European Journal of Chemistry 1 (4) (2010) 297-301

European Journal of Chemistry

Journal homepage: www.eurjchem.com

Synthesis and bioactivity of phosphorylated derivatives of stavudine

Alahari Janardhan Rao[a], Valasani Koteswara Rao[a], Pasupuleti Visweswara Rao[b], Chamarthi Naga Raju[a,*] and Sunil Kumar Ghosh[c]

[a] *Department of Chemistry, Sri Venkateswara University, Tirupati, IN-517502, India*
[b] *Department of Biotechnology, Sri Venkateswara University, Tirupati, IN-517502, India*
[c] *Bio-organic Division, Bhabha Atomic Research Centre, Mumbai, IN-400085, India*

**Corresponding author at: Department of Chemistry, Sri Venkateswara University, Tirupati, IN-517502, India. Tel.: +91.877.2249666; fax: +91.877.2225211.*
E-mail address: rajuchamarthi10@gmail.com (C. N. Raju).

ARTICLE INFORMATION

Received: 28 March 2010
Received in revised form: 13 August 2010
Accepted: 20 September 2010
Online: 31 December 2010

KEYWORDS

Phosphorylated derivatives of stavudine
Gram-positive bacteria
Gram-negative bacteria
Antibacterial activity
Antioxidant activity

ABSTRACT

Novel phosphorylated derivatives of stavudine were synthesized by the reaction of *bis*(2-chloroethyl)phosphoramidic dichloride/4-nitrophenyl phosphorodichloridate with various cyclic amines and amino acid esters in the presence of triethylamine in dry tetrahydrofuran through the corresponding monochloride intermediates 2a-l. Further reaction of the intermediates 2a-l with stavudine in tetrahydrofuran and pyridine in the presence of triethylamine formed the title compounds 4a-l. Their structures were characterized by IR, ^{1}H-, ^{13}C-, ^{31}P-NMR and mass spectral data analyses. They exhibited good antibacterial and antioxidant activities. Their bioactivity was greatly influenced by the different groups present at the phosphorus.

1. Introduction

Nucleoside analogues continue to dominate over other drugs in chemotherapy of cancer and antiviral diseases. Some of the nucleoside analogues with such an activity are ddC, FMAU, OddC, BCH-189, AZT. Stavudine (2',3'-dideoxy-2',3'-didehydrothymidine; d4T) is licensed for use in the human immunodeficiency virus (HIV) infection [1,2]. Nucleosides to become bioactive are to be phosphorylated by cellular kinases to give successively corresponding 5'-mono-, -di, and –triphosphates. It is reported that intracellular phosphorylation of stavudine via monophosphate and diphosphate, to the active triphosphate anabolite which inhibits HIV reverse transcriptase and terminates the elongating DNA chain [3-5]. The efficiency of this phosphorylation process is extremely slow. Therefore, many efforts have been made to improve therapeutic properties by taking at least the first phosphorylation step. This approach has resulted in the synthesis of potential numerous nucleotide analogues as drugs [6-10].

These potent biological activities of stavudine have stimulated great interest in the synthesis of phosphorylated compounds for extensive studies related to their biological activities. In view of these, we have focused on the synthesis of a series of novel phosphorylated derivatives of stavudine with several bioactive groups at the phosphorus and evaluated antioxidant and antibacterial properties. The oxidative stress of stavudine derivatives was assessed by estimating their activities on DPPH using natural antioxidant Vitamin C as standard. Antibacterial activity was investigated against Gram positive bacteria: *Bacillus subtilis*, *Staphylococcus aureus* and Gram negative bacteria: *Escherichia coli* and *Klebsiella pneumoniae*. The structures of all the newly synthesized compounds **4a-l** have been established by elemental analyses and spectral data (IR, ^{1}H, ^{13}C, ^{31}P NMR and MS).

2. Experimental

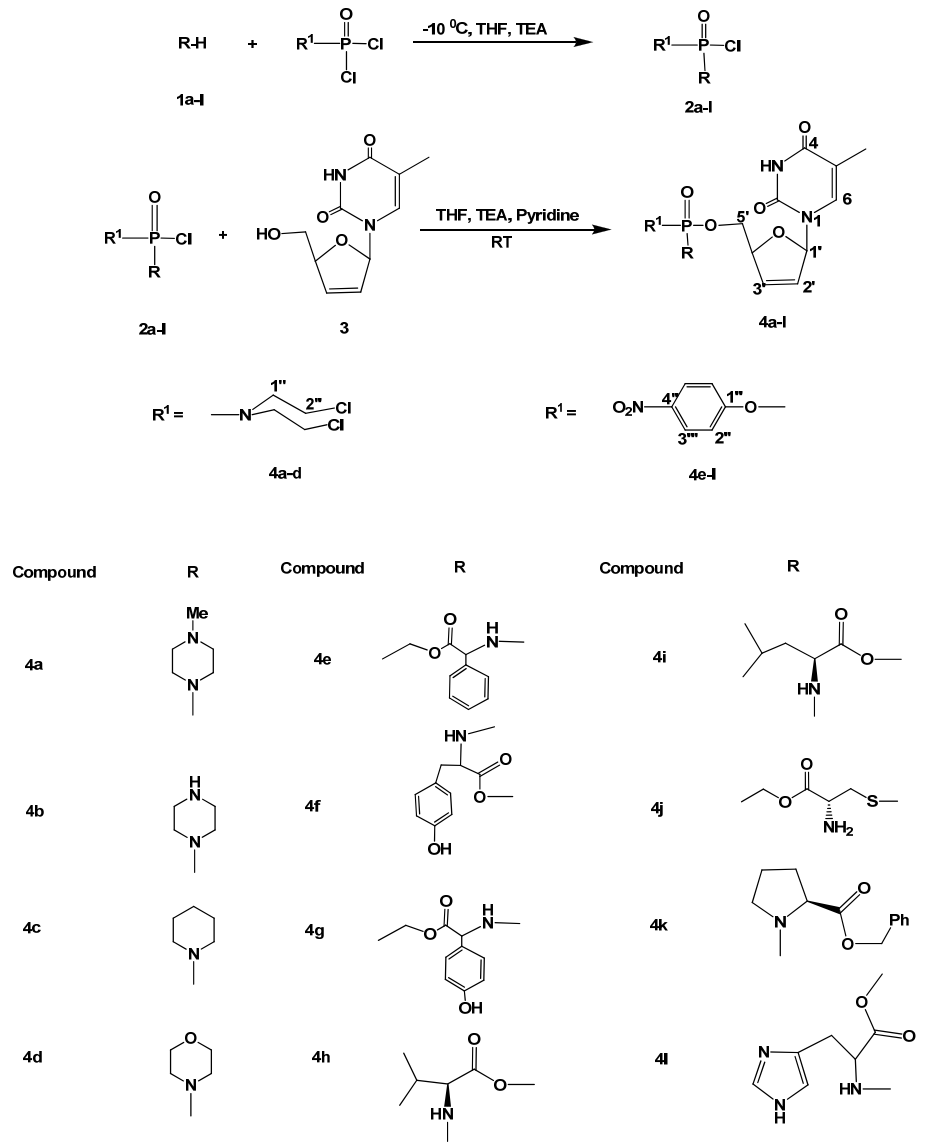
2.1. Instrumentation

Sigma-Aldrich, Merck and Lancaster chemicals were used as such without further purification. All solvents used for spectroscopic and other physical studies were reagent grade and were further purified by literature methods [11]. Melting points were determined by Guna Digital Melting Point apparatus using a calibrated centigrade thermometer. IR spectra were recorded in KBr disks on a Perkin-Elmer Model 281-B spectrophotometer in wave numbers (cm^{-1}). ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded as solutions in DMSO-d_6 on a Bruker AVANCE III 500 MHz spectrometer operating at 500 MHz for ^{1}H, 125 MHz for ^{13}C and 202.4 MHz for ^{31}P nuclei and their chemical shifts were expressed in ppm with reference to tetramethylsilane and 85 % H_3PO_4 respectively. Mass spectra were recorded on a JEOL GCMATE II Mass spectrometer. Elemental analyses were performed by Central Drug Research Institute, Lucknow, India.

2.2. Synthesis

A solution of various cyclicamines and amino acid esters (0.002 mole), in dry tetrahydrofuran (THF) (10 mL) and triethylamine (TEA) (0.002 mole), was added dropwise over a period of 15 minutes to a stirred solution of *bis*(2-chloroethyl)phosphoramidicdichloride/4-nitro phenylphos phorodichloridate (0.002 mole) in 20 mL of THF at -10-0 ^{o}C (Scheme 1).

ISSN 2153-2249 (Print) / ISSN 2153-2257 (Online)
DOI:10.5155/eurjchem.1.4.297-301.20



Scheme 1

After stirring for 2 hours, formation of intermediate monochlorides **2a-l** was ascertained by thin layer chromatography performed in 7:3 mixture of ethyl acetate and hexane. To this stavudine (0.002 mole) in 10 mL of pyridine and TEA (0.002 mole), was added dropwise at 0 °C. After completion of the addition, the reaction temperature was slowly raised to RT and continued stirring for an additional 10 h. The progress of the reaction was monitored by TLC run in 9:1 mixture of ethylacetate and hexane. The reaction mixture was filtered to remove TEA hydrochloride and the solvent in the filtrate was removed in a rotaevaporator. The crude product was purified by column chromatography on silica gel (60-120 mesh) with chloroform: methanol (9:1) as eluent to afford pure **4a-l** which were characterized by IR, ^{1}H, ^{13}C, ^{31}P NMR and mass spectral data analyses.

2.2.1. (R)-(5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl)methyl-N,N-bis(2-chloro ethyl)-P-(4-methylpiperazin-1-yl)phosphonamidate (4a)

Yield: 55%. M.p.: 155-158 °C. IR (KBr, cm^{-1}): 3393 ν(-NH), 1683 ν(C=O), 1226 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 8.5(s, 1H, NH), 7.54 (d, 1H, *J*=1.5 Hz, H-6), 6.86-6.88 (m, 1H, H-1'), 6.47-6.49 (m, 1H, H-2'), 5.95-5.97 (m, 1H, H-3'), 4.13-4.21 (m, 3H, H-4', 2H-5'), 3.95 (t, 4H, CH_2-N), 3.65 (t, 4H, CH_2-Cl), 2.74 (t, 4H, CH_2N-), 2.49 (t, 4H, CH_2N), 2.32 (s, 3H, N-Me), 1.87 (s, 3H, CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6): 164.5 (C-4), 159.2 (C-2), 137 (C-6), 135.1(C-3'), 125.1 (C-2'), 110.5 (C-5), 89.2 (C-1'), 85.5 (C-4'), 68.2 (C-5'), 55.8 (pipCH_2), 51.2 (C-2''), 47.8 (pipCH_2), 45.9 (N-CH_3), 41.6 (C-3''), 12.1 (-CH_3). ^{31}P-NMR (202 MHz, DMSO-d_6): δ -1.94, -1.24. GC-MS (m/z, %): (510.25, 45%) $[MH]^+$.

2.2.2. (R)-(5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl)methyl-N,N-bis(2-chloro ethyl)-P-(piperazin-1-yl)phosphonamidate (4b)

Yield: 62%. M.p.: 161-163 °C, Anal. Calcd. for $C_{18}H_{28}Cl_2N_5O_5P$: C, 43.56; H, 5.69; N, 14.11; Found C, 43.59; H, 5.73; N, 14.05. IR (KBr, cm^{-1}): 3386 ν(-NH), 1685 ν(C=O), 1230 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 8.9 (s, 1H, NH), 7.56 (d, 1H, *J*=1.5 Hz, H-6), 6.88-6.9 (m, 1H, H-1'), 6.47-6.49 (m, 1H, H-2'), 5.97-5.99 (m, 1H, H-3'), 4.15-4.23 (m, 3H, H-4', 2H-5'), 4.01 (t, 4H, CH_2-N), 3.69 (t, 4H, CH_2-Cl), 2.78 (t, 4H, CH_2N-), 2.54

(t, 4H, CH_2NH), 2.19 (s, 1H, NH), 1.87 (s, 3H, CH_3). ^{31}P-NMR (202 MHz, DMSO-d_6, δ) -1.03.

2.2.3. (R)-(5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl)methyl-N,N-bis(2-chloro ethyl)-P-(piperidin -1-yl) phosphonamidate (4c)

Yield: 61%. M.p.: 174-176 °C, Anal. Calcd. for $C_{19}H_{29}Cl_2N_4O_5P$: C, 46.07; H, 5.90; N, 11.31; Found C, 46.12.; H, 5.95; N, 11.25. IR (KBr, cm^{-1}): 3378 ν(-NH), 1686 ν(C=O), 1234 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 8.8 (s, 1H, NH), 7.5 (d, 1H, *J*=1.5 Hz, H-6), 6.84-6.88 (m, 1H, H-1'), 6.46-6.48 (m, 1H, H-2'), 5.93-5.95 (m, 1H, H-3'), 4.15-4.28 (m, 3H, H-4', 2H-5'), 3.94 (t, 4H, CH_2N), 3.63 (t, 4H, -CH_2Cl), 2.75(t, 4H, CH_2N-), 1.87 (s, 3H, CH_3),1.46-1.56(m, 6H, $(CH_2)3$). ^{31}P-NMR (202 MHz, DMSO-d_6, δ): 2.85.

2.2.4. (R)-(5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl)methyl-N,N-bis(2-chloro ethyl)-P-morpholinophosphonamidate (4d)

Yield: 55%. M.p.: 167-169 °C, IR (KBr, cm^{-1}): 3394 ν(-NH), 1685 ν(C=O), 1225 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 8.6 (s, 1H, NH), 7.7 (d, 1H, *J*=1.5 Hz, H-6), 6.95 -6.97 (m, 1H, H-1'), 6.45-6.47 (m, 1H, H-2'), 5.9-5.92 (m, 1H, H-3'), 4.15-4.23 (m, 3H, H-4', 2H-5'), 3.66 (t, 4H, CH_2-O), 3.6 (t, 4H, CH_2N), 3.06 (t, 4H, CH_2-Cl), 2.64 (t, 4H, CH_2N-), 1.0 (s, 3H, CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6): 163.9 (C-4), 158.4 (C-2), 136.5 (C-6), 134.5(C-3'), 125.8 (C-2'), 111.2 (C-5), 91.2 (C-1'), 86.5 (C-4'), 69.2 (C-5'), 62.8 (morCH_2), 52.5 (C-2''), 45.8 (morCH_2), 42.6 (C-3''), 12.1 (-CH_3). ^{31}P-NMR (202 MHz, DMSO-d_6, δ): -2.15, -1.51. GCMS (m/z; %): (497.25, 30%) $[MH]^+$.

2.2.5. Ethyl2-((S)-((5-(5-methyl-2,4-dioxo-3,4-dihydro pyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl)methoxy) (4-nitrophenoxy) phosphorylamino)-2-phenylacetate (4e)

Yield: 59%. M.p.: 149-152 °C, IR (KBr, cm^{-1}): 3389 ν(-NH), 1681, 1729 ν(C=O), 1236 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 11.2 (s, 1H, NH), 8.16 (d, 2H, *J*=9 Hz, Ar-H), 8.1 (d, 2H, *J*=9.5 Hz, Ar-H), 7.57 (d, 1H, *J*=1 Hz, H-6), 7.23-7.4 (m, 5H, Ar-H), 6.8-6.81 (m, 1H, H-1'), 6.31-6.33 (m, 1H, H-2'), 5.89-5.90 (m, 1H, H-3'), 6.54 (brs, 1H, Gly-NH), 3.57-3.63 (m, 4H, 2H-5', H-4', Gly-NH-CH), 3.04 (q, 2H, -OCH_2), 1.73 (s, 3H, CH_3), 1.2 (t, 3H, O-CH_2-CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6): 171 (Gly CO), 164.3 (C-4), 161.2 (C-1''), 151.2 (C-2), 142 (C-4''), 137.1 (C-6), 134.6 (GlyArC1), 129.2 (C-3'), 128.7 (GlyArC-2 & C-6), 128.1 (GlyArC-3 & C-5), 127.5 (GlyArC-4), 126.7 (C-3'' & C-5''), 125.5 (C-2'' & C-6''), 110 (C-5), 89.1 (C-1'), 85.8 (C-4'), 66.2 (C-5'), 58.14 (O-CH_2-CH_3), 45.7 (Gly-CH), 12.2 (O-CH_2-CH_3), 11.3 (CH_3). ^{31}P-NMR (202 MHz, DMSO-d_6, δ): 5.78. GCMS (m/z; %): (586.2, 35%) $[MH]^+$.

2.2.6. Methyl3-(4-hydroxyphenyl)-2-((S)-((5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl) methoxy)(4-nitrophenoxy)phosphorylamino) propanoate (4f)

Yield: 62%. M.p.: 154-156 °C IR (KBr, cm^{-1}): 3378 ν(-NH), 1678, 1723 ν(C=O), 1218 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 8.5 (s, 1H, NH), 7.92 (d, 2H, *J*=9.5 Hz, Ar-H), 7.49 (d, 1H, *J*=1.5 Hz, H-6), 7.38 (d, 2H, *J*=9 Hz, Ar-H), 7.15 (d, 2H, *J*=8.5 Hz, Ar-H), 6.89 (d, 2H, *J*=9 Hz, Ar-H), 6.78-6.79 (m, 1H, H-1'), 6.3-6.32 (m, 1H, H-2'), 5.87-5.89 (m, 1H, H-3'), 5.47 (s, 1H, Tyr-OH), 4.7 (brs, 1H, Tyr-NH), 3.91-3.97 (m, 4H, 2H-5', H-4', Tyr-NH-CH), 3.51-3.53 (m, 2H, Tyr-CH_2), 3.32 (s, 3H, -OCH_3), 4.71 (s, 3H, CH_3), ^{31}P-NMR (202 MHz, DMSO-d_6, δ): 5.91, 6.54.

2.2.7. Ethyl2-(4-hydroxyphenyl)-2-((S)-((5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl) methoxy) (4-nitrophenoxy)phosphorylamino)acetate (4g)

Yield: 57%. M.p.: 167-169 °C, Anal. Calcd. for $C_{26}H_{27}N_4O_{11}P$: C, 51.83; H, 4.52; N, 9.30; Found C, 51.79; H, 4.47; N, 9.35. IR (KBr, cm^{-1}): 3385 ν(-NH), 1683, 1725 ν(C=O), 1230 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 11.1 (s, 1H, NH), 8.18 (d, 2H, *J*=9 Hz, Ar-H), 8.11 (d, 2H, *J*=9.5 Hz, Ar-H), 7.51 (d, 1H, *J*=1.5 Hz, H-6), 7.25-7.43 (m, 5H, Ar-H), 6.79-6.81 (m, 1H, H-1'), 6.29-6.31 (m, 1H, H-2'), 5.87-5.89 (m, 1H, H-3'), 5.54 (s, 1H, Gly-OH), 4.4 (brs, 1H, GLY-NH), 3.55-3.61 (m, 4H, 2H-5', H-4', Gly-NH-CH), 3.11 (q, 2H, -OCH_2), 1.71 (s, 3H, CH_3), 1.21 (t, 3H, O-CH_2-CH_3). ^{31}P-NMR (202 MHz, DMSO-d_6, δ): 4.24.

2.2.8. (2S)-Methyl-3-methyl-2-((S)-((5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl) methoxy) (4-nitrophenoxy)phosphorylamino)butanoate (4h)

Yield: 59%. M.p.: 145-147 °C IR (KBr, cm^{-1}): 3386 ν(-NH), 1678, 1731 ν(C=O), 1220 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 8.1(s, 1H, NH), 8.0 (d, 2H, *J*=9 Hz, Ar-H), 7.51 (d, 1H, *J*=1 Hz, H-6), 7.32 (d, 2H, *J*=9 Hz, Ar-H), 6.8-6.81 (m, 1H, H-1'), 6.32-6.34 (m, 1H, H-2'), 5.89-5.90 (m, 1H, H-3'), 4.8 (brs, 1H, Val-NH), 3.94-4.00 (m, 4H, 2H-5', H-4', Val-NH-CH), 3.01 (s, 3H, -OCH_3), 2.51-2.52 (m, 1H, $(CH_3)2CH$), 1.71 (s, 3H, CH_3), 1.15-1.18 (d, 6H, $(CH_3)_2C$-). ^{13}C-NMR (125 MHz, DMSO-d_6, δ): 172.5 (Val-C=O), 164.39 (C-4), 159.8 (C-2),151.2 (C-1''), 142.3 (C-4''), 137 (C-6), 134.5 (C-3'), 126.7 (C-3'', C-5''), 125.4 (C-2'), 120.7 (C-2'', C-6''), 110.9 (C-5), 89.2 (C-1'), 85.7 (C-4'), 66.4 (C-5'), 46 (Val-CH), 43.1(-OCH_3), 33.2 $(CH_3)_2C$-), 18.9 $(CH_3)_2C$-), 12.2 (-CH_3). ^{31}P-NMR (202 MHz, DMSO-d_6, δ): 2.85. GCMS (m/z; %): (539.25, 27%) $[MH]^+$.

2.2.9. (2S)-Methyl 4-methyl-2-((S)-((5-(5-methyl-2, 4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl)methoxy)(4-nitrophenoxy)phosphorylamino)pentanoate (4i)

Yield: 63%. M.p.: 151-153 °C . IR (KBr, cm^{-1}): 3387 ν(-NH), 1675, 1728 ν(C=O), 1232 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 8.7 (s, 1H, NH), 8.21 (d, 2H, *J*=7 Hz, Ar-H), 7.6 (d, 1H, *J*=1 Hz, H-6), 7.48 (d, 2H, *J*=7.5 Hz, Ar-H), 6.94-6.95 (m, 1H, H-1'), 6.3-6.33 (m, 1H, H-2'), 5.89-5.92 (m, 1H, H-3'), 5.2 (brs, 1H, Leu -NH), 3.32-3.36 (m, 4H, 2H-5', H-4', Leu-NH-CH), 3.21 (s, 3H, -OCH_3), 1.9-1.93 (m, 2H, Leu-CH_2), 1.51-1.52 (m, 1H, $(CH_3)_2CH$), 1.71 (s, 3H, CH_3), 1.17-1.2 (d, 6H, $(CH_3)_2C$-). ^{31}P-NMR (202 MHz, DMSO-d_6, δ): 3.40, 4.11. GCMS (m/z; %): (553.35, 35%) $[MH]^+$.

2.2.10. (2R)-Ethyl 2-amino-3-((R)-((5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl) methoxy)(4-nitrophenoxy)phosphorylthio)propanoate (4j)

Yield: 59%. M.p.: 138-141 °C, Anal. Calcd. for $C_{21}H_{25}N_4O_{10}PS$: C, 45.32; H, 4.53; N, 10.07;Found C, 45.27; H, 4.49; N, 10.11. IR (KBr, cm^{-1}): 3375 ν(-NH), 1677, 1727 ν(C=O), 1228 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 9.4 (s, 1H, NH), 8.21 (d, 2H, *J*=9.5 Hz, Ar-H), 8.11(d, 2H, *J*=9 Hz, Ar-H), 7.43 (d, 1H, *J*=1 Hz, H-6), 6.95-6.97 (m, 1H, H-1'), 6.29-6.31 (m, 1H, H-2'), 5.91-5.93 (m, 1H, H-3'), 5.54 (bs, 2H, -NH_2,), 3.92-3.98 (m, 4H, 2H-5', H-4', Cys-CH- NH_2), 3.65 (q, 2H, -O-CH_2-CH_3), 2.97 -3.05 (m, 2H, Cys -CH_2), 1.73 (s, 3H, CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6, δ): 172.6 (Cys-C=O), 159.1 (C-4), 158.1 (C-2), 143.2 (C-1''), 142.6 (C-4''), 137.5 (C-6), 135.1 (C-3'), 125 (C-3'', C-5''), 124 (C-2'), 120.5 (C-2'', C-6''), 111.1 (C-5), 89.8 (C-1'), 86.1 (C-4'), 67.4 (C-5'), 61.1 (O-CH_2), 53.7 (Cys-CH), 22.1 (Cys-CH_2), 13.8 (-O-CH_2-CH_3), 12.1 (-CH_3). ^{31}P-NMR (202 MHz, DMSO-d_6, δ): 7.85.

Table 1. Antibacterial activity of **4a-l.**

Compounds	Zone of Inhibition in mm (100 µg/mL)			
	B. subtilis	*S. aureus*	*E. coli*	*K. pneumoniae*
4a	12	1 0	11	10
4b	12	12	10	11
4c	10	10	11	13
4d	11	13	14	12
4e	-	-	9	8
4f	12	11	16	9
4g	10	8	12	10
4h	11	9	12	11
4i	13	12	13	13
4j	13	11	13	14
4k	10	15	12	11
4l	11	12	17	9
Ampicillin	11	9	13	10

2.2.11. Benzyl1-((S)-((5-(5-methyl-2,4-dioxo-3,4-dihydro pyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl)methoxy) (4-nitro-phenoxy) phosphoryl)pyrrolidine-2-carboxylate **(4k)**

Yield: 62 %. M.p.: 171-173 °C. Anal. Calcd. for $C_{28}H_{29}N_4O_{10}P$: C, 54.90; H, 4.77; N, 9.15; Found C, 54.95; H, 4.79; N, 9.11. IR (KBr, cm^{-1}): 3379 ν(-NH), 1680, 1728 ν(C=O), 1234 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 9.1 (s, 1H, NH), 8.14 (d, 2H, *J*=9.5 Hz, Ar-H), 7.49 (d, 1H, *J*=1.5 Hz, H-6), 7.39 (d, 2H, *J*=9 Hz, Ar-H), 7.20-7.32 (m, 5H, Ar-H), 6.79-6.80 (m, 1H, H-1'), 6.33-6.35 (m, 1H, H-2'), 5.90-5.91 (m, 1H, H-3'), 5.38 (s, 2H, Ph-CH_2), 3.91-3.98 (m, 4H, 2H-5', H-4', Prol-CH), 2.83-2.87 (m, 2H, N-CH_2), 1.92-1.97 (m, 4H, $(CH_2)_2$), 1.71 (s, 3H, CH_3), ^{31}P-NMR (202 MHz, DMSO-d_6, δ): 2.12.

2.2.12. Methyl3-(1H-imidazol-4-yl)-2-((((2S,5R)-5-(5-methyl-2,4-dioxo-3,4dihydropyrimidin-1(2H)-yl)-2,5-dihydrofuran-2-yl) methoxy) (4-nitrophenoxy)phosphorylamino) propanoate **(4l)**

Yield: 65 %. M.p.: 164-167 °C, Anal. Calcd. for $C_{23}H_{25}N_6O_{10}P$: C, 47.92; H, 4.37; N, 14.58; Found C, 47.87; H, 4.34; N, 14.65. IR (KBr, cm^{-1}): 3385 ν(-NH), 1680, 1731 ν(C=O), 1226 ν(P=O). ^{1}H-NMR (500 MHz, DMSO-d_6, δ): 11.2 (br s, 1H, NH), 9.2 (s, 1H, NH), 8.65-8.67 (m, 1H, N=CH), 8.21 (d, 2H, *J*=9.5 Hz, Ar-H), 7.51 (d, 1H, *J*=1 Hz, H-6), 7.38-7.44 (m, 3H, NH-CH, 2Ar-H), 6.8-6.81 (m, 1H, H-1'), 6.32-6.34 (m, 1H, H-2'), 5.89-5.90 (m, 1H, H-3'), 5.2 (brs, 1H, -NH), 3.96-4.04 (m, 4H, 2H-5', H-4', His-CH), 3.34-3.38 (m,2H, His-CH_2), 3.21 (s, 3H, -OCH_3). ^{31}P-NMR (202 MHz, DMSO-d_6, δ): 3.31.

2.3. Biological Activity

2.3.1. Antibacterial activity

Compounds **4a-l** were screened for their antibacterial activity against Gram positive bacteria, *Staphylococcus aureus*, *Bacillus subtilis* and Gram negative bacteria, *Escherichia coli*, *Klebsiella pneumoniae* by the cup plate method [12] in nutrient agar medium at the concentration of 100 µg/mL in DMSO. Nutrient agar plates were prepared by pouring 20 mL of nutrient agar in sterile Petri dishes for antibacterial assay. Concentration of these organisms was prepared to contain approximately 1×10^6 cfu/mL. The tubes were then inoculated with 0.1 mL of the appropriate culture suspension of bacterium mixed gently and poured onto previously solidified nutrient agar. After setting, a cup borer (6 mm diameter) was properly sterilized by flaming and used to make four uniform cups in each Petri dish. The cups were then filled with the test compounds and allowed to diffuse for 45 minutes. Ampicillin (100 µg/disc) was used as standard for bacteria. The plates were incubated at 37 °C for 24 hours. At the end of the period, inhibition zones formed on the medium were evaluated in mm using a scale. The experiment was carried out in triplicates. The results (Table 1) were compared with that of the standard antibiotic Ampicillin (100 µg/mL).

2.3.2. Antioxidant activity

The antiradical activity of **4a-l** was measured in triplicate using a modified Burits and Bucar method [13]. A methanolic solution of DPPH (4 mL, 40 ppm) was added to 1 mL of the antioxidant solutions in 0.1 M Tris–HCl buffer (pH=7.1) at 25 °C, giving final concentrations of 10, 25, 50 and 100 µM. The absorbance read at 517 nm was measured after 60 min of the reaction in the dark and compared with the control prepared in a similar way without the addition of the antioxidants (this value was assigned arbitrarily to 100%). A 100 µM solution of ascorbic acid (a strong and natural antioxidant agent) was also tested as a control for the reaction. In this case, the violet color of DPPH disappeared immediately. It is confirmed that the test compounds showed better antioxidant activity when compared to the parent drug stavudine and moderate activity when compared with that of Vitamin C (Table 2).

Antioxidant activity (%) = $[(A_{517}\text{control} - A_{517}\text{sample}) / A_{517}\text{control}] \times 100$ (1)

Table 2. DPPH radical scavenging activity of (**4a-l**).

Compounds	IC_{50} / DPPHvalues(µg/mL)
4a	150.66 ± 6.95
4b	187.56 ± 9.31
4c	149.33 ± 4.43
4d	122.79 ± 2.51
4e	110.39 ± 7.55
4f	119.06± 0.84
4g	162.33 ± 5.45
4h	127.49 ± 6.47
4i	112.94 ± 4.73
4j	132.67 ± 7.76
4k	157.56 ± 5.28
4l	162.71 ± 3.37
Stavudine	244.82 ± 6.21
Vitamin C	71.30 ± 1.66

3. Results and discussion

The synthesis of the phosphorylated derivatives of stavudine **4a-l** is depicted in Scheme 1. Cyclic amines and amino acid esters were reacted with *bis*(2-chloroethyl) phosphoramidic dichloride and 4-nitro phenyl phosphoro dichloridate at -10 °C (Scheme 1), in the presence of TEA in THF. The progress of the reaction was monitored by TLC. After completion of the reaction in 2 h, a solution of stavudine and TEA in pyridine was added to the reaction mixture at 10 °C and the mixture was stirred for 10 h at RT. The reaction was completed as indicated by TLC, (ethylacetate:hexane, 9:1), TEA hydrochloride was removed by filtration and the solvent from the filtrate was removed in vacuum. The residue was column chromatographed on silica and eluted with 1% methanol in chloroform. All the compounds **4a-l** exhibited characteristic infrared absorption bands for P=O, C=O and NH in the regions 1218-1236, 1683-1731 and 3394-3375 cm^{-1}, respectively [14].

The chemical shifts of the NH protons in thymine ring were observed as a singlet in the region d 8.1-11.2. [15-16]. The H-6' proton signal appeared as a doublet in the region 7.43-7.7 ppm [17]. In ^{13}C NMR the chemical shifts of the carbonyl carbon of thymine ring resonated at δ 164.5-150.2 [17]. The ^{31}P NMR chemical shifts appeared in the region -1.24 to 7.85 ppm [18]. The compounds **4a**, **4d**, **4e**, **4h** and **4i** exhibited molecular ion peaks with moderate intensity in their GC mass spectra.

The compound **4j** showed high activity against Gram negative bacteria (*K. pneumoniae*). The compounds **4f** and **4l** exhibited higher activity against *Escherichia coli* when compared to that of the standard. The compound **4k** exhibited high activity, against Gram positive bacterium *Staphylococcus aureus* when compared to that of the ampicillin. Majority of the compounds exhibited significant antibacterial activity (Table 1).

Majority of the compounds showed significant activities for DPPH radical scavenging when compared to stavudine and Vitamin C (Table 2). The potency of these radical scavenging activities was mainly influenced by the fragments attached to the phosphorylated stavudine. The fragments 4-nitrophenyl phosphoryl moiety and various cyclic amines and amino acid esters linked to phosphorus led to increase in DPPH radical scavenging activity (i.e. **4e** and **4i**). 4-Nitrophenyl phosphoryl and phenyl glycine ethyl ester moieties were found to be the most potent fragments for increasing radical scavenging activity.

4. Conclusion

In conclusion, a series of novel phosphorylated derivatives of stavudine were synthesized and evaluated for their antimicrobial and antioxidant properties. Structure activity studies showed that the antioxidant and antimicrobial potency was mainly influenced by the functional groups at the end of the aliphatic chain, as well as the nature of atom attached directly to phosphorus atom and the two different types of R fragments attached to phosphorus atom, which provided additional sites of interactions between the inhibitor and the enzyme, which constitutes a key element for enhanced affinity. The results suggest that the compounds **4e**, **4f** and **4i** showed promising antioxidant activities.

Acknowledgement

We thank BRNS (DAE), BARC, Mumbai for the financial support (Project No.: (2007/37/46/BRNS/2916), dated 31-03-2008.

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