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•Original article•

Seco-cyclic phorbol derivatives and their anti-HIV-1 activities

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[ABSTRACT] Phorbol esters are recognized for their dual role as anti-HIV-1 agents and as activators of protein kinase C (PKC). The efficacy of phorbol esters in binding with PKC is attributed to the presence of oxygen groups at positions C20, C3/C4, and C9 of phorbol. Concurrently, the lipids located at positions C12/C13 are essential for both the anti-HIV-1 activity and the formation of the PKC-ligand complex. The influence of the cyclopropane ring at positions C13 and C14 in phorbol derivatives on their anti-HIV-1 activity requires further exploration. This research entailed the hydrolysis of phorbol, producing seco-cyclic phorbol derivatives. The anti-HIV-1 efficacy of these derivatives was assessed, and the affinity constant (K_d) for PKC-δ protein of selected *seco*-cyclic phorbol derivatives was determined through isothermal titration calorimetry. The findings suggest that the chemical modification of cyclopropanols could affect both the anti-HIV-1 activity and the PKC binding affinity. Remarkably, compound S11, with an EC50 of 0.27 μmol·L^{-1} and a CC_{50} of 153.92 μmol·L^{-1}, demonstrated a potent inhibitory effect on the intermediate products of HIV-1 reverse transcription (ssDNA and 2LTR), likely acting at the viral entry stage, yet showed no affinity for the PKC-δ protein. These results position compound S11 as a potential candidate for further preclinical investigation and for studies aimed at elucidating the pharmacological mechanism underlying its anti-HIV-1 activity.

[KEY WORDS] *seco*-Cyclic phorbol derivatives; Hydrolysis reaction; Cyclopropane ring; Esterification; Anti-HIV-1 agent
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Introduction

Phorbol esters are known for their wide array of biological activities, including anti-HIV virus effects, activation of protein kinase C (PKC), platelet aggregation, cell differentiation, anti-tuberculosis properties, cytotoxic actions, and the stimulation of mesenchymal stem cells [1-4]. Specifically, 12-*O*-Tetradecanoyl phorbol-13-acetate (TPA) serves as a pharmacological and biochemical agent to induce inflammation, while 12-deoxyphorbol-13-acetate (prostratin) has been identified to inhibit HIV-1 replication and activate latent, resting $CD4^+$ T cells without promoting tumor growth[5]. The mechanism underlying the biological impacts of phorbol esters is partially attributed to the interaction of oxygen groups at positions C20, C3/C4, and C9 with the diacylglycerol-binding domain of PKC through a network of hydrogen bond donors and acceptors [6, 7]. Additionally, the presence of lipids at positions C12/C13 is believed to facilitate the membrane integration of the PKC-ligand complex, influencing its conformation, binding affinity, and catalytic efficiency indirectly [5, 6]. The role of the cyclopropane ring at positions C13 and C14 in modulating the anti-HIV-1 effectiveness and the formation of PKC-ligand complexes necessitates further study. Building on our prior work, where we refined the synthesis of phorbol and developed 47 novel phorbol ester derivatives, assessing

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These authors have no conflict of interest to declare.

their cytotoxicity against human tumor cell lines and anti-HIV-1 properties [7, 8], this study aimed to hydrolyze phorbol and synthesize new *seco*-cyclic phorbol derivatives. Our objective was to investigate the influence of the cyclopropane ring on anti-HIV-1 activity and PKC binding, in pursuit of identifying promising anti-HIV-1 compounds.

Results and Discussion

Hydrolysates of phorbol

Compound **Z1-1** was isolated as a white, amorphous powder, distinct from phorbol primarily due to the opening of the cyclopropane ring, which resulted in an isopropenyl group at C-14 and a carbonyl group at C-13, as confirmed by nuclear magnetic resonance (NMR) data. In comparison to langduin A, the configuration of H-14 in Z1-1 is α, indicated by a larger coupling constant (J = 13.2 Hz) between H-8 and H-14 [9, 10]. Compound **Z1-1** has been identified as 4β,9α,20-trihydroxy-13,15-*seco*-1,6,15-tigliatriene-3,13-dione, also referred to as crotophorbolone (Fig. 1) based on a comparison of NMR data with that reported in previous studies [11, 12]. Furthermore, this compound can also be synthesized *via* the biotransformation of phorbol by human intestinal bacteria [11].

Compound **Z1-2** was obtained as a white, amorphous powder, with its molecular formula established as $C_{21}H_{30}O_6$ through high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) in positive mode, showing a peak at *m/z* 379.2110 for $[M + H]^+$($C_{21}H_{31}O_6^+$, calculated for 379.2115), and supported by NMR spectroscopic data. The NMR spectrum for **Z1-2** displayed signals for C-13 at δ 111.5, C-15 at δ 144.1, C-16 at δ 112.4, C-17 at δ 23.6, and one methoxy group at δ_H 3.53, δ_C 52.1 (Table 1). The attachment of this methoxy group to the C-13 carbonyl group in **Z1-1** was confirmed through Heteronuclear Multiple Bond Correlations (HMBCs) (Fig. 1) from the methoxy group(-OCH_3 at δ_H 3.53)toC-13(δ_C111.5).Theβ-configurationofthemethoxygroup in **Z1-2** was deduced from Rotating Frame Overhauser Enhancement Spectroscopy (ROESY) correlations between H-12β and the methoxy group (-OCH_3) (Fig. 1). Consequently, the structure of **Z1-2** was elucidated as 4β,9α,13α,20-tetrahydroxy-13-methoxy-13,15-*seco*-1,6,15-tigliatriene-3-one, representing a novel tigliane diterpenoid that arises from the hydrolysis of phorbol.

Compound **Z1-3** was isolated as a white, amorphous powder, with its molecular formula determined to be $C_{22}H_{34}O_7$ through positive mode HR-ESI-MS, showing a peak at *m/z* 411.2354 for [M + H]+ (C22H35O7+, calculated for 411.2377), and corroborated by NMR spectroscopic data. In comparison with**Z1-1**, **Z1-3** represents the product of the addition of two methanol molecules to **Z1-1**, a conclusion supported by both NMR data (Table 1) and the HMBC observation in **Z1-3** (Fig. 1). The NMR spectrum for **Z1-3** revealed signals for C-13 at δ 111.9, C-15 at δ 74.4, C-16 at δ 22.4, C-17 at δ 25.8, and two methoxy groups at δ_H 3.22, δ_C 49.2, δ_H 3.47, δ_C 51.8 (Table 1). The HMBC correlations (Fig. 1) from the methoxy group (-OCH_3 at δ_H 3.47) to C-13 (δ_C 111.9) and from another methoxy group (-OCH_3 at δ_H 3.22) to C-15 (δ_C 74.4) indicated the attachment of methanol to the C-13 carbonyl group and the C-15 alkenyl group, respectively. ROESY correlations between H-14 and the methoxy group (-OCH3 at δ_H 3.47) suggest a β-configuration for the methoxy group at C-13 (Fig. 1). Consequently, based on these spectroscopic findings, compound **Z1-3** was identified as 4β,9α,13α,20-tetrahydroxy-13,15-dimethoxy-13,15-*seco*-1,6-digliatriene-3-one, representing a novel tigliane diterpen-

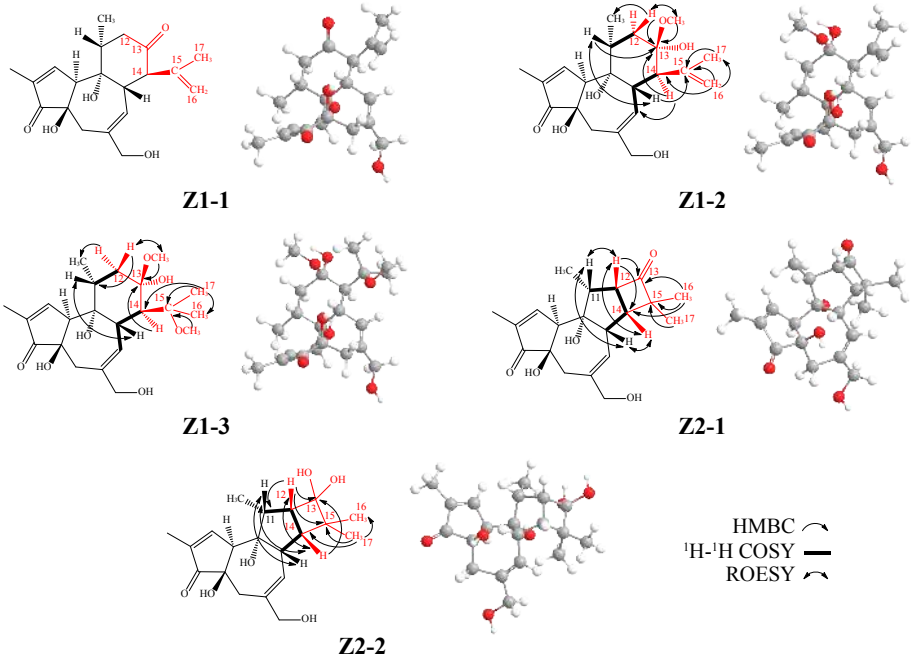


Fig. 1 Main ^{1}H-^{1}H COSY, HMBC, and ROESY correlations of compounds Z1-1–Z2-2.

Table 1 The ^{1}H and ^{13}C NMR data of hydrolysis derivatives of phorbol (CD_3OD, *J* in Hz)

No.	Z1-2		Z1-3		Z2-1		Z2-2	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	7.59 (1H, s)	161.2	7.58 (1H, s)	161.6	7.49 (1H, s)	155.9	7.49 (1H, s)	159.8
2		136.9		136.2		138.3		136.3
3		210.3		210.7		209.4		210.1
4		74.1		74.4		75.5		74.3
5	2.61 (1H, d, *J* = 18.4 Hz, H-5a) 2.25 (1H, d, *J* = 18.4 Hz, H-5b)	39.5	2.59 (1H, d, *J* = 18.9 Hz, H-5a) 2.28 (1H, d, *J* = 18.9 Hz, H-5b)	39.3	2.57 (1H, d, *J* = 19.1 Hz, H-5a) 2.40 (1H, d, *J* = 19.1 Hz, H-5b)	38.2	2.57 (1H, d, *J* = 18.9 Hz, H-5a) 2.40 (1H, d, *J* = 18.9 Hz, H-5b)	38.4
6		137.6		137.8		140.8		139.7
7	5.33 (1H, s)	129.4	5.48 (1H, s)	132.6	5.72 (1H, d, *J* = 5.4 Hz)	125.8	5.33 (1H, s)	130.2
8	3.20 (1H, d, *J* = 9.7 Hz)	50.7	3.41 (1H, d, *J* = 8.6 Hz)	48.6	3.81 (1H, dd, *J* = 10.6, 5.4 Hz)	48.4	2.46 (1H, q, *J* = 7.1 Hz)	43.3
9		86.5		86.5		89.5		87.0
10	3.47 (1H, s)	52.7	3.45 (1H, s)	52.8	2.88 (1H, m)	56.5	3.43 (1H, m)	51.8
11	2.38 (1H, m)	44.4	2.42 (1H, m)	43.5	2.45 (1H, m)	43.0	3.50 (1H, s)	47.1
12	2.16 (1H, m, H-12a) 1.23 (1H, m, H-12b)	37.1	2.67 (1H, dd, *J* = 10.0, 8.5 Hz, H-12a) 1.27 (1H, m, H-12b)	39.4	3.43 (1H, dd, *J* = 9.1, 6.1 Hz)	69.1	1.58 (1H, d, *J* = 6.8 Hz)	61.2
13		111.5		111.9		220.9		108.5
14	2.59 (1H, s)	59.4	2.02 (1H, dd, *J* = 6.9, 2.6 Hz)	62.6	2.52 (1H, dd, *J* = 10.6, 9.2 Hz)	46.1	1.93 (1H, d, *J* = 6.8 Hz)	59.5
15		144.1		74.4		58.5		34.4
16	5.01 (1H, m, H-16a) 4.91 (1H, s, H-16b)	112.4	1.30 (3H, s)	22.4	1.28 (3H, s)	25.7	1.36 (3H, s)	27.7
17	1.82 (3H, s)	23.6	1.24 (3H, s)	25.8	0.99 (3H, s)	18.3	0.95 (3H, s)	20.3
18	0.95 (3H, d, *J* = 7.0 Hz)	21.9	0.93 (3H, d, *J* = 7.0 Hz)	22.0	1.11 (3H, d, *J* = 7.0 Hz)	15.6	0.93 (3H, d, *J* = 7.1 Hz)	19.3
19	1.80 (3H, dd, *J* = 2.7, 1.4 Hz)	10.2	1.78 (3H, dd, *J* = 2.7, 1.3 Hz)	10.2	1.77 (3 H, dd, *J* = 3.0, 1.5 Hz)	10.4	1.77 (3H, d, *J* = 1.4 Hz)	10.2
20	3.94 (2H, m)	69.6	3.94 (2H, m)	69.7	3.93 (2H, m)	68.4	3.89 (2H, m)	68.8
	3.53 (3H, s,-OCH_3)	52.1	3.22 (3H, s, -OCH_3)	49.2				
			3.47 (3H, s, -OCH_3)	51.8				

oid that is synthesized through the hydrolysis of phorbol.

Compound **Z2-1** was isolated as a white, amorphous powder. Its molecular formula, $C_{20}H_{26}O_5$, was determined from positive mode HR-ESI-MS, which showed a peak at *m/z* 369.1680 $[M + Na]^+$ ($C_{20}H_{26}NaO_5^+$, Calcd. for 369.1678), supported by NMR spectroscopic data. The NMR spectrum of **Z2-1** bore resemblance to that of 4*β*,9*α*,20-trihydroxy-14(13→12)-abeo-12α_H-1,6-tigliadiene-3,13-dione [11, 13], with signals observed for C-11 at *δ* 43.0, H-11 at *δ* 2.45, C-12 at *δ* 69.1, H-12 at *δ* 3.43, C-13 at *δ* 220.9, C-14 at *δ* 46.1, H-14 at *δ* 2.52, C-15 at *δ* 58.5 (Table 1). These findings indicated that **Z2-1** was a dehydration product of phorbol. However, distinct from the aforementioned compound, the *β* configuration of H-12 in **Z2-1** was identified, supported by ROESY correlations (Fig. 1), differentiating it from the anaerobic incubation product of phorbol with human intestinal bacteria [11]. The structure of **Z2-1** was further confirmed through ^{1}H-^{1}H COSY, HSQC, and HMBC analyses, leading to its elucidation as 4*β*,9*α*,20-trihydroxy-14(13→12)-abeo-12β_H-1,6-tigliadiene-3,13-dione, a novel tigliane diterpenoid resulting from the hydrolysis of phorbol.

Compound **Z2-2**, isolated as a white, amorphous powder, was established to have a molecular formula of $C_{20}H_{28}O_6$, as determined by positive mode HR-ESI-MS, which exhibited a peak at *m/z* 387.1802 $[M + Na]^+$ ($C_{20}H_{28}NaO_6^+$, Calcd. for 387.1784), in addition to NMR data. **Z2-2** was identified as a reaction product of **Z2-1** with water, a conclusion supported by NMR data (Table 1) and HMBC correlations (Fig. 1). The NMR spectrum of **Z2-2** showed a signal for C-13 at *δ* 108.5, which signifies the addition of water to the C-13 carbonyl

group. Echoing **Z2-1**, the β configurations of H-14 and H-12 in **Z2-2** were confirmed by ROESY correlations (Fig. 1). Based on ^{1}H-^{1}H COSY, HSQC, and HMBC analyses, the structure of **Z2-2** was elucidated as 4β,9α,13,20-pentahydroxy-14(13→12)-abeo-12β_{H}-1,6-tigliadiene-3-one, marking it as another novel tigliane diterpenoid produced through the hydrolysis of phorbol.

Phorbol was subjected to hydrolysis in an H_2SO_4 environment, resulting in the formation of derivatives through the chemical cleavage of cyclopropanols (**Z1-1** to **Z2-2**). These compounds were produced via concerted mechanisms in an acidic solution, which facilitated the cleavage of the three-membered ring structures [13-15] (Fig. 2). The chemical cleavage reactions of cyclopropanol in both acidic and basic environments, known as the Flaschenträger reaction, are well documented [13-15].

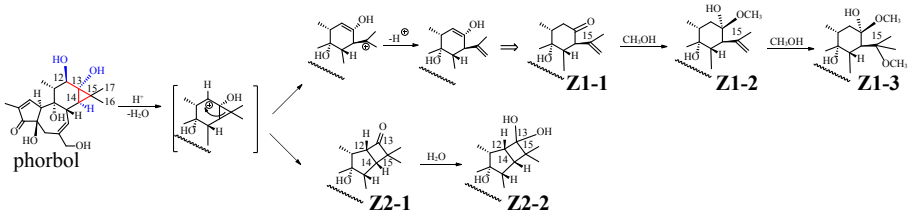


Fig. 2 Possible pathways for phorbol to compounds Z1-1-Z2-2.

We successfully synthesized five acid hydrolysates of phorbol (**Z1-1** to **Z2-2**) and further converted them into their respective esters through esterification. This process was aimed at exploring the impact of the C13/C14 cyclopropane ring on anti-HIV-1 activity and at identifying potential anti-HIV-1 candidate compounds.

Esterification of hydrolysates

The reactivity of the hydroxyl groups in phorbol follows a specific order: C20-OH > C13-OH > C12-OH > C4-OH > C9-OH [4]. Notably, the five hydrolysates (**Z1-1** to **Z2-2**) preserved the C20-OH group while lacking the C12-OH group, directing our synthesis efforts primarily towards 20-monoesters and 13,20-diesters. A total of fifteen esters (**S1**, **S2**, **S4** to **S6**, **S8** to **S17**), in addition to phorbol-20-cinnamate (**S3**) and 20-decanoyl-phorbol (**S7**), were successfully synthesized. The methods employed in synthesizing these derivatives (**S1** to **S17**) are detailed in Scheme 1.

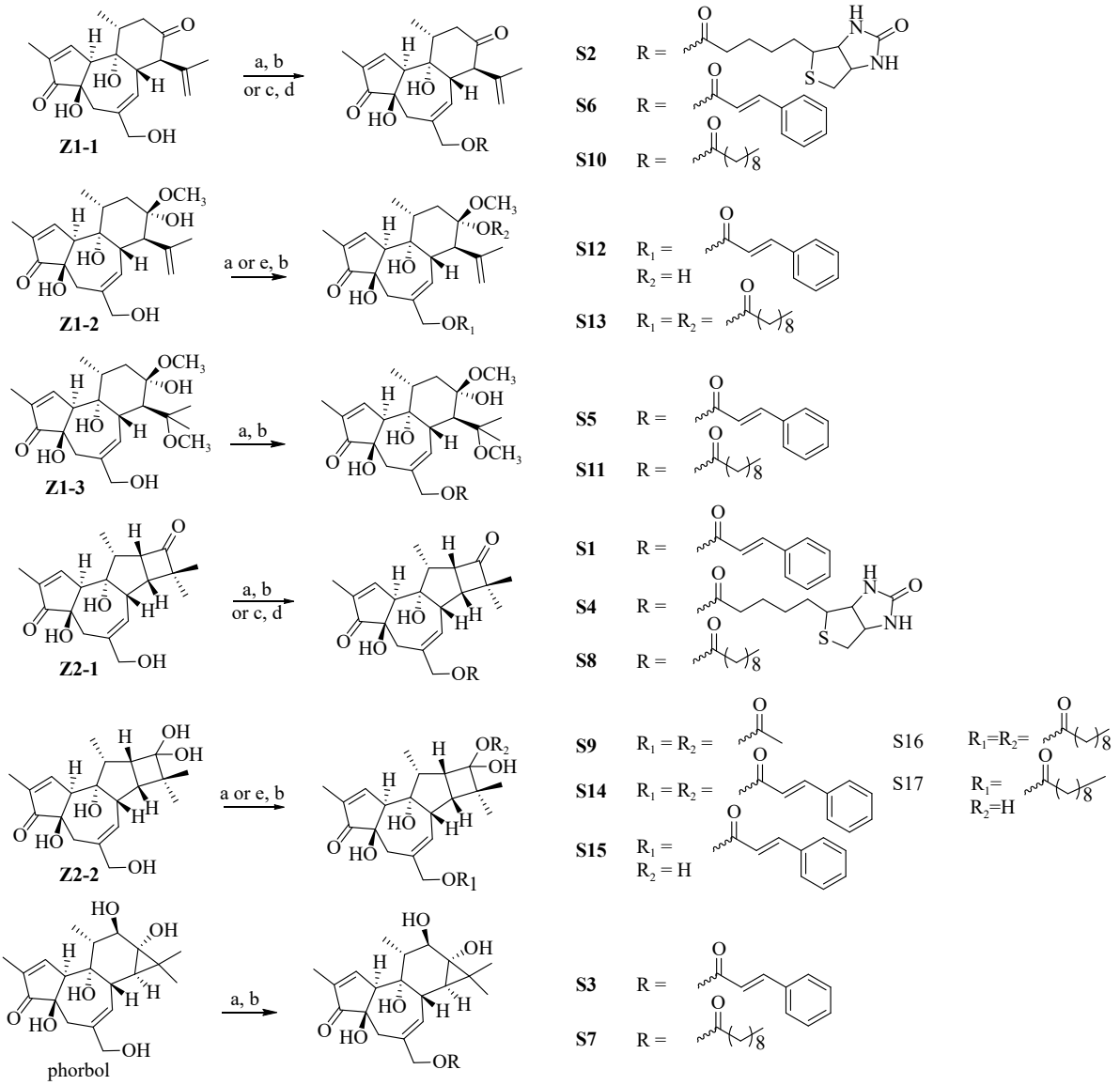
Anti-HIV-1 activities of phorbol derivatives

We evaluated the inhibitory effects of phorbol derivatives (compounds **Z1-1** to **Z2-2**, **S1** to **S17**) on syncytia formation induced by HIV-1_IIIB, aiming to explore the influence of the cyclopropane ring on anti-HIV-1 activity and to identify potential anti-HIV candidate compounds.

According to the data presented in Table 2, the hydrolysis derivatives of phorbol (compounds **Z1-1**–**Z2-2**) exhibited no cytotoxicity (CC_{50} > 200 μmol·L^{-1}). These compounds all demonstrated anti-HIV-1 activity, with EC_{50} values ranging from 108.55 and 10.00 μmol·L^{-1}, and notably, none included the C12-OH group. This observation led to the hypothesis that the presence of the C12-OH group might diminish the anti-HIV-1 activity of phorbol derivatives. Both compounds **Z1-1** and **Z2-1** exhibited relatively moderate anti-HIV-1 activities, with EC_{50} values of 88.60 μmol·L^{-1} and 108.55 μmol·L^{-1}, respectively. Interestingly, compound **Z1-1** could also result from the biotransformation of phorbol by human intestinal bacteria [11], suggesting that future studies could beneficially investigate the anti-HIV activity of phorbol metabolites.

The esterification of phorbol hydrolysates yielded insightful results on the anti-HIV-1 activities of phorbol derivatives. Particularly, derivatives from compounds **Z1-1** or **Z2-1**, when modified with biotin at the C-20 position (**S2**, **S4**), exhibited no anti-HIV-1 activity (EC_{50} > 200 μmol·L^{-1}). Conversely, linking cinnamic acid or decanoic acid to compounds **Z1-3**, **Z2-1**, or **Z2-2** significantly enhanced their anti-HIV-1 activities. For instance, the anti-HIV-1 activity of compound **Z1-2**, upon coupling with cinnamic acid or decanoic acid, slightly decreased (from an EC_{50} of 10.00 μmol·L^{-1} for **Z1-2** to an EC_{50} of 13.67 μmol·L^{-1} for **S12**, and 14.31 μmol·L^{-1} for **S13**). Notably, compound **Z1-1**, after esterification with decanoic acid at the C20-OH position, exhibited marginally weakened anti-HIV-1 activity (from an EC_{50} of 88.60 μmol·L^{-1} for **Z1-1** to an EC_{50} of 91.07 μmol·L^{-1} for **S10**). Additionally, esterifying the C20-OH of compounds **Z1-3**, **Z2-1**, or **Z2-2** with cinnamic acid increased the anti-HIV-1 activity of the resulting esters (from an EC_{50} of 15.48 μmol·L^{-1} for **Z1-3** to 10.22 μmol·L^{-1} for **S5**; from 108.55 μmol·L^{-1} for **Z2-1** to 49.02 μmol·L^{-1} for **S1**; and from 12.78 μmol·L^{-1} for **Z2-2** to 11.57 μmol·L^{-1} for **S15**), along with a slight increase in cytotoxicity (CC_{50} ranging from 87.03 to 104.74 μmol·L^{-1}). The anti-HIV-1 activity improved significantly when these compounds were esterified with decanoic acid at C20-OH, surpassing the activity of the corresponding C20-cinnamates (EC_{50} **S11** < **S5**, **S17** < **S15**, **S8** < **S1**). Remarkably, 20-decanoyl-phorbol (**S7**) displayed enhanced anti-HIV-1 activity compared to phorbol-20-cinnamate (**S3**).

Interestingly, the C20-monoesterification of compounds **Z1-1** to **Z2-2** did not universally result in cytotoxicity. Specifically, esterifying compounds **Z1-3** or **Z2-2** with decanoic acid at the C20-OH position produced compounds **S11** and **S17**, respectively. Compound **S11** demonstrated highly potent anti-HIV-1 activity with relatively lower cytotoxicity (EC_{50} 0.27 μmol·L^{-1}, CC_{50} 153.92 μmol·L^{-1}), surpassing the anti-HIV-1 activity and cytotoxicity profiles of the phorbol-20-monoesters (**S3**, **S7**). Similarly, compound **S17** showcased significant anti-HIV-1 activity while displaying no cytotoxicity (EC_{50} 6.60 μmol·L^{-1}, CC_{50} > 200 μmol·L^{-1}).



Scheme 1 Synthesis of compounds S1–S17. Reagents and conditions: (a) decanoic acid or cinnamic acid (1.5 eq), EDCI (1.0 eq), DMAP (1.0 eq), DCM, overnight; (b) Na_2CO_3, DCM; (c) biotin (1.0 eq), EDCI (1.5 eq), DMAP (1.0 eq), DMF, overnight; (d) $NaHCO_3$, EtOAc; (e) decanoic acid or cinnamic acid or acetic anhydride (2.5 eq), EDCI (1.0 eq), DMAP (1.0 eq), DCM, overnight.

These findings highlight the potential of exploring various alkyl acid derivatives at the C20-OH position of compounds, particularly **Z1-3**, with compound **S11** emerging as a notably promising candidate for further preclinical investigation.

In comparing the 20-monoesters (**S15** and **S17**), the 13,20-diesters of compound **Z2-2** exhibited enhanced anti-HIV-1 activity without demonstrating cytotoxicity (EC_{50} **S14** < **S15**, **S16** < **S17**; CC_{50} > 200 μmol·L^{-1}). Specifically, the 13,20-diacetates of compound **Z2-2** showed greater anti-HIV-1 activity than its 13,20-didecanoyl counterpart (**S9**, EC50 1.39 μmol·L^{-1}; **S16**, EC_{50} 3.05 μmol·L^{-1}). Notably, compound **Z2-2**, when modified with cinnamic acid at both the C13 and C20 positions (**S14**), displayed significant anti-HIV-1 activity without cytotoxicity (EC_{50} 0.74 μmol·L^{-1}, CC_{50} > 200 μmol·L^{-1}), rendering it an excellent control compound for assessing the impact of cinnamoyl group substituents on anti-HIV-1 activity.

The range of anti-HIV-1 activity observed in the *seco*-cyclic phorbol derivatives, including their 20-monoesters and 13,20-diesters, suggests that the chemical cleavage of cyclopropanols could play a crucial role in modulating this activity. Among these, the most potent compound, **S11** (EC_{50} 0.27 μmol·L^{-1}, CC_{50} 153.92 μmol·L^{-1}), has been identified as a promising candidate for additional preclinical studies. Future research should aim to elucidate the anti-HIV-1 mechanism of action of compound **S11**, potentially offering novel insights into therapeutic strategies against HIV-1.

*Compound **S11** might inhibit HIV-1 $_{IIIB}$ replication at the entry step*

To delve deeper into how **S11** inhibits HIV replication,

Table 2 The inhibition activity on syncytia formation induced by HIV-1_{IIIB} and the safety index of phorbol derivatives

Compd.	Anti-HIV activity (EC_{50}, μmol·L^{-1})	Cytotoxicity (CC_{50}, μmol·L^{-1})	Safety index (CC_{50}/EC_{50})	Compd.	Anti-HIV activity (EC_{50}, μmol·L^{-1})	Cytotoxicity (CC_{50}, μmol·L^{-1})	Safety index (CC_{50}/EC_{50})
Z1-1	88.60 ± 14.68	> 200	2.26	**S7**	7.04 ± 1.12	80.82 ± 7.29	11.48
Z1-2	10.00 ± 2.49	> 200	20.00	**S8**	20.22 ± 2.06	> 200	> 9.89
Z1-3	15.48 ± 0.95	> 200	12.92	**S9**	1.39 ± 0.00	> 200	> 144.38
Z2-1	108.55 ± 12.28	> 200	1.84	**S10**	91.07 ± 3.30	> 200	> 2.20
Z2-2	12.78 ± 2.86	> 200	15.65	**S11**	**0.27 ± 0.22**	**153.92 ± 3.61**	**571.69**
S1	49.02 ± 9.39	87.03 ± 1.03	1.78	**S12**	13.67 ± 2.81	96.36 ± 3.63	7.05
S2	> 200	> 200	/	**S13**	14.31 ± 3.19	> 200	> 13.98
S3	33.74 ± 3.95	106.40 ± 5.76	3.15	**S14**	0.74 ± 0.08	> 200	> 269.19
S4	> 200	> 200	/	**S15**	11.57 ± 4.62	104.74 ± 13.67	9.05
S5	10.22 ± 1.68	96.51 ± 6.17	9.44	**S16**	3.05 ± 0.56	> 200	> 57.08
S6	77.90 ± 18.04	> 200	> 2.57	**S17**	6.60 ± 2.19	> 200	> 30.32
3TC	0.18 ± 0.01	> 200	> 1137.66				

3TC: Lamivudine.

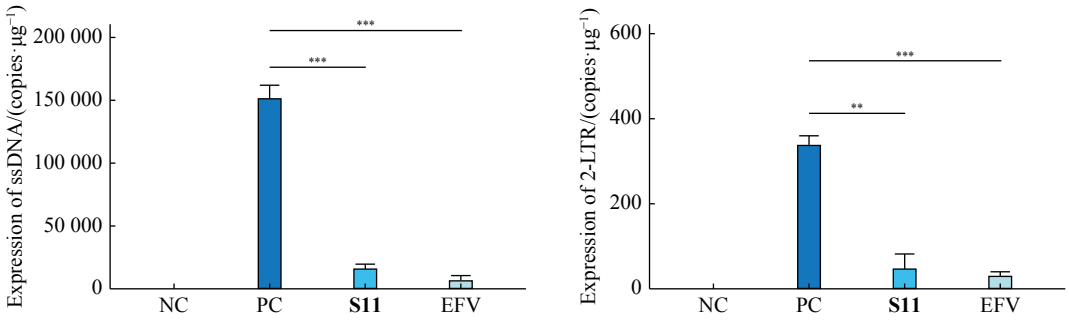


Fig. 3 The effect of compound S11 on the intermediate products of HIV-1 reverse transcription. ssDNA: single-stranded DNA, 2LTR: 2 long terminal repeat. EFV: Efavirenz, HIV-1 reverse transcriptase inhibitor, used as a positive drug control. NC: negative control, only C8166 cells, used as a negative control. PC: positive control, only virus-infected C8166 cells, without drug treatment, serve as a positive control. Data are shown as mean ± SD (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* positive control group.

we assessed its impact on HIV-1 reverse transcription intermediates (ssDNA and 2LTR). The results showed that **S11** significantly curtailed the expression of these intermediate products, hinting at **S11**'s potential action either prior to or during the reverse transcription process (Fig. 3). To substantiate this hypothesis, we explored **S11**'s effect on viral entry. C8166 cells were exposed to **S11** either concurrently with or after the entry of HIV-1 IIIB. The findings revealed that **S11**'s inhibitory effect on HIV-1 IIIB replication substantially decreased when the treatment was administered post-viral entry. This notable reduction in inhibition strongly supports the theory that **S11**'s primary mechanism of action is during the viral entry phase (Fig. 4).

PKC-δ protein affinity constant of phorbol derivatives

Phorbol esters are traditionally recognized as natural activators of PKC, attributed to their distinct structural elements, specifically the C3 carbonyl, C4-*β* hydroxyl, and C20 hydroxyl groups [6, 7]. Yet, the role of the cyclopropane ring located at the C13 and C14 positions in these molecules, particularly regarding their ability to bind to PKC, remains less explored. To delve into this aspect, we measured the affinity constant (K_d) of PKC-δ protein for a series of compounds, including phorbol, **Z1-1**, **Z1-2**, **Z1-3**, **Z2-1**, **Z2-2**, **S10**, **S17**, **S8**, and **S11**, utilizing isothermal titration calorimetry (ITC) (Fig. 5).

The investigation into the binding affinities of *seco*-cyclic phorbol derivatives (**Z1-1**, **Z1-2**, **Z1-3**, **Z2-1**, **Z2-2**) and selected esterified derivatives (**S10**, **S17**, **S8**, **S11**) for the PKC-δ protein revealed a notable decrease in affinity constants compared to phorbol itself. Remarkably, **Z1-2** and **Z1-3** exhibited no binding to PKC-δ protein, underscoring the crucial role of the cyclopropane ring at C13 and C14 in facilitating binding to PKC-δ protein. The cleavage of this cyclopropane ring appears to significantly reduce or entirely eliminate the ability of these derivatives to bind to PKC-δ. Moreover, the esterified derivatives, **S10**, **S17**, and **S8**—arising from **Z1-1**, **Z2-2**, and **Z2-1**, respectively, through esterification at the C20-OH group—also showed no

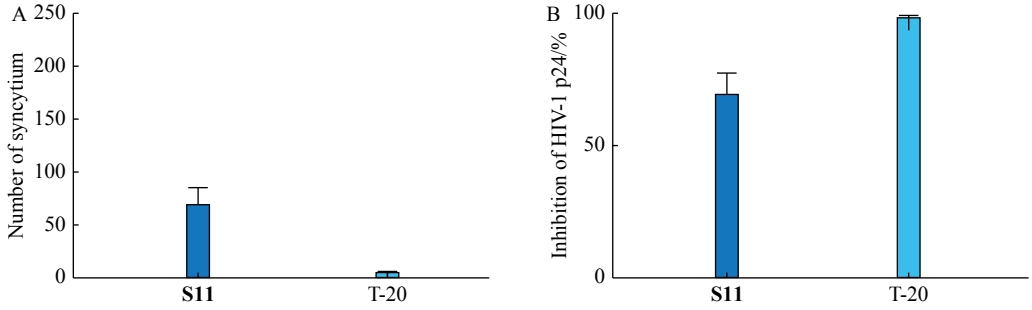


Fig. 4 The effect of compound S11 on the stage of the virus entering the cell. (A) When HIV-1_{IIIB} entered C8166 cells, the cells were treated with compound S11. (B) After the HIV-1_{IIIB} entered C8166 cells, the cells were treated with compound S11. Data are shown as mean ± SD (n = 3). T20: Enfuvirtide, HIV-1 entry inhibitor.

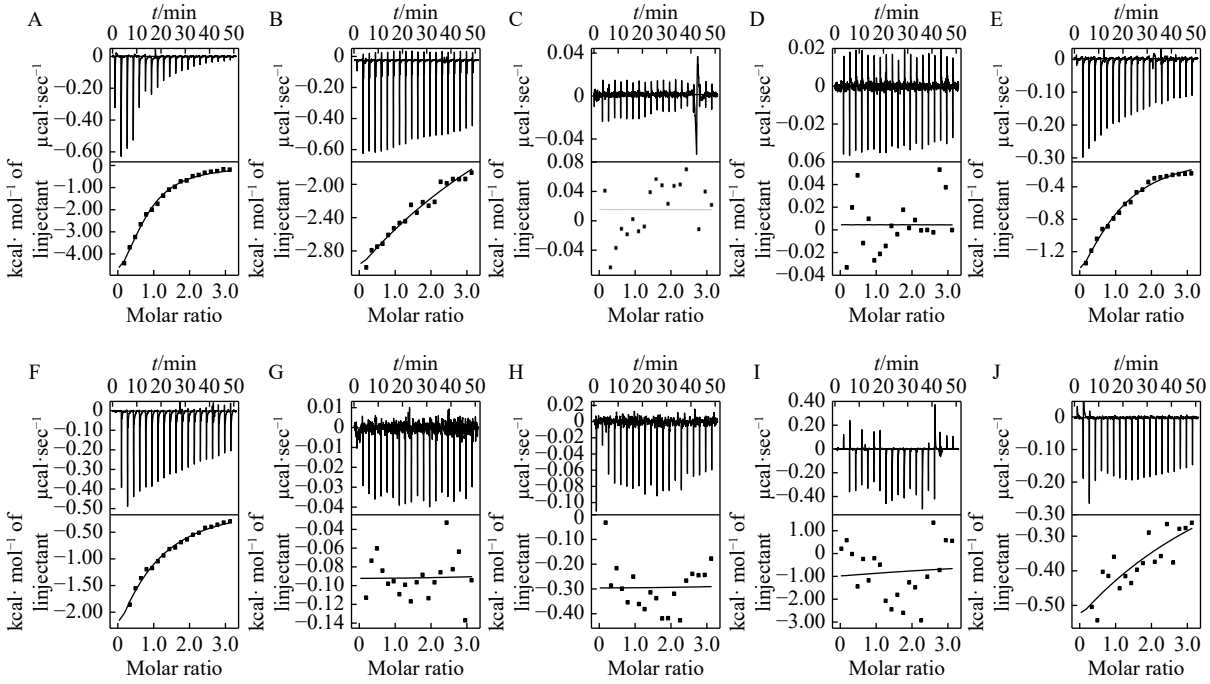


Fig. 5 PKC-δ Protein affinity constant (K_d) of compounds. (A) phorbol, K_d = 18.7 ± 2.0 μmol·L^{-1}. (B) Z1-1, K_d = 100.0 ± 15.0 μmol·L^{-1}. (C) Z1-2, no detectable binding. (D) Z1-3, no detectable binding. (E) Z2-1, K_d = 50.0 ± 9.0 μmol·L^{-1}. (F) Z2-2, K_d = 79.0 ± 2.5 μmol·L^{-1}. (G) S10, no detectable binding. (H) S17, no detectable binding. (I) S8, no detectable binding. (J) S11, no detectable binding.

binding to PKC-δ protein. **S11**, another esterified derivative, similarly demonstrated no affinity for PKC-δ protein, reinforcing the impact of structural modifications on the binding capabilities of these compounds to PKC-δ protein.

The observation that *seco*-cyclic phorbol derivatives (**Z1-2** and **Z1-3**) and their esterified forms (**S10**, **S17**, **S8**, and **S11**) did not bind to PKC-δ protein, coupled with their demonstrated anti-HIV-1 activities, especially in the case of **S11**, underscored a pivotal insight. This finding suggests that the mechanism through which these *seco*-cyclic phorbol derivatives exert their anti-HIV-1 effect is distinct from the traditional pathway of PKC activation. It highlights the potential for these compounds to act through alternative pathways or targets within the viral replication cycle that are independent of PKC activation.

Conclusions

This research delved into the properties and therapeutic potentials of *seco*-cyclic phorbol derivatives and their esterified versions. It included synthesizing new esters from *seco*-cyclic phorbol, assessing their anti-HIV-1 activities, and determining their binding affinity (K_d) for PKC-δ protein. A notable discovery was the identification of compound **S11** as exceptionally active, with an EC_{50} of 0.27 μmol·L^{-1} and a CC_{50} of 153.92 μmol·L^{-1}. Importantly, **S11** significantly inhibited the expression of HIV-1 reverse transcriptional intermediates, indicating its potential to obstruct the virus during the entry phase. Additionally, the absence of binding to PKC-δ protein by **S11** suggests that its anti-HIV-1 activity is distinct from PKC activation mechanisms. Given these insights, compound **S11** stands out as a compelling candidate for fur-

ther preclinical exploration. Its efficacy as an anti-HIV-1 agent invites a thorough investigation into its pharmacological mechanism. This study enriches the field of anti-HIV therapy development, offering new avenues for treatments that circumvent PKC activation. Such advancements hold promise for evolving strategies in combating HIV-1 infections, underscoring the importance of continuing research in this domain.

Experimental

General experimental procedures

NMR spectra were recorded on a Varian UNITY INOVA 600 spectrometer, employing tetramethylsilane (TMS) as the internal standard for precise chemical shift measurements. HR-ESI-MS spectra were acquired using an Agilent 6520 B Q-TOF spectrometer (Agilent Technologies, Santa Clara, CA, USA). For compound purification, silica gel (200−300 mesh, Qingdao Marine Chemical Co., Qingdao, China) and RP-C_{18} (50−70 μm, Daiso, Japan) were utilized as chromatographic media. These media are commonly used in column chromatography for their effectiveness in separating compounds by polarity.

Extraction and isolation of hydrolysis derivatives of phorbol

Phorbol (1 g, 0.329 mmol) was mixed with H_2SO_4/MeOH (0.25 mol·L^{-1}, 20 mL) and stirred at room temperature for 1 hour. The reaction was terminated by adding a saturated Na_2CO_3 solution. Subsequently, the mixture was extracted thrice using ethyl acetate (EtOAc). The concentrated extract was further purified *via* silica gel column chromatography, employing a gradient elution system of petroleum ether and ethyl acetate in varying ratios (2:1 to 1:1 to 0:1, *V/V*). This process yielded several hydrolysis derivatives of phorbol: **Z1-1** (20% yield), **Z1-2** (5.5% yield), **Z1-3** (4.5% yield), **Z2-1** (17% yield), and **Z2-2** (18% yield).

Compound **Z1-1**, white amorphous powder; $[\alpha]_D^{25}$ +27.50° (*c* 0.45, MeOH), HR-MS (ESI): *m/z*, Calcd. for $C_{20}H_{26}NaO_5^+$, $[M + Na]^+$: 369.1678, Found 369.1882. ^{1}H NMR (600 MHz, CD_3OD) *δ*: 7.64 (1H, s, H-1), 5.48 (1H, d, *J* = 5.9 Hz, H-7), 4.99 (1H, s, H-16a), 4.83 (1H, s, H-16b), 3.93 (2H, m, H-20), 3.72 (1H, dd, *J* = 12.8, 5.7 Hz, H-8), 3.48 (1H, d, *J* = 13.2 Hz, H-14), 3.18 (1H, m, H-10), 2.69 (1H, m, H-11), 2.54 (2H, m, H-5) , 2.53 (1H, m, H-12a), 2.22 (1H, dd, *J* = 14.6, 5.2 Hz, H-12b), 1.81 (3H, s, H-19), 1.62 (3H, s, H-17), 1.04 (3H, d, *J* = 6.7 Hz, H-18). ^{13}C NMR (150 MHz, CD_3OD) *δ*: 213.0 (C-3), 210.3 (C-13), 159.7 (C-1), 143.1 (C-15), 142.3 (C-6), 135.5 (C-2), 127.0 (C-7), 116.6 (C-16), 77.1 (C-9), 74.7 (C-4), 68.3 (C-20), 59.3 (C-10), 58.7 (C-14), 47.5 (C-12), 45.8 (C-8), 39.8 (C-5), 38.2 (C-11), 20.1 (C-17), 18.3 (C-18), 10.3 (C-19).

Compound **Z1-2**, white amorphous powder; $[\alpha]_D^{25.9}$ + 14.50° (*c* 0.30, MeOH), HR-MS (ESI): *m/z*, Calcd. for $C_{21}H_{31}O_6^+$, $[M + H]^+$: 379.2115, Found 379.2110. IR ν_{max}: 3329, 2965, 1698, 1646, 1558, 1448, 1325, 1056, 1033 cm^{-1}. ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) see Table 2.

Compound **Z1-3**, white amorphous powder; $[\alpha]_D^{25.9}$ +9.00° (*c* 0.36, MeOH), HR-MS (ESI): *m/z*, Calcd. for $C_{22}H_{35}O_7^+$, $[M + H]^+$: 411.2377, Found 411.2354. IR ν_{max}: 3523, 3244, 2967, 2939, 2921, 2879, 2844, 1696, 1635, 1457, 1385, 1305, 1192, 1102, 1055, 996 cm^{-1}. ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) see Table 2.

Compound **Z2-1**, white amorphous powder; $[\alpha]_D^{25.9}$ +2.50° (*c* 0.45, MeOH), HR-MS (ESI): *m/z*, Calcd. for $C_{20}H_{26}NaO_5^+$, $[M + Na]^+$: 369.1678, Found 369.1680. IR ν_{max}: 3232, 2963, 1763, 1689, 1635, 1456, 1053, 1033, 1003 cm^{-1}. ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) see Table 2.

Compound **Z2-2**, white amorphous powder; $[\alpha]_D^{25.9}$ +7.00° (*c* 0.46, MeOH), HR-MS (ESI): *m/z*, Calcd. for $C_{20}H_{28}NaO_6^+$, $[M + Na]^+$: 387.1784, Found 387.1802. IR ν_{max}: 3336, 2924, 2869, 1697, 1647, 1306, 1054, 1033, 1011 cm^{-1}. ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) see Table 2.

General procedures for the synthesis of target compounds

For the synthesis of esters **S12**, **S5**, **S11**, **S6**, **S10**, **S15**, **S17**, **S1**, **S8**, **S3**, and **S7**, a solution of either **Z1-1**, **Z1-3**, **Z2-2**, **Z2-1**, or phorbol (0.5 mmol) was prepared in dichloromethane (DCM, 20 mL). To this solution, either decanoic acid or cinnamic acid (1.5 equivalents), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 1.0 equivalent), and N,N-dimethyl-4-aminopyridine (DMAP, 1.0 equivalent) were added. The reaction was terminated by the addition of 5 mL of saturated Na_2CO_3 solution. Subsequently, the mixture was extracted three times with DCM [8]. The combined organic layers were then concentrated and further purified using silica gel column chromatography, utilizing a gradient elution of petroleum ether:ethyl acetate (ranging from 2 ∶ 1 to 1 ∶ 1, *V/V*). This procedure yielded the following compounds: **S12** (61%), **S5** (60%), **S11** (63%), **S6** (62%), **S10** (62%), **S15** (61%), **S17** (62%), **S1** (60%), **S8** (63%), **S3** (70%), **S7** (68%).

For the synthesis of esters **S13**, **S14**, **S16**, and **S9**, **Z1-2**, or **Z2-2** (0.5 mmol) was dissolved in an appropriate solvent. Decanoic acid, cinnamic acid, or acetic anhydride (2.5 equivalents) was added to this solution. The synthesis procedures mirrored those utilized for compounds **S12**, **S15**, and **S17**, leading to the formation of compounds **S13**, **S14**, **S16**, and **S9**, with yields ranging from 60% to 63%.

For the synthesis of esters **S2** and **S4**, **Z1-1**, or **Z2-1** (0.5 mmol) was dissolved in DMF (20 mL). Biotin (1.0 eq), EDCI (1.5 eq), and DMAP (1.0 eq) were added to this solution, and the mixture was stirred overnight. The reaction was halted by the addition of 20 mL of saturated $NaHCO_3$ solution, followed by extraction thrice with EtOAc [8]. The concentration of the combined organic layers preceded purification *via* silica gel column chromatography, employing a petroleum ether:ethyl acetate (1 ∶ 1, *V/V*) solvent system. This process resulted in compounds **S2** and **S4** with yields of 60% and 62%, respectively. These synthesis techniques are pivotal for generating specific esterified derivatives of *seco*-cyclic phor-

bol, which are subsequently evaluated for their potential biological activities, including anti-HIV-1 efficacy.

The physicochemical and spectral data of compounds S1−S17 are shown in Supplementary Materials.

The toxicity test of the samples to C8166 cells (MTT assay)

C8166 cells, utilized alongside the laboratory-adapted HIV-1 strain HIV-1IIIB (kindly donated by MRC and AIDS Reagent Project), were cultured in RPMI-1640 complete medium supplemented with 10% fetal bovine serum. To ensure the cells were in the logarithmic growth phase for the experiments, they were sub-cultured a day prior. Test compounds were dissolved in DMSO and kept at −20 °C until use. Lamivudine (3TC), serving as the positive control, was acquired from Sigma, dissolved in DMSO, and stored in a similar manner. For the assay, a cell suspension of 4×10^5/mL C8166 cells was prepared and mixed with various concentrations of the test compounds, maintaining three replicates for each concentration. Additionally, a control setup without any drugs and another with 3TC were established. The cultures were then incubated at 37 °C in a 5% CO_2 environment for three days. Post-incubation, cytotoxicity was assessed using the MTT assay, with absorbance readings taken at 570 nm and 630 nm on an ELx800 microplate reader. Based on these readings, the 50% cytotoxic concentration (CC_{50}) was calculated.[16].

The inhibition of syncytia formation assay

To assess the cytopathic effect (CPE) of HIV-1 on C8166 cells, we utilized the method described by Johnson & Byington (1990), with the tissue culture infection dose (TCID50) calculated following the Reed & Muench method. For this purpose, C8166 cells, at a concentration of 4×10^5/mL, were mixed with HIV-1IIIB (2000 TCID50/well) along with varying concentrations of the compounds under investigation. This mixture was then incubated at 37 °C in a 5% CO_2 atmosphere for 72 hours. Throughout the incubation period, the development of syncytia was closely monitored using an inverted microscope, and the resultant syncytia were quantified. The concentration at which a 50% reduction in syncytia formation was observed (EC_{50}) was determined by applying the Reed & Muench method. [16, 17].

Subsequently, dose-response curves were generated using Origin 7.5 software, facilitating the calculation of both the 50% cytotoxic concentration (CC_{50}) and the 50% effective concentration (EC_{50}) using the Reed & Muench method. The therapeutic index (TI) for each compound's anti-HIV-1 activity was calculated using the equation $TI = CC_{50}/EC_{50}$.

The inhibition of HIV-1_{IIIB} replication assay

C8166 cells were prepared at a concentration of 8×10^5 cells/mL and subsequently inoculated with the HIV-1_IIIB virus at a concentration of 2000 $TCID_{50}$ per well. The cell-virus admixture was incubated for 3 hours at 37 °C, a duration deemed sufficient for viral infection of the cells. Following incubation, non-internalized, free viral particles were eliminated by washing the cells twice with phosphate-buffered saline (PBS). The cells were then resuspended in RPMI-1640 medium, with the concentration adjusted to 4×10^5 cells/mL. Thereafter, 100 μL of this cell suspension was dispensed into each well of a 96-well plate, which already contained 100 μL of the test compounds at varying concentrations. Cultivation was carried out at 37 °C in a 5% CO_2 atmosphere. On the third day post-inoculation, the culture supernatant was harvested, and the effective concentration (EC_{50}) of the compounds was determined utilizing a p24 antigen ELISA kit, as detailed in references [16, 17].

Detection of HIV-1 replication intermediate products

To investigate the inhibitory effect of compound **S11** on HIV-1 reverse transcription, an experiment was conducted using C8166 cells treated with **S11** and subsequently infected with HIV-1_{IIIB} for 24 h. Following infection, cells were harvested for total DNA extraction, which was then subjected to quantitative PCR (qPCR) analysis using the ABI PRISM 7500 Fast Real-time PCR system (Applied Biosystems). This qPCR aimed to detect HIV-1 reverse transcriptional intermediates, specifically single-stranded DNA (ssDNA) and 2-long terminal repeats (2LTR), employing specific primers and probes. The primers and probes used were: for ssDNA, Forward Primer 5'-GCCTCAATAAAGCTTGCCTTGA-3', Reverse Primer 5'-TGACTAAAAGGGTCTGAGGGATCT-3', and Probe 5'-FAM-AGAGTCACACAACAGACGGGCACACACTA-TAMRA-3'; and for 2LTR, Forward Primer 5'-GCCTGGGAGCTCTCTGGCTAA-3', Reverse Primer 5'-AGGTAGCCTTGTGTGTGGTAGATCC-3', and Probe 5'-FAM-TAGTGTGTGCCCGTCTGTTGTGTGAC-TAMRA-3'. The reaction conditions involved a pre-denaturation phase at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 30 s. This methodological approach, leveraging the sensitivity and specificity of qPCR, was designed to precisely quantify the impact of compound **S11** on the early stages of HIV-1 replication, particularly focusing on the critical phase of reverse transcription.

Isothermal titration calorimetry of Human-PKC-δ protein binding constants of phorbol derivatives

In investigating the interactions between PKC-δ protein and various compounds, ITC was utilized, adhering to a methodology detailed in previous literature. Initially, PKC-δ protein was expressed and purified according to protocols outlined in reference [18], ensuring the procurement of a high-purity protein sample crucial for precise interaction analysis. The experiments were replicated twice, conducted at a steady temperature of 25 °C, using an iTC-200 microcalorimeter from MicroCal, Inc., USA. For ITC analysis, the concentrated protein sample was diluted in an ITC buffer containing 20 mmol·L^{-1} Tris-Cl (pH 7.5) and 50 mmol·L^{-1} NaCl, with the concentration of DMSO in the protein solution adjusted to 0.5%. The compounds being tested were initially dissolved in DMSO and then diluted in the same ITC buffer to achieve their required concentrations for the assay. A standard protein concentration of 50 mmol·L^{-1} and a compound concentration of 1.0 mmol·L^{-1} were employed. In the calorimeter,

the protein resided in the cell chamber, and the compound was introduced in 20 consecutive aliquots, with a 150-second interval between each injection, facilitating a detailed and controlled monitoring of the binding interaction. The data obtained were subsequently analyzed using the single-site binding model provided in the Origin software package by MicroCal, Inc., USA. This methodical approach allowed for an in-depth characterization of the binding interactions between the PKC-δ protein and the compounds, including the determination of affinity constants, thereby offering critical insights into the specificity and strength of these molecular interactions.

Abbreviations

DCM: dichloromethane; DMAP: *N*,*N*-dimethyl-4-aminopyridine; DMF: *N*,*N*-dimethylformamide; EtOAc: ethyl acetate; EDCI: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

Supplementary Materials

Supplementary data to this article can be obtained by sending an E-mail to the corresponding authors.

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