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Posted Date: 9 October 2025

doi: 10.20944/preprints202510.0367.v1

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Synthesis and Evaluation of Anti-HIV Activity of Fatty Acyl Conjugates of Tenofovir Alefanamide

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Abstract

The activity of nucleoside and nucleotide analogs as antiviral agents requires phosphorylation by intracellular enzymes. Phosphate-substituted analogs have low bioavailability due to the presence of ionizable negatively-charged groups. To circumvent this limitation, several prodrug approaches have been proposed and developed. Herein, we hypothesized that the conjugation of anti-HIV fatty acids with the nucleotide tenofovir alafenamide (TAF) (**1**) could improve the anti-HIV activity of the nucleotide. Several fatty acyl amide conjugates of TAF were synthesized and evaluated in comparative studies with TAF. The synthesized compounds were evaluated as racemic mixtures for anti-HIV activity in vitro in a single-round HIV-1 infection assay using TZM-bl cells at concentrations ranging from 0.01 to 100 ng/mL. Tetradecanoyl TAF conjugate **10** and palmitoyl TAF conjugate **17** had higher CLogP and displayed comparable activity to TAF (96-99% inhibition at 10–100 ng/mL) but at lower molar concentrations. The IC_{50} of conjugate **17** (0.65 nM) was lower than that of TAF (1.06 nM); however, this difference was not statistically significant.

Keywords: anti-HIV activity; conjugates of TAF; phosphonamidate; tenofovir alafenamide (TAF)

1. Introduction

Acquired immunodeficiency syndrome (AIDS), caused by the human immune deficiency virus (HIV), is still a major global health challenge. According to the Joint United Nations Programs on HIV/AIDS statistics, in the year 2024, about 31.6 million people were accessing antiretroviral therapy (UNAIDS 2025) [1]. Moreover, millions of people have died from the disease. Despite the success of highly active antiretroviral therapies, the rapid emergence of drug-resistant mutants has sharply limited the clinical applications of existing anti-HIV drugs, requiring an active pipeline of new antiretrovirals [2,3].

 doi:10.20944/preprints202510.0367.v1

The US Food and Drug Administration (FDA) has approved several drugs to treat HIV infection (HIV Treatment, 2022) [4]. An important limitation of antiviral drugs as therapeutic agents is, in many cases, their low oral bioavailability (less than 20%) and poor transport into cells, which in the case of nucleoside-based drugs is attributed to their ionizable groups [5].

A prodrug is a compound that undergoes a transformation within the body before eliciting its therapeutic action. The prodrug approach is extensively used to increase drug bioavailability, as well as drug targeting after oral administration [6]. This strategy is based on chemically modifying an active substance by attaching pro-moieties, which ideally overcome the biochemical and physical barriers associated with the parent compound. Limited oral bioavailability is usually attributed to poor membrane permeability, low aqueous solubility (in the gastrointestinal fluids), or extensive first-pass metabolism [7]. Several prodrug strategies have been applied to circumvent this problem in antiviral drugs [8–20]. Figure 1, gives the chemical structures of various antiviral prodrugs developed over the time to deal with above mentioned problems.

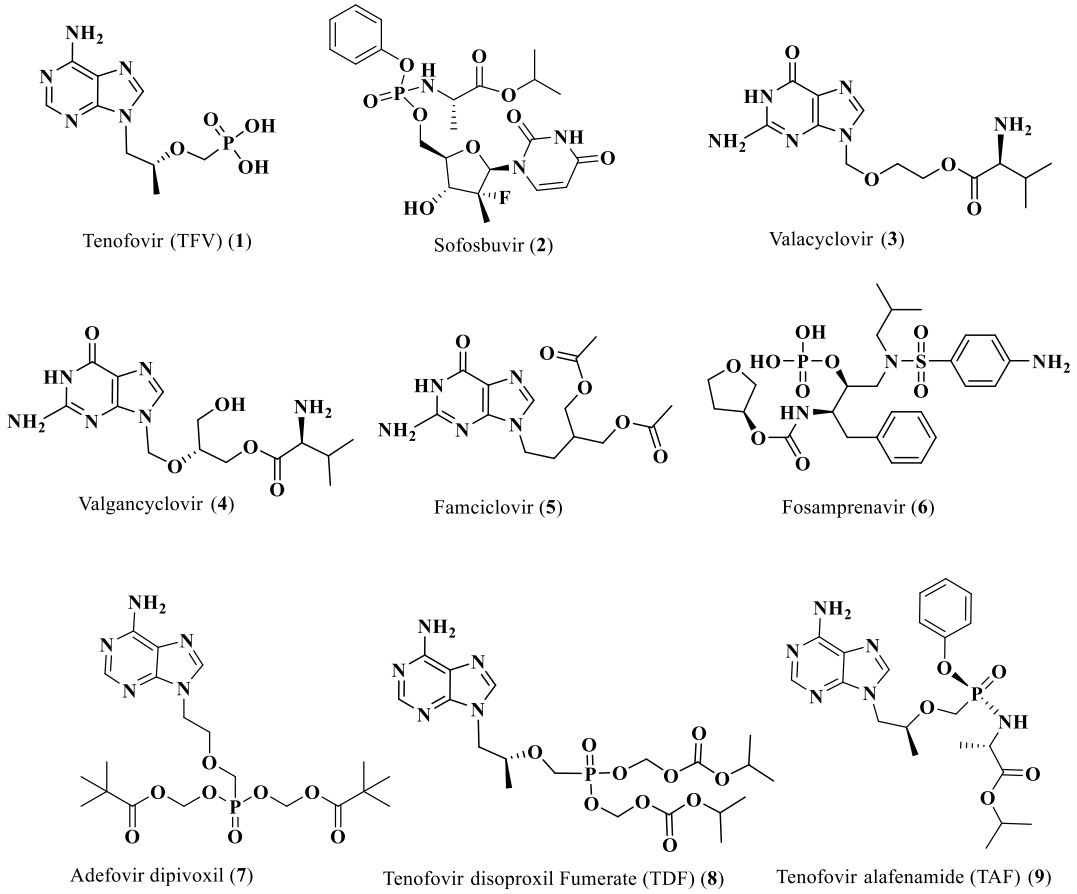

Figure 1. Chemical Structures of Antiviral Prodrugs.

Tenofovir (**1**) (TFV) (Figure 1) has activity against HIV-1, HIV-2, and hepatitis B viruses (HBV) [21]. Tenofovir disoproxil fumarate (**8**) (TDF, Viread®) (Figure 1) is an ester prodrug of TFV that is hydrolyzed to TFV intracellularly, and phosphorylated to the active metabolite, TFV diphosphate. TDF is used in combination with other antiviral medications, such as 2′,3′-dideoxy-5-fluoro-3′-thiacytidine (emtricitabine, FTC). Resistance to TDF is conferred by the reverse transcriptase (RT) K65R and/or K70E mutations. Tenofovir alafenamide fumarate (TAF) (**9**) (Figure 1) is another prodrug of tenofovir. TAF has a higher antiviral activity and distribution in the lymphatic system with fewer side effects, such as impaired kidney function [22–25]. All prodrugs are safe and effective and used as part of combination therapy or for prevention [26].

With the constant emergence of HIV mutants of clinical relevance and the need to reduce the number of ARVs for chronic treatment [27], it is logical to develop new long-acting and more potent

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nucleoside conjugates that display broad-spectrum activity, including against drug-resistant HIV. We previously demonstrated that several fatty acids, such as 2-methoxydodecanoic acid, 4-oxatetradecanoic acid, and 12-thioethyldodecanoic acid, reduced HIV-1 replication in acutely infected T-lymphocytes [28]. For example, 12-thioethyldodecanoic acid was moderately active (EC_{50} = 9.4 μM) against HIV-infected T4 lymphocytes. Protein *N*-myristoylation in HIV-1 is catalyzed by NMT, which is inhibited by myristic acid derivatives. Myristoylated proteins include PR160gag-pol, Pr55gag, p17gag, and p27nef proteins of HIV-1 [29]. Furthermore, fatty acyl derivatives of 3-fluoro-2′,3′-dideoxythymidine (FLT), 3′-azido-2′,3′-dideoxythymidine (AZT) [30], 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) [31], FTC [32], and 3TC [33] exhibited a significantly higher cellular uptake and anti-HIV profile against wild-type cell-free, cell-associated, and resistant viruses when compared with the corresponding parent nucleosides. The fatty acids were found to also have modest anti-HBV activity [34]. For example, myristic acid conjugate of FTC (IC_{90} = 15.7–16.1 nM) exhibited 6.6- and 35.2 times higher activity than FTC (IC_{90} = 103–567 nM) against multidrug-resistant viruses B-NNRTI and B-K65R, indicating that FTC conjugation with myristic acid generates a more potent analog with a better resistance profile than its parent compound. The fatty acyl conjugation changes the uptake, activity profile, and mechanism of activity, presumably by interfering with the posttranslational myristoylation of proteins in the HIV life cycle. Intracellular hydrolysis to the parent nucleoside is one of the factors that contribute to the overall anti-HIV activity.

Based on these findings, we hypothesized that the modification of the nucleotide-based TAF conjugated with different fatty acids would improve its lipophilicity, thereby leading to improved anti-HIV activity. Nine short- and long-chain amide fatty acyl conjugates of TAF **10-18** (Figure 2) were synthesized for comparative studies with TAF and the corresponding physical mixtures **19-26**. All compounds had a similar stereochemistry to TAF at the phosphorus. All compounds had larger calculated partition coefficients (CLogP ranging between 3.3-10.7) than TAF (Log P= 2.56, CLogP= 2.18). It is worth emphasizing that this work is a preliminary study to screen and identify lead compounds and templates and develop a structure–activity relationship. Further optimization and bio-stability work are planned for the future and are beyond the scope of this manuscript.

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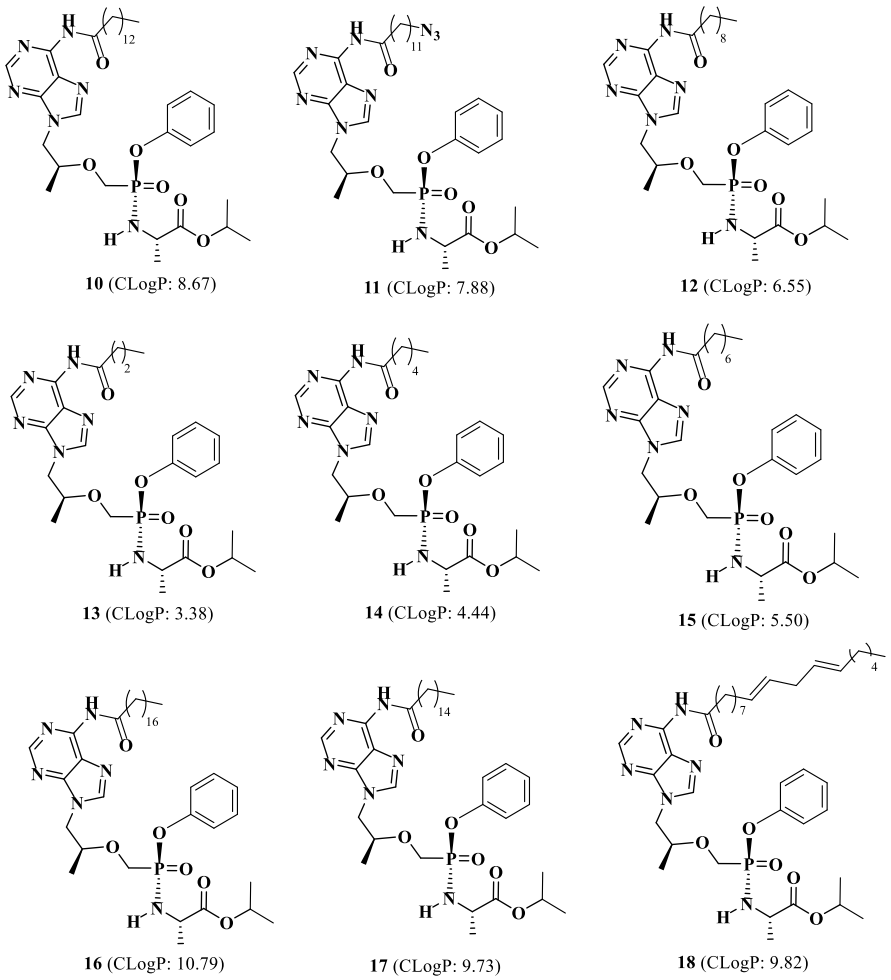

Figure 2. Chemical Structures of Synthesized TAF Conjugates **10-18**, along with their CLogP values.

This manuscript is the corrected version of our previously published article, *"Synthesis and Evaluation of Anti-HIV Activity of Mono- and Di-Substituted Phosphonamidate Conjugates of Tenofovir"*[35], that was retracted voluntarily by the authors after noticing unreliable data by one student. As noted in the retraction notice [36], the authors were allowed to resubmit a revised manuscript. This revised manuscript corrects the retracted version by removing unreliable data, incorporating new validated results, and updating the author list accordingly.

2. Results and Discussion

2.1. Chemistry

The synthesis of fatty acyl amino substituted TAF conjugates **10-18** was conducted through the reaction of TAF (**9**) with corresponding acyl chloride or acyl anhydride in the presence of *N*, *N*-diisopropylethylamine (DIPEA) in dimethylformamide (DMF) at 70 °C to afford the respective conjugate. In some cases, BOP (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate) reagent was used as the coupling agent. Alternatively, **9** was reacted with 12-azidododecanoic acid in the presence of 1-hydroxy-7-benzotriazole (HOAt) and DIPEA to yield 12-azidododecanoyl TAF conjugate **11** (Scheme 1). The anti-HIV activities of the conjugates were compared with the physical mixture of corresponding fatty acid/anhydride/chloride and TAF (**9**) (50:50 mol/mol, **19-26**).

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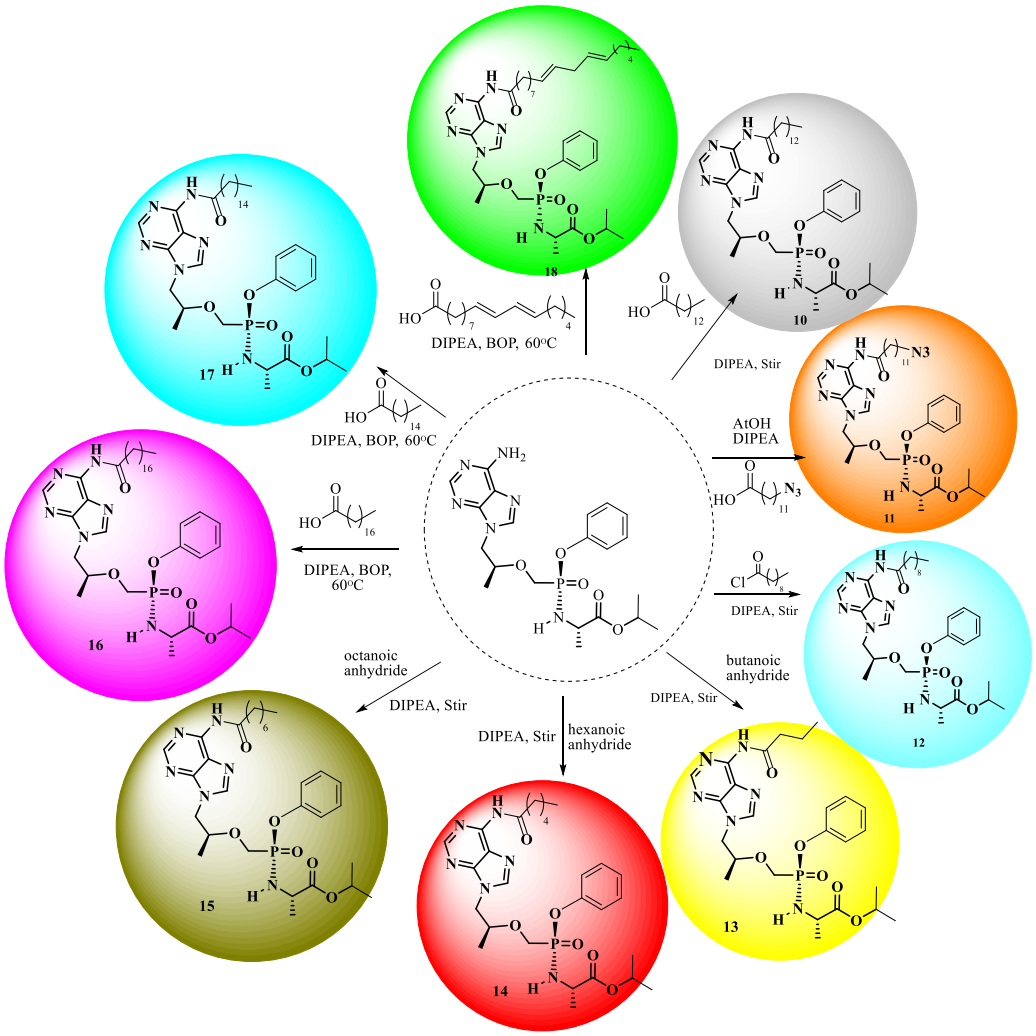

Scheme 1. Synthesis of fatty acyl amide substituted derivatives of TAF (**10**-**18**).

2.2. Biological Activities

Selected compounds were evaluated for their cytotoxicity on TZM-bl cells (Figure 3). TAF conjugates **10-18** and the physical mixtures **19-26** were found to show no toxicity at concentrations ranging between 1–100 ng/mL in TZM-bl cells (Figure 3 shows data at the highest concentration tested).

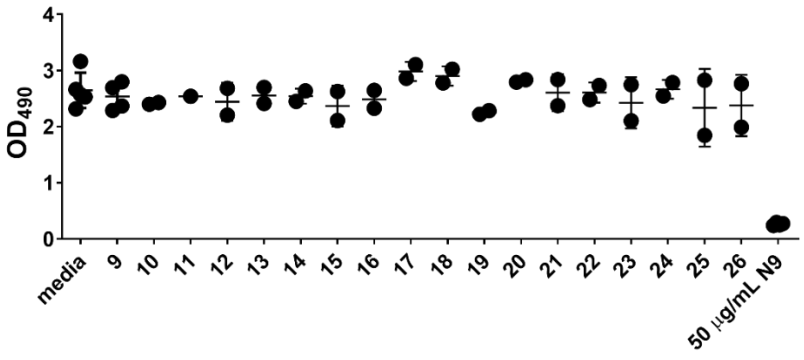

Figure 3. TZM-bl cells were exposed to TAF (**9**), TAF conjugates **10-18** and the physical mixtures of TAF (**19-26**) for 48 h. TZM-bl cells were plated in 96-well plates and exposed the following day to 1–100 ng/mL of compounds. The experiments were repeated two to four times with triplicate wells plated per concentration tested in each experiment. The cells were also exposed to nonoxynol-9 (N9) as a positive control for cytotoxicity. After 48 h

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exposure, the viability of the cells was measured by MTS assay. Only data for the highest concentration are plotted to fit all compounds in one figure, facilitating their comparison. No cytotoxicity was observed at lower concentrations.

Selected compounds were then screened for their efficacy against HIV infection in a single-round infection assay using TZM-bl cells at 0.01-100 ng/mL (Figure 4A-C). The median Relative Luminescence Unit (RLU) adjusted per assay was calculated and plotted. The experiments were repeated three or four times, and each experiment included triplicates per condition. The objective was to determine the anti-HIV activities of the conjugates in comparison to the parent molecule, TAF.

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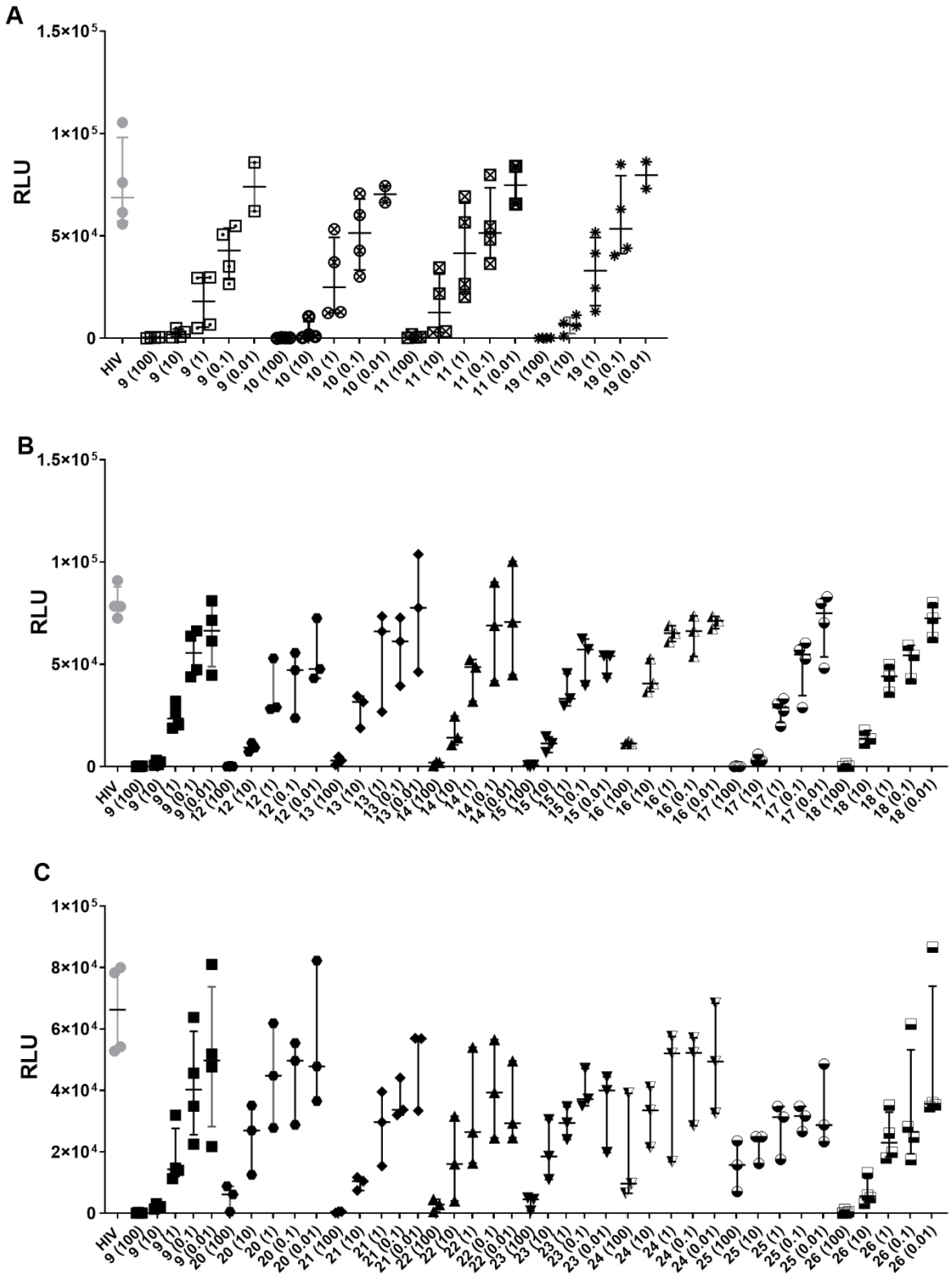

Figure 4. Anti-HIV activity of TAF (**9**) and TAF conjugates **10**, **11**, and **19** (A), **12-18** (B) and **20-26** (C) in TZMbl cells (concentrations listed below are in ng/mL). The median adjusted Relative Luminescence units (RLU) for each concentration per experiment are shown in Figure 4A, 4B and 4C (experiments were repeated 3 or 4 times, with 3 replicates per concentration in each experiment). The median percentage of HIV inhibition in cells exposed to different concentrations of TAF or TAF conjugates in presence of HIV per experiment is shown in Table 1.

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Table 1. Percentage of HIV inhibition in TZM-bl cells co-exposed to HIV$_{BAL}$ and TAF (**9**) or TAF conjugates **10-18** or the physical mixtures of TAF **19-26**.

Compound	[Concentration] ng/ml	% HIV (HIV-1$_{BAL}$) Inhibition	Compound	[Concentration] ng/ml	% HIV (HIV-1$_{BAL}$) Inhibition
9	100	99.6	**18**	100	99.6
	10	97.3		10	81.5
	1	74.6		1	40.8
	0.1	45.6		0.1	28.2
	0.01	19.5		0.01	0.7
10	100	99.6	**19**	100	99.7
	10	98.4		10	91.7
	1	70.9		1	60.4
	0.1	33		0.1	27.9
	0.01	21.1		0.01	8.6
11	100	99.4	**20**	100	92.4
	10	86.6		10	53.6
	1	43.4		1	46.1
	0.1	30.4		0.1	36.2
	0.01	17		0.01	8.6
12	100	99.7	**21**	100	98.9
	10	89.2		10	83.8
	1	57.3		1	45.7
	0.1	37.4		0.1	40
	0.01	34.5		0.01	22.3
13	100	96.4	**22**	100	94.9
	10	58.1		10	70.9
	1	18.1		1	53.5
	0.1	25.3		0.1	28.1
	0.01	0		0.01	41
14	100	97.2	**23**	100	92.8
	10	83.3		10	65
	1	36.6		1	45.6
	0.1	7		0.1	33.7
	0.01	2.8		0.01	41.4
15	100	99.3	**24**	100	81.2
	10	85.7		10	45.8
	1	55.6		1	29.5
	0.1	29.4		0.1	22.1
	0.01	34.1		0.01	11.5
16	100	84.6	**25**	100	75.4
	10	48		10	66
	1	16.6		1	55.5
	0.1	11.2		0.1	48.3
	0.01	10.8		0.01	48.6
17	100	99.85	**26**	100	99.45
	10	96.45		10	91.55
	1	61.9		1	63.85
	0.1	29.05		0.1	57.75
	0.01	4.3		0.01	35.5

At 100 ng/mL, the TAF conjugates **10**, **11**, **12**, **15**, **17**, **18**, **19**, and **26** showed inhibitory activities (more than 99%) comparable to that of TAF (Figure 4 and Table 1). At 10 ng/mL, only TAF conjugates **10** (98.4%) and **17** (96.4%) displayed similar HIV inhibition to TAF (97.3%). However, they did so at

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lower molar concentrations (145.69 nM and 139.98 nM [100 ng/mL], respectively, *vs* 209.99 nM [100 ng/mL] for TAF (**9**)), resulting in lower although not statistically significant IC_{50}, especially for compound **17** (Figure 5), and possibly suggesting a higher potency for this fatty acyl conjugate of TAF. We previously observed that fatty acyl conjugates of FTC demonstrated higher potency against resistant virus when compared with the parent FTC [32]. Further investigations are required to determine whether the fatty acyl conjugation of TAF can enhance the long-acting anti-HIV activity and potency against wild-type and tenofovir-resistant virus. Further optimization and biostability studies are also needed to determine the therapeutic relevance and added value of these conjugates.

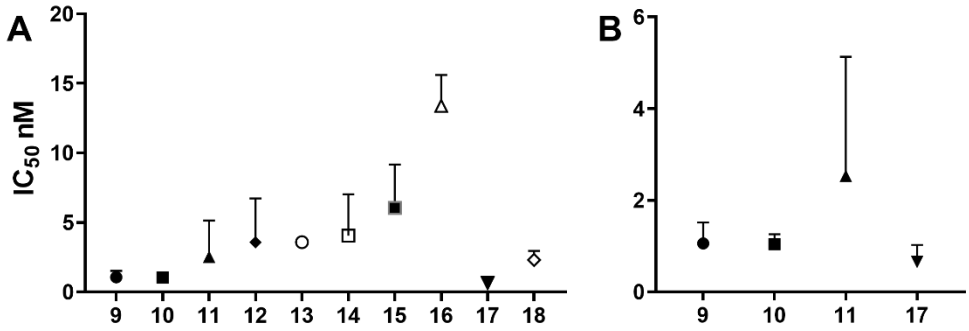

Figure 5. IC_{50} of TAF (**9**) and conjugates **10-18** for 48 h co-exposure of TAF and HIV_{BAL} in TZM-bl cells. The IC_{50} was calculated in GraphPad Prism for each individual experiment showed in Figure 5A by using log concentrations in nM and the RLU values measured for each concentration. Figure 5B shows the best three conjugates at a lower scale to facilitate the comparison with TAF.

The experiments were repeated two or four times. In each experiment, each compound was tested in triplicates.

3. Materials and Methods

3.1. General

The experimental part defines different methods and technical characteristics of the present work, which include the synthesis of fatty ester conjugates and different purification methods. Characterization of the synthesized compounds was achieved through various spectroscopic techniques, such as ^{1}H-NMR, ^{13}C-NMR, IR, UV, and mass spectrometry. The synthesized analogs were also evaluated for their anti-HIV activity.

All chemicals were of analytical grade and were directly used without any purification. TAF (**9**) was provided by Scilife Pharma (Pvt.) Ltd., Karachi, Pakistan as a gift for research purpose. Palmitic acid, acetone, methanol, hexane, ethyl acetate, and sodium azide were purchased from Sigma-Aldrich Co. (USA). Decanoyl chloride, butanoic anhydride, hexanoic anhydride, octanoic anhydride, stearic acid, linoleic acid, and myristic acid were purchased from TCI, Japan. 12-Azidodecanoic acid was prepared according to the previously reported procedure [37]. All reagents were of analytical grade and used directly without purification. Precoated silica gel plates (ALUGRAM, SIL G/UV254) were used for thin layer chromatography (TLC). TLC Chromatograms were viewed under the ultraviolet light of 254 and 365 nm. Electron impact mass spectroscopy (EI-MS) data were obtained through Jeol-jms-600H mass spectrometer (Japan). ^{1}H and ^{13}C-NMR spectra were recorded on a 300, 400 and 100 MHz Bruker Avance spectrometers (Switzerland). The chemical shifts (δ) were shown on a ppm scale and coupling constants or *J* values are expressed in Hz relative to internal standard tetramethyl silane $SiMe_4$. Buchi M-560 apparatus was used for recording the melting/boiling point (Japan). I.R.

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Spectrophotometry of the compounds was performed on FTIR-8900 (Shimadzu, Japan) by using KBr disc.

3.2. Chemistry

Isopropyl(phenoxy((((S)-1-(6-tetradecanamido-9H-purin-9-yl)propan-2-yl)oxy)methyl)phosphoryl)-L-alaninate (10).

Compound **10** was obtained by the reaction of (**9**, 9.3 mg, 2 mmol) with myristoyl chloride (1.5 mmol) using DIPEA in 5 mL of (DMF/DCM 1:1) for 24 h. The compound was purified by silica gel column chromatography. Yield (5.23 mg, 55.7%, colorless solid). ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 8.89-8.73 (1H, br-s, N*H*CO), 8.70 (1H, s, H-3 of purine ring), 8.24 (1H, s, H-8 of purine ring), 7.23 (2H, t, $J_{3'-2'}$, $J_{5'-6'}$ = 8.0 Hz, H-3′and H-5′ of phenoxy ring), 7.11 (1H, t, $J_{4'-3'}$, $J_{4'-5'}$ = 7.6 and 7.2 Hz, H-4′ of phenoxy ring), 7.00 (2H, d, $J_{2'-3'}$, $J_{6'-5'}$ = 8.8 Hz, H-2′ and H-6′ of phenoxy ring), 5.03-4.94 (1H, *m*, C*H* of propan-2-yl moiety), 4.44 (1H, *dd*, *J* =14 and 2.8 Hz, CH_2 of propan-2-yl moiety), 4.18 (1H, dd, *J* = 8.0 and 7.6 Hz, CH_2 of propan-2-yl moiety), 3.99 (2H, m, N*H* of alanine moiety and CH of propyloxy group), 3.67 (2H, m, CH_2 group), 2.83 (2H, t, *J* =7.2 Hz, α-CH_2 of the myristoyl moiety), 1.67-1.57 (2H, m, β-CH_2 of myristoyl moiety), 1.29-1.22 (32 H, m, CH_3 of propyloxy, CH_3 of alanine moiety and 2 CH_3 groups of the isopropyl moiety, and 10 CH_2 of the myristoyl moiety), 0.87 (3H, t *J* = 6.8 Hz, CH_3 of myristoyl moiety); ^{13}C-NMR (101 MHz, $CDCl_3$): δ 174.39, 173.12, 172.86, 152.53, 151.43, 149.99, 149.06, 143.77, 129.74, 125.01, 120.24, 69.26, 65.36, 63.81, 51.46, 49.85, 48.60, 37.93, 34.13, 31.93, 29.65, 29.52, 29.44, 29.37, 29.27, 29.16, 24.92, 22.70, 21.73, 21.58, 16.47, 14.14.; ^{31}P-NMR (400 MHz, DMSO-d_6) δ 22.07; ESI-MS *m*/*z* (rel. int. %): Calcd. Formula [$C_{35}H_{55}N_6O_6P$]: *m*/*z* 686.4, Found: *m*/*z* 687.4 $[M+H]^+$.

Isopropyl(((((S)-1-(6-(12-azidododecanamido)-9H-purin-9-yl)propan-2-yl)oxy)methyl)(phenoxy)phosphoryl)-L-alaninate (11).

Compound **11** was obtained by the reaction of TAF (**9**, 9.3 mg, 2 mmol) with 12-azidododecanoic acid (1.5 mmol) using HOAt as an activating agent and DIPEA as a base in 5 mL of DMF/DCM (1:1 *v*/*v*) for 24 h. The compound was purified by silica gel column chromatography. Yield (4.5 mg, 55.7%, colorless solid). ^{1}H-NMR 8.69-8.73 (1H, s, N*H*CO), 8.21 (1H, s, H-3 of purine ring), 8.15 (1H, s, H-8 of purine ring), 7.28 (2H, t, $J_{3'-2'}$, $J_{5'-6'}$ = 8.0 Hz, H-3′and H-5′ of phenoxy ring), 7.14 (1H, t, $J_{4'-3'}$, $J_{4'-5'}$ = 7.6 and 7.2 Hz, H-4′ of phenoxy ring), 7.00 (2H, d, $J_{2'-3'}$, $J_{6'-5'}$ = 8.4 Hz, H-2′ and H-6′ of phenoxy ring), 5.01-4.96 (1H, *m*, C*H* of propan-2-yl moiety), 4.41 (1H, *dd*, *J* =14 and 2.8 Hz, CH_2 of propan-2-yl moiety), 4.19 (1H, dd, *J* = 14.4 and 7.6 Hz, CH_2 of propan-2-yl moiety), 4.01-3.91 (2H, m, N*H* of alanine moiety and CH of propyloxy group), 3.72-3.66 (2H, m, CH_2 group), 3.25 (t, *J*= 6.8 Hz, 2H, CH_2N_3), 2.34 (2H, t, *J* =7.6 Hz, α-CH_2 of the 12-azidododecanoic acid moiety), 1.67-1.56 (2H, m, β-CH_2 of 12-azidododecanoic acid moiety), 1.28-1.23 (28H, m, CH_3 of propyloxy, CH_3 of alanine moiety and 2 CH_3 groups of the isopropyl moiety, and 8 CH_2 of the 12-azidododecanoic acid moiety).^{13}C-NMR (101 MHz, $CDCl_3$) δ 176.49, 173.28, 155.64, 152.65, 152.25, 151.15, 150.08, 143.81, 129.84, 125.21, 120.08, 120.04, 69.43, 65.38, 63.96, 51.50, 49.75, 48.88, 36.21, 33.61, 29.44, 29.38, 29.23, 29.13, 29.08, 28.84, 26.94, 26.71, 24.77, 21.69, 21.62, 21.49, 16.41. ESI-MS *m*/*z* (rel. int. %): Calcd. For [$C_{33}H_{50}N_9O_6P$]: (699.4), Found: *m*/*z* 700.4 $[M+H]^+$.

Isopropyl(((((S)-1-(6-decanamido-9H-purin-9-yl)propan-2-yl)oxy)methyl)(phenoxy)phosphoryl)-L-alaninate (12)

Compound **12** was obtained by the reaction of TAF (1 mmol) with decanoyl chloride (1 mmol) using DIPEA as a base in 5 mL of (DMF/DCM 1:1 v/v) for 24 h at room temperature. The compound was purified by silica gel column chromatography by using 90% EtOAc and 10% MeOH as the mobile phases. R_f = 0.71, Yield: (11 mg, 37%, colorless liquid); B.P.: 277-279 °C; FT-IR (KBr pellet cm^{-1}): υ_{max} (N-H) 3086; υ(C=O) 1710; υ(C=N) 1665. UV λ_{max}, nm (CH_3OH): 221, 262, 268; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.55 (1H, s, NHCO), 8.60 (1H, s, H-2 of purine ring), 8.40 (1H, s, H-8 of purine ring), 7.26 (2H, t, $J_{3'/2'}$, $J_{5'/6'}$ = 15.6 Hz, H-3′and H-5′ of phenoxy ring), 7.11 (1H, distorted triplet, H-4′ of phenoxy

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ring), 7.01 (2H, d, $J_{2'/3'}$, $J_{6'/5'}$ = 8.0 Hz, H-2′ and H-6′ of phenoxy ring), 5.56 (1H, t, N*H* of alanine moiety), 4.82 (1H, q, $J_{CH/(CH3)2}$ = 6.0 Hz, C*H* of propyloxy group), 4.39 (1H, dd, *J*= 14.4 and 3.2, Hz, CH_2 of propan-2-yl moiety), 4.24 (1H, dd, *J* = 14.4 and 6.4 Hz, CH_2 of propan-2-yl moiety), 3.97 (1H, m, C*H* of alanine moiety), 3.83 (3H, m, CH_2 group and, C*H* of propan-2-yl moiety), 2.54 (2H, overlapped, α-CH_2 of the decanoyl moiety), 1.58 (2H, t, *J* = 14.0 Hz, β-CH_2 of decanoyl moiety), 1.28-1.13 (12H, m, CH_3 of propyloxy, CH_3 of alanine moiety and 2 CH_3 groups of the isopropyl moiety), 1.14-1.10 (6H, m, 12 CH_2 of the decanoyl moiety), 0.83 (3H, t, $J_{CH3/CH2}$ = 13.2 Hz, CH_3 of decanoyl moiety); Positive FAB-MS *m*/*z* 631.4 $[M+H]^+$; HRFAB-MS (+ve mode) Calcd. For $[(C_{31}H_{47}N_6O_6P)+H]^+$: (631.3373) Found *m*/*z* 631.3392.

Isopropyl (((((S)-1-(6-butyramido-9H-purin-9-yl)propan-2-yl)oxy)methyl)(phenoxy)phosphoryl)-L-alaninate (13).

Compound **13** was obtained by the reaction of TAF (1 mmol) with butanoic anhydride (1 mmol) using DIPEA in 5 mL of (DMF/DCM 1:1) for 24 h at room temperature. The compound was purified by silica gel column chromatography by using 90% EtOAc and 10% MeOH as mobile phases. R_f = 0.69, Yield: (9 mg, 27%, colorless liquid); B. P.: 255-262 °C; FT-IR (KBr pellet cm^{-1}): υ_{max} (N-H) 3081; υ(C=O) 1712; υ(C=N) 1660. UV λ_{max}, nm (CH_3OH): 221, 253, 267; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.56 (1H, s, N*H*CO), 8.60 (1H, s, H-2 of purine ring), 8.40 (1H, s, H-8 of purine ring), 7.26 (2H, t, $J_{3'/2'}$, $J_{5'/6'}$ = 15.6 Hz, H-3′and H-5′ of phenoxy ring), 7.11 (1H, distorted triplet, H-4′ of phenoxy ring), 7.02 (2H, d, $J_{2'/3'}$, $J_{6'/5'}$ = 8.4 Hz, H-2′ and H-6′ of phenoxy ring), 5.60 (1H, t, N*H* of alanine moiety), 4.82 (1H, q, $J_{CH/(CH3)2}$ = 6.0 Hz, C*H* of propyloxy group), 4.39 (1H, dd, *J*= 12.4 and 3.2 Hz, CH_2 of propan-2-yl moiety), 4.24 (1H, dd, *J*= 14.4 and 6.4 Hz, CH_2 of propan-2-yl moiety), 3.98 (1H, m, C*H* of alanine moiety), 3.85 (3H, m, CH_2 group and, C*H* of propan-2-yl moiety), 2.52 (2H, overlapped, α-CH_2 of the butanoyl moiety), 1.61 (2H, sextet, *J*= 14.8 Hz, β-CH_2 of butanoyl moiety), 1.13-1.07 (12 H, m, CH_3 of propyloxy, CH_3 of alanine moiety and 2 CH_3 groups of the isopropyl moiety), 0.92 (3H, t, $J_{CH3/CH2}$ = 14.8 Hz, CH_3 of butanoyl moiety); FAB-MS (+ve) *m*/*z* 547.2 $[M+H]^+$; HRFAB-MS (+ve mode) Calcd. For $[(C_{25}H_{35}N_6O_6P)+H]^+$: (547.2434) Found *m*/*z* 547.2427.

Isopropyl (((((S)-1-(6-hexanamido-9H-purin-9-yl)propan-2yl)oxy)methyl)(phenoxy)phosphoryl)-L-alaninate (14).

Compound **14** was obtained by the reaction of TAF (1 mmol) with hexanoic anhydride (1 mmol) using DIPEA in 5 mL of (DMF/DCM 1:1) for 24 h at room temperature. The compound was purified by silica gel column chromatography by using 90% EtOAc and 10% MeOH as mobile phases. R_f = 0.72, Yield: (8 mg, 17%, colorless liquid); B. P.: 257-262 °C; FT-IR (KBr pellet cm^{-1}): υ_{max} (N-H) 3068; υ(C=O) 1718; υ(C=N) 1674. UV λ_{max}, nm (CH_3OH): 214, 220, 273; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.56 (1H, s, N*H*CO), 8.60 (1H, s, H-2 of purine ring), 8.40 (1H, s, H-8 of purine ring), 7.26 (2H, t, $J_{3'/2'}$, $J_{5'/6'}$ = 16.0 Hz, H-3′and H-5′ of phenoxy ring), 7.11 (1H, distorted triplet, H-4′ of phenoxy ring), 7.01 (2H, d, $J_{2'/3'}$, $J_{6'/5'}$ = 8.8 Hz, H-2′ and H-6′ of phenoxy ring), 5.60 (1H, t, (1H, t, N*H* of alanine moiety), 4.82 (1H, q, $J_{CH/(CH3)2}$ = 6.4 Hz, C*H* of propyloxy group), 4.37 (1H, dd, *J* =14.4 and 3.2 Hz, CH_2 of propan-2-yl moiety), 4.22 (1H, dd, *J* = 14.4 and 6.8 Hz, CH_2 of propan-2-yl moiety), 3.98 (1H, m, C*H* of alanine moiety), 3.85 (3H, m, CH_2 group and, C*H* of propan-2-yl moiety), 2.55 (2H, overlapped, α-CH_2 of the hexanoyl moiety), 1.59 (2H, t, *J* = 14.4 Hz, β-CH_2 of hexanoyl moiety), 1.30 (4H, m, 2 CH_2 of the hexanoyl moiety), 1.13- 1.07 (12H, m, CH_3 of propyloxy, CH_3 of alanine moiety and 2 CH_3 groups of the isopropyl moiety), 0.92 (3H, t, $J_{CH3/CH2}$ = 14.8 Hz, CH_3 of hexanoyl moiety); FAB-MS (-ve) *m*/*z* 573.3 $[M-H]^+$; HRFAB-MS (+ve mode) Calcd. For $[(C_{27}H_{40}N_6O_6P)+H]^+$: (575.2747) Found *m*/*z* 575.2737.

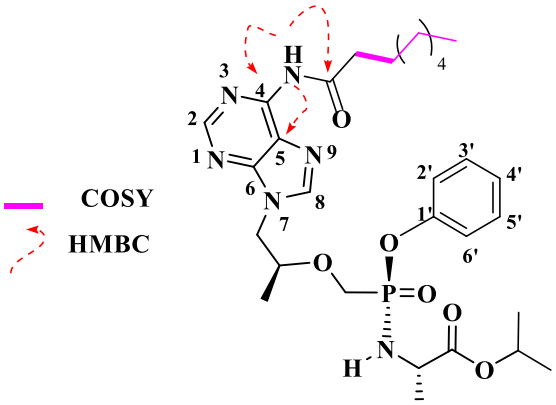
Isopropyl (((((S)-1-(6-octanamido-9H-purin-9-yl)propan-2-yl)oxy)methyl)(phenoxy)phosphoryl)-L-alaninate (15).

Compound **15** was obtained by the reaction of TAF (1 mmol) with octanoic anhydride (1 mmol) using DIPEA in 5 mL of (DMF/DCM 1:1) for 24 h at room temperature. The compound was purified

 doi:10.20944/preprints202510.0367.v1

by silica gel column chromatography by using 90% EtOAc and 10% MeOH as mobile phases. R_f = 0.74, Yield: (11 mg, 21%, colorless liquid); B. P.: 259-263 °C; FT-IR (KBr pellet cm^{-1}): υ_{max} (N-H) 3121; υ(C=O) 1708; υ(C=N) 1669. UV λ_{max}, nm (CH_3OH): 213, 221, 271; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.53 (1H, s, N*H*CO), 8.57 (1H, s, H-2 of purine ring), 8.37 (1H, s, H-8 of purine ring), 7.23 (2H, t, $J_{3'/2'}$, $J_{5'/6'}$ = 15.6 Hz, H-3′and H-5′ of phenoxy ring), 7.08 (1H, distorted triplet, H-4′ of phenoxy ring), 7.00 (2H, d, $J_{2'/3'}$, $J_{6'/5'}$ = 8.4 Hz, H-2′ and H-6′ of phenoxy ring), 5.57 (1H, t, N*H* of alanine moiety), 4.80 (1H, q, $J_{CH/(CH3)2}$ = 6.9 Hz, C*H* of propyloxy group), 4.35 (1H, dd, *J*= 14.4 and 3.2 Hz, CH_2 of propan-2-yl moiety), 4.21 (1H, dd, *J* = 6.8 and 14.8 Hz, CH_2 of propan-2-yl moiety), 3.92 (1H, m, C*H* of alanine moiety), 3.81 (3H, m, CH_2 group and, C*H* of propan-2-yl moiety), 2.51 (2H, overlapped, α-CH_2 of the octanoyl moiety), 1.55 (2H, t, *J* = 14.0 Hz, β-CH_2 of octanoyl moiety), 1.25-1.21 (8H, m, 4 CH_2 of the octanoyl moiety), 1.11- 1.05 (12 H, m, CH_3 of propyloxy, CH_3 of alanine moiety and 2 CH_3 groups of the isopropyl moiety), 0.82 (3H, t, JCH_3/CH_2 = 13.2 Hz, CH_3 of octanoyl moiety); ^{13}C-NMR (600 MHz, DMSO-d_6) δ 172.9, 171.6, 152.3, 151.5, 150.2, 150.2, 149.4, 144.8, 129.4, 124.3, 122.9, 120.5, 120.5, 118.8, 115.2, 75.4, 75.3, 67.9, 64., 63.60, 49.0, 47.1, 36.2, 33.9, 31.2, 28.5, 28.5, 24.8, 24.5, 22.1, 21.4; EI-MS *m/z* (rel. int. %): 602.1 $[M]^+$, 515.2(100), 346.0(42.5), 177.0(15.4); HREI-MS Calcd. For [$C_{29}H_{43}N_6O_6P$]: (602.2982) Found *m/z* 602.2984.

Key 2D COSY and HMBC Correlations of Compound 15:



The structure of compound **15** was further confirmed through key 2D correlations. In HMBC, NH proton attached with purine ring showed correlations with quaternary carbonyl carbon of octanoyl chain, and with quaternary carbons C-4, and C-5 of purine ring. COSY correlations were observed among the methylene protons of the long hydrocarbon chain.

Isopropyl (phenoxy((((S)-1-(6-stearamido-9H-purin-9-yl)propan-2-yl)oxy)methyl)phosphoryl)-L-alaninate (16).

Compound **16** was obtained by the reaction of TAF (1 mmol) with stearic acid (1 mmol), BOP (1 mmol) and DIPEA in 5 mL of acetone for 24 h at 60 °C. The compound was purified by silica gel column chromatography by using 90% EtOAc and 10% MeOH as mobile phases. R_f = 0.72, Yield: (15 mg, 18%, colorless solid); M. P.: 256-268 °C; FT-IR (KBr pellet cm^{-1}): υ_{max} (N-H) 3088; υ(C=O) 1750; υ(C=N) 1661. UV λ_{max}, nm (CH_3OH): 221, 269; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.53 (1H, s, N*H*CO), 8.57 (1H, s, H-2 of purine ring), 8.37 (1H, s, H-8 of purine ring), 7.24 (2H, t, $J_{3'/2'}$, $J_{5'/6'}$ = 16.0 Hz, H-3′and H-5′ of phenoxy ring), 7.08 (1H, distorted triplet, H-4′ of phenoxy ring), 7.01 (2H, d, $J_{2'/3'}$, $J_{6'/5'}$ = 8.4 Hz, H-2′ and H-6′ of phenoxy ring), 5.58 (1H, t, N*H* of alanine moiety), 4.80 (1H, p, $J_{CH/(CH3)2}$ = 6.4 Hz, C*H* of propyloxy group), 4.39 (1H, dd, *J* =14.4 and 3.2 Hz, CH_2 of propan-2-yl moiety), 4.24 (1H, dd, *J* = 14.4 and 6.4 Hz, CH_2 of propan-2-yl moiety), 3.94 (1H, m, C*H* of alanine moiety), 3.80 (3H, m, CH_2 group and, C*H* of propan-2-yl moiety), 2.57 (2H, overlapped, α-CH_2 of the stearoyl moiety), 1.14-1.19 (30 H, m, 1 CH_2 of β-CH_2 of stearoyl moiety moiety and 14 CH_2 of stearoyl moiety), 1.15-1.05 (12 H, m, CH_3 of propyloxy, CH_3 of alanine moiety and 2 CH_3 groups of the isopropyl moiety), 0.83 (3H, t,

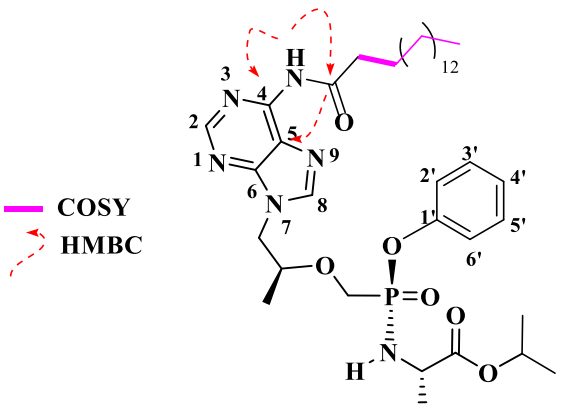
 doi:10.20944/preprints202510.0367.v1

$J_{CH3/CH2}$ = 13.2 Hz, CH_3 of stearoyl moiety moiety); EI-MS *m*/*z* (rel. int. %): 742.1 $[M]^+$, 94.1(100), 346.1(98); HREI-MS Calcd. For [($C_{39}H_{63}N_6O_6P$)]: (742.4547) Found *m*/*z* 742.4543.

Isopropyl (((((S)-1-(6-palmitamido-9H-purin-9-yl)propan-2-yl)oxy)methyl)(phenoxy)phosphoryl)-L-alaninate (17)

Compound **17** was obtained by the reaction of TAF (1 mmol) with palmitic acid (1 mmol), BOP (1 mmol), and DIPEA in 5 mL of acetone for 24 h at 60 °C. The compound was purified by silica gel column chromatography by using 90% EtOAc and 10% MeOH as mobile phases. R_f = 0.63, Yield: (9 mg, 25%, colorless solid); M. P.: 263-268 °C; FT-IR (KBr pellet cm^{-1}): υ_{max} (N-H) 3215; υ(C=O) 1698; υ(C=N) 1669. UV λ_{max}, nm (CH_3OH): 201, 208, 230; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.53 (1H, s, N*H*CO), 8.57 (1H, s, H-2 of purine ring), 8.37 (1H, s, H-8 of purine ring), 7.24 (2H, t, $J_{3'/2'}$, $J_{5'/6'}$ = 15.6 Hz, H-3′and H-5′ of phenoxy ring), 7.08 (1H, distorted triplet, H-4′ of phenoxy ring), 7.00 (2H, d, $J_{2'/3'}$, $_{6'/5'}$ = 8.4 Hz, H-2′ and H-6′ of phenoxy ring), 5.57 (1H, t, N*H* of alanine moiety), 4.80 (1H, q, $J_{CH/(CH3)2}$ = 6.4 Hz, CH of propyloxy group), 4.35 (1H, dd, *J* =14.0 and 2.8 Hz, CH_2 of propan-2-yl moiety), 4.21 (1H, dd, *J* = 14.8 and 6.8 Hz, CH_2 of propan-2-yl moiety), 3.95 (1H, m, C*H* of alanine moiety), 3.86 (3H, m, CH_2 group and, C*H* of propan-2-yl moiety), 2.46 (2H, overlapped, α-CH_2 of the palmitoyl moiety), 1.54 (2H, m, β-CH_2 of palmitoyl moiety), 1.24-1.19 (22H, m, 11 CH_2 of the palmitoyl moiety), 1.11-1.04 (12 H, m, CH_3 of propyloxy, CH_3 of alanine moiety and 2 CH_3 groups of the isopropyl moiety), 0.80 (3H, t, JCH_3/CH_2 = 13.2 Hz, CH_3 of palmitoyl moiety); ^{13}C-NMR (600 MHz, DMSO-d_6) δ 172.9, 172.9, 171.7, 152.4, 152.3, 151.5, 151.3, 150.2, 149.4, 149.2, 145.5, 145.0, 129.4, 128.6, 124.3, 123.0, 121.4, 120.5, 120.5, 75.4, 75.3, 70.0, 68.0, 65.0, 64.0, 49.0, 47.2, 36.2, 31.3, 29.0, 28.9, 28.7, 28.5, 24.8; EI-MS *m*/*z* (rel. int. %): 714.3 $[M]^+$, 94.0(100), 346.0(80.0), 177.0(75.4); HREI-MS Calcd. For [$C_{37}H_{59}N_6O_6P$]: (714.4234) Found *m*/*z* 714.4187.

Key 2D COSY and HMBC Correlations of Compound 17.



The structure of compound **17** was further confirmed through key 2D correlations. In HMBC, NH proton attached with purine ring showed correlations with quaternary carbonyl carbon of palmitoyl group, and also with quaternary carbons C-4, and C-5 of purine ring. Several COSY correlations were observed for the methylene protons of the palmitoyl group.

Isopropyl (((((S)-1-(6-((9E,12E)-octadeca-9,12-dienamido)-9H-purin-9-yl)propan-2-yl)oxy)methyl)(phenoxy)phosphoryl)-L-alaninate (18).

Compound **18** was obtained by the reaction of TAF (1 mmol) with linoleic acid (1 mmol), BOP (1 mmol) and DIPEA in 5 mL of acetone for 24 h at 60 °C. The compound was purified by silica gel column chromatography by using 90% EtOAc and 10% MeOH as mobile phases. R_f = 0.70, Yield: (14 mg, 15%, colorless liquid); B. P.: 264-265 °C; FT-IR (KBr pellet cm^{-1}): υmax (N-H) 3128; υ(C=O) 1708; υ(C=N) 1669. UV λmax, nm (CH_3OH): 0.621 (214), 0.805 (273); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.53 (1H, s, N*H*CO), 8.57 (1H, s, H-2 of purine ring), 8.37 (1H, s, H-8 of purine ring), 7.24 (2H, t, $J_{3'/2'}$, $J_{5'/6'}$ = 15.6 Hz, H-3′ and H-5′ of phenoxy ring), 7.08 (1H, distorted triplet, H-4′ of phenoxy ring), 7.00 (2H,

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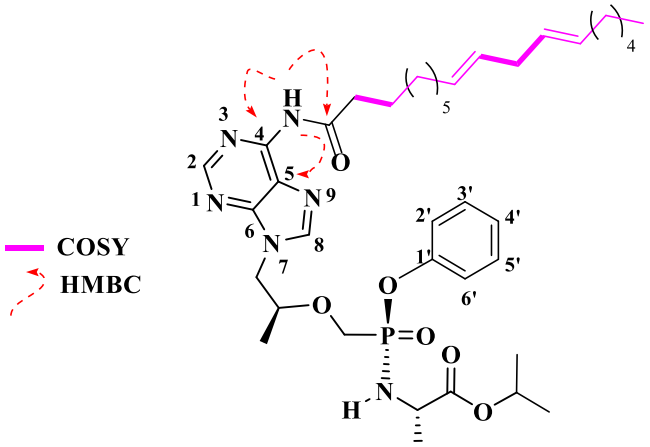
d, $J_{2'/3'}$, $J_{6'/5'}$ = 8.4 Hz, H-2′ and H-6′ of phenoxy ring), 5.57 (1H, t, N*H* of alanine moiety), 4.80 (3H, m, C*H* of propyloxy group, and CH_2 of linoleoyl moiety), 4.80 (1H, m, C*H* of C-10′′ of linoleoyl moiety), 4.35 (1H, dd, *J* = 14.4 and 3.2 Hz, CH_2 of propan-2-yl moiety), 4.21 (1H, dd, *J* = 14.5 and 6.5 Hz, CH_2 of propan-2-yl moiety), 3.95 (1H, m, C*H* of alanine moiety), 3.78 (4H, m, CH_2 group, C*H* of propan-2-yl moiety, and 1 *CH* of C-9′′ of linoleoyl moiety), 2.69 (1H, t, $J_{13''/14''}$ = 11.6 Hz, C*H* of C-13′′ of linoleoyl moiety), 2.51 (3H, overlapped, α-CH_2 of the linoleoyl moiety, and C*H* of C-12′′ of linoleoyl moiety), 1.98-1.19 (20H, m, 10-CH_2 of linoloeyl moiety), 1.13-1.04 (12H, m, CH_3 of propyloxy, CH_3 of alanine moiety and 2 CH_3 groups of the isopropyl moiety), 0.79 (3H, t, $J_{CH3/CH2}$ = 13.2 Hz, CH_3 of linoleoyl moiety); ^{13}C-NMR (600 MHz, DMSO-d_6) δ 172.9, 171.6, 152.3, 151.4, 150.2, 150.2, 149.4, 144.8, 129.7, 129.6, 129.4, 127.7, 124.3, 122.8, 120.5, 120.5, 75.3, 75.3, 67.9, 64.6, 63.6, 49.0, 47.1, 36.2, 31.2, 30.9, 29.1, 29.0, 28.8, 28.7, 28.5, 26.6, 26.5; EI-MS *m*/*z* (rel. int. %): 738.3 $[M]^+$, 94.0(100), 106.9(85.0), 149.9(60.0); HREI-MS Calcd. For [$C_{39}H_{59}N_6O_6P$]: (738.4234) Found *m*/*z* 738.4196.

Key 2D COSY and HMBC Correlations of Compound 18:



The structure of compound **18** was further confirmed through key 2D correlations. In HMBC, NH proton attached with purine ring showed correlations with quaternary carbonyl carbon of linoloyl chain, and with quaternary carbons C-4, and C-5 of purine ring. Several COSY correlations were also observed among the methylene and methane protons of the linoloyl chain.

The Preparation of Physical Mixtures

The physical mixture of TAF with myristic acid (19). The physical mixture was prepared by mixing 1 mmol of TAF (**9**) with 1 mmol of myristic acid. The mixture was dissolved in 6 mL of THF: MeOH (1:2 *v*/*v*), and then the samples were completely dried under a vacuum overnight.

The physical mixture of TAF with decanoyl chloride (20). The physical mixture was prepared by mixing 1 mmol of TAF (**9**) with 1 mmol of decanoyl chloride. The mixture was dissolved in 6 mL of THF: MeOH (1:2 *v*/*v*), and then the samples were completely dried under a vacuum overnight.

The physical mixture of TAF with butanoic anhydride (21). The physical mixture was prepared by mixing 1 mmol of TAF (**9**) with 1 mmol of butanoic anhydride. The mixture was dissolved in 6 mL of THF: MeOH (1:2 *v*/*v*), and then the samples were completely dried under a vacuum overnight.

The physical mixture of TAF with hexanoic anhydride (22). The physical mixture was prepared by mixing 1 mmol of TAF (**9**) with 1 mmol of hexanoic anhydride. The mixture was dissolved in 6 mL of THF: MeOH (1:2 *v*/*v*), and then the samples were completely dried under a vacuum overnight.

The physical mixture of TAF with octanoic anhydride (23). The physical mixture was prepared by mixing 1 mmol of TAF (**9**) with 1 mmol of octanoic anhydride. The mixture was dissolved in 6 mL of THF: MeOH (1:2 *v*/*v*), and then the samples were completely dried under a vacuum overnight.

The physical mixture of TAF with stearic acid (24). The physical mixture was prepared by mixing 1 mmol of TAF (**9**) with 1 mmol of stearic acid. The mixture was dissolved in 6 mL of THF: MeOH (1:2 *v*/*v*), and then the samples were completely dried under a vacuum overnight.

 doi:10.20944/preprints202510.0367.v1

The physical mixture of TAF with palmitic acid (25). The physical mixture was prepared by mixing 1 mmol of TAF (**9**) with 1 mmol of palmitic acid. The mixture was dissolved in 6 mL of THF: MeOH (1:2 *v*/*v*), and then the samples were completely dried under a vacuum overnight.

The physical mixture of TAF with linoleic acid (26). The physical mixture was prepared by mixing 1 mmol of TAF (**9**) with 1 mmol of linoleic acid. The mixture was dissolved in 6 mL of THF: MeOH (1:2 *v*/*v*), and then the samples were completely dried under a vacuum overnight.

3.3. Cytotoxicity and Anti-HIV Assays

Compounds were dissolved in DMSO and then stored at −20 °C. TZM-bl cells were plated (10^4 cells per well in 96-well plate), and concentrations ranging between 0.01 to 100 ng/mL) of the compounds were applied to the cells the following day with or without HIV in triplicates. For toxicity testing, 100 µL of medium with or without compounds was added to each well for 48 h. The surfactant nonoxynol-9 (N9) was used as a cytotoxic positive control. The media was removed and replaced with 20 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA), and 100 µL of cDMEM media for 3–4 h. Absorbance was read at 490 nm. An HIV-1_{BAL} strain, generously obtained from Dr. Susana Asin's lab (originally from the NIH AIDS repository) was amplified in our lab using Interleukin-2 stimulated human peripheral blood mononuclear cells and also tittered in our lab in TZMbl cells (ATCC). The $TCID_{50}$ (s) were calculated according to the Kaerber formula (Kärber 1931). For antiviral activity (inhibition) testing, we used the Bright-Glo Luciferase Assay System (Promega, USA) following the manufacturer's instructions. Briefly, 100 µL of medium +/- TAF compounds containing HIV-1_{BaL} (5×10^3 $TCID_{50}$) was added to each well. After 48 h, the cells were lysed with 100 µL of Glo Lysis buffer. Lysate 50 µL was transferred into a 96-well black microtiter plate, after which 50 µL of Bright-Glo assay reagent was added, and the luminescence was measured and expressed in relative luminescence units (RLU). The average percentage of infection of the HIV-1_{BaL} growth in three wells exposed to TAF compounds was calculated, and compared to the control (cells exposed to growth medium and HIV; no compounds) 100% infection was detected. The % HIV inhibition is 100 minus % HIV infection.

4. Conclusions

Fatty acyl amide conjugation generated more lipophilic compounds with comparable activity to the parent molecule TAF. Thus, this strategy may be used to improve the cellular penetration, formulation and possibly anti-HIV activity of TAF. This work represents a preliminary study to show anti-HIV of TAF fatty acyl conjugates. However, more research is needed based on the identified templates for the optimization and development of long-acting TFV-based anti-HIV agents.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: H.S., G.F.D., M.I.C., and K.P., planned and designed the experiments; H.S, M.S. A.V. and N.S.E.-S. performed the chemistry; L.A.O. conducted the antiviral and cytotoxicity assays; K.P. and H.S. contributed reagents/materials/analysis tools; L.A.O, G.F.D., H.S., M.I.C. and K.P. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: The biological activities reported in this study was supported from funds from Chapman University School of Pharmacy (KP) and Director's Funds from Eastern Virginia Medical School (LAO).

Institutional Review Board Statement: This study did not involve human subject research and thus did not require IRB review.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

 doi:10.20944/preprints202510.0367.v1

Acknowledgments: The authors also acknowledge the support of the core facility at the Chapman University School of Pharmacy (USA). We also acknowledge the financial support of the Searle Company (Pakistan).

Conflicts of Interest: The authors declare no conflict of interest.

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