12-*O*-Acetylphorbol-13-decanoate Potently Inhibits Cytopathic Effects of Human Immunodeficiency Virus Type 1 (HIV-1), without Activation of Protein Kinase C

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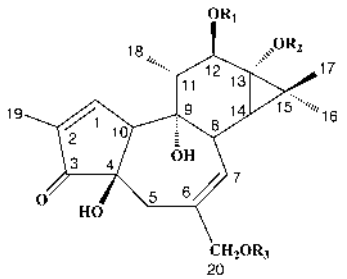
Through bioactivity-guided fractionation, eight phorbol diesters, including five new ones (1—5), were isolated from the seeds of *Croton tiglium* collected in Egypt. 12-*O*-Acetylphorbol-13-decanoate (6) and 12-*O*-decanoylphorbol-13-(2-methylbutyrate) (4) potently inhibited the HIV-1-induced cytopathic effect on MT-4 cells (IC_{100} values of 7.6 ng/ml and 7.81 μg/ml, and CC_0 values of 62.5 μg/ml and 31.3 μg/ml, respectively) without activating protein kinase C.

Key words *Croton tiglium*; phorbol ester; anti-HIV-1; protein kinase C

Although phorbol esters elicit a wide range of biochemical effects,[1,2] a prevailing concern is their possible tumor-promoting effects. Different experiments indicated that tumor-promoting phorbol esters interact with and activate protein kinase C (PKC).[3,4] It has been reported that the PKC family plays an important role in signal transduction in normal cells and its elevated activation has been detected in several cancer cell lines. Chowdhury *et al*.[4] found that the tumor-promoting and the HIV-1 inhibitory effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were mediated through its direct activation of PKC. However, there are many phorbol esters that are biologically active without being tumor-promoters. 12-*O*-Tigloylphorbol-13-decanoate,[5] ostodin, and 12-*O*-undecadienoylphorbol-13-acetate,[6] are potent antitumor agents. Prostratin, 12-deoxyphorbol-13-acetate, is a potent anti-HIV-1 agent without tumor-promoting action.[7] We previously reported that extracts of the seeds of *Croton tiglium* L. collected in Egypt significantly inhibited HIV-1.[8] Therefore extracts of this plant were investigated to isolate a selective inhibitor of HIV-1 that does not activate PKC. From the MeOH extract, we have now isolated 8 phorbol diesters (Chart 1). Compounds **1**—**5** are new[9] and **6**—**8** were known compounds.[10,11] The structures of these compounds were established by 2D-NMR spectroscopy (^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY) and selective hydrolysis of acyl groups followed by GC/MS. The details of the structure elucidation will be reported elsewhere.

Table 1 shows the inhibitory activity of **1**—**8** against the cytopathic effects (CPE) of HIV-1 in MT-4 cells,[12] and their activation of PKC. The 13,20-diesters, **1** and **2**, demonstrated complete inhibition of CPE at concentrations (IC_{100}) of 15.6 μg/ml and 7.81 μg/ml, with minimum cytotoxic concentrations (CC_0) of 62.5 μg/ml for both. However, phorbol 12,13-diesters contained long-chain and short-chain acyl groups (long-chain at C-12 as in **8**, or at C-13 as in **6**) and demonstrated potent inhibition of HIV-1-induced CPE at very low



	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

Chart 1

Table 1. Inhibition of HIV-1-induced CPE[a)] and Activation of PKC[b)] by **1**—**8**

Compound	Anti-HIV-1(μg/ml)		% Activation of PKC at
	IC_{100}[c)]	CC_0[d)]	10 ng/ml
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**)*	0.0076	62.5	0
12-*O*-(2-Methyl butyroyl)phorbol-13-dodecanoate (**7**)	15.6	62.5	16
12-*O*-Tetradecanoylphorbol-13-acetate (**8**)	0.00048	31.3	96
DS 8000	3.90	>1000	

a) Measured by the method of Harada *et al*.[12] using HTLV-I-carrying cell line MT-4. *b*) Assayed by measuring the incorporation of ^{32}P radioactivity from [γ-^{32}P]ATP into a peptide, Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-OH, using a Biotrak PKC enzyme assay system code RPN 77 kit except that the TPA in the kit was replaced by **1**—**8** (2.7 μg/ml and 10 ng/ml) in DMSO at a final concentration of 0.02%. *c*) IC_{100}: the minimal concentration for complete inhibition of HIV-1-induced CPE in MT-4 cells, determined by microscopic observation. *d*) CC_0: the minimal concentration for the appearance of MT-4 cell toxicity, determined by microscopic observation. * Activation of PKC was not observed at 100 ng/ml. DS 8000: Dextrine sulfate.

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concentrations of 0.00048 μg/ml and 0.0076 μg/ml, respectively. Compound **4**, with C_{10} and C_5 acyl groups, completely inhibited HIV-1 at 7.81 μg/ml, while **7**, with C_5 and C_{12}, demonstrated the same inhibitory potency as that of **1**. Phorbol diesters containing two short acyl residues (**3** and **5**) showed no appreciable anti-HIV CPE activity. These compounds were tested for their abilities to activate PKC by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into a peptide. It was found that TPA (**8**), the most potent tumor promoter known so far, activated PKC by almost 100% at a concentration of 10 ng/ml. However, **6**, which demonstrated potent anti-HIV-1 effects, showed no activation of PKC at concentrations of 10—100 ng/ml. Compounds **1** and **4** did not activate PKC at 10 ng/ml, while **2**, **3**, **5**, and **7** demonstrated 10—16% increases in the activity of PKC. Although the experimental conditions were not identical to ours, prostratin (potent anti-HIV-1 12-deoxyphorbol-13-acetate) was reported to show an $IC_{100} \geq 1$ μM (0.39 μg/ml) and weak PKC activation.[7] Compound **4** was also a selective inhibitor of HIV-1, and the very potent effects of **6** led us to assume that the effects of phorbol esters were influenced by chain length and acyl group position. This observation encouraged us to carry out further modification of these compounds to clarify the structural features required for their selective anti-HIV-1 effects. The results of this study will be reported in a forthcoming paper.

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9) Compound **1** (13-*O*-acetylphorbol-20-linoleate): an oily substance, $[\alpha]_D$ +50.0° (*c*, 0.05 $CHCl_3$), IR: 3400 (OH), 1700 (ester C=O), 1650 (α,β-unsaturated ketone) cm^{-1}; API-MS (positive mode) *m/z* 691 $[M+Na]^+$. ^{1}H-NMR ($CDCl_3$): δ 0.88 (3H, m, C$\underline{H}_3$, linoloyl), 1.02 (1H, d, *J*=5.3 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$\underline{H}_2$, linoloyl), 1.60 (2H, t, *J*=7.6 Hz, –CO–CH_2–C$\underline{H}_2$, linoloyl), 1.78 (3H, s, H_3-19), 1.99 (1H, m, H-11), 2.04 (4H, m, C$\underline{H}_2$–CH=CH–CH_2–CH=CH–C$\underline{H}_2$, linoloyl), 2.12 (3H, s, –COC$\underline{H}_3$), 2.29 (2H, t, *J*=7.6 Hz, –CO–C$\underline{H}_2$, 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$\underline{H}_2$–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$\underline{H}$=C$\underline{H}$)$_2$–, linoloyl], 5.67 (1H, br d, H-7), and 7.58 (1H, br s, H-1). Compound **2** (13-*O*-tigloylphorbol-20-linoleate): an oily substance, $[\alpha]_D$ +98.3° (*c*, 0.05 $CHCl_3$), IR: 3400 (OH), 1730 (ester C=O), 1650 (α,β-unsat. C=O) cm^{-1}; API-MS (positive mode) *m/z* 731 $[M+Na]^+$. ^{1}H-NMR ($CDCl_3$): δ 0.88 (3H, m, C$\underline{H}_3$, linoloyl), 1.02 (3H, d, *J*=6.8 Hz, H_3-18), 0.91 (1H, br d, H-14), 1.19 (3H, s, H_3-16), 1.25 (3H, s, H_3-17), 1.29 (14H, m, C$\underline{H}_2$, linoloyl), 1.60 (2H, br t, C$\underline{H}_2$, linoloyl), 1.80 (3H, d, *J*=1.3 Hz, C$\underline{H}_3$, tigloyl), 1.81 (3H, d, *J*=1 Hz, H_3-19), 1.82 (3H, d, *J*=1.3 Hz, C$\underline{H}_3$, tigloyl), 2.05 (4H, m, C$\underline{H}_2$–CH=CH–CH_2–CH=CH–C$\underline{H}_2$, linoloyl), 2.16 (1H, m, H-11), 2.30 (2H, t, *J*=7.5 Hz, –CO–C$\underline{H}_2$, 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*=7.0 Hz, =CH–C$\underline{H}_2$–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$\underline{H}$=C$\underline{H}$)$_2$, linoloyl], 5.67 (1H, br d, H-7), 6.87 (1H, m, C=C$\underline{H}$, tigloyl), and 7.61 (1H, dd, *J*=2.5 and 1.0 Hz, H-1). Compound **3** (12-*O*-acetylphorbol-13-tigliate): an oily substance, $[\alpha]_D$ +17.2° (*c*, 0.05 $CHCl_3$), IR: 3400 (OH), 1720 (ester C=O), 1650 (α,β-unsat. C=O) cm^{-1}; EI-MS *m/z* 488 $[M]^+$. ^{1}H-NMR ($CDCl_3$): 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 Hz, H_a-5), 2.57 (1H, d, *J*=19 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 Hz, H_a-20), 4.05 (1H, d, *J*=13 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, 1.3 Hz, H-1). Compound **4** [12-*O*-decanoylphorbol-13-(2-methylbutyrate)]: an oily substance, $[\alpha]_D$ +56.0° (*c*, 0.05 $CHCl_3$), IR: 3360 (OH), 1730 and 1710 (ester C=O), 1650 (α,β-unsat. C=O) cm^{-1}; EI-MS *m/z* 602 $[M]^+$. ^{1}H-NMR ($CDCl_3$): 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, $\underline{H}_3$-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, H-10), 4.02 (2H, ABq, *J*=13.0 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, 1.3 Hz, H-1). Compound **5** [12-*O*-tigloylphorbol-13-(2-methylbutyrate)]: an oily substance, $[\alpha]_D$ +20.0° (*c*, 0.05 $CHCl_3$), IR: 3360 (OH), 1710 (ester C=O), 1650 (α,β-unsat. C=O) cm^{-1}; EI-MS *m/z* 530 $[M]^+$. ^{1}H-NMR ($CDCl_3$): 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 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).
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