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Anti-HIV-1 phorbol esters from the seeds of *Croton tiglium*

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Abstract

Five phorbol diesters, together with three known ones, were isolated from a MeOH extract of the seeds of *Croton tiglium*, and their structures were determined by spectroscopic methods and selective hydrolysis of acyl groups. These compounds were assessed for their abilities to inhibit an HIV-induced cytopathic effect (CPE) on MT-4 cells and to activate protein kinase C (PKC) associated with tumor-promoting action. 12-*O*-Acetylphorbol-13-decanoate and 12-*O*-decanoylphorbol-13-(2-methylbutyrate) effectively inhibited the cytopathic effect of HIV-1 [complete inhibitory concentration (IC_{100}) values of 7.6 ng/ml and 7.81 μg/ml, and minimum cytotoxic concentration (CC_0) value of 62.5 and 31.3 μg/ml, respectively]; however, 12-*O*-acetylphorbol-13-decanoate showed no activation of PKC at concentrations of 10 and 100 ng/ml. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was found to be not only the most potent inhibitor of HIV-1-induced CPE (IC_{100} value of 0.48 ng/ml), but also the most potent activator of PKC (100% activation at 10 ng/ml).

Keywords: 12-*O*-Acetylphorbol-13-decanoate; HIV-1; *Croton tiglium*; Phorbol esters; Protein kinase C

1. Introduction

Since the discovery of human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS), significant progress has been made towards the development of anti-HIV agents. A variety of chemically disparate compounds derived from plants has been shown to be effective in either inhibiting the replication of HIV-1 or its essential enzymes (Che, 1991; Schinazi, 1992; Nasr, Cradock & Johnson, 1992; El-Mekkawy, Meselhy, Kusumoto, Kadota, Hattori & Namba, 1995; El-Mekkawy et al., 1998; Ng, Huang, Fong & Yeung, 1997; Kusumoto & Hattori, 1999). In the course of our search for anti-HIV-1 agents from natural sources, a number of plant extracts used in Egyptian folk medicine were evaluated for possible anti-HIV properties, and it was apparent that the MeOH and water extracts of the seeds of *Croton tiglium* significantly inhibited the infectivity and HIV-1-induced cytopathic effect (CPE) on MT-4 cells at concentrations (IC_{50} values of 0.025 and 2.0 μg/ml, respectively) below the cell toxic concentration (selectivity indices of 34.4 and 50.0, respectively) (Kawahata et al., 1996). *C. tiglium* belongs to the family Euphorbiaceae and is known to contain tigliane phorbol esters. These compounds have been shown to be responsible for eliciting a remarkable range of biochemical effects. Although the ability of these compounds to promote tumors presents one potential limitation to their utility (Evans & Taylor, 1983; Evans & Soper, 1978; de Chaffoy de Courcelles, Roevens & van Belle, 1984; Hecker, 1978; Blumberg, 1980, 1981, 1988; Gschwendt & Hecker, 1974), it should be stressed that there are many phorbol esters that exert profound beneficial biological effects without tumorigenesis. 12-*O*-Tigloylphorbol-13-decanoate isolated from croton oil demonstrated antileukemic activity against the P-388 leukemia in mice (Kupchan, Uchida, Branfman, Daily

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& Yu-Fei, 1976). Ostodin and 12-*O*-undecadienoylphorbol-13-acetate are potent antitumor agents (Handa, Kinghorn, Cordell & Farnsworth, 1983). Prostratin, 12-deoxyphorbol-13-acetate, was isolated from a Samoan plant, *Homalanthus nutans*, as an anti-HIV principle (Gustafson et al., 1992). Although structurally belonging to the phorbol esters, it does not exhibit tumor-promoting effects, while 12-deoxyphorbol-13-tetradecanoate isolated from *Euphorbia triangularis* possesses potent tumor-promoting activity (Gschwendt & Hecker, 1974). It has been reported that tumor-promoting phorbol esters interact with and activate protein kinase C (PKC), which phosphorylates a variety of substrates within the cell (Nishizuka, 1984; Castagna, Takai, Kaikuchi, Sano, Kikkawa & Nishizuka, 1982). The tumor-promoting and HIV-1 inhibitory effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were found to be mediated through its direct activation of PKC (Chowdhury et al., 1990).

In an effort to isolate a selective anti-HIV agent that does not activate PKC, we investigated the HIV-1 inhibitory components in a MeOH extract of the seeds of *C. tiglium*, from which we isolated eight phorbol diesters. Herein, we present the inhibitory effects of these compounds against HIV-1, as well as their activation of PKC.

2. Results and discussion

By bioassay directed fractionation of the ether-soluble portion of the MeOH extract of the seeds of *C. tiglium*, we isolated eight compounds (**1**–**8**) which inhibited HIV-1-induced CPE. All were phorbol diesters as determined by analysis of their NMR and MS data. The acyl groups and the sites of acylation were determined by GC/MS after selective hydrolysis. Compounds **6**–**8** were identified as 12-*O*-acetylphorbol-13-decanoate (**6**), 12-*O*-(2-methylbutyryl)phorbol-13-dodecanoate (**7**), and 12-*O*-tetradecanoylphorbol-13-acetate (**8**, TPA) (Hecker, 1971; Hecker & Schmidt, 1974). Compounds **1**–**5** are new and their structures were determined as follows.

Compound **1** (API-MS m/z 691 $[M+Na]^+$) showed absorption bands in the IR (ν_{max} 1650 cm^{-1}) and UV spectra (λ_{max} 243 nm) characteristic for an α,β-conjugated C═O group. The ^1H- and ^{13}C-NMR spectra analyzed by the aid of ^1H–^1H COSY and HMQC experiments showed patterns similar to those of **6**–**8**, with signals at δ 7.58 for H-1 (C-1 at δ 160.4), 1.78 for H-19 (C-19 at δ 10.1), and 208.7 for C-3. Signals for two ester carbonyls were also seen at δ 174.0 and 173.5. The downfield shift of C-13 (δ 68.0) and C-20 (δ 69.2; H_2-20 at δ_H 4.46) suggested acylation at the two carbons. Long-range correlations observed in the HMBC spectrum of **1** between H_2-20 and a carbonyl carbon signal at δ 173.5 confirmed acylation at C-20 with an octadecadienoyl group. The 9*Z*, 12*Z* configuration of this residue was evident from the ^{13}C-NMR spectrum [CH_2–CH═CH–CH_2–CH═CH–$\underline{C}H_2$ appeared at δ 27.2]. Furthermore, hydrolysis of **1** with $HClO_4$ gave two products. The first product was identified as a monoester, **9** (m/z 406 $[M]^+$, H_2-20 at δ 4.00), and the second product was, after methylation, identified as 9*Z*,12*Z*-octadecadienoic acid methyl ester by GC/MS (R_t 19.25 min, m/z 294 $[M]^+$). The chemical shifts of H-1, H-7 (δ 5.70), H-8 (δ 3.20) and H-10 (δ 3.14) are diagnostic for the A/B *trans*-ring junction (Sakata, Kawazu & Mitsui, 1971). Furthermore, we observed NOESY correlations of H-1 with H-10, H-18 (δ 1.04) and H-19 (δ 1.78), and between H_2-5 (δ 2.52, 2.38) and H-8, which led us to propose the structure of **1** as 13-*O*-acetylphorbol-20-(9*Z*,12*Z*-octadecadienoate).

Compound **2** (API-MS, m/z 731 $[M+Na]^+$) provided spectral data quite similar to that of **1**. However, signals for an acetyl group in **1** were replaced by signals for a tigloyl group in **2** [δ_H 6.87 (δ 138.7, $C(CH_3)$═$\underline{C}$H–), 128.1 (*s*, $\underline{C}$═CH), 1.80 and 1.82 (δ 14.5 and 10.1, 2 × $\underline{C}H_3$)]. The HMBC spectrum indicated acylation at C-13 with a tigloyl group. The 9*Z*,12*Z*-octadecadienoyl group (identified by GC/MS after selective hydrolysis with $HClO_4$ followed by methylation) was assigned to C-20. The structure of **2** was established to be 13-*O*-tigloylphorbol-20-(9*Z*,12*Z*-octadecadienoate).

By analysis of NMR and MS data of **3** (EI-MS, m/z 488 $[M]^+$ and fragment ions at 428 and 389), the compound was expected to be a phorbol-12,13-diester possessing two acyl groups with molecular weights of 59 and 99 (see Section 3). On the basis of HMBC and GC/MS, an acetyl group (δ_H at 2.1, 3H, *s*) was assigned to C-12 (δ_H 5.43/δ_C 76.6), and a tigloyl group (a methine proton signal at δ 6.85 for $C(CH_3)$═$C\underline{H}$, rather than at δ 6.15 in its isomer, angeloyl group) (Evans, 1978) to C-13 (δ_C 65.7). Thus, **3** was established as a new phorbol diester, 12-*O*-acetylphorbol-13-tigliate.

Most of the spectral data of **4** (EI-MS m/z 602 $[M]^+$) were similar to those of **6** except that the signals of an acetyl group in **6** were replaced by signals for a 2-methylbutyryl group in **4**. Selective hydrolysis with 0.1 M KOH/MeOH (Taylor, Gafur, Choudhury & Evans, 1981; Taylor, Williamson & Evans, 1983) gave 2-methylbutyric acid methyl ester, which was identified by GC/MS (R_t 9.40 min, m/z 116 $[M]^+$) and confirmed acylation at C-13. Similarly, a decanoyl moiety was assigned to C-12 after hydrolysis with 0.2 M NaOMe and GC/MS (decanoic acid methyl ester at R_t 10.16 min, m/z 186 $[M]^+$). The sites of acylation were further confirmed by HMBC. The structure of **4** was determined to be 12-*O*-decanoylphorbol-13-(2-methylbuty-

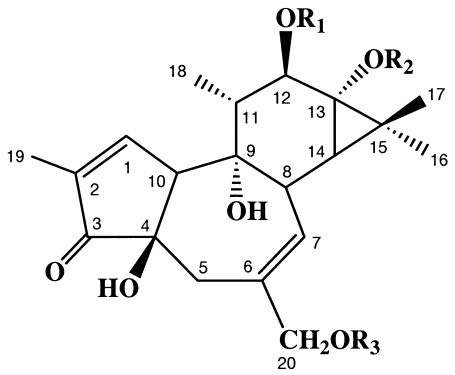
rate), a new positional isomer of a compound obtained previously from the plant under investigation (Evans & Taylor, 1983; Hecker & Schmidt, 1974).

The decanoyl group at C-12 of **4** was replaced by a tigloyl group in **5** (EI-MS *m/z* 530 $[M]^+$). Assignment of the position of acylation was made on the basis of HMBC and GC/MS experiments. Accordingly, **5** was confirmed as 12-*O*-tigloylphorbol-13-(2-methylbutyrate), a new positional isomer of the compound isolated previously from *Euphorbia frankiana* Berger and *E. coerulescens* Haw (Evans & Taylor, 1983; Hecker & Schmidt, 1974). To the best of our knowledge, **1**–**5** have not been previously isolated as natural products from plant sources.

The phorbol esters **1**–**8** were tested for their ability to inhibit the replication and infectivity of HIV-1 on MT-4 cells, a human CD_4-positive cell line carrying HTLV-I, and the results are presented in Table 1. The most potent compounds, **8** and **6**, had complete inhibition of CPE at concentrations (IC_{100}) of 0.48 and 7.6 ng/ml, with minimum cytotoxic concentrations (CC_0) of 31.3 and 62.5 μg/ml, respectively. Next in potency were **2** and **4** (IC_{100} values of 7.8 μg/ml; CC_0 values of 62.5 and 31.3 μg/ml, respectively). Complete inhibition of CPE by **1** and **7** was observed at concentrations of 15.6 μg/ml (CC_0 values of 62.5 μg/ml). Complete inhibition by **5** was seen at a concentration of 31.3 μg/ml (CC_0 value of 62.5 μg/ml), while complete inhibition by **3** was only seen at 125.0 μg/ml.

Under the conditions where PKC from mouse brain was activated by calcium, phosphatidylserine and TPA, the effects of **1**–**8** on PKC were investigated at a standard concentration of 10 ng/ml (Table 1). TPA (**8**) showed almost 100% activation of PKC, while **6,** which demonstrated potent anti-HIV-1 effects, showed no activation at 10–100 ng/ml. Other compounds (**1** and **4**) did not activate PKC at 10 ng/ml, while **2**, **3**, **5** and **7** demonstrated a 10–16% increase in the activity of PKC at 10 ng/ml.

In the present experiment, TPA (**8**) was found to be an equipotent inhibitor of HIV-1-induced CPE and an activator of PKC (similar results were previously reported by Chowdhury et al., 1990). They found that 0.4 ng/ml of TPA acts through the activation of PKC in down-modulating CD_4 molecules and syncytia formation. Of greatest interest was the fact that compound **6** demonstrated anti-HIV activity as low as 7.6 ng/ml without activating PKC (no activation was seen at concentrations of 10–100 ng/ml). Although not directly translatable to dose response and therapeutic index, the results obtained suggested that **6,** which demonstrated HIV inhibition without PKC activation, is worthy of further investigation. Structural modifications of **6** and **8** are currently underway in our laboratories in order to obtain a selective anti-HIV-1 phorbol ester.



	R_1=	R_2=	R_3=
1	H	Ac	$C_{18}H_{31}O$
2	H	Tigloyl	$C_{18}H_{31}O$
3	Ac	Tigloyl	H
4	$C_{10}H_{19}O$	2-Me butyryl	H
5	Tigloyl	2-Me butyryl	H
6	Ac	$C_{10}H_{19}O$	H
7	2-Me butyryl	$C_{12}H_{23}O$	H
8	$C_{14}H_{27}O$	Ac	H
9	H	Ac	H

3. Experimental

3.1. General

Optical rotations were obtained on a DIP-360 automatic polarimeter (JASCO, Kyoto, Japan), whereas IR spectra were recorded on a FT/IR-230 spectrophotometer (JASCO, Kyoto, Japan). UV spectra were measured with a UV-2200 UV-VIS recording spectrophotometer (Shimadzu, Kyoto, Japan). NMR spectra were obtained on a Varian Unity plus 500 (^1H, 500 MHz; ^{13}C, 125 MHz) spectrometer and chemical shifts are given in δ ppm relative to tetramethylsilane (TMS). Electron impact (EI) mass spectra were obtained with a JMS-AX 505 HAD spectrometer (JEOL) at an ionization voltage of 70 eV. Atmospheric pressure ionization (API) mass spectra were measured with a PE SCIEX API III biomolecular mass analyzer.

3.2. Chromatography

TLC: Silica gel 60 F_{254} and RP-18 F_{254} S plates (Merck, Darmstadt, Germany); spots were detected under UV light or after spraying with anisaldehyde-H_2SO_4 reagent, followed by heating. Column chroma-

Table 1
Inhibition of HIV-1-induced CPE and activation of PKC by **1**–**8**

Compound	Anti-HIV-1 (μg/ml)		%Activation of PKC at 10 ng/ml
	IC_{100}	CC_0	
13-*O*-Acetylphorbol-20-linoleate (**1**)	15.6	62.5	0
13-*O*-Tigloylphorbol-20-linoleate (**2**)	7.81	62.5	14
12-*O*-Acetylphorbol-13-tigliate (**3**)	125	500	16
12-*O*-Decanoylphorbol-13-(2-methylbutyrate) (**4**)	7.81	31.3	0
12-*O*-Tigloylphorbol-13-(2-methylbutyrate) (**5**)	31.3	62.5	10
12-*O*-Acetylphorbol-13-decanoate (**6**)[a]	0.0076	62.5	0
12-*O*-(2-Methylbutyroyl)phorbol-13-dodecanoate (**7**)	15.76	62.5	16
12-*O*-Tetradecanoylphorbol-13-acetate (**8**)	0.00048	31.3	96
DS 8000[b]	3.9	> 1000	

[a] Activation of PKC was not observed at 100 ng/ml.
[b] Dextran sulphate (prepared from average M_r 8000; Moriya et al., 1993).

tography: Silica gel 60 (70–230 mesh, Merck), and ODS Cosmosil 140 C18-OPN (Nacalai Tesque, Kyoto, Japan). Medium pressure liquid chromatography (MPLC) was performed on a LiChroprep Si 60 column or LiChroprep RP-18 column (both size A, Merck, Darmstadt). Gas chromatography-mass spectra (GC-MS) were obtained using a GC-17A gas chromatograph (Shimadzu, Kyoto, Japan) fitted with a DB-1 column [0.25 mm (i.d) × 30 m] (J & W Scientific, USA), coupled to an automass system II benchtop quadrapole mass spectrometer (JEOL, Japan) under the following conditions: column temperature, 50°C for 10 min and gradient to 250°C (10°C/min) for 20 min; injection temperature, 250°C, or isothermal 30°C for 30 min (for methyl esters of short-chain fatty acids, injection temperature 170°C; carrier gas, He (flow rate 15 ml/min).

3.3. Plant material

Seeds of *Croton tiglium* were purchased at Harraz Herbal drug store, Cairo, Egypt and were authenticated by Professor El-Sayed E. Aboutabl, Faculty of Pharmacy, Cairo University, Egypt. A voucher specimen was deposited at the Museum of Materia Medica of Toyama Medical and Pharmaceutical University, Toyama, Japan.

3.4. Chemicals and enzymes

Rat brain protein kinase C (PKC, specific activity, 100 units/ml), staurosporine and L-α-phosphatidyl-L-serine were purchased from Sigma (St. Louis, MO, USA). Protein kinase enzyme assay system code RPN 77 kit was purchased from Amersham International plc (Buckinghamshire, England). The assay system components are calcium buffer (12 mM calcium acetate in 50 mM Tris/HCl and 0.05% sodium azide, pH 7.5), lipid (0.3 mg/ml L-α-phoshatidyl-L-serine and 24 μg/ml phorbol 12-tetradecanoate-13-acetate (TPA) in 50 mM Tris/HCl containing 0.05% sodium azide, pH 7.5), peptide buffer (900 μM peptide, Arg–Lys–Arg–Thr–Leu–Arg–Arg–Leu–OH, in 50 mM Tris/HCl, containing 0.05% sodium azide, pH 7.5), DTT buffer (30 mM dithiothreitol (DTT) in 50 mM Tris/HCl containing 0.05% sodium azide, pH 7.5), magnesium ATP buffer (1.2 mM ATP in a buffer containing 30 mM Hepes, 72 mM magnesium chloride, pH 7.4), stop reagent (300 mM orthophosphoric acid containing carmosine red), and peptide binding papers (assay discs, 3.0 cm diameter). [γ-^{32}P]ATP was supplied at specific activity of 370 MBq/ml (10 mCi/ml) from Amersham. Benzamidine HCl was obtained from Tokyo Kasei Org. Chemicals (Tokyo, Japan). Ethylene diamine tetra acetic acid (EDTA), ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra acetic acid (EGTA), phenyl methylsulphonyl flouride, β-mercaptoethanol, Tris/HCl and orthophosphoric acid were from Wako Pure Chemical and Industries (Osaka, Japan). Fatty acid methyl esters were from Nacalai Tesque (Kyoto, Japan).

3.5. Isolation of **1**–**8**

The air-dried seeds (3 kg) were first homogenized with an electric mill, and then the homogenate was extracted with MeOH (10l × 3) under reflux for 3 h. The MeOH extracts were combined and evaporated under reduced pressure to give an oily residue (763 g). The residue was suspended in 90% aqueous MeOH (7 l) and extracted with hexane (4 l × 3) and then ether (4 l × 3). The ether solutions were combined and evaporated under reduced pressure to give a resinous residue (150 g). The residue was chromatographed on a

silica gel column (2 kg). After washing with hexane (5l), elution was started with hexane–EtOAc (9:1 → 6:4) and then $CHCl_3$–MeOH (9:1, 8:2, 7:3) to furnish 20 fractions. Column chromatography (RP-18, MeOH–MeCN, 1:1) of Fr. 13 (1.3 g) followed by MPLC (RP-18, MeOH–H_2O, 9.5:0.5) afforded **2** (60 mg). Fr. 17 (2.9 g) was applied to a column of RP-2, which was then eluted with MeOH–H_2O (9:1) to give two subfractions, Fr. 17-A (683 mg) and 17-B (217 mg). MPLC (Si60, hexane–ether–EtOAc, 1:1:1) of Fr. 17-A afforded **1** (153 mg), while MPLC (RP-18, MeOH–H_2O, 9.5:0.5) of Fr. 17-B gave **4** (21 mg) and **7** (30 mg). A portion (10 g) of Fr. 18–20 was further fractionated by column chromatography (RP-2, MeOH–H_2O, 6:4) to give 10 subfractions (I–X). Through MPLC (RP-18), **3** (35 mg) was obtained from Fr. III using MeOH–H_2O (8:2), **6** (74 mg) was from Fr. V using MeOH–H_2O (9:1) and additional amount of **4** (57 mg) was from Fr. VII. In a similar manner, **5** (12 mg) was obtained from Fr. IX using MeOH–MeCN (4:6), while **8** (110 mg) was obtained from Fr. X after elution with MeOH–H_2O (9.4:0.6).

*3.5.1. 13-O-Acetylphorbol-20-(9Z,12Z-octadecadienoate) (**1**)*

Oil, $[\alpha]_D$ + 50° (*c* 0.05, $CHCl_3$); IR ν_{max} cm^{-1}: 3400 (OH), 1700 (ester C=O, *sh*), 1650 (α,β-unsaturated C=O); UV λ_{max} (log ε) nm: 243 (3.74); APIMS (positive mode): *m/z* 691 $[M+Na]^+$, 631 $[M+Na-CH_3COOH]^+$ and 351 $[M+Na-CH_3COOH-$linoleic acid$]^+$; EIMS: *m/z* 632 $[M-2H_2O]^+$, 590 $[M-H_2O-CH_3COOH]^+$, and 388 [M-linoleic acid]$^+$; HR-MS: *m/z* 669.4354 $[M+H]^+$ (Calculated for $C_{40}H_{61}O_8$: 669.4367); ^{1}H-NMR spectral data ($CDCl_3$) δ: 0.88 (3H, *m*, C<u>H</u>$_3$, linoloyl), 1.02 (1H, *d*, *J* = 2.8 Hz, H-14), 1.04 (3H, *d*, *J* = 6.8 Hz, H$_3$-18), 1.23 (3H, *s*, H$_3$-16), 1.25 (3H, *s*, H$_3$-17), 1.30 (14H, *m*, C<u>H</u>$_2$, linoloyl), 1.60 (2H, *t*, *J* = 7.6 Hz, –CO–CH$_2$–C<u>H</u>$_2$, linoloyl), 1.78 (3H, *s*, H$_3$-19), 1.99 (1H, *m*, H-11), 2.04 [4H, *m*, C<u>H</u>$_2$–CH=CH–CH$_2$–CH=CH–C<u>H</u>$_2$, linoloyl], 2.12 (3H, *s*, –COC<u>H</u>$_3$), 2.29 (2H, *t*, *J* = 7.6 Hz, –CO–C<u>H</u>$_2$, linoloyl), 2.38 (1H, *d*, *J* = 19.0 Hz, H$_a$-5), 2.52 (1H, *d*, *J* = 19.0 Hz, H$_b$-5), 2.78 (2H, *t*, *J* = 6.8 Hz, =CH–C<u>H</u>$_2$–CH=, linoloyl), 3.14 (1H, *br s*, H-10), 3.20 (1H, *t*, *J* = 5.3 Hz, H-8), 3.98 (1H, *dd*, *J* = 7.7, 2.2 Hz, H-12), 4.46 (2H, *ABq*, *J* = 12.4 Hz, H$_2$-20), 5.35 (4H, *m*, 2 × –(C<u>H</u>=C<u>H</u>)$_2$–, linoloyl), 5.67 (1H, *br d*, *J* = 4.0 Hz, H-7) and 7.58 (1H, *br s*, H-1); ^{13}C-NMR spectral data ($CDCl_3$) δ: 10.1 (C-19), 14.1 (C<u>H</u>$_3$, linoloyl), 15.0 (C-18), 16.9 (C-16), 21.1 (CO<u>C</u>H$_3$), 23.6 (C-17), 22.6 and 24.9 (2 × <u>C</u>H$_2$, linoloyl), 25.6 (=CH–<u>C</u>H$_2$–CH=, linoloyl), 27.2 [<u>C</u>H$_2$–CH=CH–CH$_2$–CH=CH–<u>C</u>H$_2$, linoloyl], 29.1–31.9 (7 × <u>C</u>H$_2$, linoloyl), 34.2 (<u>C</u>H$_2$, linoloyl), 35.2 (C-14), 38.9 (C-5), 39.2 (C-8), 44.9 (C-11), 56.7 (C-10), 68.0 (C-13), 69.2 (C-20), 73.3 (C-4), 77.5 (C-12), 78.2 (C-9), 127.9, 128.0, 130.0 and 130.2 [–(<u>C</u>H=<u>C</u>H)$_2$–, linoloyl), 132.3 (C-7), 133.1 (C-2), 136.1 (C-6), 160.4 (C-1), 173.5 (<u>C</u>=O, linoloyl), 174.0 (<u>C</u>OCH$_3$), and 208.7 (C-3).

*3.5.2. 13-O-Tigloylphorbol-20-(9Z,12Z-octadecadienoate) (**2**)*

Oil, $[\alpha]_D$ + 98.3° (*c* 0.05, $CHCl_3$); IR ν_{max} cm^{-1}: 3400 (OH), 1730 (ester C=O), 1650 (α,β-unsaturated C=O); UV λ_{max} (log ε) nm: 243 (3.97); APIMS (positive mode): *m/z* 731 $[M+Na]^+$, 631 $[M+Na-$tiglic acid$]^+$ and 351 $[M+Na-$tiglic acid$-$linoleic acid$]^+$; EIMS: *m/z* 590 $[M-$tiglic acid$-H_2O]^+$, 410 [M−linoleic acid]$^+$ and 328 [M−tiglic acid−linoleic acid$-H_2O$]$^+$; HR-MS: *m/z* 709.4737 $[M+H]^+$ (Calculated for $C_{43}H_{65}O_8$: 709.4794); ^{1}H-NMR spectral data ($CDCl_3$) δ: 0.88 (3H, *m*, C<u>H</u>$_3$, linoloyl), 1.02 (3H, *d*, *J* = 6.8 Hz, H$_3$-18), 0.91 (1H, *m*, H-14), 1.19 (3H, *s*, H$_3$-16), 1.25 (3H, *s*, H$_3$-17), 1.29 (14H, *m*, C*H*$_2$, linoloyl), 1.60 (2H, *br t*, C<u>H</u>$_2$, linoloyl), 1.80 (3H, *d*, *J* = 1.3 Hz, C<u>H</u>$_3$, tigloyl), 1.81 (3H, *d*, *J* = 1 Hz, H$_3$-19), 1.82 (3H, *d*, *J* = 1.3 Hz, C<u>H</u>$_3$, tigloyl), 2.05 (4H, *m*, C<u>H</u>$_2$–CH=CH–CH$_2$–CH=CH–C<u>H</u>$_2$, linoloyl), 2.16 (1H, *m*, H-11), 2.30 (2H, *t*, *J* = 7.5 Hz, –CO–C<u>H</u>$_2$, linoloyl), 2.37 (1H, *d*, *J* = 18.0 Hz, H$_a$-5), 2.54 (1H, *d*, *J* = 18.0 Hz, H$_b$-5), 2.77 (2H, *t*, *J* = 7 Hz, =CH–C<u>H</u>$_2$–CH=, linoloyl), 3.12 (1H, *br t*, H-8), 3.18 (1H, *t*, *J* = 2.5 Hz, H-10), 4.49 (1H, *d*, *J* = 12.4 Hz, H$_a$-20), 4.45 (1H, *d*, *J* = 12.4 Hz, H$_b$-20), 4.87 (1H, *d*, *J* = 9.9 Hz, H-12), 5.39 (4H, *m*, (C<u>H</u>=C<u>H</u>)$_2$, linoloyl), 5.67 (1H, *br d*, H-7), 6.87 (1H, *m*, C=C<u>H</u>, tigloyl), and 7.61 (1H, *dd*, *J* = 2.5 and 1.0 Hz, H-1); ^{13}C-NMR spectral data ($CDCl_3$) δ: 10.1 (C-19), 12.1 (<u>C</u>H$_3$, tigloyl), 14.1 (<u>C</u>H$_3$, linoloyl), 14.5 (<u>C</u>H$_3$, tigloyl), 17.0 (C-18), 22.2, 22.6, 24.9 (4 × –<u>C</u>H$_2$, linoloyl), 25.6 (=CH–<u>C</u>H$_2$–CH=, linoloyl), 27.2 [<u>C</u>H$_2$–CH=CH–CH$_2$–CH=CH–<u>C</u>H$_2$, linoloyl], 27.7, 29.1–29.6, 31.5, 34.2 (9 × –<u>C</u>H$_2$, linoloyl), 35.0 (C-14), 39.0 (*t*, C-5), 39.2 (C-8), 43.5 (C-11), 56.6 (C-10), 60.7 (C-13), 69.2 (C-20), 73.3 (C-4), 79.0 (C-9), 87.3 (C-12), 127.9, 128.0 (<u>C</u>H=<u>C</u>H, linoloyl), 128.1 (<u>C</u>=CH, tigloyl), 130.0, 130.2 (<u>C</u>H=<u>C</u>H, linoloyl), 132.5 (C-7), 133.4 (C-2), 135.9 (C-6), 138.7 (<u>C</u>H, tigloyl), 160.4 (C-1), 170.4 (<u>C</u>=O, tigloyl), 173.5 (<u>C</u>=O, linoloyl), and 208.7 (C-3).

*3.5.3. 12-O-Acetylphorbol-13-tigliate (**3**)*

Oil, $[\alpha]_D$ + 17.0° (*c* 0.05, $CHCl_3$); IR ν_{max} cm^{-1}: 3400 (OH), 1720 (ester C=O), 1650 (α,β-unsaturated C=O); UV λ_{max} (log ε) nm: 241 (3.51); EIMS: *m/z* 488 $[M]^+$, 470 $[M-H_2O]^+$, 428 $[M-AcOH]^+$, 410 $[M-AcOH-H_2O]^+$, 389 $[M+H-$tiglic acid$]^+$ and 328 $[M-AcOH-$tiglic acid$]^+$; HRMS: *m/z* 488.2374 $[M]^+$ (Calculated for $C_{27}H_{36}O_8$: 488.2411); ^{1}H-NMR spectral data ($CDCl_3$) δ: 0.89 (3H, *d*, *J* = 6.5 Hz, H$_3$-18), 1.10 (1H, *d*, *J* = 5.5 Hz, H-14), 1.21 (3H, *s*, H$_3$-16), 1.28

(3H, *s*, H_3-17), 1.77 (3H, *d*, *J* = 1.3 Hz, H_3-19), 1.80 (3H, *m*, CH_3, tigloyl), 1.85 (3H, *m*, CH_3, tigloyl), 2.11 (3H, *s*, $COC\underline{H}_3$), 2.18 (1H, *m*, H-11), 2.49 (1H, *d*, *J* = 19.0 Hz, H_a-5), 2.57 (1H, *d*, *J* = 19.0 Hz, H_b-5), 3.25 (1H, *t*, *J* = 2.4 Hz, H-10), 3.27 (1H, *t*, *J* = 5.5 Hz, H-8), 4.00 (1H, *d*, *J* = 13.0 Hz, H_a-20), 4.05 (1H, *d*, *J* = 13.0 Hz, H_b-20), 5.43 (1H, *d*, *J* = 10.2 Hz, H-12), 5.69 (1H, *d*, *J* = 5.5 Hz, H-7), 6.85 (1H, *m*, $C(CH_3)$=C$\underline{H}$–CH_3, tigloyl), and 7.60 (1H, *dd*, *J* = 2.4 and 1.3 Hz, H-1). ^{13}C-NMR spectral data ($CDCl_3$) δ: 10.1 (C-19), 12.2 (2 × $\underline{C}H_3$, tigloyl), 14.4 (C-18), 16.9 (C-16), 21.1 ($CO\underline{C}H_3$), 23.8 (C-17), 25.7 (C-15), 36.3 (C-14), 38.5 (C-5), 39.0 (C-8), 43.1 (C-11), 56.1 (C-10), 65.7 (C-13), 68.0 (C-20), 73.5 (C-4), 76.6 (C-12), 78.3 (C-9), 128.4 (CO–$\underline{C}(CH_3)$=CH, tigloyl), 129.2 (C-7), 132.8 (C-2), 137.7 (CO–C=$\underline{C}$H, tigloyl), 140.4 (C-6), 160.8 (C-1), 167.8 ($\underline{C}$=O, tigloyl), 173.9 ($\underline{C}OCH_3$), and 209.0 (C-3).

3.5.4. 12-O-Decanoylphorbol-13-(2-methylbutyrate) (**4**)

Oil, $[\alpha]_D$ + 56° (*c* 0.05, $CHCl_3$); IR ν_{max} cm^{-1}: 3360 (OH), 1730 and 1710 (ester C=O), 1650 (α,β-unsaturated C=O); UV λ_{max} (log ε) nm: 241 (3.83); EIMS: *m*/*z* 602 $[M]^+$, 584 $[M-H_2O]^+$, 501 [M−(2-methylbutyric acid) + H]$^+$, 430 [M−decanoic acid]$^+$, 412 $[M-\text{decanoic acid}-H_2O]^+$, 328 [M−decanoic acid-(2-methylbutyric acid)]$^+$; HRMS: *m*/*z* 602.3820 $[M]^+$ (Calculated for $C_{35}H_{54}O_8$: 602.3830); ^{1}H-NMR spectral data ($CDCl_3$) δ: 0.86–0.94 [9H, *m*, H_3-18, $C\underline{H}_3$ decanoyl, and CH_2–$C\underline{H}_3$ (2-methylbutyryl)], 1.07 (1H, *d*, *J* = 5.1 Hz, H-14), 1.15 [3H, *d*, *J* = 7 Hz, CH–$C\underline{H}_3$ (2-methylbutyryl)], 1.20 (3H, *s*, H_3-16), 1.25 (3H, *s*, H_3-17), 1.26 (12H, *m*, 6 × –$C\underline{H}_2$–, decanoyl), 1.40 (2H, *m*, CO–CH_2–$C\underline{H}_2$), 1.66 (H, *m*, CH–$C\underline{H}_2$–CH_3), 1.7 (3H, *m*, H_3-19), 2.14 (1H, *dd*, *J* = 10.5 and 6.5 Hz, H-11), 2.33 (2H, *m*, –CO–$C\underline{H}_2$–$(CH_2)_7$–, decanoyl), 2.38 [1H, *m*, C$\underline{H}$–CH_3 (2-methylbutyryl)], 2.48 (1H, *d*, *J* = 19.0 Hz, H_a-5), 2.54 (1H, *d*, *J* = 19.0 Hz, H_b-5), 3.25 (2H, *m*, H-8 and H-10), 4.02 (2H, *ABq*, *J* = 13 Hz, H_2-20), 5.42 (1H, *d*, *J* = 10.5 Hz, H-12), 6.00 (1H, *br d*, H-7), and 7.60 (1H, *dd*, *J* = 2.3 and 1.3 Hz, H-1). ^{13}C-NMR spectral data ($CDCl_3$) δ: 10.1 (C-19), 11.6 (C-18), 14.1 ($\underline{C}H_3$, decanoyl), 14.4 [$\underline{C}H_3$ (2-methylbutyryl)], 16.9 (C-16), 17.0 [$\underline{C}H_3$ (2-methylbutyryl)], 22.7 ($\underline{C}H_2$, decanoyl), 23.9 (C-17), 24.5 [$\underline{C}H_2$–CH_3 (2-methylbutyryl)], 25.6 ($\underline{C}H_2$, decanoyl), 26.7 ($\underline{C}H_2$, decanoyl), 29.0–29.7 (4 × $\underline{C}H_2$, decanoyl), 31.8 ($\underline{C}H_2$, decanoyl), 34.3 ($\underline{C}H_2$, decanoyl), 36.3 (C-14), 38.6 (C-5), 39.0 (C-8), 41.8 (C-11), 56.1 (C-10), 65.3 (C-13), 68.0 (C-20), 73.7 (C-4), 76.3 (C-12), 78.2 (C-9), 129.2 (C-7), 132.8 (C-2), 140.3 (C-6), 160.9 (C-1), 176.1 [$\underline{C}$=O, (2-methylbutyryl)], 176.4 ($\underline{C}$=O, decanoyl), and 209.0 (C-3).

3.5.5. 12-O-Tigloylphorbol-13-(2-methylbutyrate) (**5**)

Oil, $[\alpha]_D$ + 20.0° (*c* 0.3, $CHCl_3$); IR ν_{max} cm^{-1}: 3360 (OH), 1710 (ester C=O), 1650 (α,β-unsaturated C=O); UV λ_{max} (log ε) nm: 241 (3.56): EIMS: *m*/*z* 530 $[M]^+$, 428 [M-(2-methylbutyric acid)]$^+$, 410 [M−(2-methylbutyric acid)$-H_2O]^+$, 398, 370, 328 [M−(2-methylbutyric acid)−tiglic acid]$^+$ and 310; HRMS: *m*/*z* 530.2914 $[M]^+$ (Calculated for $C_{30}H_{42}O_8$: 530.2949); ^{1}H-NMR spectral data ($CDCl_3$) δ: 0.89 (3H, *d*, *J* = 6.5 Hz, H_3-18), 1.05 (1H, *d*, *J* = 5.4 Hz, H-14), 1.10–1.2 [12H, *m*, H_3-16, H_3-17, and 2 × CH_3 (2-methylbutyryl)], 1.60 [2H, *m*, -$C\underline{H}_2$–CH_3 (2-methylbutyryl)], 1.80 and 1.83 (6H, 2 × $C\underline{H}_3$, tigloyl), 1.77 (3H, *m*, H_3-19), 2.20 (1H, *m*, H-11), 2.30 [1H, *m*, –C$\underline{H}$–CH_2–CH_3 (2-methylbutyryl)], 2.50 (1H, *d*, *J* = 18.0 Hz, H_a-5), 2.58 (1H, H_b-5, unclear due to overlapping), 3.26 (1H, *br t*, H-10), 3.29 (1H, *br t*, H-8), 4.00 (1H, *d*, *J* = 12.9 Hz, H_a-20), 4.05 (1H, *d*, *J* = 12.9 Hz, H_b-20), 5.45 (1H, *d*, *J* = 10.2 Hz, H-12), 5.70 (1H, *br d*, H-7), 6.83 (1H, *m*, C=C$\underline{H}$, tigloyl) and 7.60 (1H, *t*, *J* = 2.2 Hz, H-1). ^{13}C-NMR spectral data ($CDCl_3$) δ: 10.1 (C-19), 12.2 ($\underline{C}H_3$, tigloyl), 14.4 (2 × $\underline{C}H_3$, C-18 and $\underline{C}H_3$, tigloyl), 17.0 (C-16), 18.5 [$\underline{C}H_3$, (2-methylbutyryl)], 18.6 [$\underline{C}H_3$, (2-methylbutyryl)], 23.8 (C-17), 25.9 [$\underline{C}H_2$–CH_3, (2-methylbutyryl)], 34.1 (CO–$\underline{C}$H–CH_2–, 2-methylbutyryl), 36.5 (C-14), 38.5 (C-5), 39.0 (C-8), 43.3 (C-11), 56.1 (C-10), 65.3 (C-13), 68.0 (C-20), 73.9 (C-4), 76.6 (C-12), 78.4 (C-9), 127.4 [(C-7), 128.5 (–$\underline{C}$=CH–CH_3, tigloyl)], 132.8 (C-2), 137.5 (C=$\underline{C}$H–, tigloyl), 140.4 (C-6), 161.1 (C-1), 167.6 ($\underline{C}$=O, tigloyl), 179.5 [$\underline{C}$=O (2-methylbutyryl)] and 209.4 (C-3).

3.6. Cells

Cells of the HTLV-I-carrying cell line MT-4 were used. They were maintained at 37°C under 5% CO_2 in RPMI-1640 medium (Flow Laboratories, Irvine, Scotland), supplemented with 10% fetal calf serum (FCS, Flow laboratories, North Ryde, Australia), 100 μg/ml of streptomycin (Meiji Seika, Tokyo, Japan) and 100 U/ml of penicillin G (Banyu Pharmaceutical, Tokyo, Japan).

3.7. Virus

HIV-1 was obtained from culture supernatant of MOLT-4 cells that had been persistently infected with LAV-1.

3.8. Assay of cytopathic effect (CPE) of HIV-1 on MT-4 cells

The inhibitory effect of test compounds on HIV-1-induced cytopathogenicity was measured by the method of Harada, Koyanagi and Yamamoto (1985).

MT-4 cells were infected for 1 h with HIV-1 at $TCID_{50}$ of 0.001/cell (determined by MT-4 cells on day 5 after infection), and nonadsorbed virus was removed by washing. Then, the cells were resuspended at 1.5×10^5 cells/ml in RPMI-1640 medium. 200 μl/well of the cell suspension was cultured for five days in a 96-well culture plate containing various concentrations of the tested compounds. Control assays were performed in the absence of test compounds with HIV-1-infected and uninfected cultures. On day 5, the IC of the test compound required for completely preventing HIV-1-induced CPE (IC_{100}) was determined through an optical microscope, and the cell growth was examined to give the CC_0 that reduces the viability of MT-4 cells.

3.9. Activation of protein kinase C by 1–8

PKC activation was assayed by measuring the incorporation of ^{32}P radioactivity from [γ-^{32}P]ATP into peptide, Arg–Lys–Arg–Thr–Leu–Arg–Arg–Leu–OH, using a Biotrak PKC enzyme assay system code RPN 77 kit, except that TPA in the kit was replaced by **1–8** (10 ng/ml) dissolved in DMSO (final concentration of DMSO not to exceed 0.02%). The reaction mixture contained, in a total volume of 55 μl, 0.002 units PKC, 50 mM Tris/HCl, pH 7.5, 0.13% w/v mercaptoethanol, 2.1 mM EDTA, 4.18 mM EGTA, 20.9 μg/ml phenyl methyl sulphonyl flouride, 4.2 mM benzamidine, 1.3 μM calcium acetate, 75 μM peptide, 34 μg/ml L-α-phosphatidyl-L-serine, 3.4 mM DTT, 0.68 μM sodium azide, and 6.5 nM $MgCl_2$. After addition of 0.55 nM [γ-^{32}P]ATP (50×10^3 cpm/nmol), the reaction mixture was mixed and kept at 37°C for 30 min. Reactions were terminated by the addition of 10 μl of ice-cold stop reagent. Aliquots of 35 μl were transferred to the center of peptide binding discs. After 10 min, the discs were washed two times with 75 mM orthophosphoric acid. The radioactivity of ^{32}P-labeled samples was counted in 10 ml of a scintillation fluid for 1 min. In the presence of TPA, lipid and $CaCl_2$, PKC activation represents 100% and corresponds to 8400 nmol/mg/min (positive control). Values for the activation of PKC by **1–8** are given as the mean of duplicate determinations, and calculated relative to that of the positive control. Blank (in the absence of PKC) and control (in the absence of TPA or tested samples) were also carried out. One unit of PKC was defined as that amount of enzyme which incorporated 1 nmol of phosphate from ATP into its substrate, peptide, per minute under the assay conditions described above. In the presence of 34 μg/ml L-α-phosphatidyl-L-serine, 1.3 μM calcium acetate and 2.7 μg/ml TPA, PKC activity was completely inhibited by staurosporine at a concentration of 180 nM.

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