. (Kanagawa, Japan). A two-stage carcinogenesis experiment on mouse skin was performed as described previously.[5] Initiation was achieved by a single application of 100 $\mu$g DMBA. From one week after this initiation, repeated topical applications of **1** (400 nmol) or TPA (4 nmol) were provided twice a week, until week 20. As controls, mice were treated with DMBA alone, compound **1** alone, or TPA alone. DMBA, compound **1**, and TPA were dissolved in $Me_2CO$. Each experimental group consisted of 15 female CD-1 mice.

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