Bioactive Compounds from Euphorbia usambarica Pax. with HIV-1 Latency Reversal Activity

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Abstract: Euphorbia usambarica is a traditional medicine used for gynecologic, endocrine, and urogenital illnesses in East Africa; however, its constituents and bioactivities have not been investigated. A variety of compounds isolated from Euphorbia species have been shown to have activity against latent HIV-1, the major source of HIV-1 persistence despite antiretroviral therapy. We performed bioactivity-guided isolation to identify 15 new diterpenoids (1–9, 14–17, 19, and 20) along with 16 known compounds from E. usambarica with HIV-1 latency reversal activity. Euphordracuolate C (1) exhibits a rare 6/6/3-fused ring system with a 2-methyl-2-cyclopentenone moiety. Usambariphanes A (2) and B (3) display an unusual lactone ring constructed between C-17 and C-2 in the jatrophane structure. 4β-Crotignoid K (14) revealed a 250-fold improvement in latency reversal activity compared to crotignoid K (13), identifying that configuration at the C-4 of tigliane diterpenoids is critical to HIV-1 latency reversal activity. The primary mechanism of the active diterpenoids 12–14 and 21 for the HIV-1 latency reversal activity was activation of PKC, while lignans 26 and 27 that did not increase CD69 expression, suggesting a non-PKC mechanism. Accordingly, natural constituents from E. usambarica have the potential to contribute to the development of HIV-1 eradication strategies.

Keywords: Euphorbia usambarica; diterpenoid; usambariphane; HIV; latency reactivation; latency reversal agent; PKC

1. Introduction

Antiretroviral therapy (ART) durably blocks HIV-1 transcription by targeting viral enzymes; however, these drugs do not result in viral eradication due to the presence of replication-competent proviruses that are stably integrated into the genomes of a small population of long-lived memory T cells, known as the latent reservoir [1]. A promising strategy to address HIV-1 persistence is to use small molecules to reactivate latent proviruses in order to expose these cells to immune clearance and/or viral cytopathic effect. Natural products offer much promise regarding the discovery of new latency reversal agents (LRAs) for HIV-1 eradication [2–4].

The Euphorbia is one of the largest genera in Euphorbiaceae [5,6]. There are many bioactive secondary metabolites in the genus Euphorbia, including more than 20 different types of diterpenoids (abietane, atisane, casbane, daphnane, ingenane, jatrophane, karane, lathyrane, tigliane, and others) [7]. Moreover, sesquiterpenoids, triterpenoids, flavonoids, alkaloids, polyphenols, tannins, volatile compounds, and phytosterols have...
also been discovered in *Euphorbia* species, many of which are in active use as traditional medicines [8–10]. The pharmacological effects of *Euphorbia* species are related to anti-inflammatory [11], multidrug-resistance-reversing [12,13], antiviral [14,15], cytotoxic [16,17], anti-arrhythmic [18], antifungal [19], anti-thrombotic [20], antiallergic [21], and muscle relaxant [22] properties.

In past, several *Euphorbia* plants have previously been evaluated to determine their efficacy as LRAs [23–30]. For instance, Liu et al. reported the effects on HIV-1 transcription of ingenane esters 3-angeloylingenol and 3-(2-naphtho)ingenol from *E. kansui*, which can reactivate latent HIV with EC\textsubscript{50} values at 4.2 and 2.4 nM, respectively [28]. Yan et al. published atisane diterpenoids euphorneroid D and ent-3-oxoatisan-16α,17-acetonide from *E. neriifolia* which showed anti-HIV-1 activities with EC\textsubscript{50} values at 34 and 24 µM, respectively [29]. Valadão et al. established deoxyphorbol esters from *E. umbellata* which increased HIV-1 latency reactivation through NF-κB activation, nuclear translocation, and HIV-1 LTR promoter [30].

*Euphorbia usambarica* Pax. distributes mainly in East Africa [31] and is a large branching shrub as well as used as a traditional medicine for gynecologic, endocrine, and urogenital illnesses [32,33]. In our preliminary study, we found that the whole plant extract of *E. usambarica* showed a significant HIV-1 latency reversal activity. However, there was no study related to the chemical constituents and bioactivities of *E. usambarica* in the reported literature. In addition, the prevention and treatment of HIV infection and acquired immune deficiency syndrome (AIDS) are still the central issues around the world. Therefore, we would investigate the active constituents and the pharmacological effect of *E. usambarica*. Further, we sought to test its aqueous, and organic fractions for HIV-1 latency reversal activity and cytotoxicity. Dichloromethane and n-hexane fractions showed increased activity compared to the whole plant extract (EU) in dose-response analysis. Further sub-fractionation of the active fractions was followed by compounds purification and identification using multistep chromatography, NMR, and mass spectroscopy to yield 31 purified compounds. Six of those compounds demonstrated HIV-1 anti-latency activity. Extended dose-response curves were then generated for these compounds. Several of these compounds have no previously described anti-HIV-1 or anti-latency activity. These results support further exploration of medicinal plants, and *Euphorbia* species in particular, as sources of new means to address HIV-1 persistence.

2. Results
2.1. Structure Elucidation of New Compounds

The partitioned n-hexane (EU-H) and dichloromethane (EU-C) phases significantly improved upon reactivation efficacy compared to the EU. The EU-H reactivated latent HIV-1 to 91% at concentrations of 50 and 100 µg/mL. The EU-C phase reactivated latent HIV-1 up to 86% at 10 µg/mL concentration and 98% at 50 µg/mL. The partitioned ethyl acetate (EU-E) and water-soluble residue (EU-W) phases did not appear to have any activity (Figure 1A). Cell viability declined steeply above concentrations of 100 µg/mL. Significant toxicity at concentrations above 100 µg/mL limits conclusions about reactivation. The lower concentrations of the EU-H and EU-C fractions did not affect toxicity but markedly improved viral reactivation (Figure 1B). Due to the high reactivation ratio (86%) at the lowest tested concentration (10 µg/mL), the EU-C phase was selected for bioactivity-guided isolation. This led to identification of 15 new diterpenoids (1–9, 14–17, 19, and 20) along with 16 known compounds (10–13, 18, and 21–31) (Figure 2).
Figure 1. HIV-1 latency reversal activity of methanolic crude extract (EU), partitioned n-hexane (EU-H), dichloromethane (EU-C), ethyl acetate (EU-E), and water-soluble residue (EU-W) phases. (A) dose-response experiments conducted with Jurkat T cells that were latently infected with full-length HIV-1 reporter construct (J-Lat 10.6 cells), HIV-1 reactivation quantified as % of positive control (PMA); (B) cell viability of each sample at 10, 50, 100, 500, and 1000 μg/mL.

Figure 2. Structures of compounds 1–31 isolated from E. usambarica.
2.1.1. Euphordraculoate C (1)

Compound 1 was purified as a colorless gum with $[\alpha]^{28}_D = -63$ (c 0.05, CHCl$_3$). The molecular formula was identified as C$_{29}$H$_{33}$O$_7$ by HR-ESIMS $m/z$ 493.2237 [M + H]$^+$ (calcd. for C$_{29}$H$_{33}$O$_7$ 493.2221), indicating 14 unsaturated degrees. The $^1$H NMR spectrum of 1 revealed six methyls, an oxygenated methine, two unsaturated methines, and a mono-substituted aromatic group (Table 1). The $^{13}$C-JMOD spectrum of 1 evidenced 29 carbon signals, including six methyls, one methylene, seven olefinic methines, one oxygenated methine, four saturated methines, one quaternary, three olefinic quaternary, two oxygenated quaternary, and four carbonyl carbons (Table 2). According to the combination of the 1D and 2D NMR spectra, one benzoxyloxy group (OBz) [δ$_H$ 8.02 (2H), 7.58, 7.46 (2H); δ$_C$ 166.2, 133.2, 130.4, 129.9 (2C), 128.6 (2C)] and one acetoxy (OAc) (δ$_H$ 1.97; δ$_C$ 170.4, 21.1) could be identified in 1. Based on the COSY and HSQC spectra of 1, a series of COSY correlations between an olefinic methine (δ$_H$ 7.52, CH-1)/a methine (δ$_H$ 3.80, CH-15)/methylene (δ$_H$ 2.52, 2.24, CH$_2$-4), together with the allylic four-bond coupling between H-1 and a methyl group [δ$_H$ 1.74 (3H), CH$_3$-16]. Key HMBC correlations from H-1 and H-16 to an olefinic quaternary carbon C-2 (δ$_C$ 141.3) and a ketone carbon C-3 (δ$_C$ 206.8), and H-4 to C-3, indicated the presence of an α-methyl-α,β-unsaturated cyclopentanone moiety. Moreover, a series of COSY correlations between a methine (δ$_H$ 2.37, CH-8), an olefinic methine (δ$_H$ 6.68, CH-7), and an allylic coupled methyl group [δ$_H$ 2.02 (3H), CH$_3$-17], together with the key HMBC correlations from H-7 to a carbonyl carbon C-5 (δ$_C$ 163.4) and an oxygenated quaternary carbon C-14 (δ$_C$ 85.8), H-8 to an olefinic quaternary carbon C-6 (δ$_C$ 127.8), and H$_2$-17 to C-5, C-6, and C-7, indicated the presence of an α-methyl-α,β-unsaturated-δ-lactone moiety. A gem-dimethylcyclopropane moiety could be identified by the key HMBC correlations from two methyl groups [δ$_H$ 1.43 (3H), δ$_C$ 16.5, CH$_3$-18; δ$_H$ 1.16 (3H), δ$_C$ 24.8, CH$_3$-19] to a methine C-9 (δ$_H$ 1.02, δ$_C$ 34.0), a quaternary carbon C-10 (δ$_C$ 24.9), an oxygenated quaternary carbon C-11 (δ$_C$ 63.4) and each other, and H-9 to C-10 and C-11. In addition, the $^1$H$^1$H COSY cross peak between H-8/H-9, a methyl group [δ$_H$ 0.91 (3H), CH$_3$-20]/a methine (δ$_H$ 2.10, CH-13)/an oxygenated methine (δ$_H$ 5.86, CH-12), as well as the HMBC correlations from H-7 to C-14, H-8 to C-11 and C-13, H-9 to C-11 and C-14, H-12 to C-10 and C-11, and C-13 to C-14, demonstrated the presence of a six-membered ring fusion with the gem-dimethyl-cyclopropane moiety at C-9 and C-11, and the α-methyl-α,β-unsaturated-δ-lactone moiety at C-8 and C-14. The HMBC correlations from H-4 to C-14 and H-8 to C-15 indicated that the α-methyl-α,β-unsaturated cyclopentanone moiety was linked to C-14. The OAc and OBz groups should be connected to C-11 and C-12, respectively, based on HMBC correlations (Figure 3). Additionally, comparing the NMR data of 1 with those of euphordraculoate A [34] suggested the same rare diterpenoid skeleton of both compounds. According to the NOESY cross-peaks between H-8/H-13, H-8/H$_2$-18, H-8/H-15, H-9/H$_3$-19, 11-OAc/H$_3$-19, H-12/H$_3$-20, and H-13/H$_3$-18, as well as comparing with euphordraculoate A [34] and euphodendriane A [35], the relative configuration of 1 was established as shown on structural formula (Figure 3), and the compound was named as euphordraculoate C.
Table 1. $^1$H NMR data of compounds 1–6 in CDCl$_3$ at 500 MHz ($\delta_H$ in ppm, mult. $J$ in Hz).

| Position | 1          | 2          | 3          | 4          | 5          | 6          |
|----------|------------|------------|------------|------------|------------|------------|
| 1        | 7.52, br s | a: 2.87, d (16.5) | b: 2.09, d (16.5) | a: 2.89, d (17.0) | b: 2.18, d (16.5) | a: 2.76, d (16.0) |
| 2        | 4.26, dd (9.5, 3.5) | 4.25, dd (9.5, 3.5) | 4.52, dd (12.5, 4.0) | 5.80, dd (4.5, 10.0) | 5.82, dd (4.0, 1.0) |
| 3        | 2.52, dd (18.5, 6.5) | 2.54, m | 2.54, m | 2.55, m | 3.97, m | 3.98, m |
| 4        | 6.53, d (2.0) | 6.55, d (2.0) | 6.09, m | 5.70, d (10.0) | 5.70, d (10.0) |
| 5        | 5.73, d (4.5) | 5.75, d (4.5) | 5.77, d (5.0) | 5.72, s | 5.75, s |
| 6        | 6.53, d (2.0) | 6.55, d (2.0) | 6.09, m | 5.70, d (10.0) | 5.70, d (10.0) |
| 7        | 4.26, dd (9.5, 3.5) | 4.25, dd (9.5, 3.5) | 4.52, dd (12.5, 4.0) | 5.80, dd (4.5, 10.0) | 5.82, dd (4.0, 1.0) |
| 8        | 2.52, dd (18.5, 6.5) | 2.54, m | 2.54, m | 2.55, m | 3.97, m | 3.98, m |
| 9        | 6.53, d (2.0) | 6.55, d (2.0) | 6.09, m | 5.70, d (10.0) | 5.70, d (10.0) |
| 10       | 4.26, dd (9.5, 3.5) | 4.25, dd (9.5, 3.5) | 4.52, dd (12.5, 4.0) | 5.80, dd (4.5, 10.0) | 5.82, dd (4.0, 1.0) |
| 11       | 2.52, dd (18.5, 6.5) | 2.54, m | 2.54, m | 2.55, m | 3.97, m | 3.98, m |
| 12       | 6.53, d (2.0) | 6.55, d (2.0) | 6.09, m | 5.70, d (10.0) | 5.70, d (10.0) |
| 13       | 4.26, dd (9.5, 3.5) | 4.25, dd (9.5, 3.5) | 4.52, dd (12.5, 4.0) | 5.80, dd (4.5, 10.0) | 5.82, dd (4.0, 1.0) |
| 14       | 2.52, dd (18.5, 6.5) | 2.54, m | 2.54, m | 2.55, m | 3.97, m | 3.98, m |
| 15       | 6.53, d (2.0) | 6.55, d (2.0) | 6.09, m | 5.70, d (10.0) | 5.70, d (10.0) |

Table 2. $^{13}$C NMR data of compounds 1–6 in CDCl$_3$ at 125 MHz ($\delta_C$ in ppm).

| Position | 1          | 2          | 3          | 4          | 5          | 6          |
|----------|------------|------------|------------|------------|------------|------------|
| 1        | 160.2      | 49.1       | 49         | 45.7       | 52.5       | 52.5       |
| 2        | 141.3      | 89.5       | 89.5       | 90.1       | 87.7       | 87.7       |
| 3        | 206.8      | 80.5       | 80.5       | 80.7       | 80         | 79.8       |
| 4        | 37.9       | 43.3       | 43.1       | 44.9       | 45.2       | 45.1       |
| 5        | 163.4      | 72.8       | 73.1       | 73         | 73.2       | 72.9       |
| 6        | 127.8      | 92.5       | 92.4       | 84.4       | 81.1       | 81.6       |
| 7        | 141.4      | 70.8       | 70.5       | 69.1 $^e$  | 68.2       | 68.1       |
| 8        | 35.2       | 68.9       | 69.0       | 69.2 $^e$  | 68.1       | 68.1       |
| 9        | 34         | 79.4       | 78.9       | 78.8       | 81.7       | 81.7       |
| 10       | 24.9       | 41.2       | 41.2       | 40.6       | 40.3       | 40.4       |
| 11       | 63.4       | 134.6      | 134.6      | 135.3      | 137.4      | 137.3      |
| 12       | 76.2       | 133        | 132.9      | 134.1      | 128.9      | 128.9      |
| 13       | 38.9       | 37.4       | 37.4       | 39         | 43.9       | 43.6       |
| 14       | 85.8       | 81.6       | 81.5       | 82.2       | 211.4      | 211.6      |
2.1.2. Usambariphane A (2)

Compound 2 was obtained as a white amorphous powder. Its molecular formula was calculated as C_{40}H_{52}O_{16} by the analysis of HR-ESIMS (m/z 789.3327 [M + H]^+) (calcd. for C_{40}H_{53}O_{16} 789.3328). The NMR spectra of 2 revealed clearly four OAc (δ_C 2.56, δ_C 174.3, 21.4; δ_C 170.2, 22.7; δ_C 2.14, δ_C 169.8, 21.5; δ_C 2.08, δ_C 170.0, 21.6), one OBz (δ_C 7.93 (2H), 7.52, 7.39 (2H), δ_C 164.5, 133.5, 129.7, 128.9, 128.8), one propionate group [δ_C 2.41 (2H), 1.16 (3H), δ_C 174.2, 27.7, 8.9; OPr], four methyls [δ_C 0.93 (3H), δ_C 26.0, CH_3-11; δ_C 1.13 (3H), δ_C 21.7, CH_3-19; δ_C 1.14 (3H), δ_C 23.2, CH_3-20], a trans-disubstituted C=C (δ_C 5.49, δ_C 134.6, CH-11; δ_C 5.83, δ_C 133.0, CH-12), and a lactone carbonyl carbon (δ_C 175.1, C-22). Further, comparing the 1D NMR data of 2 (Tables 1 and 2) with those of isotterracinolide A (10) [36], the skeleton of 2 was established as a dihomojatrophane type diterpenoid [7] with a double bond at δ_C 11,12, and a lactone moiety. An OH group was located at C-3 based on a 1H-1H COSY cross peak between H-3 and 3-OH, as well as the HBMC correlations from 3-OH to C-3 and C-4. Another OH group was connected to C-15 by the confirmation of the HMBC correlations from 15-OH to C-4, C-14, and C-15. Moreover, the HMBC correlations from H-5 to δ_C 164.5, H-7 to δ_C 174.2, H-8 to δ_C 170.0, H-9 to δ_C 170.2, H-14 to δ_C 174.3, indicated that OBz and OPr were located at C-5 and C-7 respectively, and three OAc were linked to C-8, C-9, and C-14 each. The last OAc was located apparently to C-6 based on NOESY correlations between the acetyl proton signal δ_H 2.14 (6-OAc) with H-5 and H-17a. The remaining lactone ring was proposed to be constructed between C-17 and C-2 in structure 2. According to the δ_C 92.5 of 2 was close to the signal in sororianolide A (δ_C 93.0, C-6-βOAc) and different from sororianolide B (δ_C 80.9, C-6-αOAc), suggesting the OAc at C-6 in 2 can be assigned as β-oriented [37]. Moreover, the NOESY correlations of H-3/H-27, H-3/H-4, H-4/H-7, H-4/H-8, H-8/H-19, H-19/H-13, H-19/H-14, H-16/3-OH, H-16/H-1b, H-1b/15-OH, 15-OH/H-9, and H-9/H-18 indicated the configurations of 3β-OH, Ha-4, Hb-5, 6β-OAc, 7β-OPr, 8β-OAc, 9α-OAc, 14β-OAc, 15-βOH, βCH_3-16, and βCH_3-20. Thus, the structure of 2 was established and named as usambariphane A.

### Table 2. Cont.

| Position | 1  | 2  | 3  | 4  | 5  | 6  |
|----------|----|----|----|----|----|----|
| 15       | 45.8 | 86.3 | 86.3 | 87.1 | 84.6 | 84.6 |
| 16       | 10.2 | 19.1 | 19 | 20 | 18.5 | 18.5 |
| 17       | 17.4 | 24.5 | 24 | 23.4 | 26.5 | 26.6 |
| 18       | 16.5 | 26 | 26 | 26.8 | 25.8 | 26 |
| 19       | 24.8 | 21.7 | 21.2 | 21.9 | 23.2 | 23.1 |
| 20       | 12.5 | 23.2 | 23.1 | 22.4 | 21.7 | 21.6 |
| 21       | 26 | 28.3 | 26 | 29.1 | 29.2 |
| 22       | 175.1 | 174.9 | 168.1 | 172.7 | 172.7 |

2-OAc 169.7, 22.7; 169.6, 22.4; 169.6, 22.4

3-OAc 169.1, 20.6 b; 169.2, 21.0 i

5-OBz 164.5, 133.5, 129.7, 129.6, 128.7

6-OAc 169.8, 21.5 a

7-OBu 176.4, 34.2

7-OPr 174.2 b, 27.7, 8.9

8-OAc 170.2, 22.7; 169.8 b, 21.4 c

9-OAc 170.2, 22.7; 169.8 b, 22.6

11-OAc 170.4, 21.1

12-OBz 130.4, 129.9

14-OAc 174.3 b, 21.4 a; 174.3, 21.3 c

170.8, 20.8

a i: Exchangeable.
2.1.3. Usambariphane B (3)

Compound 3 was obtained as a white amorphous powder. The molecular formula was determined as C₄₁H₆₄O₁₆ based on HR-ESIMS m/z 803.3488 [M + H]⁺ (calcd. for C₄₁H₆₂O₁₆ 803.3485). The 1D NMR data (Tables 1 and 2) of 3 were highly similar to those of 2, except for an isobutyryl group (OiBu) [δ_H 2.61, 1.21 (3H), 1.20 (3H), δ_C 176.4, 34.2, 18.8, 18.5] instead of propanoyl. The isobutyryl group was connected to C-7 in 3 based on the HMBC correlation from H-7 (δ_H 5.38) to the OiBu carbonyl carbon (δ_C 176.4). The NOESY correlations of 3 revealed the same relative configuration as that of 2. The structure of 3 was established and named as usambariphane B.

2.1.4. Usambariphane C (4)

Compound 4 was purified as a colorless crystal. The molecular formula was identified as C₄₀H₅₂O₁₆ by HR-ESIMS m/z 789.3346 [M + H]⁺ (calcd. for C₄₀H₅₀O₁₆ 789.3328). Based on the comparison of the ¹H and ¹³C NMR data (Tables 1 and 2) for 4 with those of usambariphane B (2), the skeleton of 4 was suggested to be a C₂₂ dihydrojatrophane with a double bond at Δ₁₁,₁₂ (δ_H 5.41, δ_C 135.3, CH-11; δ_H 5.72, δ_C 134.1, CH-12) and a lactone moiety (δ_H 3.09, 2.40, δ_C 23.4, CH₂-17; δ_H 2.65, 2.16, δ_C 26.0, CH₂-21; δ_C 168.1, C-22). A δ-lactone ring was constructed at C-5 and C-6 supporting by the ¹H-¹H COSY cross peak between H-2₁7 and H₂-2₁, as well as the HMBC correlations from H-5 to C-17 and C-2₂, H₂-17 to C-5, C-6, and C-2₂, and H₂-2₁ to C-5 and C-2₂. Moreover, the 1D NMR data of 4 were highly close to those of euphosorophane D [38] except for an OPr group [δ_H 2.57, 2.50, 1.22 (3H); δ_C 174.6, 27.5, 8.9] at C-7 according to an HMBC correlation from H-7 (δ_H 5.40) to δ_C 174.6. The NOESY cross-peaks of 4 demonstrated the same relative orientations to those of euphosorophane D [38]. Therefore, the structure of 4 was established and named as usambariphane C.

2.1.5. Usambariphane D (5)

Compound 5 was purified as a colorless crystal. The molecular formula was identified as C₄₀H₅₀O₁₆ by HR-ESIMS m/z 787.3193 [M + H]⁺ (calcd. for C₄₀H₅₁O₁₆ 787.3172). The inspection of 1D (Tables 1 and 2) and 2D NMR data suggested that compound 5 was a bishomojatrophane type diterpenoid with a double bond at Δ₁₁,₁₂ (δ_H 6.16, δ_C 137.4, CH-11; δ_H 5.43, δ_C 128.9, CH-12), a lactone moiety (δ_H 2.72, 2.01, δ_C 26.5, CH₂-17; δ_H 3.43, 2.50, δ_C 29.1, CH₂-2₁; δ_C 172.7, C-2₂), and a ketone unit δ_C 211.4 (C-1₄). A δ-lactone ring was constructed at C-5 and C-6 supporting by the ¹H-¹H COSY cross peak between H₂-17 and H₂-2₁, and the HMBC correlations from H-5 to C-1₇ and C-2₂, H₂-17 to C-5, C-6, and C-2₂, and H₂-2₁ to C-5 and C-2₂. Moreover, the HMBC correlations from H-5 to C-1₇ and C-2₂, H₂-17 to C-5, C-6, and C-2₂, and H₂-2₁ to C-5 and C-2₂. The ketone unit in 5 was located at C-1₄ based on the HMBC correlations from H-1₁, H₁₂, H₁₃, and H₃-2₀ to C-1₄, respectively. An OH group was connected to C-1₅ by the confirmation of HMBC correlations from 1₅-OH to C-4, C₁₄, and C-1₅. Moreover, four OAc [δ_H 2.26 (3H), δ_C 169.6, 22.4; δ_H 2.05 (3H), δ_C 169.1, 20.6; δ_H 2.03 (3H), δ_C 169.9, 20.9; δ_H 2.00 (3H), δ_C 170.0, 21.2], one OBz [δ_H 7.88 (2H), 7.65, 7.51 (2H), δ_C 165.8, 133.8, 130.6, 129.7 (2C), 128.5 (2C)], and one OPr [δ_H 2.49, 2.31, 1.23 (3H), δ_C 173.4, 27.6, 8.6] moieties were identified clearly by the examination of the NMR spectra. The HMBC correlations from H-3 to δ_C 169.1, H-7 to δ_C 173.4, H-8 to δ_C 170.0, and H-9 to δ_C 169.9, indicated the OPr was located at C-7, and three OAc were linked to C-3, C₈, and C-9, respectively. The location of the OBz at C-6 was confirmed by the NOESY correlations between the benzoyl proton signal δ_H 7.88 with H-5, H-8, and H-1₂. The last OAc was connected to C-2 based on the NOE cross-peak between the acetyl proton signal δ_H 2.26 with H₃-1₆. The relative configuration of 5 was evaluated by the NOESY spectrum and comparison with a similar structure terracinolide [39] to assign 2α-OAc, 3β-OAc, Ha-4, Hβ-5, 6β-OBz, 7β-OPr, 8α-OAc, 9α-OAc, βCH₃-2₀, and 15β-OH. Above all, the structure of 5 was established and named as usambariphane D.
2.1.6. Usambariphane E (6)

Compound 6 was obtained as a colorless crystal. The molecular formula was identified as C41H39O15 by HR-ESIMS m/z 801.3356 [M + H]+ (calcd. for C41H39O15 801.3328). The 1D (Tables 1 and 2) and 2D NMR data of 6 were almost identical with those of 5, except for the ester group at C-7. In 6, an OMe [δH 2.63, 1.26 (3H), 1.22 (3H), δC 175.2, 34.5, 19.0, 18.1] was presented at C-7 as confirmed by the HMBC correlation from H-7 (δH 6.39) to δC 175.2. The NOESY correlations of 6 revealed the same relative configuration as that of 5. The structure of 6 was established and named as usambariphane E.

2.1.7. Usambariphane F (7)

Compound 7 was obtained as a colorless crystal. The molecular formula was identified as C39H32O15 by HR-ESIMS m/z 761.3383 [M + H]+ (calcd. for C39H32O15 761.3379). The 1D (Table 3) and 2D NMR spectra of 7 revealed four OAc [δH 2.43 (3H), δC 170.1, 21.0; δH 2.12 (3H), δC 170.9, 22.5; δH 2.06 (3H), δC 172.1, 20.9; δH 1.70 (3H), δC 172.2, 20.4], one OMe [δH 3.75 (3H), δC 55.0] revealed the same relative configuration as that of 6. Comparison of the NMR data with those of known jatrophane-type diterpenoids [38,40]. The NOESY correlations of H-1/H-4, H-3/H-4, and H-4/H-7 indicated the trans-orientation of H-1, H-3, and H-7; meanwhile, the NOESY cross-peaks of H-5/15-OH, H-5/H-8, H-7 to C-6 and C-9, H-8 to C-6 and C-10, H-9 to C-8 and C-11, H-11 to C-10 and C-13, H-12 to C-10, H-14 to C-1, C-4, C-12, C-13, and C-15, H-12 to C-5, C-6, and C-7, H-13 and H-12 to C-9, C-10 and C-11, H-20 to C-12, C-13, and C-14. The presence of the 3-OH group was deduced by the 1H-1H COSY cross-peak between H-3 and 3-OH, and the HMBC correlations from 3-OH to C-2, C-3, and C-4. Another OH group was located at C-8 by the COSY cross-peak between H-8 and 8-OH, and the HMBC correlations from 8-OH to C-7 and C-8. The third OH group was connected to C-15 by the confirmation of the HMBC correlations from 15-OH to C-1, C-4, and C-15. The HMBC correlations of H-1/C-17 and H-5/C-16 (OAc), H-5/C-16 (OAc), H-7/C-17 and H-7/C-17 (OAc), and H-14/C-17 (OAc), demonstrated the locations of the acyl groups, and of necessity, the last OAc was located at C-2. The relative configuration of 7 was deduced by the NOESY spectrum. The H-4 and 15-OH in 7 can be assigned as α- and β-oriented, respectively, according to the comparison of the NMR data with those of known jatrophane-type diterpenoids [38,40].

The NOESY cross-peaks of H-1/4-H, H-3/4-H, and H-4/H-7 indicated the α-orientation of H-1, H-3, and H-7; meanwhile, the NOESY cross-peaks of H-5/15-OH, H-5/H-8, H-8/H-19, H-9/H-19, H-14/15-OH and H-14/H-20 indicated the β-orientation of H-5, H-8, H-9, H-14, and H-19, and H-20. Above all, the structure of 7 was established and named as usambariphane F.

2.1.8. Usambariphane G (8)

Compound 8 was obtained as a colorless crystal. The molecular formula was identified as C41H39O13N by HR-ESIMS m/z 764.3230 [M + H]+ (calcd. for C41H39O13N 764.3277), indicating 18 degrees of molecular unsaturation. The 1D (Table 3) and 2D NMR spectra of 8 revealed two OAc [δH 2.07 (3H), δC 169.7, 20.7; δH 2.00 (3H), δC 169.9, 20.8], an OMe [δH 3.76 (3H), δC 52.0], and two OH groups at C-3 and C-4, δC 157.8, 34.0 19.7, 18.4, one OMe [δH 3.76 (3H), δC 52.0] revealed the same relative configuration as that of 6. Comparison of the NMR data with those of known jatrophane-type diterpenoids [38,40]. The NOESY correlations of H-1/H-4, H-3/H-4, and H-4/H-7 indicated the trans-orientation of H-1, H-3, and H-7; meanwhile, the NOESY cross-peaks of H-5/15-OH, H-5/H-8, H-8/H-19, H-9/H-19, H-14/15-OH and H-14/H-20 indicated the β-orientation of H-5, H-8, H-9, H-14, and H-19, and H-20. Above all, the structure of 7 was established and named as usambariphane F.
Table 3. $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR data of compounds 7–9 in CDCl$_3$ (δ in ppm).

| Position | 7 | 8 | 9 |
|----------|---|---|---|
| δ$_H$, mult. (f in Hz) | δ$_C$ | δ$_H$, mult. (f in Hz) | δ$_C$ | δ$_H$, mult. (f in Hz) | δ$_C$ |
| 1 | 5.46, s | 79.8 | a: 2.95, d (15.5) | 51.4 | a: 2.83, d (16.5) | 52.0 |
| 2 | 90.6 | 4.67, dd (10.0, 4.5) | 79.1 | 5.48, d (4.0) | 84.7 |
| 3 | 4.36, dd (10.5, 5.5) | 78.0 | 5.18, s | 70.7 | 5.71, d (6.5) | 70.0 |
| 4 | 2.76, m | 41.4 | 3.32, m | 47.9 | 2.97, dd (4.0, 3.5) | 44.2 |
| 5 | 6.00, br s | 71.0 | 5.67, br s | 69.2 | 6.58, d (3.5) | 77.9 |
| 6 | 144.4 | 144.9 | 141.6 |
| 7 | 5.21, s | 68.8 | 5.41, br s | 68.5 | 5.27, s | 68.4 |
| 8 | 4.30, d (11.0) | 70.2 | 5.18, s | 70.7 | 5.71, d (6.5) | 70.0 |
| 9 | 4.79, s | 86.6 | 4.96, s | 80.6 | 4.96, d (6.5) | 78.4 |
| 10 | 40.1 | 41.1 | 40.1 |
| 11 | 5.93, d (16.5) | 134.0 | 5.87, d (15.5) | 137.6 | 5.50, d (16.0) | 134.7 |
| 12 | 5.76, d (16.5) | 130.9 | 5.57, dd (15.5, 9.5) | 129.6 | 5.79, d (16.0) | 134.1 |
| 13 | 2.76, m | 36.9 | 3.75 | 44.4 | 2.69, m | 36.9 |
| 14 | 4.78, s | 76.9 | 21.2 | 5.04, s | 80.4 |
| 15 | 84.8 | 89.0 | 84.8 |
| 16 | 1.55, s | 17.1 | 1.89, s | 20.9 | 1.75, s | 19.8 |
| 17 | a: 5.26, s, b: 3.10, s | 131.4 | a: 5.41, a: 5.16, s | 111.6 | a: 1.85, m; b: 1.73, m | 32.0 |
| 18 | 1.03, s | 27.6 | 0.91, s | 26.5 | 0.98, s | 26.4 |
| 19 | 1.40, s | 23.4 | 1.36, s | 23.2 | 1.04, s | 20.8 * |
| 20 | 1.06, d (7.0) | 23.9 | 1.24, d (6.5) | 19.6 | 1.11, d (7.0) | 22.4 |
| 21 | a: 3.21, m; b: 2.33, m | 28.1 | 173.5 |
| 22 | 2.04, s | 170.9, 22.5 | 2.19, s | 169.6, 22.9 |

Notes: 1-OAc: 2.24, s 170.1, 21.0, 2.19, s 169.6, 22.9
2-OAc: 2.12, s 170.9, 22.5
1-OH: 8.48, s 164.9, 153.4
2-OH: 8.79, d (5.0, 2.0) 151.5, 137.6
3: 3.36, d (10.5) 85.2, m; 7.39, m 127.5, 123.2
5: 8.00, m 165.4, 133.4 8.06, m 164.7, 133.4 8.07, m 168.3, 133.9
6: 7.56, m 130.1, 129.7 7.84, m 131.1, 130.0 131.5, 129.7
7: 7.84, m 128.7 7.44, m 128.7 7.46, m 128.6
8: 3.57, d (10.0) 3.57, d (10.0) 3.57, d (10.0) 3.57, d (10.0)
9: 2.14, s 171.0, 20.9
10: 1.97, s 172.1, 20.9
11: 1.20, s 172.2, 20.4
12: 4.34, s 172.0, 20.7

* Exchangeable.
2.1.9. Isoterracinolides C (9)

Compound 9 was obtained as a white amorphous powder with a molecular formula of C_{29}H_{35}O_{16} determined based on HR-ESIMS m/z 775.3172 [M + H]^{+} (calcd. for C_{29}H_{35}O_{16} 775.3172). The 1D (Table 3) and 2D NMR spectra of 9 revealed five OAc [δ\_H 2.36 (3H), δ\_C 172.0, 20.7; δ\_H 2.19 (3H), δ\_C 169.6, 22.9; δ\_H 2.16 (3H), δ\_C 170.4, 21.4; δ\_H 2.15 (3H), δ\_C 171.2, 21.7; δ\_H 2.14 (3H), δ\_C 171.0, 20.9], one OBz [δ\_H 8.07 (2H), 7.57, 7.46 (2H), δ\_C 168.3, 133.9, 130.1 (2C), 128.8 (2C), 128.6], five methyls [δ\_H 2.11 (3H), 1.20 (3H), 1.19 (3H), 1.17 (3H), 1.16 (3H), δ\_C 26.4, CH\_3-18; δ\_H 1.04 (3H), δ\_C 20.8, CH\_3-19; δ\_H 1.11 (3H), δ\_C 22.4, CH\_3-20], and a trans-disubstituted C=C (δ\_C 5.50, δ\_C 134.7, CH-11; δ\_H 5.79, δ\_C 134.1, CH-12), and a lactone carbonyl carbon (δ\_C 173.5, C-22). Further, comparing the NMR data of 9 with those of isoterracinolide A (10) [36] indicated that the structure of 9 is very similarly to 10, except for the OiBu which was replaced in 10 by an OAc. The HMBC correlation from H-7 to δ\_C 171.0 suggested that the OAc was located at C-7 in 9. Compound 9 was thus established and named as isoterracinolides C.

2.1.10. 4β-Crotignoid K (14)

Compound 14 was obtained as a white amorphous powder. The molecular formula was determined as C_{29}H_{35}O_{17} by HR-ESIMS m/z 495.2385 [M + H]^{+} (calcd. for C_{29}H_{35}O_{17} 495.2377). The 1D (Tables 4 and 5) and 2D NMR of 14 revealed one OAc [δ\_H 2.14 (3H), δ\_C 173.9, 21.3], one OBz [δ\_H 8.02 (2H), 7.59, 7.47 (2H), δ\_C 166.4, 133.4, 130.1, 129.9 (2C), 128.7 (2C)], four methyls [δ\_H 1.21 (3H), δ\_C 23.9, CH\_3-16; δ\_H 1.33 (3H), δ\_C 17.1, CH\_3-17; δ\_H 0.98 (3H), δ\_C 15.3, CH\_3-18; δ\_H 1.73 (3H), δ\_C 10.3, CH\_3-19], an oxygenated methylene [δ\_H 4.05 (2H), δ\_C 67.6, CH\_2-20], an oxygenated methine (δ\_H 5.68, δ\_C 77.8, CH-12), two unsaturated methines (δ\_H 7.57, δ\_C 159.7, CH-1; δ\_H 5.56, δ\_C 126.6, CH-7), and a ketone unit (δ\_C 208.7, C-3). The interpretation of HMBC correlations suggested the skeleton of 14 was a tigiane-type diterpenoid [7] with an α-methyl-α,β-unsaturated cyclopentanone ring fused between C-4 and C-10, an OH (δ\_H 5.62) connected to C-9, the OBz connected to C-12, the OAc connected to C-13 and a hydroxymethyl linked to C-6. Moreover, according to the NOESY correlations of H-4/H-8/H-11/H-3-17 and H-12/H-14/9-OH/H\_3-18, as well as comparing the 1D NMR data of 14 with those of crotignoid K (13) [42] and 4-deoxyphorbole 12, 13-bis(isobutyrate) [43]. The structure of 14 was established as a 4β proton against the 4α proton of crotignoid K, thus named as 4β-crotignoid K.

2.1.11. Euphodendriane B (15)

Compound 15 was obtained as a white amorphous powder. The molecular formula was determined as C_{29}H_{35}O_{17} by HR-ESIMS m/z 495.2396 [M + H]^{+} (calcd. for C_{29}H_{35}O_{17} 495.2377). The 1D (Tables 4 and 5) and 2D NMR of 15 revealed one OAc [δ\_H 2.11 (3H), δ\_C 174.1, 21.2], one OBz [δ\_H 8.06 (2H), 7.61, 7.49 (2H), δ\_C 166.4, 133.4, 130.1, 129.9 (2C), 128.7 (2C)], five methyls [δ\_H 1.20 (3H), δ\_C 24.3, CH\_3-16; δ\_H 1.33 (3H), δ\_C 16.7, CH\_3-17; δ\_H 1.16 (3H), δ\_C 11.9, CH\_3-18; δ\_H 1.83 (3H), δ\_C 10.6, CH\_3-19; δ\_H 1.90 (3H), δ\_C 27.2, CH\_3-20], two oxygenated methines (δ\_H 4.46, δ\_C 71.1, CH-5; δ\_H 5.73, δ\_C 75.7, CH-12), two unsaturated methines (δ\_H 7.06, δ\_C 154.6, CH-1; δ\_H 4.88 δ\_C 125.5, CH-7), and a ketone unit (δ\_C 207.5, C-3). The interpretation of HMBC correlations demonstrated that 15 was a tigiane-type diterpenoid with an α-methyl-α,β-unsaturated cyclopentanone ring fused between C-4 and C-10, with two OH (δ\_H 5.92 and 5.95) connected to C-5 and C-9 respectively, one OBz connected to C-12, and one OAc connected to C-13 and 20-methyl group. The NMR data of 15 was highly close to those of euphodendriane A [35], except for the substitution at C-13 where an OiBu in euphodendriane A was replaced in 15 by the OAc. The relative configuration of 15 was deduced by inspection of the NOESY spectrum, showing the same orientations to euphodendriane A [35]. Thus, the structure of 15 was established and named as euphodendriane B.
2.1.12. 16-Nor-abieta-8,11,13-trien-3,7,15-trione (16)

Compound 16 was obtained as a colorless crystal with a molecular formula of C_{19}H_{22}O_{3} identified by HR-ESIMS m/z 299.1648 [M + H]^+ (calcd. for C_{19}H_{22}O_{3} 299.1642). The 1D (Tables 4 and 5) and 2D NMR data of 16 revealed an acetyl moiety (δ_C 197.3, C-15; δ_H 2.64 (3H), δ_C 26.9, CH-17), three methyls [δ_H 1.17 (3H), δ_C 25.2, CH-18; δ_H 1.23 (3H), δ_C 21.7, CH-19; δ_H 1.48 (3H), δ_C 22.8, CH-20], three methylenes [δ_H 2.68, 2.05, δ_C 36.8, CH-2; δ_H 2.91, 2.59, δ_C 34.6, CH-2; δ_H 2.83, 2.75, δ_C 36.5, CH-2], a methine (δ_H 2.36, δ_C 49.2, CH-5), a set of trisubstituted aromatic ring (δ_C 130.8, C-8; δ_C 158.2, C-9; δ_H 7.49 (d, J = 8.5), δ_C 125.2, CH-11; δ_H 8.17 (dd, J = 8.5, 2.5), δ_C 133.4, CH-12; δ_C 135.9, C-13; δ_H 8.57 (d, J = 2.5), δ_C 128.3, CH-14), two ketone units (δ_C 214.0, C-3; δ_C 197.4, C-7), and two quaternary carbons (δ_C 47.6, C-4; δ_C 38.3, C-10). The HMBC correlations of 16 from H-2 to C-3, C-9, and C-20, H-2 to C-3 and C-4, H-5 to C-1, C-4, C-9, and C-10, H-6 to C-7, C-8, and C-10, H-12 to C-15, H-14 to C-7 and C-15, H-17 to C-13, H-18 and H-19 to C-3, C-4, and C-5, and H-20 to C-5 and C-10, suggested that 16 was an abietane-type diterpenoid [7] and was structurally similar to a known compound abieta-8,11,13-triene-3,7-dione [44,45], except for the substitution of the acetyl moiety at C-15–C-17. The relative configuration of 16 was the same as the typical abieta-8,11,13-triene diterpenoids [45] based on the NOESY correlations of H-5/H-3 and H-3/H-20 as well as the comparison of the NMR data of 16 with those of literature [44,45]. The structure of 16 was identified as 16-nor-abieta-8,11,13-trien-3,7,15-trione.

Table 4. 1H NMR data of compounds 14–17, 19, and 20 in CDCl3 at 500 MHz (δ_H in ppm, mult. J in Hz).

| Position | 14   | 15   | 16   | 17   | 19   | 20   |
|----------|------|------|------|------|------|------|
| 1        | 7.57, s |       | a: 2.68, m | a: 2.42, m | a: 2.05, m | a: 1.98, m |
| 2        |       | 7.06, br s | b: 2.05, m | b: 1.73, m | b: 1.75, m | b: 1.24, m |
| 3        |       |       | a: 2.91, m | a: 1.92, m | a: 2.65, ddd (15.4, 14.0, 6.0) | a: 1.73, m |
| 4        | 2.52, m | 3.13, dd (6.5, 4.5) | b: 2.59, ddd (15.5, 5.5, 3.0) | b: 1.87, m | b: 2.37, ddd (15.5, 4.8, 3.2) | b: 1.62, m |
| 5        | a: 2.87, ddd (18.5, 9.5) | 4.46, dd (11.5, 4.5) | 2.36, dd (14.0, 3.5) | 1.88, m | 1.67, m | 1.05, m |
| 6        | b: 2.19, ddd (18.5, 4.0) |       | a: 2.83, dd (17.5, 14.0) | 2.79, dd (18.0, 13.5) | a: 1.79, m | a: 1.79, m |
| 7        | 5.56, m | 4.88, br s |       | a: 2.17, m | a: 1.98, m | a: 1.98, m |
| 8        | 2.46, t (5.5) | 2.06, m |       | a: 2.16, m | a: 1.66, m | a: 1.66, m |
| 9        | 3.28, m | 3.65, m |       | 2.70, d (5.0) | 1.95, m |       |
| 10       | 1.75, m | 1.86, dd (10.5, 6.5) | 7.49, d (8.5) | 7.47, d (8.0) | 5.44, d (5.0) | a: 2.27, dd (13.5, 5.5) |
| 11       | 5.68, d (10.0) | 5.73, d (10.5) | 8.17, dd (8.5, 2.5) | 8.14, dd (8.0, 2.0) |       | b: 1.41, m |
| 12       | 1.14, d (5.5) | 0.89, d (6.5) | 8.57, d (2.5) | 8.55, d (2.0) | 3.76, br s | 3.77, s |
| 13       | 1.21, s | 1.20, s |       |       |       |       |
| 14       | 1.33, s | 1.33, s | 2.64, s | 2.63, s | 2.09, s | 1.97, d (2.0) |
| 15       | 0.98, d (6.5) | 1.16, d (6.5) | 1.17, s | 0.99, s | 1.17, s | 1.06, s |
| 16       | 1.73, dd (2.5, 1.0) | 1.83, br s | 1.23, s | 1.08, s | 1.09, s | 0.88, s |
| 17       | 4.05, m | 1.90, s | 1.48, s | 1.27, s | 0.95, s | 1.08, s |
| 18       | 5-OH | 5.92, d (11.5) |       |       |       |       |
| 19       | 5.62, s | 5.95, s |       |       |       |       |
| 20       | 8.02, m | 8.06, m |       |       |       |       |
| 21       | 7.59, m | 7.61, m |       |       |       |       |
| 22       | 7.47, m | 7.49, m |       |       |       |       |
| 23       | 2.14, s | 2.11, s |       |       |       |       |
2.1.13. 16-Nor-3β-hydroxy-abieta-8,11,13-trien-7,15-dione (17)

Compound 17 was obtained as a colorless crystal with a molecular formula of C_{19}H_{24}O_3 identified by HR-ESIMS m/z 301.1803 [M + H]^+ (calcld. for C_{19}H_{25}O_3 301.1798). The inspection of 1D (Tables 4 and 5) and 2D NMR data revealed that 17 was a 16-nor-abieta-8,11,13-triene diterpenoid [45]. The 1H and 13C NMR data of 17 was close to those of 16, except for a hydroxy group that was situated at C-3 (δ_C 78.0) by the confirmation of the 1H-1H COSY cross-peaks between H-2/H-12/H-5/H-3 as well as the HMBC correlations from H-21 to C-3 and H-22 to C-3. The H-3 [δ_H 3.37 (dd, j = 11.5, 4.0)] was identified as α-oriented by the NOESY correlations of H-3/H-5/H-18 and the comparison of the proton signals for 17 with those of the similar compound 3β-hydroxy-abieta-8,11,13-trien-7-one [46]. The structure of 17 was identified as 16-nor-3β-hydroxy-abieta-8,11,13-trien-7,15-dione.

2.1.14. ent-8β,14β-Epoxyabieta-3-one-11,13(15)-dien-16,12-olide (19)

Compound 19 was purified as a colorless gum. The molecular formula was calculated as C_{20}H_{34}O_4 by HR-ESIMS m/z 329.1753 [M + H]^+ (calcld. for C_{20}H_{25}O_4 329.1747). The 1D (Tables 4 and 5) and 2D NMR data of 19 revealed four methyls [δ_H 2.09 (3H), δ_C 9.0, CH_3-17; δ_H 1.17 (3H), δ_C 25.9, CH_3-18; δ_H 1.09 (3H), δ_C 22.4, CH_3-19; δ_H 0.95 (3H), δ_C 15.0, CH_3-20], four methylenes [δ_C 2.05, 1.75, 1.53, 1.75, CH_2=1; δ_C 2.65, 2.37, 3.42, CH_2=2; δ_C 1.79, 1.70, 21.7, CH_2=6; δ_H 2.17, 1.68, 33.9, 21.7, CH_2=6], four methine (δ_C 1.67, 54.1, CH_5; δ_C 2.70, 50.1, CH_9; δ_C 5.44, 102.9, CH-11; δ_C 3.76, 54.5, CH-14), six quaternary carbons (δ_C 48.1, C-4; δ_C 61.0, C-8; δ_C 40.9, C-10; δ_C 148.1, C-12; δ_C 144.7, C-13; δ_C 126.2, C-15), and two carbonyl carbons (δ_C 215.0, C-3; δ_C 175.0, C-16). The structure of 19 was suggested an abietane-type diterpenoid with a ketone carbon at C-3, an epoxy ring fused at C-8 and C-14, a double bond at A_{11,12}, and an α-methyl-α,β-unsaturated δ-lactone ring formed as D ring according to the analysis of the COSY cross-peaks of H_2-11/H_2-2, H_2-5/H_2-6/H_2-7, and H_9/H_11, as well as the HMBC correlations from H_2-1 to C_3, C_5, and C_10, H_2-2 to C_3 and C_10, H_5 to C_4, C_18, C_19, and C_20, H_2-7 to C_5 and C_8, H_9 to C_1, C_5, C_8, C_12, C_14, and C_20, H_11 to C_8, C_9, C_12, and C_13, H_14 to C_7, C_8, C_12, and C_13, H_3-17 to C_13, C_15, and C_16, H_3-18 and H_3-19 to C_3 and C_4.

| Position | 14  | 15  | 16  | 17  | 19  | 20  |
|----------|-----|-----|-----|-----|-----|-----|
| 1        | 159.7         | 154.6 | 36.8 | 35.9 | 38.1 | 38.7 |
| 2        | 136.7         | 144.3 | 34.6 | 27.5 | 34.2 | 27.3 |
| 3        | 208.7         | 207.5 | 214.0 | 78.0 | 215.0 | 78.6 |
| 4        | 44.4          | 56.3  | 47.6  | 39.1  | 48.1  | 39.0  |
| 5        | 29.8          | 71.1  | 49.2  | 48.3  | 54.1  | 53.6  |
| 6        | 142.3         | 138.0 | 36.5  | 36.1  | 21.7  | 20.8  |
| 7        | 126.6         | 125.5 | 197.4  | 198.5  | 33.9  | 34.8  |
| 8        | 42.3          | 40.2  | 130.8  | 130.8  | 61.0  | 61.0  |
| 9        | 78.0          | 78.7  | 158.2  | 159.9  | 50.1  | 49.2  |
| 10       | 54.3          | 48.0  | 38.3  | 38.5  | 40.9  | 39.2  |
| 11       | 42.8          | 43.5  | 125.2  | 124.8  | 102.9 | 24.0  |
| 12       | 77.8          | 75.7  | 133.4  | 133.2  | 148.1 | 75.6  |
| 13       | 65.5          | 65.4  | 135.9  | 135.5  | 144.7 | 155.6 |
| 14       | 36.0          | 38.5  | 128.3  | 128.1  | 54.5  | 56.2  |
| 15       | 26.0          | 25.1  | 197.3 a | 197.4 a | 126.2 | 128.9 |
| 16       | 23.9          | 24.3  |         |         | 170.5 | 174.1 |
| 17       | 17.1          | 16.7  | 26.9  | 26.8  | 9.0   | 8.9   |
| 18       | 15.3          | 11.9  | 25.2  | 27.6  | 25.9  | 29.1  |
| 19       | 10.3          | 10.6  | 21.7  | 15.2  | 22.4  | 16.1  |
| 20       | 67.6          | 27.2  | 22.8  | 23.3  | 15.0  | 19.3  |
| 12-OBz   | 133.4         | 133.4 |       |       |       |       |
| 13-OAc   | 21.3          | 21.2  |       |       |       |       |

a: Exchangeable.
together with H3-20 to C-1, C-9, and C-10. Comparison of the $^{13}$C NMR data with those of the related compounds jolkinolide A [47] and gelomulide C [48] further evidenced the presence of an ent-abieta skeleton with $8\beta, 14\beta$-epoxide in 19. Thus, 19 was established as ent-$8\beta, 14\beta$-epoxyabieta-3-one-11,13(15)-dien-16,12-olide.

2.1.15. ent-$8\beta, 14\beta$-Epoxyabieta-$3\alpha$-hydroxy-$13(15)$-en-16,12-olide (20)

Compound 20 was purified as a colorless gum. The molecular formula was calculated as C$_{20}$H$_{28}$O$_{4}$ by HR-ESIMS $m/z$ 333.2067 [M + H]$^+$ (calcd. for C$_{20}$H$_{29}$O$_{4}$ 333.2060). The $^1$H and $^{13}$C NMR data (Tables 4 and 5) of 20 were close to those of 19 suggesting that the skeleton of 20 was an ent-abieta with $8\beta, 14\beta$-epoxide. Instead of the ketone carbon at C-3 in 19, a hydroxyl group was connected to C-3 ($\delta$ C 78.6) based on the inspection of the $^1$H–$^1$H COSY cross-peaks between H$_2$-1/H$_2$-2/H-3 together with the HMBC correlations from H-3 to C-1, C-4, C-18, and C-19. Saturated methylene ($\delta$ H 2.27, 1.41, $\delta$ C 24.0) and an oxygenated methine ($\delta$ H 4.99, $\delta$ C 75.6) were assigned to be at C-11 and C-12, respectively, according to the analysis of the $^1$H–$^1$H COSY cross-peaks between H$_2$-11/H-12 together with the HMBC correlations from H$_2$-11 to C-8, C-10, and C-13, as well as H-12 to C-13 and C-15. The structure was found to be highly similar to the NMR features of gelomulide A [48], except for instead of the hydroxyl group at C-3 in 20. The configuration of 3-OH was deduced to be $\alpha$-oriented as the proton signal of H-3 at 3.30 (dd, $J = 12.0, 4.0$ Hz) [49,50]. It was also supported by the NOESY correlations of H-3/H-5/H-9/H$_3$-18. The structure of 20 was established as ent-$8\beta, 14\beta$-epoxyabieta-$3\alpha$-hydroxy-$13(15)$-en-16,12-olide.

2.2. HIV-1 Latency Reversal Activity of Isolated Compounds in Vitro

Jurkat cells with a full-length integrated HIV-1 provirus that have been modified to contain a GFP coding region in place of the env gene (J-lat 10.6 cells) were used for HIV-1 anti-latency activity, cytotoxicity, and cellular activation testing. All 31 compounds were tested at 1, 10, and 100 $\mu$M. Through the GFP expression of J-lat 10.6 cells, it was determined that compounds 12, 13, 14, 21, 26, and 27 showed HIV-1 latency reversal activity (Figure 4). These compounds were further tested at additional concentrations to determine dose response and toxicity curves (Figure 5A–F). Cell viability for all isolated compounds is presented in Figure S106 in the supplementary material.

4$\beta$-Crotignoid K (14) showed high reactivation levels into nM concentrations, ~250-fold less than crotignoid K (13), which is a stereoisomer of 14, differing only in the configuration on C-4 (Figure 6). The striking difference between these compounds isolated from E. usambarica demonstrated a structure-activity relationship (SAR) of an important cellular trigger to induce HIV-1 proviral transcription. A similar SAR has recently been described between protein kinase C (PKC) agonists, 4-deoxyphorbol (4$\beta$-dPEA), phorbol myristate acetate (PMA), and prostratin [63].
Figure 4. HIV-1 latency reversal activity of compounds 1–31 on J-Lat 10.6 cells in vitro.

Figure 5. HIV-1 latency reversal activity dose-response (green bar) and cytotoxicity (red curve) of active compounds 12–14, 21, 26, and 27 at additional concentrations. (A) dose-response experiments of 12 in a series concentration from 0.468 to 20 μM; (B) dose-response experiments of 13 in a series concentration from 0.158 to 160 μM; (C) dose-response experiments of 14 in a series concentration from 0.0025 to 320 μM; (D) dose-response experiments of 21 in a series concentration from 0.156 to 320 μM; (E) dose-response experiments of 26 in a series concentration from 1.88 to 320 μM; (F) dose-response experiments of 27 in a series concentration from 0.625 to 320 μM.
Euphorbia species have been shown to be enriched for compounds capable of protein kinase C (PKC) activation in human cells [5,9,11,14,15]. In order to determine whether our active compounds were acting through PKC, we evaluated the latency reversal activity of each of these compounds in the presence and absence of a pan-PKC inhibitor, Gö6983 (Figure 7). Compounds 12, 13, 14, and 21 all showed reduced efficacy, indicating likely activation of PKC as their primary mechanism of action. In contrast, compounds 26 and 27 did not show a significant reduction in their activity when PKC was inhibited. In addition, these compounds did not increase CD69 expression (a hallmark of PKC activation), further suggesting an alternative (non-PKC) mechanism of latency reversal.

3. Discussion

In this study, 15 new diterpenoids, together with 16 known compounds, were isolated from the dichloromethane phase of methanolic extract of the medicinal plant E. usambarica. Compound 1 exhibited a 6/6/3-fused ring system with an α-methyl-α,β-unsaturated cyclopentanone moiety to construct a rare diterpenoid lactone, this skeleton was the second time discovered from nature [34]. The other compounds could be summarized in 4 types of
diterpenoids, including jatrophanes (2–11), tiglianes (12–15), abietanes (16–23), and kaurane (24), alone with coumarinolignoid (25), lignan (26), coniferyl acetate (27), and benzenoids (28–31). Especially, usambariphanes A (2) and B (3) displayed an unusual lactone ring constructed between C-17 and C-2 in the jatrophane structure, which is different from such lactone ring commonly constructed between C-17 and C-3 or between C-17 and C-5.

Furthermore, compounds 12–14, 21, 26, and 27 showed significant HIV-1 latency reversal activity demonstrated by the GFP expression of J-lat 10.6 cells. 4β-Crotignoid K (14) showed the reactivation of HIV-1 latency at a very low concentration of EC50 about 0.015 μM and a higher CC50 concentration than 160 μM. The stereoisomer, crotignoid K (13), showed the EC50 and CC50 concentrations about 3.75 and 40 μM, respectively, indicating that 4β-crotignoid K (14) was provided with higher safety and efficacy. There is a 250-fold difference in EC50 and ~1000-fold difference in selectivity index (CC50/EC50) between these compounds. The structural difference between 13 and 14 is only in the relative configuration on C-4. However, they demonstrated dramatically different biological activity, indicating that the configuration on C-4 of tigliane-type diterpenoids is critical to HIV-1 latency reversal activity and likely reflects improved PKC activation. The primary mechanism of the active compounds 12–14 and 21 for the HIV-1 latency reversal activity was activation of PKC.

Currently, LRAs are still under investigation and have not been approved by the US Food and Drug Administration (US-FDA). Therefore, the intensive study of LRAs is an important topic, especially to discover new candidates from natural sources. For example, a known LRA, ingenol, is isolated originally from Euphorbia peplus [64] and is a US-FDA-approved topical treatment for actinic keratosis (AK) [65], showed a significant effect in the reactivation of HIV-1 latency through the PKC pathway [65]. Both compound 14 and ingenol mebutate are Euphorbia diterpenoids and the potent PKC agonists. In addition, compound 14 presented the lower cytotoxicity, indicating 14 is a promising candidate for the development of an LRA.

In contrast, (+)-syringaresinol (26) and dimeric coniferyl acetate (27) did not increase CD69 expression, further suggesting a non-PKC mechanism of latency reversal that merits further exploration.

4. Materials and Methods
4.1. General Experimental Procedures

Optical rotation was performed on a Perkin-Elmer 341 polarimeter. 1D and 2D NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer at 500 MHz (1H) and 125 MHz (13C). Chemical shifts were reported in parts per million (δ), and the coupling constants (J) were expressed in Hertz. The residual peaks of the deuterated solvents were taken as reference points. The NMR data were acquired and processed with MestReNova v12.0.0–20080 software. High-resolution MS spectra were acquired on an FTRMS-Orbitrap (Thermo-Finnigan) mass spectrometer equipped with an ESI ion source (Thermo-Finnigan) mass spectrometer equipped with an ESI ion source. HPLC analyses were performed with a Shimadzu LC-10AS pump interface equipped with a Shimadzu SPD-10A UV–VIS detector (Shimadzu Inc., Kyoto, Japan) using a Phenyl-Hexyl column (5 μm, 100 Å, 250 × 4.6 mm), Phenyl-Hexyl column (5 μm, 100 Å, 250 × 4.6 mm), Phenyl-Hexyl column (5 μm, 100 Å, 250 × 4.6 mm), Phenyl-Hexyl column (5 μm, 100 Å, 250 × 4.6 mm), and/or Luna® Phenyl-Hexyl column (5 μm, 250 × 10 mm) (Phenomenex Inc., Torrance, CA, USA) using a mixture of acetonitrile–H2O or mixture of methanol–H2O as mobile phase. Rotational planar chromatography (RPC) was performed on self-coated silica plates (Kieselgel 60 GF254, 15 μm, Merck, Germany) using a Chromatotron apparatus (Harrison Research, Palo Alto, CA, USA). Silica gel (Kieselgel 60, 63–200 μm, Merck, Darmstadt, Germany), polyamide (MP Polyamide, 50–160 μm, MP Biomedicals, Irvine, CA, USA), and Sephadex LH-20 gel (Pharmacia Fine Chemicals AB, Uppsal, Sweden) were used for column chromatography (CC). Thin-layer chromatography (TLC) was carried out using silica gel (Kieselgel 60 F254, Merck) and RP-C18 (F254s, Merck) pre-coated plates, and the preparative TLC (pre-TLC) was performed on glass sheet silica gel pre-coated plates.
(20 × 20 cm, Kieselgel 60 F254, Merck). The compounds were detected with a developer (20% H2SO4 (v/v) with 5% vanillin (w/v) in ethanol) followed by heating (120 °C).

4.2. Plant Material

Euphorbia usambarica Pax. (Euphorbiaceae) was collected in Taita Taveta county, Kenya in 2019. Identification was performed by Peter Waweru Mwangi (Department of Medical Physiology, School of Medicine, University of Nairobi, Nairobi, Kenya). A voucher specimen (No. EU-001) has been deposited in the Herbarium of the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

4.3. Extraction and Isolation

The dried stem and root part (2.7 kg) were chopped and extracted with methanol (MeOH, 15 L) at room temperature. After removing the solvent, the crude methanolic extract (EU, 220.0 g) was dissolved in 50% MeOHaq and subjected to liquid–liquid partition to afford n-hexane (EU-H), dichloromethane (CH2Cl2, EU-C), ethyl acetate (EtOAc, EU-E), and water-soluble residue (EU-W) phases. The EU-C (25.7 g) was subjected to polyamide CC with MeOH–H2O mixture solvent system (40%, 60%, 80%, and 100% MeOHaq; EU-C-P1–P4). The EU-C-P1 (8.7 g) was further subjected to normal phase CC (silica gel, 63–200 μm) with a gradient solvent system of n-hexane–EtOAc–MeOH mixtures (from 40:5:1 to 0:8:1) to obtain ten subfractions (EU-C-P1-1–10) based on the TLC monitoring. EU-C-P1-2 (29.7 mg) was subjected to Sephadex LH-20 CC with the eluent of CH2Cl2–EtOAc–MeOH (1:1:6) to yield 6 subfractions (EU-C-P1-2/1–6), and EU-C-P1-2/2 was further separated by RP-HPLC on Kinetex XB-C18 column with an isocratic solvent system of MeCN–H2O (60:40, 2.0 mL/min) to yield compound 18 (1.1 mg). EU-C-P1-3 (895.5 mg) was separated by Sephadex LH-20 CC using CH2Cl2–EtOAc–MeOH (1:1:6) as eluent to obtain 5 subfractions (EU-C-P1-3/1–5). EU-C-P1-3/2 (245.5 mg) was further subjected to RPC (thickness 2 mm) using a gradient system of CH2Cl2–MeOH (from 100:0 to 15:1) to obtain 5 subfractions (EU-C-P1-3/2/1–5). Compound 21 (63.5 mg) was purified by recrystallization (MeOH) from EU-C-P1-3/2/1 (133.8 mg), and the residue of this fraction was further purified by RP-HPLC on Kinetex XB-C18 column with an isocratic system of MeCN–H2O (53:47, 2.0 mL/min) to yield 6 subfractions (EU-C-P1-3/2/1–6) and EU-C-P1-3/2/2 (38.6 mg) was subjected to prep-TLC using CH2Cl2–MeOH (60:1) as eluent to obtain 5 subfractions (EU-C-P1-3/2/2/1–5), then the second and third subfractions were purified by RP-HPLC on Kinetex XB-C18 column with an isocratic system of MeCN–H2O (65:35, 2.0 mL/min) to yield compounds 10 (4.7 mg) and 15 (1.0 mg), respectively. EU-C-P1-3/3 (178.5 mg) was subjected to RPC (thickness 2 mm) using a gradient system of n-hexane–CH2Cl2–MeOH (from 5:1:0 to 20:1) to obtain 9 subfractions (EU-C-P1-3/3/1–9), then the second subfraction (23.0 mg) was purified by RP-HPLC on Kinetex Biphenyl column with an isocratic system of MeOH–H2O (75:25, 1.0 mL/min) to yield compound 24 (2.7 mg). EU-C-P1-3/4 (39.3 mg) was separated by prep-TLC using CH2Cl2–MeOH (60:1) as eluent to yield compound 28 (11.0 mg). EU-C-P1-3/5 (10.2 mg) was purified by prep-TLC using CH2Cl2–MeOH (35:1) as eluent to yield compound 29 (1.8 mg). EU-C-P1-4 (1060.4 mg) was subjected to Sephadex LH-20 gel chromatography eluting with CH2Cl2–EtOAc–MeOH (1:1:6) to obtain 9 subfractions (EU-C-P1-4/1–9). EU-C-P1-4/2 (442.8 mg) was further separated by RPC (thickness 2 mm) using a gradient system of n-hexane–CH2Cl2–MeOH (from 1:1:0 to 10:1) to obtain 8 subfractions (EU-C-P1-4/2/1–9). EU-C-P1-4/2/2 (14.1 mg) was further purified by RP-HPLC on Kinetex XB-C18 column with an isocratic solvent system of MeCN–H2O (50:50, 2.0 mL/min) to yield compound 27 (4.6 mg). EU-C-P1-4/2/4 (68.6 mg) was further purified by RP-HPLC on Kinetex XB-C18 column with an isocratic solvent system of MeCN–H2O (55:45, 2.0 mL/min) to yield compounds 1 (1.1 mg), 2 (4.0 mg), 3 (1.5 mg), 4 (2.0 mg), 5 (5.2 mg), 6 (2.5 mg), 7 (3.2 mg), 8 (1.4 mg), 9 (1.7 mg), 11 (3.7 mg), and 22 (2.2 mg). EU-C-P1-4/2/6 (60.4 mg) was purified by RP-HPLC on Kinetex XB-C18 column with an isocratic solvent system of MeCN–H2O (50:50, 2.0 mL/min) to yield compounds 12 (2.3 mg) and 13 (4.2 mg). EU-C-P1-4/2/7 (40.9 mg) was purified by RP-HPLC on
Luna® Phenyl-Hexyl column with an isocratic system of MeCN–H₂O (51:49, 2.0 mL/min) to yield compound 14 (1.4 mg). EU-C-P1-4/4 (218.3 mg) was further separated by RPC (thickness 2 mm) using a gradient system of CH₂Cl₂–MeOH (from 100:0 to 20:1) to obtain 6 subfractions (EU-C-P1-4/4/1–6). EU-C-P1-4/4/2 (52.4 mg) was further chromatographed by Sephadex LH-20 CC eluting with CH₂Cl₂–EtOAc–MeOH (1:1:6) to obtain 3 subfractions, then the second subfraction was purified by RP-HPLC on Kinetex XB-C18 column with an isocratic solvent system of MeCN–H₂O (53:47, 2.0 mL/min) to yield compounds 17 (2.8 mg), 20 (2.7 mg), and 23 (14.2 mg). EU-C-P1-4/8 (11.6 mg) was purified by RP-HPLC on Kinetex XB-C18 column with an isocratic solvent system of MeCN–H₂O (35:65, 2.0 mL/min) to yield compounds 30 (1.3 mg) and 31 (1.6 mg). EU-C-P1-7 (480.1 mg) was subjected to Sephadex LH-20 CC eluting with CH₂Cl₂–EtOAc–MeOH (1:1:6) to obtain 8 subfractions (EU-C-P1-7/1–8). EU-C-P1-7/6 was further purified by RP-HPLC on Kinetex XB-C18 column with an isocratic system of MeOH–H₂O (48:52, 2.0 mL/min) to yield compound 26 (2.6 mg). Compound 25 (8.3 mg) was yielded by re-crystallization (MeOH) from EU-C-P1-7/7.

### 4.4. Physical Characteristic of New Compounds

**Euphordraculoate C (1):** Colorless gum; [α]°D +63 (c 0.05, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Tables 1 and 2; HR-ESIMS m/z 493.2237 [M + H]⁺ (calcd. for C₂₉H₃₅O₂ 493.2221), m/z 515.2046 [M + Na]⁺ (calcd. for C₂₉H₃₅O₂Na 515.2040).

**Usambaricinophane A (2):** White amorphous powder; [α]°D −17 (c 0.20, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Tables 1 and 2; HR-ESIMS m/z 789.3327 [M + H]⁺ (calcd. for C₄₀H₃₅O₁₆ 789.3328), m/z 811.3169 [M + Na]⁺ (calcd. for C₄₀H₃₅O₁₆Na 811.3148).

**Usambaricinophane B (3):** White amorphous powder; [α]°D −7 (c 0.07, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Tables 1 and 2; HR-ESIMS m/z 803.3488 [M + H]⁺ (calcd. for C₄₁H₃₇O₁₆ 803.3485), m/z 825.3333 [M + Na]⁺ (calcd. for C₄₁H₄₃O₁₆Na 825.3304).

**Usambaricinophane C (4):** colorless crystal; [α]°D +34 (c 0.10, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Tables 1 and 2; HR-ESIMS m/z 789.3346 [M + H]⁺ (calcd. for C₄₀H₃₅O₁₆ 789.3328) m/z 811.3171 [M + Na]⁺ (calcd. for C₄₀H₄₀O₁₆Na 811.3148).

**Usambaricinophane D (5):** Colorless crystal; [α]°D +54 (c 0.30, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Tables 1 and 2; HR-ESIMS m/z 787.3193 [M + H]⁺ (calcd. for C₄₀H₃₅O₁₆ 787.3172), m/z 809.3011 [M + Na]⁺ (calcd. for C₄₀H₃₇O₁₆Na 809.2991).

**Usambaricinophane E (6):** Colorless crystal; [α]°D +54 (c 0.15, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Tables 1 and 2; HR-ESIMS m/z 801.3356 [M + H]⁺ (calcd. for C₴₁H₃₇O₁₆ 801.3348), m/z 823.3170 [M + Na]⁺ (calcd. for C₄₁H₄₃O₁₆Na 823.3148).

**Usambaricinophane F (7):** Colorless crystal; [α]°D +15 (c 0.20, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Table 3; HR-ESIMS m/z 761.3383 [M + H]⁺ (calcd. for C₃₉H₃₅O₁₅ 761.3379), m/z 783.3215 [M + Na]⁺ (calcd. for C₃₉H₃₇O₁₆Na 783.3198).

**Usambaricinophane G (8):** Colorless crystal; [α]°D +15 (c 0.08, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Table 3; HR-ESIMS m/z 764.3230 [M + H]⁺ (calcd. for C₄₁H₃₇O₁₃Na 764.3277), m/z 786.3098 [M + Na]⁺ (calcd. for C₄₁H₄₃O₁₃NaNa 786.3096).

**Isoterracinalide C (9):** White amorphous powder; [α]°D −2 (c 0.10, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Table 3; HR-ESIMS m/z 775.3172 [M + H]⁺ (calcd. for C₃₉H₃₅O₁₅Na 775.3172), m/z 797.3011 [M + Na]⁺ (calcd. for C₃₉H₃₇O₁₆NaNa 797.2991).

**4β-Crotignoid K (14):** White amorphous powder; [α]°D +48 (c 0.05, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Tables 4 and 5; HR-ESIMS m/z 495.2385 [M + H]⁺ (calcd. for C₂₉H₂₅O₇ 495.2377), m/z 517.2202 [M + Na]⁺ (calcd. for C₂₉H₂₆O₇Na 517.2197).

**Euphodrantrione B (15):** White amorphous powder; [α]°D +12 (c 0.03, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Tables 4 and 5; HR-ESIMS m/z 495.2396 [M + H]⁺ (calcd. for C₂₉H₂₅O₇ 495.2377), m/z 517.2209 [M + Na]⁺ (calcd. for C₂₉H₂₆O₇Na 517.2197).

**16-Nor-abiet-8,11,13-trien-3,7,15-triene (16):** Colorless crystal; [α]°D +15 (c 0.20, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Tables 4 and 5; HR-ESIMS m/z 299.1648 [M + H]⁺ (calcd. for C₁₉H₂₉O₂ 299.1642), m/z 321.1466 [M + Na]⁺ (calcd. for C₁₉H₂₉O₂Na 321.1461).

**16-Nor-3β-hydroxy-abiet-8,11,13-trien-7,15-dione (17):** Colorless crystal; [α]°D −9 (c 0.20, CHCl₃); the ¹H and ¹³C NMR spectroscopic data, see Tables 4 and 5; HR-ESIMS m/z
301.1803 [M + H]+ (calcd. for C_{19}H_{25}O_{3} 301.1798), m/z 323.1623 [M + Na]+ (calcd. for C_{19}H_{24}O_{3}Na 323.1618).

ent-8β,14β-Epoxyabieta-3-one-11,13(15)-dien-16,12-olide (19): Colorless gum; [α]_{D}^{28} +90 (c 0.20, CHCl_{3}); the ¹H and ¹³C NMR spectroscopic data, see Tables 4 and 5; HR-ESIMS m/z 329.1753 [M + H]+ (calcd. for C_{20}H_{25}O_{3} 329.1747), m/z 351.1572 [M + Na]+ (calcd. for C_{20}H_{24}O_{3}Na 351.1567).

ent-8β,14β-Epoxyabieta-3α-hydroxy-13(15)-en-16,12-olide (20): Colorless gum; [α]_{D}^{28} +57 (c 0.20, CHCl_{3}); the ¹H and ¹³C NMR spectroscopic data, see Tables 4 and 5; HR-ESIMS m/z 333.2067 [M + H]+ (calcd. for C_{20}H_{29}O_{4} 333.2060), m/z 355.1886 [M + Na]+ (calcd. for C_{20}H_{28}O_{4}Na 355.1880).

4.5. Cell Isolation and Culture

The HIV-1-infected Jurkat T cell line (J-Lat 10.6) was obtained in January 2019 from the NIH/ATCC HIV-1 Reagent Program (www.hivreagentprogram.org) and cultured in RPMI-based media supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin.

4.6. Flow Cytometry

After in vitro culture, J-lat cells were washed with phosphate-buffered saline (1× PBS) prior to staining with 0.1 µL fixable viability dye Live/Dead Aqua (Cat L34957, www.thermofisher.com) per 10⁵ cells for 30 min at 4 °C. Simultaneously, J-lat cells were also stained with antibodies against CD69 conjugated to an APC fluorophore (APC anti-human CD69 antibody, biolegend.com). Cells were then washed and re-suspended in 1× PBS prior to flow cytometry acquisition evaluating for cellular viability, green fluorescent protein (GFP) expression, and CD69 expression. Flow cytometry was performed with a BD FACSCelesta or FACSCanto flow cytometer with FACSDiva acquisition software (Becton Dickinson, Mountain View, CA) prior to analysis with FlowJo (TreeStar Inc., Ashland, OR, USA).

4.7. Compound Screening

Isolated compounds were resuspended in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Burlington, USA), at a concentration of 10 mM, and diluted with PBS, and tested with J-Lat 10.6 cells at concentrations of 100, 10, and 1 µM. J-lat 10.6 cells were tested with compounds at a concentration of 2.5 × 10⁵ cells/mL. We performed a minimum of three replicates of each condition for all experiments. Negative controls contained 1% DMSO to account for any effect of DMSO in the highest dilution of compounds. Additional dilutions were tested for those compounds that showed reactivation through increased GFP production in J-lat 10.6 cells.

4.8. Statistical Analysis

Statistical significance was analyzed using software from GraphPad Prism Version 7.0c (GraphPad Software, San Diego, CA, USA). The mean values and standard deviations for all replicate J-lat results were calculated and used to create Figures 4–7. Where applicable, Students t-test was used to determine statistical significance of experimental mean results relative to negative controls.

5. Conclusions

In this study, 4β-crotignoid K (14) revealed a very higher effect improvement compared to crotignoid K (13), indicating that configuration at the C-4 of tigliane diterpenoids is critical to HIV-1 latency reversal activity. (+)-Syringaresinol (26) and dimeric coniferyl acetate (27) showed no exhibition of CD69 expression, suggesting a non-PKC mechanism of latency reversal. Our results provide insights into the stereochemistry importance of bioactive diterpenoids and suggest that isolated compounds from *E. usambarica* can further research and development into therapeutic strategies for HIV-1 management, particularly as reactivators of latent HIV-1.
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