RAJESHWARI & ASSOCIATES
Trademark & patent attorneys

S – 357, First Floor
Near HDFC Bank
Panchsheel Park
New delhi – 110017, India
Tel: + +91-11-41038911
Fax: +91-11-43851067

December 05, 2020

The Controller of Patent
The Patent Office
Boudhik Sampada Bhawan, Plot No. 32
Sector 14, Dwarka, New Delhi-110078

Re: **REPRESENTATION u/s 25(1) of the Patent act – By SANKALP REHABILITATION TRUST against Indian Patent Application No. 201617031899 filed on 19/09/2016**
Applicant: **MERCK SHARP & DOHME CORP.**

Respected Sir,

We submit herewith Pre-Grant Opposition under Section 25(1) of the Patent Act, 2005 along with evidence and Form 7A.

The Controller is requested to take the documents on record and proceed further in the matter and keep the Petitioner advised of each and every step taken in the matter.

We crave the leave of the Controller to submit additional documents or evidence or if necessary to support any of the averments in the representation as may be necessitated in the proceeding.

Lastly, we request the Controller to grant an opportunity of being heard before the above representation is finally decided.

Thanking you,

Yours faithfully,

RAJESHWARI H. IN/PA - 0358
FOR RAJESHWARI AND ASSOCIATES
AGENT FOR THE OPPONENT

Encl: As stated

C.C.: ANAND AND ANAND
B-41, NIZAMUDDIN EAST
NEW DELHI – 110013
Email.: email@anandandanand.com

BEFORE THE CONTROLLER OF PATENTS, NEW DELHI

IN THE MATTER OF:

The Patents Act, 1970 as amended by the Patents (Amendment) Act 2005, and The Patents Rules, 2003, as amended by The Patents (Amendment) Rules, 2006
AND
IN THE MATTER of Pre-grant opposition under Section 25(1)
AND
IN THE MATTER of Indian Patent Application No. 201617031899

IN THE MATTER OF:

SANKALP REHABILITATION TRUST ….. OPPONENT

VS.

MERCK SHARP & DOHME CORP. .....APPLICANT

PRE-GRANT OPPOSITION BY SANKALP REHABILITATION CENTRE

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Dated this day 05th of December, 2020

RAJESHWARI H.
AGENT FOR THE OPPONENT
RAJESHWARI AND ASSOCIATE

To,
The Controller of Patents
The Patent Office, New Delhi

FORM 7A
THE PATENTS ACT,
1970 (39 OF 1970)
AND
THE PATENTS RULES, 2003
REPRESENTATION FOR OPPOSITION TO GRANT OF PATENT
[See Rule 55]

We, **SANKALP REHABILITATION TRUST**, having its registered office at SS Bengali Municipal School, First Floor, Thakurdwar Road, Charni Road East, Mumbai – 400002, hereby give Notice of opposition to the grant of patent in respect of Indian Patent Application No. 201617031899 dated 19th September, 2016 made by MERCK SHARP & DOHME CORP. on the grounds.

(a) Section 25(1)(b): Lack of novelty
(b) Section 25(1)(e): Lack of inventive step
(c) Section 25(1)(f): Invention is not patentable under section 3 (d)
(d) Section 25(1)(g): The complete specification does not sufficiently and clearly describe the invention or the method by which it is to be performed.
(e) Section 25(1)(h): Failed to disclose to the Controller the information required by section 8.

(Detailed grounds are set out in the Opposition)

Our address for service in India is:

RAJESHWARI H.
RAJESHWARI & ASSOCIATES
A – 202, FIRST FLOOR
SHIVALIK ENCLAVE
MALVIYA NAGAR
NEW DELHI – 110017
INDIA
Tel: + 91-11-41038911
Fax: +91-11-43851067
Mobile No. 9910206718
Email: rajeshwari@ralegal.co.in; patent@ralegal.co.in

Dated this 05th day of December, 2020

RAJESHWARI H. IN/PA – 0358
AGENT FOR THE OPPONENT
OF RAJESHWARI AND ASSOCIATES

To
The Controller of Patents
Patent Office, New Delhi

BEFORE THE CONTROLER OF PATENTS, THE PATENT OFFICE, NEW DELHI

In the matter of Section 25(1) of The Patents Act,1970 as amended by The Patents (Amendment) Act 2005;

And

In the matter of Rule 55 of The Patents Rules 2003 as amended by thePatent (Amendment) Rules, 2006

And

IN THE MATTER of Indian Patent Application 201617031899 filed on 19/09/2016 in the name of MERCK SHARP & DOHME CORP.

REPRESENTATION BY:

SANKALP REHABILITATION TRUST ..... OPPONENT

VS.

MERCK SHARP & DOHME CORP. .....APPLICANT

REPRESENTATION BY WAY OF PRE-GRANT OPPOSITION UNDER SECTION 25(1) OF THE PATENTS ACT, 1970

We, **SANKALP REHABILITATION TRUST,** an Indian citizen, hereby submit my representation by way of oppostion to the grant of patent in respect of Indian Patent Application 201617031899 filed on 19/09/2016 in the name of MERCK SHARP & DOHME CORP. entitled "4 SUBSTITUTED NUCLEOSIDE DERIVATIVES AS HIV REVERSE TRANSCRIPTASE INHIBITORS".

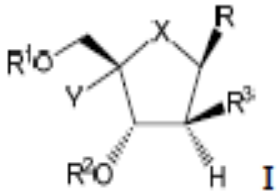
STATEMENT OF CASE OF OPPONENT

1. The Opponent has learnt that the Applicant has filed an Indian Patent Application No. 201617031899 (hereinafter "the Impugned Application") on 19/09/2016. The Impugned application was published in the Official Journal of the patent office on 13/01/2017, which is currently pending before the Patent Office.

2. The Impugned application is entitled "4 SUBSTITUTED NUCLEOSIDE DERIVATIVES AS HIV REVERSE TRANSCRIPTASE INHIBITORS".

3. The opponent by way of this present pre-grant opposition submits that the claims currently pending on record are not patentable under the provisions provided in this Act. The claims as filed and currently on record are annexed herewith as **Annexure-1** and reproduced herein below for ready reference:

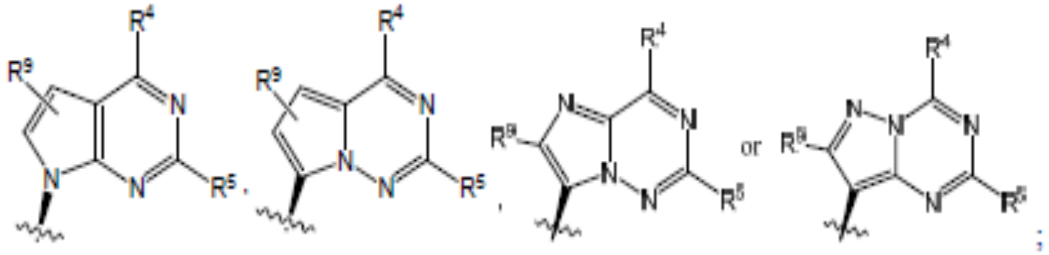
1. A compound of structural Formula I



I

or a pharmaceutically acceptable salt thereof, wherein:

R is

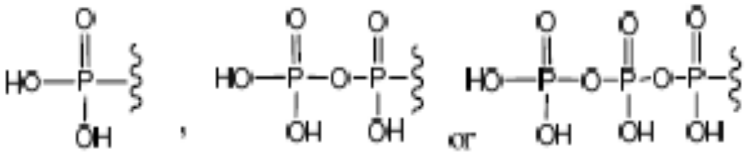


;

X is O, S, CH_2 or CF_2;

Y is $-C\equiv C-R^8$ or $-C\equiv N$;

$\mathbf{R^1}$ is -H, $-C(O)R^6$, $-C(O)OR^6$, $-C(O)N(R^6)_2$,



,

or a pro-drug modification of the mono-, di- or triphosphate;

$\mathbf{R^2}$ is -H, $-C(O)R^{6a}$, $-C(O)OR^{6a}$ or $-C(O)N(R^{6a})_2$;

$\mathbf{R^3}$ is –H;

$\mathbf{R^4}$ is -H, $-C_1$-C_6 alkyl, $-C_2$-C_6 alkenyl, $-C_2$-C_6 alkynyl, $-C_1$-C_6 haloalkyl, $-C_3$-C_7 cycloalkyl, 5- or 6-membered monocyclic heteroaryl, a 9- or 10-membered bicyclic heteroaryl, halo, -CN, $-NO_2$, $-N(R^X)_2$, $-NH(C_1$-C_6alkylene)-(5- or 6-membered monocyclic heteroaryl), $-NH(C_1$-C_6 alkylene)-(9- or 10-membered bicyclic heteroaryl), aryl, $-NHC(O)OR^{6b}$, $-N(C(O)OR^{6b})_2$, $-NHC(O)N(R^{6b})_2$, or $-NHC(O)R^{6b}$, wherein each of said $-C_1$-C_6 alkyl group, said $-C_2$-C_6 alkenyl group or said $-C_2$-C_6 alkynyl group can be optionally substituted with halo;

$\mathbf{R^5}$ is -H, $-C_1$-C_6 alkyl, $-C_2$-C_6 alkenyl, $-C_2$-C_6 alkynyl, $-C_1$-C_6 haloalkyl, $-C_3$-C_7 cycloalkyl, 5- or 6-membered monocyclic heteroaryl, a 9- or 10-membered bicyclic heteroaryl, halo, $-OR^X$, -CN, $-NO_2$, $-N(R^X)_2$, $-NH(C_1$-C_6alkylene)-(5- or 6-membered monocyclic heteroaryl), $-NH(C_1$-C_6 alkylene)-(9- or 10-membered bicyclic heteroaryl), aryl, $-NHC(O)OR^{6b}$, $-N(C(O)OR^{6b})_2$, $-NHC(O)N(R^{6b})_2$, or $-NHC(O)R^{6b}$, wherein each of said $-C_1$-C_6 alkyl group, said $-C_2$-C_6 alkenyl group or said $-C_2$-C_6 alkynyl group can be optionally substituted with halo;

$\mathbf{R^6}$, $\mathbf{R^{6a}}$ and $\mathbf{R^{6b}}$ are each independently selected at each occurrence from -H, $-C_1$-C_6 alkyl, $-C_1$-C_6 haloalkyl, $-(C_1$-C_3 alkylene$)_m$-(C_3-C_7 cycloalkyl), $-(C_1$-C_3 alkylene$)_m$-(aryl), $-(C_1$-C_3 alkylene$)_m$-(4 to 7-membered heterocycloalkyl), $-(C_1$-C_3 alkylene$)_m$-(5- or 6-membered monocyclic heteroaryl) or

-(C_1-C_3 alkylene)$_m$-(9- or 10-membered bicyclic heteroaryl), wherein each of said -C_1-C_6 alkyl, said C_3-C_7 cycloalkyl group, said aryl group, said 4 to 7-membered heterocycloalkyl group, said -(5- or 6-membered monocyclic heteroaryl group or said 9- or 10-membered bicyclic heteroaryl group can be optionally substituted with R^7;

m is an integer selected from 0 (zero) or 1;

R^7 represents from one to five substituent groups, each independently selected from -C_1-C_6alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_1-C_6 haloalkyl, aryl, or a 5-6-member heteroaryl;

R^8 is -H, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, -C_3-C_7 cycloalkyl, aryl, 5- or 6-membered monocyclic heteroaryl or 9- or 10-membered bicyclic heteroaryl;

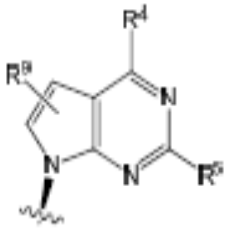
R^9 is -H, halo, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, -CN, -OR^Y or -$N(R^Y)_2$;

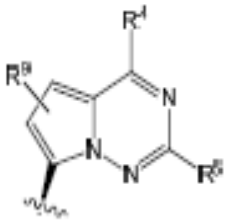
R^X is independently selected at each occurrence from -H, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, aryl, or 5- or 6- membered monocyclic heteroaryl;

or when either or both of R^4 or R^5 is -$N(R^X)_2$, each R^X may optionally be joined together with the nitrogen to which they are both attached to form a 5- or 6-membered monocyclic heteroaryl or 9- or 10-membered bicyclic heteroaryl; and

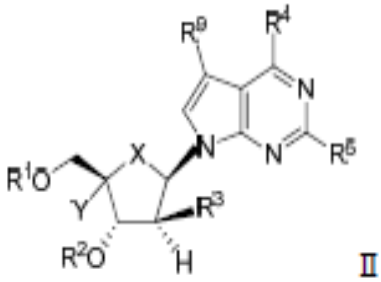
R^Y is -H, -C_1-C_6 alkyl or -C_1-C_6 haloalkyl.

2. The compound as claimed in claim 1 or a pharmaceutically acceptable salt thereof wherein R is



or


3. The compound as claimed in claim 1 or a pharmaceutically acceptable salt thereof having structural formula II



II

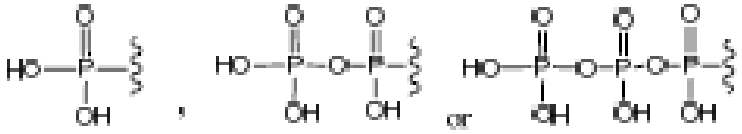
wherein X, Y, R^1, R^2, R^3, R^4, R^5 and R^9 are as claimed in claim 1.

4. The compound as claimed in any one of claims 1 to 3 or a pharmaceutically acceptable salt thereof wherein

X is O;

Y is -C=C-R^8 or -C=N;

R^1 is -H, $-C(O)R^6$, $-C(O)OR^6$, $-C(O)N(R^6)_2$ or



or a pro-drug modification of the mono-, di- or triphosphate;

R^2 is -H, $-C(O)R^{6a}$, $-C(O)OR^{6a}$ or $-C(O)N(R^{6a})_2$;

R^3 is -H;

R^4 is $-N(R^X)_2$, $-NHC(O)OR^{6b}$, $-N(C(O)OR^{6b})_2$, $-NHC(O)N(R^{6b})_2$, or $-NHC(O)R^{6b}$;

R^5 is -H, halo, $-C_1$-C_6 alkyl, $-C_1$-C_6 haloalkyl, -CN, $-OR^x$, $-N(R^X)_2$, $-C_3$-C_7 cycloalkyl, $-NHC(O)OR^{6b}$, $-N(C(O)OR^{6b})_2$, $-NHC(O)N(R^{6b})_2$, or $-NHC(O)R^{6b}$;

R^6, R^{6a} and R^{6b} are each independently selected at each occurrence from -H, $-C_1$-C_6 alkyl, or $-C_1$-C_6 haloalkyl;

R^8 is -H, $-C_1$-C_6 alkyl, $-C_1$-C_6 haloalkyl, $-C_3$-C_7 cycloalkyl;

R^9 is -H, halo, $-C_1$-C_6 alkyl, $-C_1$-C_6 haloalkyl, -CN, $-OR^Y$ or $-N(R^Y)_2$;

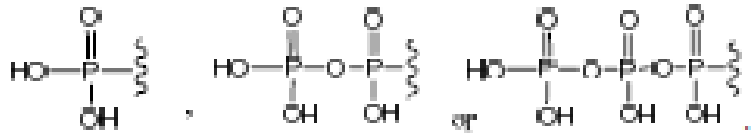
R^X is independently selected at each occurrence from -H, $-C_1$-C_6 alkyl, $-C_1$-C_6 haloalkyl, aryl, or 5- or 6- membered monocyclic heteroaryl;

or when either or both of R^4 or R^5 is $-N(R^X)_2$, each R^X may optionally be joined together with the nitrogen to which they are both attached to form a 5- or 6-membered monocyclic heteroaryl or 9- or 10-membered bicyclic heteroaryl; and

R^Y is -H, $-C_1$-C_6 alkyl or $-C_1$-C_6 haloalkyl.

5. The compound as claimed in any one of claims claim 1 to 4 or a pharmaceutically acceptable salt thereof wherein:

R^1 is –H,



; and

R^4 is $-N(R^X)_2$.

6. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein R^2 is -H.

7. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein Y is $-C\equiv C-R^8$.

8. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein R^4 is $-N(R^X)_2$.

9. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein R^5 is –H, halo, $-C_1$-C_6 alkyl, $-OR^X$, or $-N(R^X)_2$.

10. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein R^8 is–H.

11. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein R^9 is –H, halo, -C_1-C_3 alkyl, -C_1-C_3 haloalkyl, -CN, -OR^Y or -$N(R^Y)_2$.

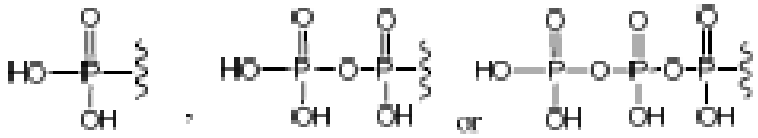
12. The compound as claimed in any one of claims 1 to 11 or a pharmaceutically acceptable salt thereof wherein
R^X is -H, -C_1-C_6 alkyl or -C_1-C_6 haloalkyl.

13. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein:
Y is –C≡C-R^8;
R^5 is –H, halo, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, -CN, -OR^X, -$N(R^X)_2$, -NHC(O)OR^{6b}, -$N(C(O)OR^{6b})_2$, -NHC(O)$N(R^{6b})_2$, or -NHC(O)R^{6b}; and
R^9 is –H, halo, -C_1-C_3 alkyl, -C_1-C_3 haloalkyl, -CN, -OR^Y or -$N(R^Y)_2$.

14. The compound as claimed in claim 13 or a pharmaceutically acceptable salt thereof wherein R^4 is -$N(R^X)_2$.

15. The compound as claimed in claim 13 or a pharmaceutically acceptable salt thereof wherein R^1 is –H and R^4 is -$N(R^X)_2$.

16. The compound as claimed in any one of claims 1 to 3 or a pharmaceutically acceptable salt thereof, wherein:
X is O;
Y is –C≡CH or -C≡N;
R^1 is -H, -C(O)R^6, -C(O)OR^6, -C(O)$N(R^6)_2$,



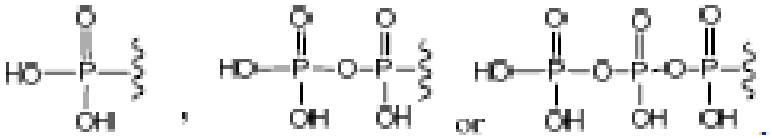
or a pro-drug modification of the mono-, di- or triphosphate;
R^2 is H, -C(O)R^{6a}, -C(O)OR^{6a} or -C(O)$N(R^{6a})_2$;
R^3 is –H;
R^4 is -$N(R^X)_2$, -NHC(O)OR^{6b} or -NHC(O)$N(R^{6b})_2$;
R^5 is –H, halo, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, -CN, -OR^X or -$N(R^X)_2$;
R^6, R^{6a}, and R^{6b} are each independently selected at each occurrence from -H, -C_1-C_6 alkyl, or -C_1-C_6 haloalkyl;
R^9 is –H, halo, -C_1-C_3 alkyl, -C_1-C_3 haloalkyl, -CN, -OR^Y, or -$N(R^Y)_2$;
R^X is independently selected at each occurrence from -H, -C_1-C_6 alkyl, or -C_1-C_6 haloalkyl; and
R^Y is independently selected at each occurrence from -H, -C_1-C_6 alkyl or -C_1-C_6 haloalkyl.

17. The compound as claimed in any one of claims 1 to 3, or a pharmaceutically acceptable salt

thereof, wherein:

Y is -C≡CH or -C≡N;

R^1 is -H,



;

R^2 is H;

R^3 is -H;

R^4 is NH_2;

R^5 is –H, halo or $-NH_2$; and

R^9 is –H, halo or $-CH_3$.

18. The compound as claimed in any one of claims 1 to 3 or a pharmaceutically acceptable salt thereof, wherein one or more of R^1, R^2, R^4 or R^5 is selected as follows:

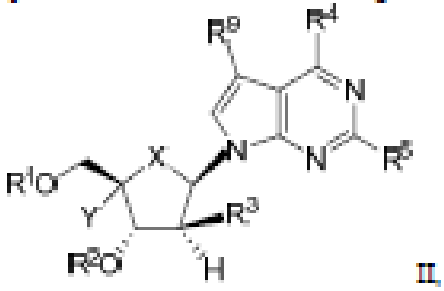
R^1 is $-C(O)R^6$, $-C(O)OR^6$, $-C(O)N(R^6)_2$; and/or

R^2 is $-C(O)R^{6a}$, $-C(O)OR^{6a}$ or $-C(O)N(R^{6a})_2$; and/or

R^4 is $-NHC(O)OR^{6b}$, $-N(C(O)OR^{6b})_2$, $-NHC(O)N(R^{6b})_2$, or $-NHC(O)R^{6b}$, and/or

R^5 is $-NHC(O)OR^{6b}$, $-N(C(O)OR^{6b})_2$, $-NHC(O)N(R^{6b})_2$, or $-NHC(O)R^{6b}$.

19. The compound as claimed in claim 1 having structural Formula II,



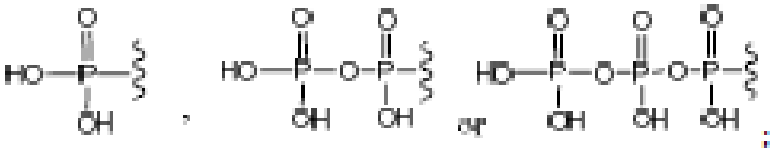
II,

or a pharmaceutically acceptable salt thereof, wherein:

X is O;

Y is –C≡CH;

R^1 is –H,



;

R^2 is –H;

R^3 is –H;

R^4 is $–NH_2$;

R^5 is –H, halo or $-NH_2$; and

R^9 is –H, halo or $–CH_3$.

20. The compound as claimed in claim 1 that is:

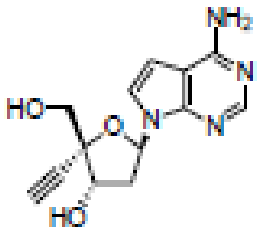
1)	(2*R*,3*S*,5*R*)-5-(4-amino-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;

2)	(2*R*,3*S*,5*R*)-5-(4-amino-5-fluoro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
3)	(2*R*,3*S*,5*R*)-5-(4-amino-5-methyl-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
4)	(2*R*,3*S*,5*R*)-5-(4-amino-2-chloro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
5)	(2*R*,3*S*,5*R*)-5-(4-amino-5-bromo-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
6)	(2*R*,3*S*,5*R*)-5-(4-amino-5-chloro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
7)	(2*R*,3*S*,5*R*)-5-(4-amino-5-iodo-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
8)	(2*R*,3*S*,5*R*)-5-(2,4-diamino-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
9)	(2*R*,3*S*,5*R*)-5-(4-amino-2-fluoro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
10)	(2*R*,3*S*,5*R*)-5-(4-amino-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-3-hydroxy-2-(hydroxymethyl)tetrahydrofuran-2-carbonitrile ;
11)	(2*R*,3*S*,5*R*)-5-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
12)	(2*R*,3*S*,5*R*)-5-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-3-hydroxy-2-(hydroxymethyl)tetrahydrofuran-2-carbonitrile ;
13)	((2*R*,3*S*,5*R*)-5-(4-amino-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
14)	((2*R*,3*S*,5*R*)-5-(4-amino-5-fluoro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
15)	((2*R*,3*S*,5*R*)-5-(4-amino-5-methyl-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
16)	((2*R*,3*S*,5*R*)-5-(4-amino-2-chloro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
17)	((2*R*,3*S*,5*R*)-5-(4-amino-5-chloro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
18)	((2*R*,3*S*,5*R*)-5-(4-amino-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-cyano-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
19)	((2*R*,3*S*,5*R*)-5-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
20)	((2*R*,3*S*,5*R*)-5-(4-aminopyrazolo[1,5-a][1,3,5]triazin-8-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
21)	((2*R*,3*S*,5*R*)-5-(4-aminoimidazo[2,1-f][1,2,4]triazin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
22)	(2*R*,3*S*,5*R*)-5-(4-aminoimidazo[2,1-f] [1,2,4]triazin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol;

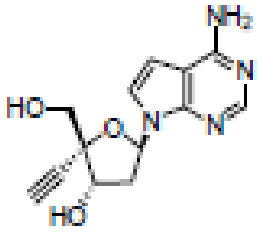
23)	(2*R*,3*S*,5*R*)-5-(4-aminopyrazolo[1,5-a][1,3,5]triazin-8-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol;

or a pharmaceutically acceptable salt thereof

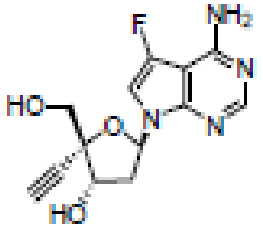
21. The compound as claimed in claim 1 that is:



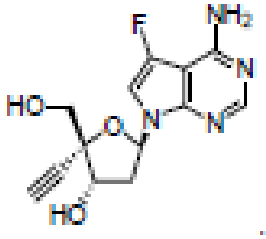
22. The compound as claimed in claim 1 that is a pharmaceutically acceptable salt of:



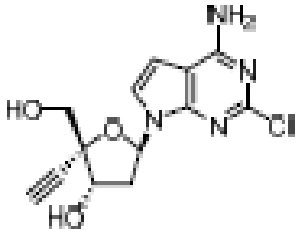
23. The compound as claimed in claim 1 that is:



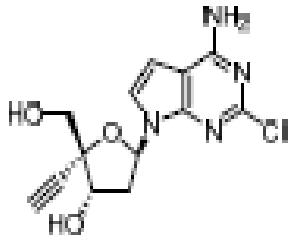
24. The compound as claimed in claim 1 that is a pharmaceutically acceptable salt of:



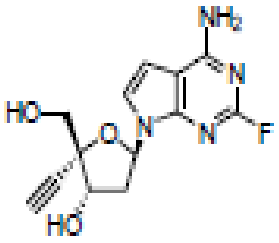
25. The compound as claimed in claim 1 that is:



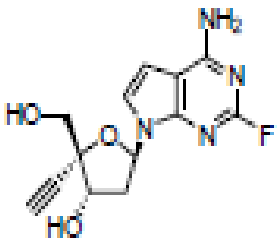
26. The compound as claimed in claim 1 that is a pharmaceutically acceptable salt of:



27. The compound as claimed in claim 1 that is:



28. The compound as claimed in claim 1 that is a pharmaceutically acceptable salt of:



29. A pharmaceutical composition comprising a compound as claimed in any one of claims 1 to 20, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier and optionally comprising at least one anti-HIV agent selected from an HIV antiviral agent, an immunomodulator, and an anti-infective agent such as herein described.

4. **Impugned Patent Application:** The present pre-grant opposition is against Indian Patent Application 201617031899, entitled "4 SUBSTITUTED NUCLEOSIDE DERIVATIVES AS HIV REVERSE TRANSCRIPTASE INHIBITORS" and is drawn towards the compounds having HIV reverse transcriptase inhibition properties.

5. **Disclosure in the impugned patent application:** As per the Applicant, the present invention relates to 4'-substituted nucleoside derivatives and their use in the inhibition of HIV

reverse transcriptase, the prophylaxis of infection by HIV, the treatment of infection by HIV, and the prophylaxis, treatment, and delay in the onset or progression of AIDS and/or ARC.

6. **PRIOR ARTS:** The opponent wishes to rely on the following prior arts as evidence in support of the grounds of opposition.

i. **D1**- EP 2177527A1 (EP'527)
ii. **D2**-Ei-Ichi Kodama Et Al; 4'-Ethynyl Nucleoside Analogs: Potent Inhibitors of Multidrug-Resistant Human Immunodeficiency Virus Variants In Vitro; Antimicrobial Agents And Chemotherapy,May 2001, p. 1539–1546.
iii. **D3**-Hiroshi Ohrui; 2′-Deoxy-4′-C-Ethynyl-2-Fluoroadenosine, a Nucleoside Reverse Transcriptase Inhibitor, Is Highly Potent Against All Human Immunodeficiency Viruses Type 1 and Has Low Toxicity;The Chemical Record, Vol. 6, 133–143 (2006).
iv. **D4**-Kristjan S. Gudmundsson et al; Phosphoramidate Protides of Carbocyclic 2′,3′-Dideoxy-2′,3′-Didehydro-7-Deazaadenosine with Potent Activity Against HIV and HBV;NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDSVol. 23, No. 12, pp. 1929–1937, 2004.
v. **D5**-Karen A. Kirby et al; Effects of Substitutions at the 4' and 2 Positions on the Bioactivity of 4'-Ethynyl-2-Fluoro-2'-Deoxyadenosine; Antimicrob. Agents Chemother. 2013, 57(12):6254.

7. It is submitted that all claims of the impugned patent application are liable to be refused on following grounds as below:

(a) Section 25(1)(b): Lack of novelty
(b) Section 25(1)(e): Lack of inventive step

(c) Section 25(1)(f): Invention is not patentable under section 3 (d)

(d) Section 25(1)(g): The complete specification does not sufficiently and clearly describe the invention or the method by which it is to be performed.

(e) Section 25(1)(h): Failed to disclose to the Controller the information required by section 8.

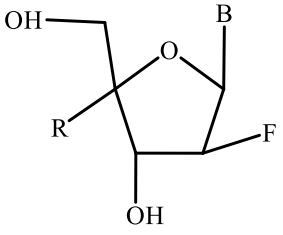
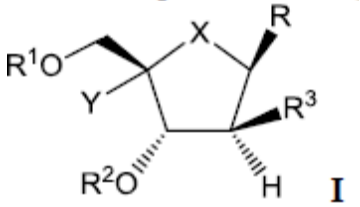
(a) GROUND 1: LACK OF NOVELTY

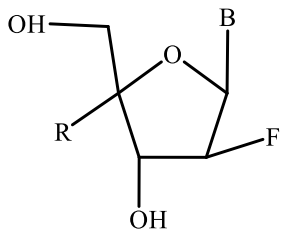
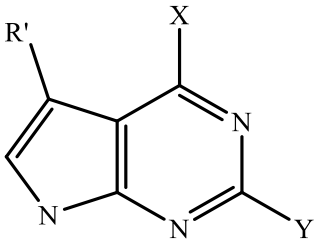
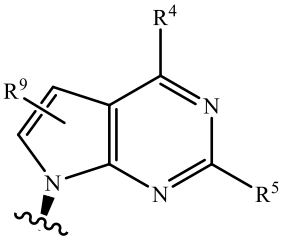
8. CLAIMS 1 TO 29 ARE NOT NOVEL, AND THEREFORE HAVE TO BE REJECTED UNDER SECTION 25(1)(b) OF THE PATENTS ACT.

9. The impugned patent application lacks novelty in view of EP 2177527A1 (EP'527). This document was published on 21.04.2010 which is prior to priority date of impugned patent application i.e. 28/03/2014. The impugned patent application claims compounds which are already covered in EP'527 patent. The table below sets out the manner in which the compounds of impugned patent application are anticipated by EP'527.

10. A similarity table is given below which clearly discloses that impugned patent application lacks novelty.

Table 1:

S.No.	Prior art (EP 2177527A1) (EP'527)	Impugned patent application (201617031899)
1		 I

		R1=H, X=O, Y=R (R from prior art), R2=H, R3=F, R=B (B from prior art) page 3 Now reach to prior art Formula (I) 
2	Wherein B=  R'= H, F X= NH_2 Y= F	Wherein R  Here R' is equivalent to R9, X is equivalent to R4 and Y is equivalent to R5 R9= H, Halo (F) page 3 R4= -N(R^x), where R^x=H means –NH_2page 3 R5= Halo (F) Page 4

11. From the above mentioned table, it is crystal clear that not only the structure of formula (I) of the prior art but its different substituents also present in the impugned patent application. Therefore the present impugned patent application lacks novelty over EP'527. Therefore, the impugned patent application ought to be rejected on this ground alone.

(b) GROUND 2: LACK OF INVENTIVE STEP:

12. It is submitted that the invention as claimed is obvious and does not involve any inventive step in view of whatever was known and published in India or elsewhere prior to the priority date of impugned patent application i.e. prior to 28/03/2014 the earliest claimed priority.

13. It is submitted that all the claims of the impugned patent application are not inventive and are obvious in view of common general knowledge in art and combined with teachings of above mentioned prior arts.

14. The claimed compound of formula I can be divided in three sections and all these sections were known in the art before the priority of this impugned patent application. Applicant has only assembled all these sections in one box. These sections are (A) Nitrogenous Base (B) Sugar (C) Phosphate group. A figure has been given below to understand it.

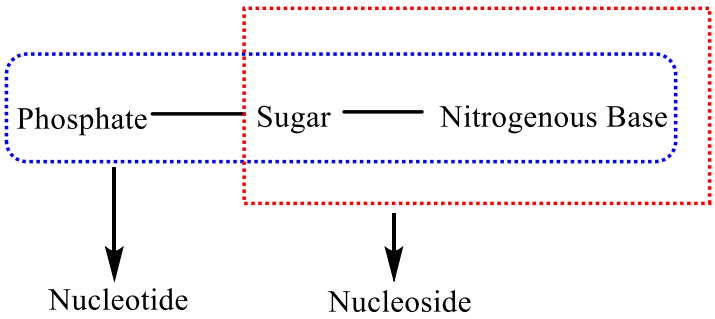


Figure 1: Basic concept of Nucleotide and Nucleoside

15. The impugned patent application claims 4'-substituted nucleoside derivatives and their use in the inhibition of HIV reverse transcriptase. There are various compounds in the impugned patent applications that have been claimed, some are given below.

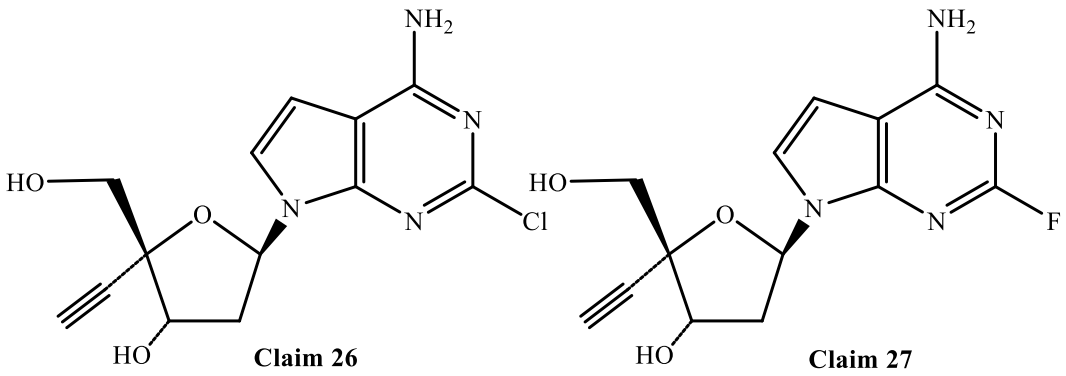


Figure 2: Claimed compounds

16. Opponent states that there are various prior arts available that discloses the importance of 4' position in the development of antiviral agents. Kodama et al. has clearly discloses that 4'-Ethynyl nucleoside analogs are potent inhibitors of multidrug-resistant human immunodeficiency virus. Some important paragraphs are reproduced here-

"Sugimoto et al. have reported that 4'-substituted nucleosides including 4'-ethynylthymidine exhibited potent activity against not only HIV-1, but also herpes simplex virus type 1 (38). We recently designed and synthesized a series of 4'-ethynyl (4'-E)-2'-deoxynucleosides and their analogs and identified several highly potent anti-HIV-1 compounds, including 4'-E-2'-deoxycytidine (4'-E-dC), 4'-E-2'-deoxyadenosine (4'-E-dA), 4'-E-2'-deoxyribofuranosyl-2,6-diaminopurine (4'-E-dDAP), and 4'-E-2'-deoxyguanosine (4'-E-dG). These 4'-E compounds, unlike all of the currently available nucleoside RTIs (NRTIs), lack the 2',3'-dideoxyribose configuration but have a 2'-deoxyribose configuration (Fig. 1 and Table 1)."

"All of these 4'-E compounds blocked the replication ofa wide spectrum of laboratory and clinical HIV-1 strainsin vitro with

low cellular toxicities. These compoundsalso suppressed the replication of various drug-resistantHIV-1 clones, including HIV-$1_{M41L/T215Y}$, HIV-1_{L74V},HIV-1_{K65R}, HIV-$1M41_{L/T69S-S-G/T215Y}$, HIV-1_{Y181C}, andHIV-$1_{A62V/V75I/F77L/F116Y/Q151M}$."

"It is likely that these 4'-substitutednucleoside analogs serve as NRTIs but not as NNRTIs. First,the ***4'-substituted nucleoside analogs were active against thevirus with any pyrimidines or purines****."*

The teaching of above paragraphs are-

- Ethynyl is essential at 4' position for anti HIV activity
- Deoxy condition at 2' position
- Bases like cytidine, adenosine,diaminopurine and guanosine shows excellent anti HIV activity.
- The combined teaching of this article suggest that any bases can be choose to get anti HIV activity but ethynyl group should be present at 4' position and 2' position should be free.
- Such combinations will be potent HIV inhibitors for different strains of HIV.

The present impugned patent application has followed the same teaching. The figure 3 clearly depicts the teaching of Kodama et al. The sugar portion of this figure is exactly same as disclosed in this prior art. The only difference in between this article and impugned patent application is nitrogenous base. This article also suggests that any bases can be choose to get anti HIV activity. Therefore, varying the bases would not affect too much to anti HIV activity. However, upcoming

paragraphs will show that even changing this nitrogenous base was also obvious.

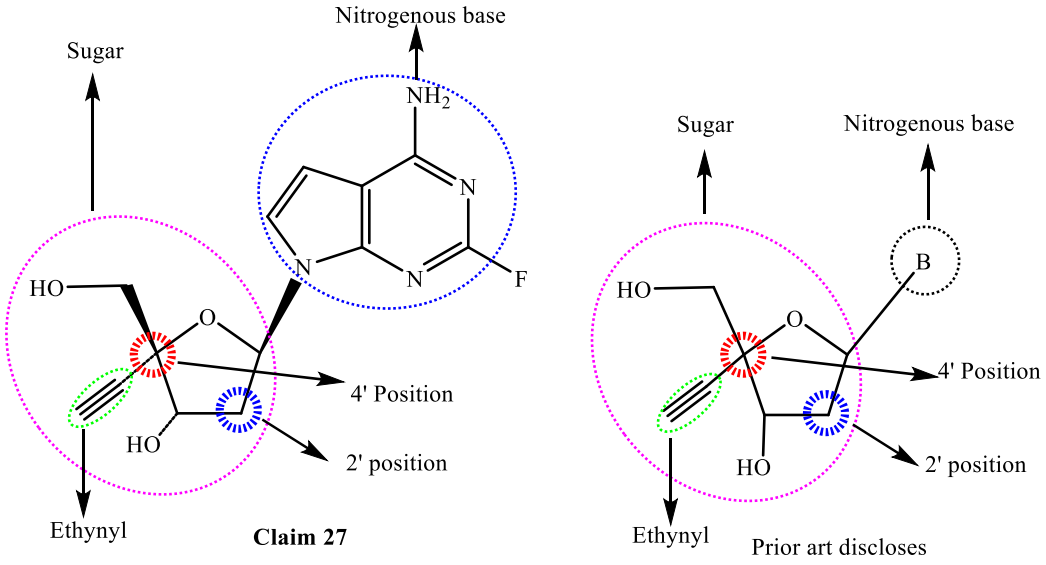


Figure 3

17. Hiroshi Ohrui has published an article in 2006 that discloses about a compound (2′-Deoxy-4′-C-Ethynyl-2-Fluoroadenosine) that has exceptionally very high anti HIV activity. It further discloses that this substance is highly potent against all HIV-1s and is expected to prevent the emergence of drug-resistant mutants, has a low toxicity, and is stable to enzymatic catabolism.

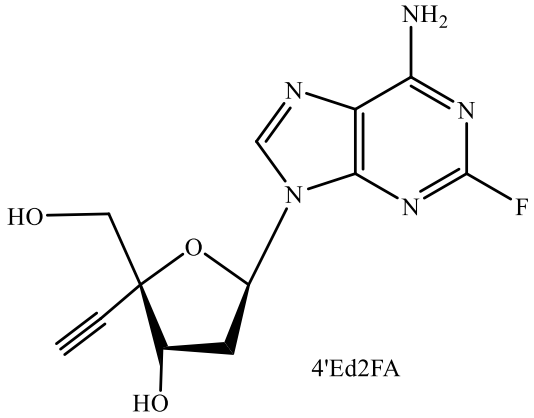


Figure 4: Structure of 2′-deoxy-4′-C-ethynyl-2-fluoroadenosine (4′Ed2FA)

18. It is noted that the difference between 4′Ed2FA and claim 27 compounds is very minor. A structural similarity is given below-

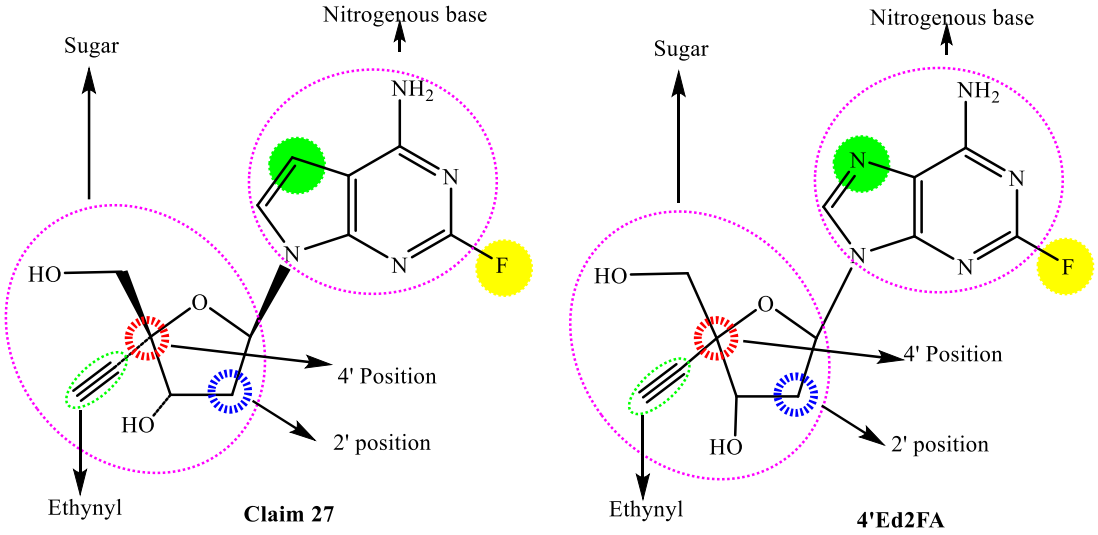


Figure 4: similarity between claim 27 compound and 4′Ed2FA

19. The above figure clearly discloses plethora of similarities. However, the difference between these two compounds is nitrogenous base (shown in green colour). Opponent further states that it was also known in prior art that these two nitrogenous base are analogues to each other. Therefore, using nitrogenous base as claimed in impugned patent application (4 amino pyrrolo pyrimidine) in place of nitrogenous base (adenine) as disclosed in 4'Ed2FA is so obvious for a person ordinary skilled in the art.

20. Gudmundsson has published an article in 2004 that discloses the anti HIV activities by 7-deazaadenosine derivatives. These compounds have same nitrogenous base as disclosed by impugned application i.e. 4 amino pyrrolo pyrimidine.

21. The teaching of above cited prior arts suggest that the impugned patent application could be mere a combination of D3 (Ohrui, 2006) and D4 (Gudmundsson, 2004) where D3 discloses the

sugar (ethynyl group should be present at 4' position and 2' position should be free) and an halo (F) substitution on nitrogenous base at specific position i.e. 2 where as D4 teaches an adenine analogue that has anti HIV activity.

22. Furthermore, the halo substitution at 2 position can be fluoro (claim 27) or Cl (claim26) because both belongs to same family and will show similar activity. Furthermore, Kirby et al also discloses the chloro derivatives of 4'Ed2FA. This article further discloses the importance of the 4' and 2 positions by varying different groups.

23. In addition to this, EP'527 discloses the same nitrogenous base as mentioned in impugned patent application. It also discloses that if nitrogenous base is 4 amino pyrrolo pyrimidine then there is one more possibility for the substitution and i.e. halo substitution at 5th position (R') where R'=H, F; X= NH_2; Y= H, F. The impugned application further claims some compounds that fall in this category where the nitrogenous base are same as disclosed in EP'527 and some have substitution at 5th position (R').

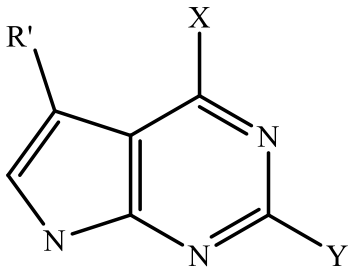


Figure 6

24. Just like above mentioned paragraph, the teaching of above cited prior arts further suggest that the impugned patent application is mere a combination of D1 and D3 whereas D1 teaches an adenine analogue (4 amino pyrrolo pyrimidine) that has anti HIV

activity and D3 discloses the sugar (ethynyl group should be present at 4' position and 2' position should be free) and a halo (F) substitution on nitrogenous base at specific position i.e. 2. The teaching directs toward the impugned invention.

25. The combined teaching is given below to understand the obviousness story of present impugned patent application.

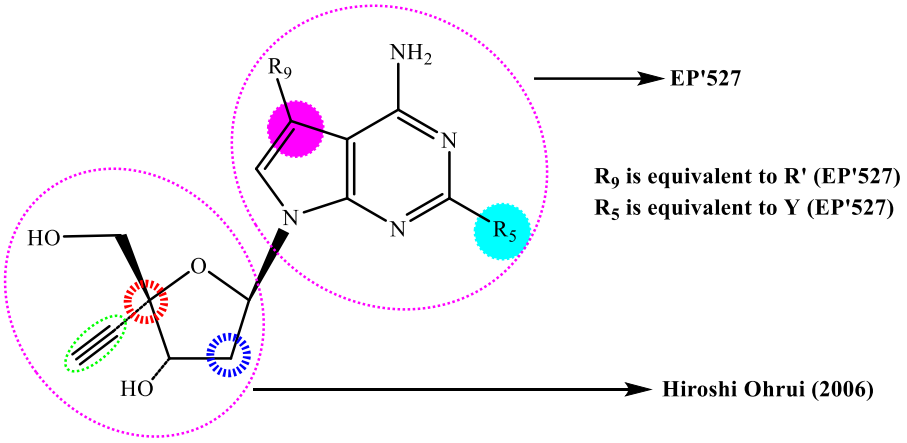


Figure 7

26. Furthermore, it is common in practice to make nucleotide from nucleoside by attaching phosphate group. Therefore, if nucleoside of impugned application is obvious then its phosphate derivative.ie. nucleotides are also obvious in nature.

27. In light of above, the present impugned patent application lack inventive steps and therefore, this application should be rejected on this ground alone.

(c) <u>GROUND 3: Claims not patentable under Section 25(1)(f)</u>

28. It is submitted that the impugned patent application should not be allowed under Section 3(d) which states that "*the mere discovery of a new form of a known substance which does not result in the enhancement of the known efficacy of that substance*

or the mere discovery of any new property or new use for a known substance or of the mere use of a known process, machine or apparatus unless such known process results in a new product or employs at least one new reactant.

Explanation -For the purposes of this clause, salts, esters, ethers, polymorphs, metabolites, pure form, particle size, isomers, mixtures of isomers, complexes, combinations and other derivatives of known substance shall be considered to be the same substance, unless they differ significantly in properties with regard to efficacy."

29. The Opponent states that the compounds claimed in impugned patent application are a derivative of known compound 4'Ed2FA. The opponent submits that the applicant has failed to demonstrate any enhancement in the therapeutic efficacy with respect to the known efficacy of compound 4'Ed2FAas disclosed by Hiroshi Ohrui (2006). The Opponent states that the Applicant miserably failed to provide data demonstrating enhanced 'therapeutic' efficacy as there is no RT polymerase comparative data disclosed in the impugned patent application showing improved efficacy of substance of impugned patent application over known substance disclosed in Hiroshi Ohrui (2006).

30. Therefore, the claimed compounds does not show any enhancement of known efficacy and thus not patentable under section 3 (d). Thus, the subject matter of impugned patent application squarely falls within the purview of Section 3(d) of the Act. Hence the impugned patent application should be rejected under section 3(d) of the act.

(d) <u>GROUND 4: INSUFFICIENCY OF DISCLOSURE</u>

31. The complete specification does not sufficiently and clearly describe the invention or the method by which it is to be performed.

32. The Applicant has not shown any standard compound data in table 2 that discloses the RT Polymerase assay. Applicant has mentioned only IC_{50} data of some synthesized compounds in table 2.

33. In table 3 and 4, Applicant used AZT as a standard drug to show a comparative data but it was already known that 4'Ed2FA has excellent anti HIV activity and show closest similarity. Therefore, Applicant must provide a data in comparison to 4'Ed2FA.

34. The impugned patent application does not provide adequate teaching to a person skilled in the art to practice the invention. In light of above, it is clear that impugned patent application does not sufficiently and clearly describe the invention. Therefore, the impugned patent application should be refused on this ground alone.

(e) <u>GROUND 5 -Section 25(1)(h)</u>

35. The patentee has failed to disclose to the Controller the information required under Section 8.The applicant is required to provide all the information regarding the prosecution of the equivalent applications till the grant of the Indian application to the Controller in writing from time to time and also within the prescribed time. It is observed that applicant has not updated the status of corresponding application in the Form-3 which information has not been provided to the learned Controller.

36. Therefore, the applicant has failed to comply with the requirements of the section 8 of the act and the opponent demands rejection on this ground also. It is submitted that the Applicant has failed to disclose the details of corresponding foreign applications and impugned patent application to be refused.

CONCLUSION

In view of the above, the claims are not inventive, not patentable and insufficient. The pre-grant opposition as filed may be allowed and the subject patent application may be refused.

P R A Y E R

In the fact and circumstances of the case, the Opponent prays as follows:

i. that the Indian Patent application number 201617031899made by Merck Sharp & Dohme Corp., be refused under Section 25(1) of the Patents (Amendment) Act, 2005;
ii. the Opponent may be allowed to file further documents as evidence if necessary to support its averments;
iii. the Opponent may be allowed to make further submissions in case the applicant makes any amendments in the claims;

Dated this day 05th of December, 2020

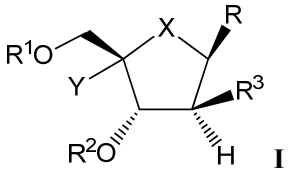
RAJESHWARI H.
AGENT FOR THE OPPONENT
RAJESHWARI AND ASSOCIATE

To,
The Controller of Patents
The Patent Office, New Delhi

Clean Copy
26.03.2019

We Claim:

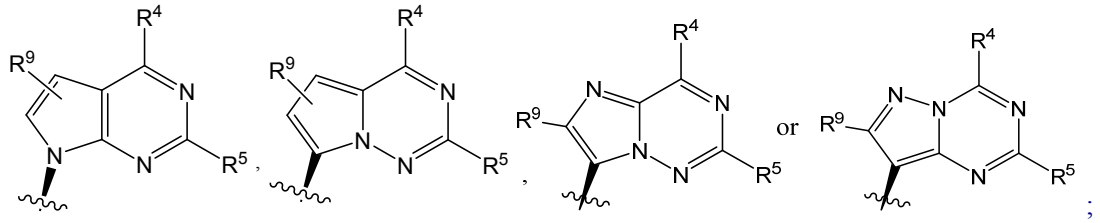
1. A compound of structural Formula I



I

or a pharmaceutically acceptable salt thereof, wherein:

R is

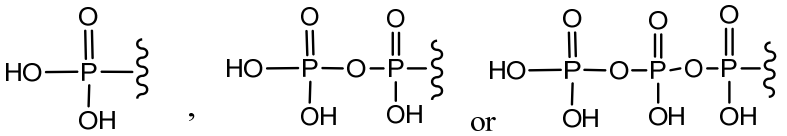


;

X is O, S, CH_2 or CF_2;

Y is –C≡C-R^8 or -C≡N;

R^1 is -H, -C(O)R^6, -C(O)OR^6, -C(O)N(R^6)$_2$,



,

or a pro-drug modification of the mono-, di- or triphosphate;

R^2 is -H, -C(O)R^{6a}, -C(O)OR^{6a} or -C(O)N(R^{6a})$_2$;

R^3 is –H;

R^4 is -H, -C_1-C_6 alkyl, -C_2-C_6 alkenyl, -C_2-C_6 alkynyl, -C_1-C_6 haloalkyl, -C_3-C_7 cycloalkyl, 5- or 6-membered monocyclic heteroaryl, a 9- or 10-membered bicyclic heteroaryl, halo, -CN, -NO_2, -N(R^X)$_2$, -NH(C_1-C_6alkylene)-(5- or 6-membered monocyclic heteroaryl), -NH(C_1-C_6 alkylene)-(9- or 10-membered bicyclic heteroaryl), aryl, -NHC(O)OR^{6b}, -N(C(O)OR^{6b})$_2$, -NHC(O)N(R^{6b})$_2$, or -NHC(O)R^{6b}, wherein each of said -C_1-C_6 alkyl group, said -C_2-C_6 alkenyl group or said -C_2-C_6 alkynyl group can be optionally substituted with halo;

R^5 is -H, -C_1-C_6 alkyl, -C_2-C_6 alkenyl, -C_2-C_6 alkynyl, -C_1-C_6 haloalkyl, -C_3-C_7 cycloalkyl, 5- or 6-membered monocyclic heteroaryl, a 9- or 10-membered bicyclic heteroaryl, halo, -OR^X, -CN, -NO_2, -N(R^X)$_2$, -NH(C_1-C_6alkylene)-(5- or 6-membered monocyclic heteroaryl), -NH(C_1-C_6 alkylene)-(9- or 10-membered bicyclic heteroaryl), aryl, -NHC(O)OR^{6b}, -N(C(O)OR^{6b})$_2$, -NHC(O)N(R^{6b})$_2$, or -NHC(O)R^{6b}, wherein each of said -C_1-C_6 alkyl group, said -C_2-C_6 alkenyl group or said -C_2-C_6 alkynyl group can be optionally substituted with halo;

R^6, **R^{6a}** and **R^{6b}** are each independently selected at each occurrence from -H, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, -(C_1-C_3 alkylene)$_m$-(C_3-C_7 cycloalkyl), -(C_1-C_3 alkylene)$_m$-(aryl), -(C_1-C_3 alkylene)$_m$-(4 to 7-membered heterocycloalkyl), -(C_1-C_3 alkylene)$_m$-(5- or 6-membered monocyclic heteroaryl) or

-(C_1-C_3 alkylene)$_m$-(9- or 10-membered bicyclic heteroaryl), wherein each of said -C_1-C_6 alkyl, said C_3-C_7 cycloalkyl group, said aryl group, said 4 to 7-membered heterocycloalkyl group, said -(5- or 6-membered monocyclic heteroaryl group or said 9- or 10-membered bicyclic heteroaryl group can be optionally substituted with R^7;

m is an integer selected from 0 (zero) or 1;

R^7 represents from one to five substituent groups, each independently selected from -C_1-C_6alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_1-C_6 haloalkyl, aryl, or a 5-6-member heteroaryl;

R^8 is -H, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, -C_3-C_7 cycloalkyl, aryl, 5- or 6-membered monocyclic heteroaryl or 9- or 10-membered bicyclic heteroaryl;

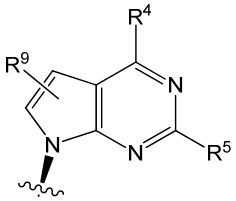
R^9 is -H, halo, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, -CN, -OR^Y or -N(R^Y)$_2$;

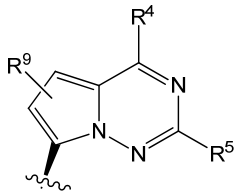
R^X is independently selected at each occurrence from -H, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, aryl, or 5- or 6- membered monocyclic heteroaryl;

or when either or both of **R^4** or **R^5** is -N(R^X)$_2$, each R^X may optionally be joined together with the nitrogen to which they are both attached to form a 5- or 6-membered monocyclic heteroaryl or 9- or 10-membered bicyclic heteroaryl; and

R^Y is -H, -C_1-C_6 alkyl or -C_1-C_6 haloalkyl.

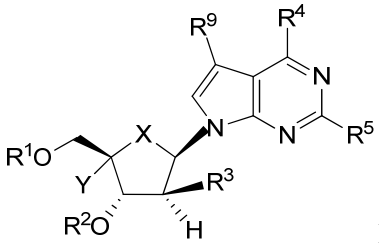
2. The compound as claimed in claim 1 or a pharmaceutically acceptable salt thereof wherein **R** is



or


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3. The compound as claimed in claim 1 or a pharmaceutically acceptable salt thereof having structural formula II



II

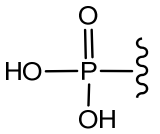
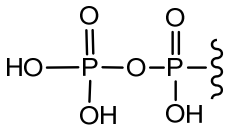
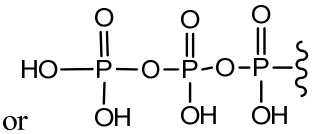
wherein **X**, **Y**, **R^1**, **R^2**, **R^3**, **R^4**, **R^5** and **R^9** are as claimed in claim 1.

4. The compound as claimed in any one of claims 1 to 3 or a pharmaceutically acceptable salt thereof wherein

X is O;

Y is -C≡C-R^8 or -C≡N;

R1 is -H, -C(O)R6, -C(O)OR6, -C(O)N(R6)2 or


,

or


or a pro-drug modification of the mono-, di- or triphosphate;

R2 is -H, -C(O)R6a, -C(O)OR6a or -C(O)N(R6a)2;

R3 is -H;

R4 is -N(RX)2, -NHC(O)OR6b, -N(C(O)OR6b)2, -NHC(O)N(R6b)2, or -NHC(O)R6b;

R5 is -H, halo, -C1-C6 alkyl,-C1-C6 haloalkyl, -CN, -ORx, -N(RX)2, -C3-C7 cycloalkyl, -NHC(O)OR6b, -N(C(O)OR6b)2, -NHC(O)N(R6b)2, or -NHC(O)R6b;

R6, **R6a** and **R6b** are each independently selected at each occurrence from -H, -C1-C6 alkyl, or -C1-C6 haloalkyl;

R8 is -H, -C1-C6 alkyl, -C1-C6 haloalkyl, -C3-C7 cycloalkyl;

R9 is -H, halo, -C1-C6 alkyl, -C1-C6 haloalkyl, -CN, -ORY or -N(RY)2;

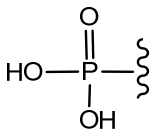
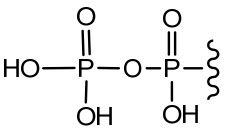
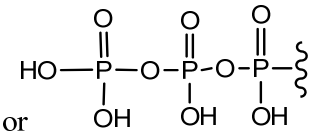
RX is independently selected at each occurrence from -H, -C1-C6 alkyl, -C1-C6 haloalkyl, aryl, or 5- or 6- membered monocyclic heteroaryl;

or when either or both of R4 or R5 is -N(RX)2, each RX may optionally be joined together with the nitrogen to which they are both attached to form a 5- or 6-membered monocyclic heteroaryl or 9- or 10-membered bicyclic heteroaryl; and

RY is -H, -C1-C6 alkyl or -C1-C6 haloalkyl**.**

5. The compound as claimed in any one of claims claim 1 to 4 or a pharmaceutically acceptable salt thereof wherein:

R1 is –H,


,

or

; and

R4 is -N(RX)2.

6. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein **R2** is -H.

7. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein **Y** is –C≡C-R8.

8. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein **R4** is -N(RX)2.

9. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein **R5** is –H, halo, -C1-C6 alkyl, -ORX, or -N(RX)2.

10. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein **R8** is–H.

11. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein **R9** is –H, halo, -C_1-C_3 alkyl, -C_1-C_3 haloalkyl, -CN, -OR^Y or -$N(R^Y)_2$.

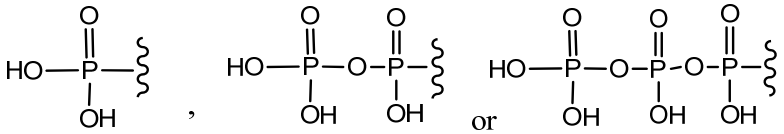
12. The compound as claimed in any one of claims 1 to 11 or a pharmaceutically acceptable salt thereof wherein
$\mathbf{R}^X$ is -H, -C_1-C_6 alkyl or -C_1-C_6 haloalkyl.

13. The compound as claimed in any one of claims 1 to 4 or a pharmaceutically acceptable salt thereof wherein:
Y is –C≡C-R^8;
R5 is –H, halo, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, -CN, -OR^X, -$N(R^X)_2$, -NHC(O)OR^{6b}, -$N(C(O)OR^{6b})_2$, -NHC(O)$N(R^{6b})_2$, or -NHC(O)R^{6b}; and
R9 is –H, halo, -C_1-C_3 alkyl, -C_1-C_3 haloalkyl, -CN, -OR^Y or -$N(R^Y)_2$.

14. The compound as claimed in claim 13 or a pharmaceutically acceptable salt thereof wherein **R4** is -$N(R^X)_2$.

15. The compound as claimed in claim 13 or a pharmaceutically acceptable salt thereof wherein **R1** is –H and **R4** is -$N(R^X)_2$.

16. The compound as claimed in any one of claims 1 to 3 or a pharmaceutically acceptable salt thereof, wherein:
X is O;
Y is –C≡CH or -C≡N;
R1 is -H, -C(O)R^6, -C(O)OR^6, -C(O)$N(R^6)_2$,



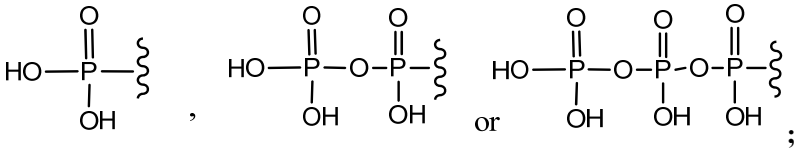
or a pro-drug modification of the mono-, di- or triphosphate;
R2 is H, -C(O)R^{6a}, -C(O)OR^{6a} or -C(O)$N(R^{6a})_2$;
R3 is –H;
R4 is -$N(R^X)_2$, -NHC(O)OR^{6b} or -NHC(O)$N(R^{6b})_2$;
R5 is –H, halo, -C_1-C_6 alkyl, -C_1-C_6 haloalkyl, -CN, -OR^X or -$N(R^X)_2$;
R6, **R6a**, and **R6b** are each independently selected at each occurrence from -H, -C_1-C_6 alkyl, or -C_1-C_6 haloalkyl;
R9 is –H, halo, -C_1-C_3 alkyl, -C_1-C_3 haloalkyl, -CN, -OR^Y, or -$N(R^Y)_2$;
$\mathbf{R}^X$ is independently selected at each occurrence from -H, -C_1-C_6 alkyl, or -C_1-C_6 haloalkyl; and
$\mathbf{R}^Y$ is independently selected at each occurrence from -H, -C_1-C_6 alkyl or -C_1-C_6 haloalkyl.

17. The compound as claimed in any one of claims 1 to 3, or a pharmaceutically acceptable salt

thereof, wherein:

Y is -C≡CH or -C≡N;

R1 is -H,



;

R2 is H;

R3 is -H;

R4 is NH_2;

R5 is –H, halo or -NH_2; and

R9 is –H, halo or -CH_3.

18. The compound as claimed in any one of claims 1 to 3 or a pharmaceutically acceptable salt thereof, wherein one or more of R^1, R^2, R^4 or R^5 is selected as follows:

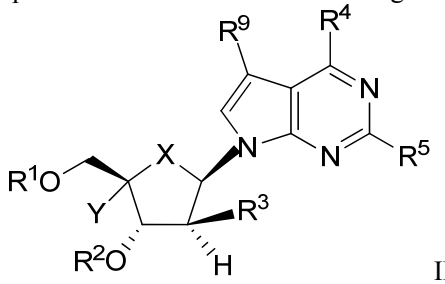
R1 is -C(O)R^6, -C(O)OR^6, -C(O)N(R^6)$_2$; and/or

R2 is -C(O)R^{6a}, -C(O)OR^{6a} or -C(O)N(R^{6a})$_2$; and/or

R4 is -NHC(O)OR^{6b}, -N(C(O)OR^{6b})$_2$, -NHC(O)N(R^{6b})$_2$, or -NHC(O)R^{6b}; and/or

R5 is -NHC(O)OR^{6b}, -N(C(O)OR^{6b})$_2$, -NHC(O)N(R^{6b})$_2$, or -NHC(O)R^{6b} .

19. The compound as claimed in claim **1** having structural Formula II,



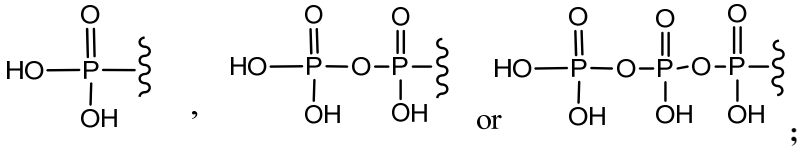
II,

or a pharmaceutically acceptable salt thereof, wherein:

X is O;

Y is –C≡CH;

R1 is –H,



;

R2 is –H;

R3 is –H;

R4 is –NH_2;

R5 is –H, halo or -NH_2; and

R9 is –H, halo or –CH_3.

20. The compound as claimed in claim 1 that is:

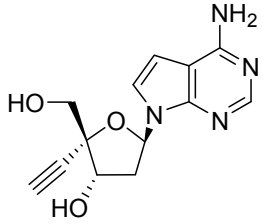
1)	(2*R*,3*S*,5*R*)-5-(4-amino-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;

2)	(2*R*,3*S*,5*R*)-5-(4-amino-5-fluoro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
3)	(2*R*,3*S*,5*R*)-5-(4-amino-5-methyl-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
4)	(2*R*,3*S*,5*R*)-5-(4-amino-2-chloro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
5)	(2*R*,3*S*,5*R*)-5-(4-amino-5-bromo-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
6)	(2*R*,3*S*,5*R*)-5-(4-amino-5-chloro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
7)	(2*R*,3*S*,5*R*)-5-(4-amino-5-iodo-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
8)	(2*R*,3*S*,5*R*)-5-(2,4-diamino-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
9)	(2*R*,3*S*,5*R*)-5-(4-amino-2-fluoro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
10)	(2*R*,3*S*,5*R*)-5-(4-amino-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-3-hydroxy-2-(hydroxymethyl)tetrahydrofuran-2-carbonitrile ;
11)	(2*R*,3*S*,5*R*)-5-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol ;
12)	(2*R*,3*S*,5*R*)-5-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-3-hydroxy-2-(hydroxymethyl)tetrahydrofuran-2-carbonitrile ;
13)	((2*R*,3*S*,5*R*)-5-(4-amino-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
14)	((2*R*,3*S*,5*R*)-5-(4-amino-5-fluoro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
15)	((2*R*,3*S*,5*R*)-5-(4-amino-5-methyl-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
16)	((2*R*,3*S*,5*R*)-5-(4-amino-2-chloro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
17)	((2*R*,3*S*,5*R*)-5-(4-amino-5-chloro-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
18)	((2*R*,3*S*,5*R*)-5-(4-amino-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)-2-cyano-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
19)	((2*R*,3*S*,5*R*)-5-(4-aminopyrrolo[2,1-f][1,2,4]triazin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
20)	((2*R*,3*S*,5*R*)-5-(4-aminopyrazolo[1,5-a][1,3,5]triazin-8-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
21)	((2*R*,3*S*,5*R*)-5-(4-aminoimidazo[2,1-f][1,2,4]triazin-7-yl)-2-ethynyl-3-hydroxytetrahydrofuran-2-yl)methyl tetrahydrogen triphosphate;
22)	(2*R*,3*S*,5*R*)-5-(4-aminoimidazo[2,1-f] [1,2,4]triazin-7-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol;

23)	(2*R*,3*S*,5*R*)-5-(4-aminopyrazolo[1,5-a][1,3,5]triazin-8-yl)-2-ethynyl-2-(hydroxymethyl)tetrahydrofuran-3-ol;

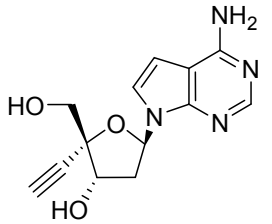
or a pharmaceutically acceptable salt thereof

21. The compound as claimed in claim 1 that is:



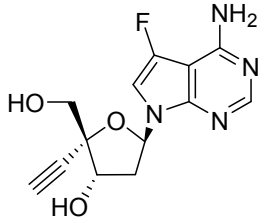
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22. The compound as claimed in claim 1 that is a pharmaceutically acceptable salt of:



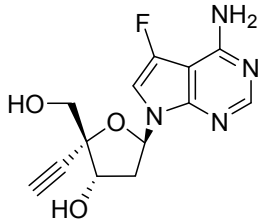
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23. The compound as claimed in claim 1 that is:



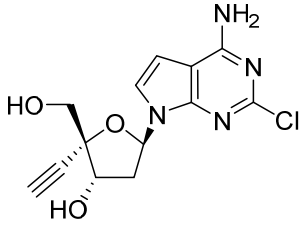
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24. The compound as claimed in claim 1 that is a pharmaceutically acceptable salt of:



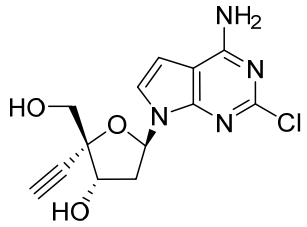
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25. The compound as claimed in claim 1 that is:



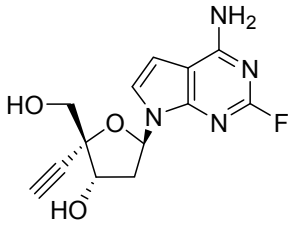
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26. The compound as claimed in claim 1 that is a pharmaceutically acceptable salt of:



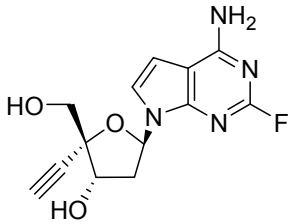
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27. The compound as claimed in claim 1 that is:



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28. The compound as claimed in claim 1 that is a pharmaceutically acceptable salt of:



.

29. A pharmaceutical composition comprising a compound as claimed in any one of claims 1 to 20, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier and optionally comprising at least one anti-HIV agent selected from an HIV antiviral agent, an immunomodulator, and an anti-infective agent such as herein described.

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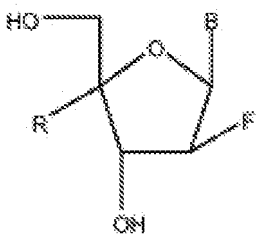
(71) Applicants:
- **Zhengzhou University**
  High-tech Development Zone
  Zhengzhou
  Henan 450001 (CN)
- **Analyse Testing And Research Center Of Henan Province**
  Jinshui
  Zhengzhou
  Henan 450002 (CN)

(72) Inventor: **CHANG, Junbiao**
Henan 450001 (CN)

(74) Representative: **von Kreisler Selting Werner**
Deichmannhaus am Dom
Bahnhofsvorplatz 1
50667 Köln (DE)

(54) **2'-FLUORO-4'-SUBSTITUTED NUCLEOSIDES, THE PREPARATION AND USE**

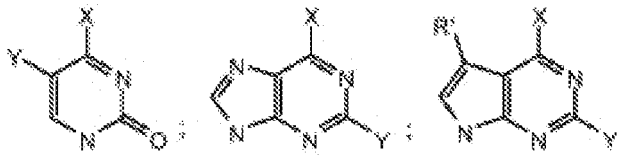
(57) The present invention provides 2'-fluorine-4'-substituted-nucleoside analogues or their pro-drugs or 5'-phosphate esters (including the pro-drugs of the 5'-phosphate esters), preparation methods and uses thereof. The compounds have the general formula as follows:



(I)

wherein:

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B= 

R = CH_3, CH, N_3, C≡CH;
R' = H, F;
X = F, OH, NH_2;
Y = H, CH_3, F, OH, NH_2

The compounds are used in the synthesis of drugs for the treatment of virus infection, especially for the treatment of HBV, HCV or HIV infection.

Description

Field of the Invention

[0001] This invention relates to nucleoside analogues, preparation methods and uses thereof, and more particularly, relates to 2'-fluorine -4'substituted nucleoside analogues, preparation methods and uses thereof.

Background of the Invention

[0002] The first case of AIDS patient was discovered in the United States in 1981. It was until in 1983 that the AIDS virus (HIV) is isolated and identified by scientists, revealing that HIV composed of 9749 nucleotides only is categorized into the Lentivirinae in Retroviridae. At present, the replication cycle of HIV virus has been made generally clear. According to relevant research findings, the reproduction process of the HIV virus can be divided into the following steps: the viral adsorption, invasion and uncoating, the reverse transcription, the integration of virus as well as the synthesis, the assembly, the release and the maturing of viral RNA and protein. Each of these steps can be used as a target for screening the Anti-HIV drugs, and the protein synthesis and the viral genome DNA replication are the most critical steps. At present, the screening of anti-HIV drugs is focused on finding the inhibitors for these specific enzymes, including the reverse transcriptase (RT) inhibitors, the protein synthesis inhibitors and the reverse transcriptase initiation inhibitors. HIV-reverse transcriptase is a multifunctional enzyme, which also has the activities of RNA dependent DNA polymerase, DNA dependent DNA polymerase and Rhase H. Initially, DNA chain(-) synthesis of HIV is processed with the virus genome RNA acting as a template and the host cell RNA as a primer, and then the synthesis of DNA chain (+) is completed in the same way. After the completion of the reverse transcription, all the genetic information carried by HIV is transformed from single-stranded RNA into double-stranded mDNA. Reverse transcriptase inhibitors can prevent the extension of mDNA and interfere with the HIV reverse transcription process, and thus become the drugs for the chemotherapy of AIDS. According to different mechanisms of the inhibitors, these drugs could be divided into two categories: 1) the nucleoside reverse transcriptase inhibitors which realize anti-HIV actions by being inserted into the viral DNA to promote the viral DNA to become defective DNA, resulting in the invalid duplication after integration of HIV with host cell during HIV DNA reverse transcription, and the available drugs include Zidovudine (AZT), Didanosine (ddI), Zalcitabine (ddC), Stavudine (d4T), Lamivudine (3TC), Abacavir; 2) non-nucleoside reverse transcriptase inhibitors, and mechanisms thereof are to prevent HIV RNA from being directly connected to the RT and thus being encoded into DNA. The available drugs include Nevirapine, Delavirdine and Efavirenz.

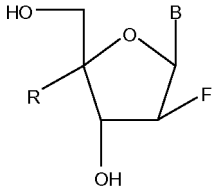
[0003] Zidovudine, the first commercial anti-HIV drug that was put into market in 1987, is a nucleoside reverse transcriptase inhibitor. This drug can alleviate the symptoms and prolong the lives of the patients, although it has much toxicity and failed to cure any patient. Later, several anti-HIV nucleoside analogue agents have also become available in the market. Therefore, nucleoside analogues are considered as an important class of compounds with anti-HIV activity. However, these drugs have some shortcomings at present. On the one hand, their effect is limited; on the other hand, severe toxic and side effects and drug resistance will be generated in a long-term administration. Accordingly, the synthesis of new nucleoside analogues is still an important trend in the research. In order to find more effective nucleoside anti-viral agents, diversified chemical modifications have been made for the nucleosides, including the fluorin-containing nucleoside analogues (Clark, J. PCT Patent Appl., WO 2005003174; Ismaili, H.M.A. PCT Patent Appl., WO 0160315A22001). Relevant studies have shown that these compounds have anti-viral activity at different levels, and are a new type of compounds with anti-viral activities. However, in the currently available literatures, it is not found any relevant report on the synthesis of 2'-fluoro-4 '-substituted-D-and L-nucleoside analogues of the present invention as well as on their applications in the preparation of anti-HIV, anti-HBV and anti-HCV drugs.

Summary of the invention

[0004] It is the primary object of the present invention to provide 2'-fluoro-4 '-substituted nucleoside analogues. It is another object of the present invention to provide the synthesis method for such compounds. It is a further object of the present invention to provide the uses of such compounds in pharmacology.

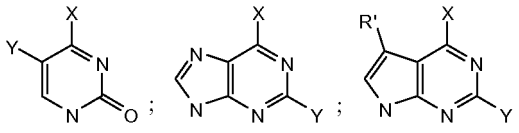
[0005] To achieve the objects of the present invention, the technical solutions are provided as follows:

2'-fluoro-4'- substituted nucleoside analogues of the present invention has the structure of the general formula (I):



(I)

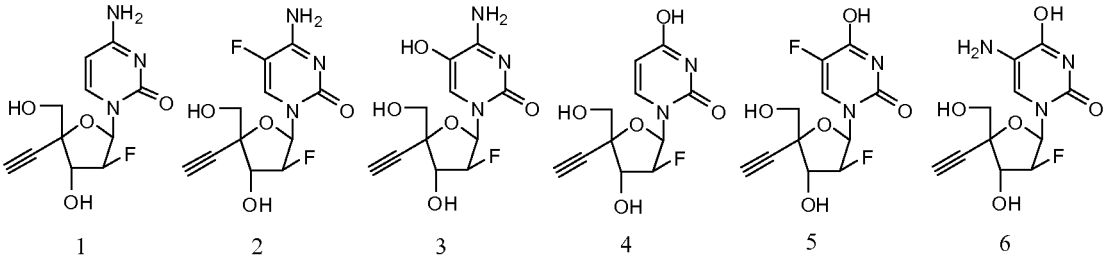
wherein :

B= 

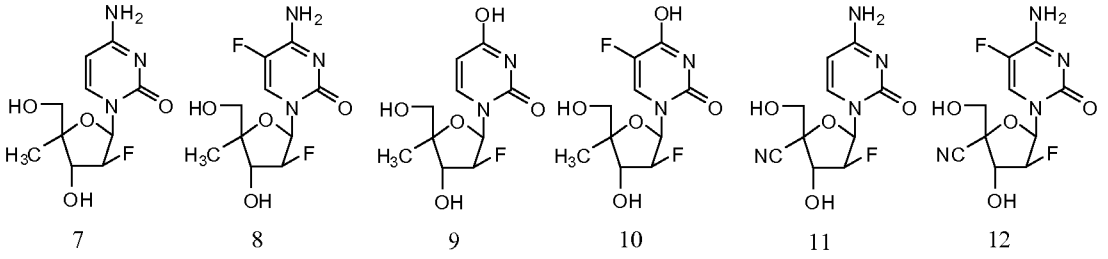
R = CH_3, CN, N_3, C≡CH;
R' = H, F;
X = F, OH, NH_2;
Y = H, CH_3, F, OH, NH_2

[0006] The above compounds may exist in the form of salts, which are generated through the reaction between the active compounds (I) or their pro-drugs or their 5'-phosphate esters and organic or inorganic acid.

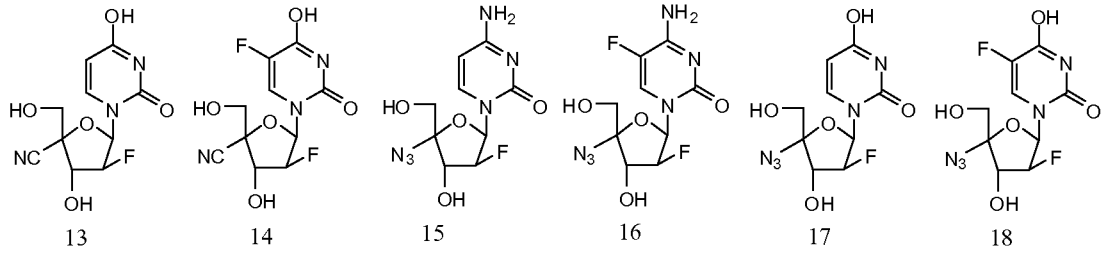
[0007] The above compounds may be one of the following compounds, but are not limited to these compounds:



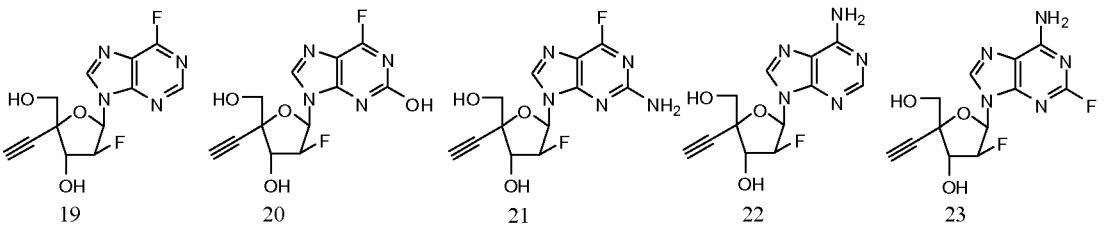
1 2 3 4 5 6

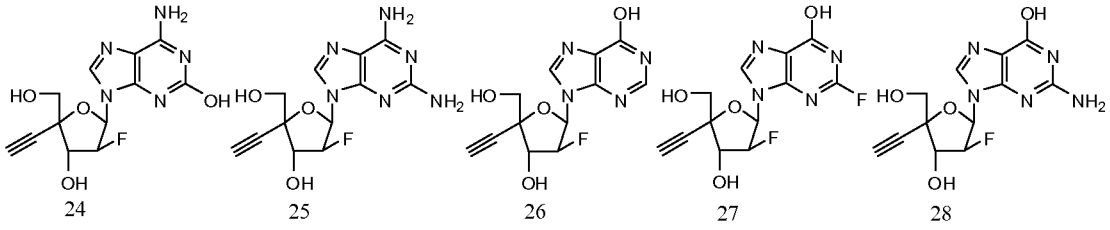


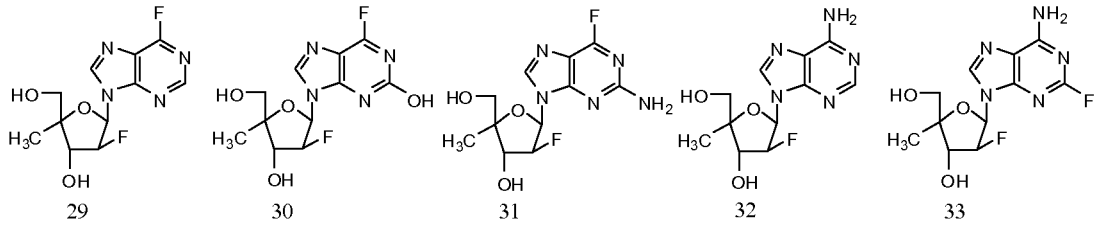
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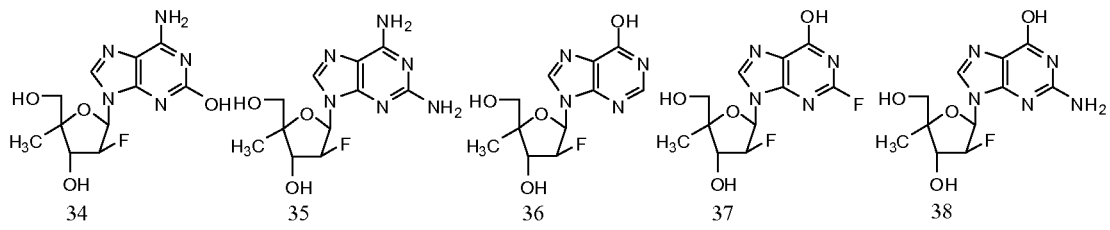


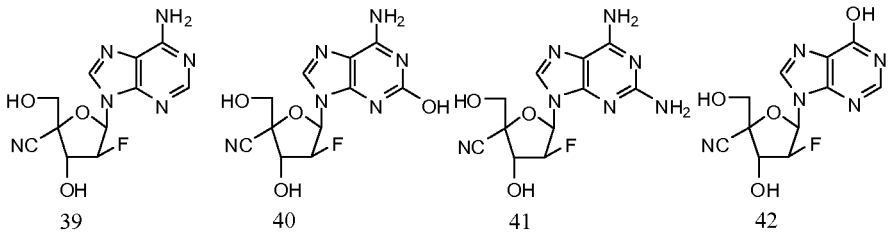
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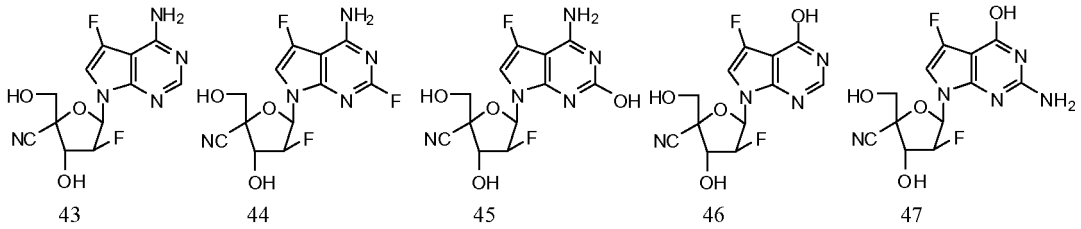

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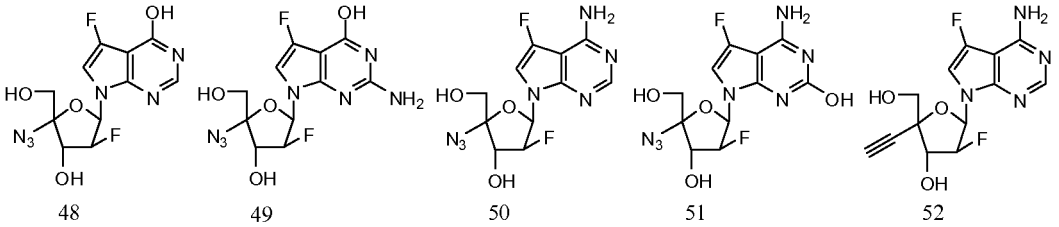

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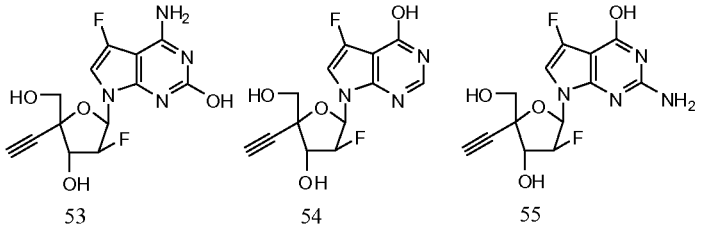

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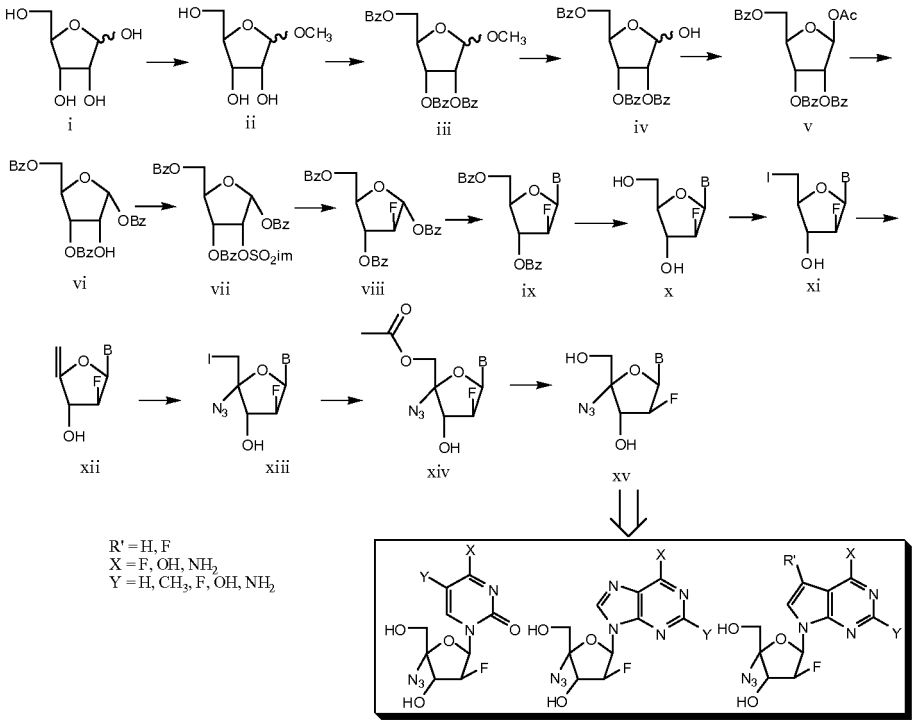

39 40 41 42


43 44 45 46 47





[0008] The present invention also relates to the uses of 2'-fluoro-4 '-substituted nucleoside analogues in the preparation of medicine such as anti-viral drugs for HBV or HCV or HIV, wherein said anti-viral drugs for HBV are anti-hepatitis B virus drugs; said anti-viral drugs for HIV are anti-AIDS drugs; and said anti-viral drugs for HCV are anti-hepatitis C drugs. The present invention also relates to the preparation reaction of 2'-fluoro-4 '-substituted nucleoside analogues. Where $R=N_3$, the reaction path is as follows:



[0009] The synthesis of compound ii: The compound i (D-ribose or L-ribose) is dissolved in HCl/MeOH. The solution is stirred in a water bath under hermetization and warm preservation. Pyridine is added into the solution to terminate the reaction, and then the solution is drawn off under a reduced pressure, so as to obtain light yellow syrup-like compound ii.

[0010] The synthesis of compound iii: The compound ii is dissolved in dry pyridine, and the solution is slowly added dropwise with benzoyl chloride in an ice-salt bath, and then stirred for reaction at a room temperature. After the reaction is completed, the reaction solution is poured into ice water, and extracted with chloroform. The organic layer is washed

sequentially with ice water, pre-cooled sulfuric acid and saturated sodium bicarbonate until the water layer shows weak alkaline, then washed with ice water until the water layer shows neutral. The solution is dried over anhydrous sodium sulfate, and then drawn off under a reduced pressure, so as to obtain light yellow syrup-like compound iii.

[0011] The synthesis of compound iv: Benzoyl compound iii is dissolved in chloroform, red phosphorus is added, bromine is slowly added dropwise while stirring the solution in an ice-water bath. It is stirred for about 30 minutes. Then, the solution is slowly added dropwise with ice water and stirred at a room temperature. Broken ice is added into the solution and the solution is stirred until the ice is melted, and then the reaction solution is poured into ice water and extracted with chloroform. The organic layer is washed with saturated sodium bicarbonate until the water layer shows weak alkaline, and then washed with ice water until the water layer shows neutral. The solution is dried over anhydrous sodium sulfate, and then drawn off under a reduced pressure, so as to obtain dark yellow syrup. Finally the syrup is separated by silica gel column, so as to obtain white dry syrup compound iv.

[0012] The synthesis of compound v: The compound iv is dissolved in dry pyridine, the solution is slowly added dropwise with acetic anhydride in an ice water bath, and then stirred for about 30 minutes. Then, the ice water bath is removed, and the solution is stirred at a room temperature for about 7 hours, and then is heated up to 40°C. This temperature is kept for about 1 hour. Broken ice is added into the solution, and the solution is stirred until the broken ice is melted. Then, the reaction solution is poured into ice water and extracted with chloroform. The organic layer is washed sequentially with ice water, pre-cooled sulfuric acid and saturated sodium bicarbonate until the water layer shows weak alkaline, then washed with ice water until the water layer shows neutral, dried over anhydrous sodium sulfate, and then drawn off under a reduced pressure, so as to obtain light yellow syrup-like compound v.

[0013] The synthesis of compound vi: The compound v is dissolved in dry dichloromethane and the dry HCl gas is slowly introduced into an ice-water bath. The solution is washed with ice water so hat an organic layer is separated. The organic layer is washed with saturated sodium bicarbonate until the water layer shows weak alkaline, and then washed with ice water until the water layer shows neutral, dried over anhydrous sodium sulfate, and drawn off under a reduced pressure to obtain light yellow syrup. The syrup is recrystallized to obtain white solid compound vi.

[0014] The synthesis of compound vii: The compound vi is dissolved in dry dichloromethane and dry DMF, and SO_2Cl_2 is slowly added into the solution while stirring the solution at about -15 °C. The solution is stirred at -15°C for about 30 minutes, and naturally warmed up to a room temperature for reaction. Then, imidazole is added into the solution for three times at 0°C, and the mixed solution is stirred at a room temperature. After completion of the reaction, the reaction solution is diluted by adding CH_2Cl_2 and then is washed with ice water. The water layer is extracted with CH_2Cl_2. The organic layers are combined and then dried over anhydrous sodium sulfate, and drawn off under a reduced pressure to obtain light yellow syrup. The syrup is separated and purified by silica gel column, so as to obtain white solid compound vii.

[0015] The synthesis of compound viii: The compound vii is dissolved in ethyl acetate, and Et3N • 3HF is added while stirring the solution. The solution is heated up to about 60°C and stirred for about 3h, and then heated up to 70 °C and stirred for about 1.5h. It is added with ice-salt water to terminate the reaction, and then extracted with dichloromethane. Then the organic layers are combined and then washed sequentially with saline, water and saturated sodium bicarbonate, dried over anhydrous sodium sulfate, and drawn off under a reduced pressure to obtain dark yellow syrup. The syrup is purified to obtain light yellow syrup. Finally the crude product is crystallized to obtain white crystalline compound viii.

[0016] The synthesis of compound ix: The compound viii is dissolved in anhydrous dichloromethane and a mixed solution of HBr-AcOH is added. The solution is stirred for reaction at a room temperature. After completion of the reaction, the mixture is evaporated to dryness, and the residue is dissolved by dichloromethane. The dichloromethane solution is washed with sodium bicarbonate solution, and dichloromethane is removed through evaporation to obtain a syrup-like product. Meanwhile, the protected cytosine and $(NH_4)_2SO_4$ are refluxed under nitrogen protection in HMDS. After the completion of reaction, the solvent is removed through evaporation under reduced pressure to obtain the silylated cytosine. The syrup obtained in the above reaction is dissolved in dichloroethane, and then added into the silylated cytosine. Then the mixture is refluxed under N_2 protection, and the reaction is terminated by ice. Then the solution is extracted by dichloromethane, and the dichloromethane layer is washed sequentially with saturated sodium bicarbonate and saline, and dried over anhydrous sodium sulfate. After drying the solvent is removed through evaporation to get white solid, the white solid is separated and purified by column chromatography to obtain the compound ix.

[0017] The synthesis of compound x: The compound ix is dissolved in saturated NH_3-CH_3OH and is stirred for reaction at room temperature. After completion of the reaction, the solvent is evaporated to dryness, and the obtained residue is purified by column chromatography to obtain the compound x.

[0018] The synthesis of compound xi: The compound x, imidazole and triphenylphosphine are dissolved in tetrahydrofuran, and the solution is slowly added dropwise with iodiferous tetrahydrofuran. The solution is stirred for reaction at a room temperature. After completion of the reaction, the solvent is removed through evaporation, and the residue is added with ethyl acetate and filtered. The ethyl acetate is removed through evaporation and the residue is separated by column chromatography to obtain the compound xi.

[0019] The synthesis of compound xii: The compound xi is dissolved in tetrahydrofuran, and then the solution is added with DBU, being stirred for reaction at about 60°C. After completion of the reaction, the solvent is evaporated to dryness,

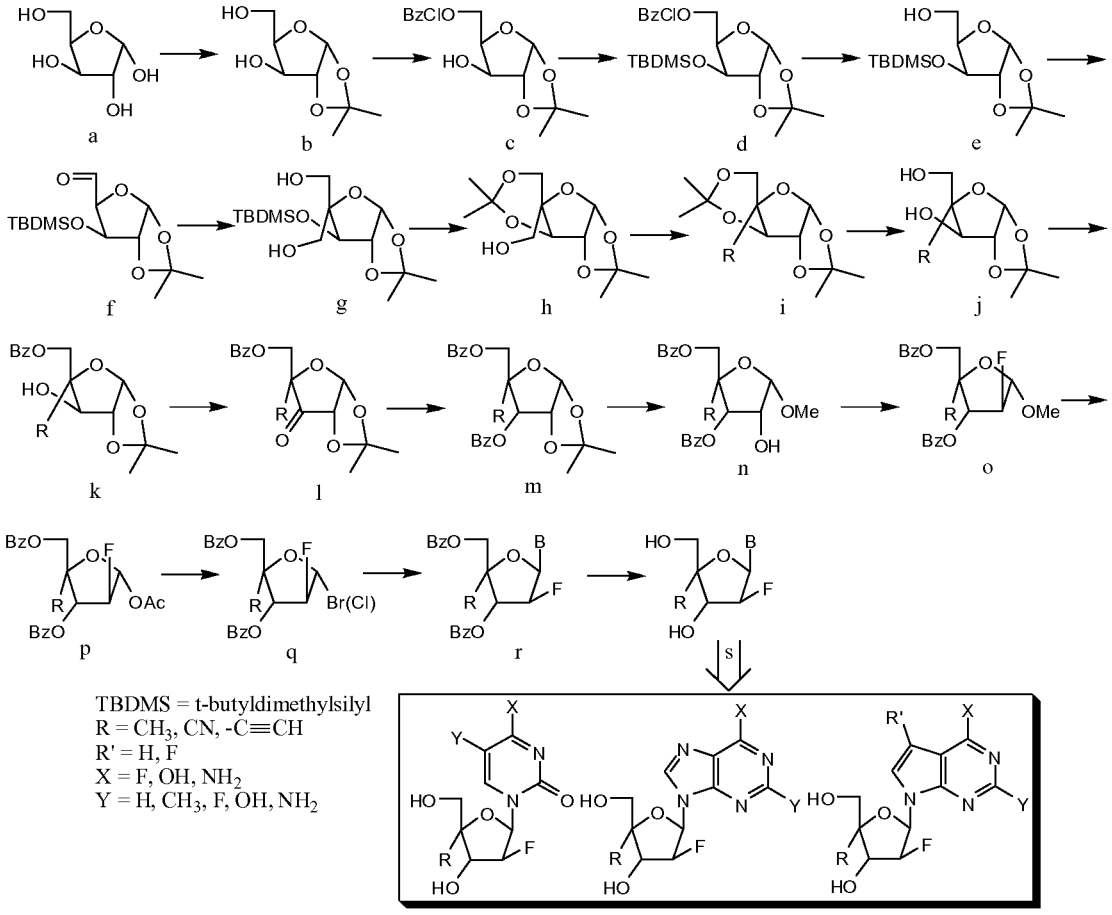
and the residue is separated by column chromatography to obtain the compound xii.

[0020] The synthesis of compound xiii: The DMF solution dissolved with ICl is added in the DMF solution dissolved with NaN_3 at about 0 °C. The solution is stirred at the freezing point for about 10 minutes and is slowly added dropwise with DMF solution dissolved with the compound xii to continue the reaction. After completion of the reaction, the mixed solution is added with sodium sulfite until the color of iodine disappears completely. The solvent is removed through evaporation under a reduced pressure, and the residue is separated and purified by column chromatography, so as to obtain the compound xiii.

[0021] The synthesis of compound xiv: The compound xiii is dissolved in DMF, silver acetate is added while stirring, and the reaction is carried out at a room temperature. After completion of the reaction, the solution is filtered. The solvent is removed under a reduced pressure and the residue is purified by column chromatography, so as to obtain the compound xiv.

[0022] The synthesis of compound xv: The compound xiv is dissolved in the solution of methanol-triethylamine, and the solution is stirred for reaction at a room temperature. After completion of the reaction, the solvent is removed through evaporation and the residue is purified by column chromatography, so as to obtain the compound xv.

[0023] The present invention also relates to the preparation reaction for 2'-fluoro-4 '-substituted nucleoside analogues. Where R=CH_3, CN, C≡CH, the reaction path is as follows:



[0024] The synthesis of compound b: Compound a is added in acetone, the concentrated sulfuric acid is slowly added while stirring the solution at a room temperature, and the solution is stirred for reaction at a room temperature. After the reaction is detected by TLC as complete, the solution is added with the concentrated ammonia to adjust pH, filtered, and evaporated under a reduced pressure to remove most of the acetone. Then 0.4% dilute hydrochloric acid is added while stirring, and the reaction is completed under the acetone reflux condition until the compound a has been completely hydrolyzed into the compound b. Then the reaction solution is neutralized by solid $NaHCO_3$, then filtered, and evaporated to remove the solvent under a reduced pressure. The residue is dissolved by dichloromethane, dried over anhydrous Na_2SO_4 for overnight and then filtered, and then evaporated to remove the solvent, so as to obtain the yellow viscous oily compound b.

[0025] The synthesis of compound c: The compound b and triethylamine are dissolved in dichloromethane. The solution is slowly added dropwise with parachlorobenzoyl chloride in an ice salt bath, and mechanically stirred for reaction at a temperature below 0 °C. After completion of the reaction, the solution is added with a saturated $NaHCO_3$ solution. The dichloromethane layer is washed with water and saturated salt water, dried over anhydrous $MgSO_4$, filtered, and then evaporated under a reduced pressure to remove the solvent. The residue is recrystallized to obtain the white crystalline compound c.

[0026] The synthesis of compound d: The compound c is dissolved in anhydrous dichloromethane. The solution is added with imidazole and tert-butyl-2-methyl chloride silicon (TBDMSCl) under nitrogen protection for the reaction at a room temperature. After completion of the reaction, the reaction is neutralized by hydrochloric acid, and two layers are obtained. The organic layer is washed with water and saturated salt water respectively, dried over anhydrous Na_2SO_4, and then evaporated to remove the solvent. The residue is separated and purified by column chromatography, so as to obtain the compound d.

[0027] The synthesis of compound e: The compound d is dissolved in the mixed solution of sodium methoxide and methanol for reaction at a room temperature. After completion of the reaction, the solution is neutralized by dilute acetic acid, filtered, washed with methanol and then evaporated under a reduced pressure to remove the solvent. Finally the residue is separated and purified by column chromatography, so as to obtain the compound e.

[0028] The synthesis of the compound f: DMSO is added dropwise into the dichloromethane solution of oxalyl chloride at about -60 °C. The solution is stirred at the same temperature for about 15 minutes, and then added dropwise with the dichloromethane solution of the compound e. After the solution is stirred for reaction at about -65 °C for about 30 minutes, triethylamine is added, and the solution is stirred for reaction at a room temperature. After completion of the reaction, water is added into the reaction solution so that the organic layer is separated, and the organic layer is dried over anhydrous magnesium sulfate. The solvent is removed through evaporation and the residue is separated and purified by column chromatography, so as to obtain the compound f.

[0029] The synthesis of compound g: Sodium hydroxide and water are added in the reaction bottle. The solution is stirred evenly and then added with a formaldehyde solution, 95% ethanol, and then the compound f. The solution is stirred for reaction at about 35 °C. After completion of the reaction, the reaction bottle is cooled with ice water while stirring until the product is completely precipitated. After the suction filtration, the solid product is washed with water to neutral, dried, dissolved in anhydrous methanol, and then sodium borohydride is added for reflux reaction. After completion of the reaction, the reaction solution is neutralized by dilute hydrochloric acid and then extracted with dichloromethane, dried over anhydrous Na_2SO_4. The solvent is removed by evaporation so that the compound g is obtained.

[0030] The synthesis of compound h: The compound g is dissolved in methanol, and the solution is added with Dowex H^+ (washed with methanol in advance) for reaction at a room temperature. After completion of the reaction, the resin is removed from the solution through filtration and repeatedly washed with methanol. The solution is evaporated to obtain the solid substance. The obtained solid substance is dissolved in acetone, and then concentrated sulfuric acid (catalytic amount) is slowly added into the solution for reaction at a room temperature while stirring. The reaction solution is added with concentrated ammonia water to adjust pH value, and then filtered and evaporated under a reduced pressure to remove acetone, so as to obtain the compound h.

[0031] The synthesis of compound i: DMSO is added dropwise into the dichloromethane solution of oxalyl chloride at -60 °C. Then, the solution is stirred for about 15 minutes and then is added dropwise with the dichloromethane solution of the compound h. After the reaction is carried out for 30 minutes at -60 °C, triethylamine is added in the solution to continue the reaction at a room temperature for 30 minutes. Then, water is added to terminate the reaction, and the organic layer is separated and dried over anhydrous Na_2SO_4. The solvent is removed through evaporation and the residue is separated and purified by column chromatography to obtain the solid product. $ClCH_2P(C_6H_5)_3Cl$ is added into anhydrous tetrahydrofuran. The solution is cooled to about -78 °C, added with n-BuLi hexane solution, stirred for reaction for about 1h, then added with the dry tetrahydrofuran solution of the solid product. The temperature of the solution is gradually increased to about 0 °C to continue the reaction. After completion of the reaction, saturated NH_4Cl solution is carefully added dropwise in the solution, and the solution is subsequently extracted with ethyl acetate. The organic layer is washed with saline twice, dried over anhydrous $MgSO_4$, and evaporated to remove the solvent. The residue obtained is dissolved into anhydrous tetrahydrofuran, cooled to about -78 °C, and slowly added dropwise with n-BuLi hexane solution. After the solution is stirred for about 2 hours, the reaction is carefully terminated by using saturated NH_4Cl. The organic layer is washed with saturated salt water, dried over anhydrous MgSO4, and evaporated under a reduced pressure to remove the solvent. Finally the residue is separated and purified by rapid silica gel column, so as to obtain the compound i.

[0032] The synthesis of compound j: The compound i is added in the diluted hydrochloric acid solution, the solution is stirred at a room temperature for reaction. After completion of the reaction, the solution is neutralized by solid $NaHCO_3$, and then filtered. The filtrate is evaporated under a reduced pressure to remove the solvent, and the residue is dissolved by dichloromethane and dried over anhydrous Na_2SO_4, and then filtered and evaporated to remove the solvent, so as to obtain the compound j.

[0033] The synthesis of compound k: The compound j is dissolved in pyridine, and the solution is added dropwise with BzCl for reaction at a room temperature. After completion of the reaction, the solvent is evaporated to dryness, and the residue is purified to obtain the compound k.

[0034] The synthesis of compound 1: The compound k and 2,2,6,6 - tetramethyl piperidine-N-oxide are dissolved in dichloromethane, the solution is cooled to about 0 °C and added with the mixed solution of sodium hypochlorite solution, $NaHCO_3$ and water for reaction at about 0 °C for about 30 minutes. Isopropanol is added into the reaction solution and stirred at a room temperature. The separated dichloromethane layer is washed with water twice, dried over anhydrous $MgSO_4$, filtered, and evaporated to remove the solvent. The obtained residue is recystalized to obtain the compound 1.

[0035] The synthesis of compound m: The compound 1 is dissolved in the mixed solution of absolute ethanol and ethyl acetate. The solution is cooled to about 0 °C, and then added with sodium borohydride in batches, being stirred for reaction. After completion of the reaction, the reaction solution is neutralized with a dilute acetic acid solution, filtered, and the filtrate is evaporated. The residue is dissolved in dichloromethane and then washed with water. The dichloromethane layer is dried over anhydrous $MgSO_4$, filtered, and evaporated to remove the dichloromethane under a reduced pressure. The residue is recrystallized by the mixed solvent of dichloromethane and petroleum ether to obtain solid substance. Then the solid product is dissolved in pyridine. BzCl is added dropwise at about 0°C, and after that the reaction goes on at a room temperature. After completion of the reaction, the solvent is removed through evaporation and the residue is extracted with ethyl acetate, washed with saturated $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, filtered, evaporated to remove the solvent, so that the compound m is obtained.

[0036] The synthesis of compound n: The compound m is dissolved in methanol, and then the solution is added with the mixed solution of concentrated hydrochloric acid / dioxane for reaction at a room temperature. After completion of the reaction, the reaction solution is neutralized with $NaHCO_3$, and filtered. The solvent is removed from the filtrate by vacuum distillation, and the residue is dissolved in an appropriate amount of dichloromethane and dried over anhydrous Na_2SO_4 for overnight and then filtered, evaporated to dryness, and finally separated and purified by column chromatography to obtain the compound n.

[0037] The synthesis of compound o: The compound n is dissolved in dichloromethane, and the solution is added with DAST under a room temperature and stirred for reaction. After completion of the reaction, the solution is poured into saturated $NaHCO_3$ solution and the organic layer is separated. The organic layer is washed with saturated $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, and separated and purified by column chromatography to obtain the compound o.

[0038] The synthesis of compound p: The compound o is added in formic acid solution, and the solution is stirred at a room temperature for reaction. After completion of the reaction, the solution is evaporated under a reduced pressure to remove the solvent. The residue and toluene are evaporated together to obtain the solid product. The obtained solid product is dissolved in the solution of dry pyridine and acetic anhydride for reaction at a room temperature. After the reaction is completed, the reaction solution is concentrated and then evaporated together with toluene, so as to obtain the compound p.

[0039] The synthesis of compound q: The compound p is dissolved in anhydrous dichloromethane and cooled to about 0°C. The HCl gas is slowly introduced into the solution to a saturation level and the solvent is removed by vacuum evaporation at a room temperature. The residue is dried in vacuum to obtain the compound q.

[0040] The synthesis of compound r: With the existence of $(N_4)_2SO_4$, 2,6-diamino-purine and Hexamethyldisilazane (HMDS) are heated and refluxed to be a transparent solution. The solvent is removed under vacuum to obtain a white solid product, and the solid product is subsequently dissolved in 1, 2-dichloroethane. Then, 1,2-dichloroethane solution of compound q and molecular sieve are added, the solution is stirred for reaction under nitrogen protection at a room temperature. After the reaction is detected by TLC as complete, the reaction solution is added with dichloromethane and filtered by diatomite. The filtrate is washed with saturated Na_2SO_4 and saline respectively. The organic layer is dried over anhydrous Na_2SO_4 overnight and the solvent is removed through evaporation to obtain the residue. The residue is separated under a reduced pressure through silica gel column, so as to obtain the compound r.

[0041] The synthesis of compound s: The compounds r is dissolved in saturated NH_3-CH_3OH solution, and the solution is stirred for reaction at a room temperature. After completion of the reaction, the solvent is removed through evaporation and the residue is separated and purified by column chromatography, so as to obtain the compound s.

[0042] The beneficial effects of the present invention lie in that, by means of chemical modifications made to the glycosyl and base, D-and L-nucleoside analogue would contain special structures such as fluoric group, alkynyl, azide and cyano etc. and thus can overcome the deficiencies of the present D-and L - nucleoside analogues, including more toxic side effect and less activity. Furthermore, the synthesis method is simple and feasible with high yield ratio, having a good value when it is used in the preparation of anti-HBV or anti-HCV or anti-HIV drugs.

Specific Embodiments

[0043] The present invention is further described hereinafter according to the synthetic route of the compound expressed in the general formula I in combination with the preferred embodiments. However, these preferred embodiments

provided are not to limit the scope of the present invention.

Example 1. The synthesis of the compound 15 wherein the base is cytosine

[0044] The synthesis of compound ii: The compound i (D-ribose or L-ribose) (8.66mmol) was dissolved in 31.2m HCl/MeOH (0.2mmol/L), and the solution was stirred in a water bath at 27°C for 3 hours under hermetization and warm preservation. The solution was added with 7.8ml pyridine to terminate the reaction, and then drawn off under a reduced pressure, so as to obtain the light yellow syrup-like compound ii (96%);

[0045] The synthesis of compound iii: The compound ii (7.92mmol) was dissolved in 21 ml dry pyridine, and 5.2 ml benzoyl chloride (0.0447mol) was slowly added dropwise in the solution in an ice-salt bath. The solution was stirred at a room temperature for 17 hours. The reaction solution was poured into 39 ml ice water, and extracted with chloroform. The organic layer was washed sequentially with ice water, pre-cooled sulfuric acid (3mol/L) and saturated sodium bicarbonate solution until the water layer shows weak alkaline, and then washed with ice water until the water layer shows neutral, dried over anhydrous sodium sulfate for more than 4 hours, and drawn off under a reduced pressure, so as to obtain the light yellow syrup-like compound iii (80.0%). ^{1}H NMR($CDCl_3$) δppm: 7.28~8.10 (m, 15H, OBz), 5.87 (m, 1H, H-3), 5.68 (d, 1H, H-2), 5.16 (s, 1H, H-1), 4.72 m, 2H, H-5), 4.52 (q, 1H, H-4), 3.42 (s, 3H, OCH_3).

[0046] The synthesis of compound iv: Benzoyl compound iii (0.0027mol) was dissolved in chloroform (13mol), red phosphorus (390mg, 0.0126mol) was added, and bromine (1.3ml., 0.025mmol) was slowly added dropwise while stirring the solution in an ice-water bath. After stirring it for 30 minutes, 1.3 ml ice water was slowly added dropwise and the solution was stirred at a room temperature for 4 hours. 7g broken ice was added and stirred to melt the broken ice. Then the reaction solution was poured into 14 ml ice water and extracted with chloroform. The organic layer was washed with saturated sodium bicarbonate until the water layer shows weak alkaline, and then washed with ice water until the water layer shows neutral, and then dried over anhydrous sodium sulfate for over 4 hours, and then drawn off under a reduced pressure to obtain the dark yellow syrup. Finally the syrup was separated by silica gel column (gradient elution, petroleum ether: acetone), so as to obtain the white dry syrup compound iv (64.2%). ^{1}H NMR($CDCl_3$) δppm: 7.32~8.10 (m, 15H, OBz), 5.90 (m, 1H, H-3), 5.70 (d, 1H, H-2), 5.63 (s, 1H, H-1), 4.55~4.79 (m, 3H, H-5, H-4).

[0047] The synthesis of compound v: The compound iv (2.73mmol) was dissolved in 5ml dry pyridine, and the solution was slowly added dropwise with acetic anhydride (0.0276mol) in an ice water bath, then stirred for 30 minutes. Then, the ice water bath was removed, and the solution was stirred at a room temperature for 7 hours, and then heated up to 40°C. This temperature was kept for an hour. The solution was added with 6.5g broken ice and then stirred until the broken ice was melted. The reaction solution was poured into 13 ml ice water and extracted with chloroform. The organic layer was washed sequentially with ice water, pre-cooled sulfuric acid (3mol/L) and saturated sodium bicarbonate until the water layer shows weak alkaline, then washed with ice water until the water layer shows neutral, dried over anhydrous sodium sulfate for more than 4 hours, and then drawn off under a reduced pressure, so as to obtain the light yellow syrup-like compound v (92.1%). ^{1}H NMR($CDCl_3$) δppm: 7.30~8.10 (m, 15H, OBz), 6.43 (s, 1H, H-1), 5.90 (m, 1H, H-3), 5.80 (d, 1H, H-2), 4.50~4.80 (m, 2H, H-5), 2.00 (s, 3H, CH_3COO-).

[0048] The synthesis of compound vi: The compound v (2.57mmol) was dissolved in 26 ml dry dichloromethane and dry HCl gas was slowly introduced into an ice-water bath for 2.5 hours. The solution was washed with 19.5ml ice water and the organic layer was separated. The organic layer was washed with saturated sodium bicarbonate until the water layer shows weak alkaline, and then washed with ice water until the water layer shows neutral, dried over anhydrous sodium sulfate for more than 4 hours, and then drawn off under a reduced pressure to obtain light yellow syrup. The syrup was recrystallized using the mixed solvent of hexane and dichloromethane, so as to obtain the white solid compound vi (65.1%). ^{1}H NMR($CDCl_3$) δppm: 7.36~8.14 (m, 15H, OBz), 6.68 (d, *J*=4.4Hz, 1H, H-1), 5.59 (dd, 1H, H-3), 4.64~4.80 (m, 4H, H-2, H-4 and H-5). M,p, 139~140°C.

[0049] The synthesis of compound vii: The compound vi (2.81mmol) was dissolved in 13 ml dry dichloromethane and 3.5ml dry DMF, and SO_2Cl_2 (0.0079mmol) was slowly added while stirring the solution at -15°C. After it was stirred at -15°C for about 30 minutes, the solution was naturally warmed up to a room temperature, reacting for 3 hours. Then the solution was added with imidazole (0.0407mmol) for three times at 0°C. The mixed solution was stirred at a room temperature for 15 hours. The reaction solution was diluted with CH_2Cl_2 (26ml) added, and washed with ice water (35ml). The water layer was extracted with CH_2Cl_2. Then the organic layers were combined and then dried over anhydrous sodium sulfate for over 4 hours, and then drawn off under a reduced pressure to obtain light yellow syrup. The syrup was separated and purified by silica gel column (gradient elution, petroleum ether: acetone) to obtain the white solid compound vii (76.0%). ^{1}H NMR($CDCl_3$) δppm: 7.00~8.10 (m, 15H, OBz), 6.71 (d, *J*=4.4Hz, 1H, H-1), 5.59 (dd, 1H, H-3), 5.25 (dd, 1H, H-2), 4.56~4.81 (m, 3H, H-4, H-5). M.p.128~129°C.

[0050] The synthesis of compound viii: The compound vii (2.2mmol) was dissolved in ethyl acetate (54ml), and Et_3N • 3HF (2.08ml, 0.013mmol) was added while stirring the solution. The solution was heated up to 60°C, stirred for 3h, and then heated to 70 °C and stirred for 1.5h. The solution was added with ice-salt (10 ml) to terminate the reaction, and extracted with dichloromethane. The organic layers were combined and then washed sequentially with saline, water

and saturated sodium bicarbonate, and dried over anhydrous sodium sulfate for over 4 hours. The solvent was drawn off under a reduced pressure to obtain dark yellow syrup. The syrup was purified (dichloromethane elution) by means of a silica gel funnel (5cm × 5cm) to yield light yellow syrup(86.8%). Finally the crude product was crystallized in 95% ethanol solution to obtain the white compound viii crystalline (66.4%). ^{1}H NMR($CDCl_3$) δppm: 7.31~8.10 (m, 15H, OBz), 6.71 (d, *J*=9.0Hz, 1H, H-1), 5.68 (dd, *J*=19.44Hz, 1H, H-3), 5.32 (d, *J*=48.2Hz, 1H, H-2), 4.65~4.77 (m, 3H, H-4, H-5). M.p.80~82°C.

[0051] The synthesis of compound ix: The compound viii (6.0mmol) was dissolved in anhydrous dichloromethane (20ml), and a mixed solution of HBr-AcOH (45%, V/V, 4.6ml, 25mmol) was added in the solution. The solution was stirred for reaction at a room temperature for 20 hours. The mixture was evaporated to dryness. The residue was dissolved by dichloromethane (50ml). The dichloromethane solution was washed with sodium bicarbonate solution (3×30ml), and dichloromethane was removed through evaporation to obtain a syrup-like product. Meanwhile, the protected cytosine (15mmol) and $(NH_4)_2SO_4$ (0.1g) were refluxed for 17 hours under nitrogen protection in HMDS (30ml). After completion of reaction, the solvent was removed through evaporation under a reduced pressure to obtain silylated cytosine. The syrup obtained in the above reaction was dissolved in dichloroethane (25ml), and added into the silylated cytosine. Then the mixture was refluxed for 15 hours under N_2 protection and the reaction was terminated by ice. Then, the solution was extracted with dichloromethane (3×45ml) and the dichloromethane layer was washed sequentially with saturated sodium bicarbonate and saline, and dried over anhydrous sodium sulfate. After drying, the solvent was removed through evaporation to yield white solid, and the solid was separated and purified by column chromatography (1% MeOH-$CHCl_3$) to obtain the compound ix(71%). ^{1}H NMR($CDCl_3$) δppm: 8.07 (d, J=9.86Hz, 1H), 7.45 (d, J=9.82Hz, 1H), 7.26~8.10 (m, 10H, OBz), 6.03 (d, *J*=9.0Hz, 1H, H-1), 5.64 (dd, *J*=19.44Hz, 1H, H-3), 5.26 (d, *J*=48.2Hz, 1H, H-2), 4.65~4.77 (m, 3H, H-4, H-5).

[0052] The synthesis of compound x: The compound ix (3.60mmol) was dissolved in saturated NH_3-CH_3OH (30ml), and the solution was stirred for reaction at a room temperature for 15 hours. After completion of the reaction, the solvent was evaporated to dryness to obtain the residue, and the residue was purified by column chromatography (15:1 $CHCl_3$-MeOH) to obtain the compound x(80%). ^{1}H NMR($CDCl_3$) δppm: 8.10 (d, J=9.86Hz, 1H), 7.40(d, J=9.82Hz, 1H), 6.03 (d, *J*=9.0Hz, 1H, H-1), 5.64 (dd, *J*=19.44Hz, 1H, H-3), 5.26 (d, *J*=48.2Hz, 1H, H-2), 4.50 (m, 1H, H-4), 3.70~3.77 (m, 2H, H-5).

[0053] The synthesis of compound xi: The compound x (9.46mmol), imidazole (18.93mmol) and triphenylphosphine (14.19mmol) were dissolved in tetrahydrofuran (50ml). The solution was slowly added dropwise with 15ml iodiferous tetrahydrofuran (14.18mmol). The solution was stirred for reaction at a room temperature for 3 hours. After completion of the reaction, the solvent was removed through evaporation to obtain the residue, and the residue was added with ethyl acetate (100ml) and filtered. The ethyl acetate was removed through evaporation and the residue was separated by column chromatography to obtain the compound xi(83.9%). ^{1}H NMR($CDCl_3$) δppm: 8.06 (d, J=9.86Hz, 1H), 7.43 (d, J=9.82Hz, 1H), 6.01 (d, *J*=9.0Hz, 1H, H-1), 5.66 (dd, *J*=19.44Hz, 1H, H-3), 5.22 (d, *J*=48.2Hz, 1H, H-2), 4.57 (m, 1H, H-4), 3.58~3.69 (m, 2H, H-5).

[0054] The synthesis of compound xii: The compound xi (5.88mmol) was dissolved in tetrahydrofuran (50ml), and the solution was added with DBU (6.44mmol) and then stirred at 60°C for 3 hours. The solvent was evaporated to dryness, and the residue was separated by column chromatography to obtain the compound xii(75.1%) which is directly used in the next reaction.

[0055] The synthesis of compound xiii: The 15m DMF solution dissolved with ICl (13.9mmol) was added in the DMF (15ml) solution dissolved with NaN_3 (9.75mmol) at 0°C. The solution was stirred at 0°C for 10 minutes and was slowly added dropwise with DMF (20ml) dissolved with the compound xii (6.8mmol) to continue the reaction at 0°C for an hour. After completion of the reaction, the mixed solution was added with sodium sulfite until the color of iodine disappears completely. The solvent was removed through evaporation under a reduced pressure, and the residue was separated and purified by column chromatography to obtain the compound xii (77.6%) which is directly applied in the next reaction.

[0056] The synthesis of compound xiv: The compound xiii (5mmol) was dissolved in DMF (15ml), and the solution was added with silver acetate (6mmol) and stirred. After the reaction was carried out at a room temperature for 8 hours, the solution was filtered and the solvent was removed under a reduced pressure (at a temperature below 50°C). The residue was purified by column chromatography to obtain the compound xiv (71.3%). ^{1}H NMR(DMSO-d_6, 300 MHz) δ: 8.12 (d, 1H), 7.40 (d, 1H), 7.26 (br, 2H), 6.12 (dd, 1H), 5.87 (d, 1H), 5.09 (t, 1H), 4.90 (dt, 1H), 4.18 (dt, 1H), 3.74-3.87 (m, 2H), 2.12 (s, 3H, CH_3).

[0057] The synthesis of compound xv: The compound xiv (5mmol) was dissolved in 5% methanol-triethylamine (100ml), and the solution was stirred at a room temperature for 12 hours. After completion of the reaction, the solvent was removed through evaporation and the residue was purified by column chromatography to obtain the compound xv (89.0%).ESI-MS: 287[M+H]. ^{1}H NMR(DMSO-d_6, 300 MHz)δ: 8.14 (d, 1H), 7.32 (d, 1H), 7.21 (br, 2H), 6.08 (dd, 1H), 5.82 (d, 1H), 5.11 (t, 1H), 4.92 (dt, 1H), 4.16 (dt, 1H), 3.62-3.69 (m, 2H).

Example 2. The synthesis of the compounds 25, wherein the 4'-substitued group is alkynes and the base is 1,6 - diamino-purine

[0058] The synthesis of compound b: Compound a (60g) was added in acetone (2L) and the concentrated sulfuric acid (40ml) was slowly added while stirring. The solution was stirred for reaction at a room temperature for 40 minutes. After the reaction was detected by TLC as complete, the solution was added with the concentrated ammonia to adjust pH=7-8, filtered, and evaporated under a reduced pressure to remove most of the acetone. The remaining acetone and water were about 150 ml. Then the solution was added with 0.4% dilute hydrochloric acid (150m1) while being stirred, and the reaction was completed under the acetone reflux condition for 20 minutes until the compound a has been completely hydrolyzed into the compound b. After completion of the reaction, the reaction solution was neutralized to pH=7-8 by solid $NaHCO_3$, filtered, and evaporated to remove the solvent under a reduced pressure. The residue was dissolved with dichloromethane (200ml), dried over anhydrous Na_2SO_4 overnight and then filtered. Finally the solvent was removed through evaporation to obtain the yellow viscous oily b (73g, 95%), ESI-MS: 191 [M+H].

[0059] The synthesis of compound c: The compound b (154g, 0.81mol) and triethylamine (339ml, 2.43mol) were dissolved in dichloromethane (1.50L). The solution was cooled to 0 °C in an ice salt bath, slowly added dropwise with parachlorobenzoyl chloride (113ml, 0.891mol) and mechanically stirred for reaction at a temperature below 0 °C for 4 hours. After completion of the reaction, the solution was added with a saturated $NaHCO_3$ solution (500ml). The dichloromethane layer was washed with water and saturated salt water, dried over anhydrous $MgSO_4$, filtered, and evaporated under a reduced pressure to remove the solvent. The residue was recrystallized to obtain white crystalline compound c (196g, yield=74.6%), ESI-MS: 330[M+H].

[0060] The synthesis of compound d: The compound c (0.114ml) was dissolved in anhydrous dichloromethane (600ml), and the solution was added with imidazole (15.5g, 0.228mol) and tert-butyl-dimethyl chloride silicon (TBDMSCI) (18.9g, 0.125ml) under nitrogen protection for the reaction for 3 hours at a room temperature. After completion of the reaction, 1N hydrochloric acid was added to neutralize the reaction and two reaction layers were obtained. The organic layer was washed with water and saturated salt water, dried over anhydrous Na_2SO_4, and evaporated to remove the solvent. The residue was separated and purified by column chromatography to obtain compound d (yield=69.2%), ESI-MS: 444[M+H].

[0061] The synthesis of compound e: The compound d (0.64mmol) was dissolved in the mixed solution (0.2N, 15 ml) of sodium methoxide and methanol for reaction for 4 hours at a room temperature. After completion of the reaction, the solution was neutralized by dilute acetic acid, filtered, washed with methanol and evaporated under a reduced pressure to remove the solvent. Finally the residue was separated and purified by column chromatography to obtain compound e (yield=75%), ESI-MS: 305[M+H].

[0062] The synthesis of compound f: DMSO (5.5ml, 77.5mmol) was added dropwise into the dichloromethane (80.0ml) solution of oxalyl chloride (3.4ml, 38.7mmol) at -60 °C. The solution was stirred at the same temperature for 15 minutes and then added dropwise with the dichloromethane solution (100ml) of the compound e (25.7mmol). After the solution was stirred for reaction at -65°C for 30 minutes, triethylamine (10.9ml, 78.2mmol) was added to continue the reaction for 30 minutes and the solution was stirred at a room temperature. After completion of the reaction, water was added in the reaction mixture so that the organic layer is separated. The organic layer was dried over anhydrous magnesium sulfate. Finally the solvent was removed through evaporation and the residue was separated and purified by column chromatography to obtain the compound f (yield=94.6%), ESI-MS: 303[M+H].

[0063] The synthesis of compound g: Sodium hydroxide (1.3g) and water (11.5ml) were added in the reaction bottle. The solution was stirred evenly and added with formaldehyde solution (30%), 7.2 ml 95% ethanol, and then the compound f (25mmol). Then, the solution was stirred for reaction at a controlled reaction temperature of 30~35°C for 2 hours. After completion of the reaction, the reaction bottle was cooled with ice water while stirring until the product was completely precipitated. After the suction filtration, the solid product was washed with water to neutral, dried, dissolved in anhydrous methanol, then added into sodium borohydride (0.925g, 25mmol) and refluxed for an hour. After completion of the reaction, the reaction solution was neutralized by dilute hydrochloric acid and extracted with dichloromethane (3×50ml), dried over anhydrous Na_2SO_4. The solvent was removed by evaporation, so that the compound g was obtained (yield= 90.5%, ESI-MS: 335[M+H]).

[0064] The synthesis of compound h: The compound g (9.7mmol) was dissolved in methanol (260ml), and the solution was added with 40ml Dowex H^+ (washed with methanol in advance) for reaction at a room temperature for 4 hours. The resin was removed from the solution through filtration and repeatedly washed with methanol. The solvent was dried to obtain solid substance. The obtained solid substance was dissolved in acetone (200ml). Then concentrated sulfuric acid (catalytic amount, 2 ml) was slowly added dropwise for reaction at a room temperature for about 0.5 hours. Then the solution was added with concentrated ammonia water to adjust pH value to 7~8, filtered, evaporated under a reduced pressure to remove most of acetone. The reaction solution was added with diluted hydrochloric acid (0.4%, 40ml) and reacted 20 minutes under acetone refluxing. Then the reaction solution was neutralized by $NaHCO_3$ to pH=7~8, filtered, evaporated under a reduced pressure to remove the solvent. The residue was dissolved by appropriate amount of dichloromethane, dried over anhydrous Na_2SO_4, and evaporated to remove the solvent, so as to obtain the compound

h (yield=80.2%, ESI-MS: 261 [M+H])

[0065] The synthesis of compound i: DMSO (5.5ml, 77.5mmol) was added dropwise into the dichloromethane(80ml) solution of oxalyl chloride(3.4ml, 38.7mmol) at -60 °C. Then, the solution was stirred for 15 minutes and added dropwise with the dichloromethane (100ml) solution of compound h (25.7mmol). After the reaction was carried out for 30 minutes at -60 °C, triethylamine (10.9ml, 78.2mmol) was added in to continue the reaction at a room temperature for 30 minutes. Then, water was added to terminate the reaction, and the organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was removed through evaporation and the residue was separated and purified by column chromatography to obtain a solid product. $ClCH_2P(C_6H_5)_3Cl$ (2.15g, 6.19mmol) was added into anhydrous tetrahydrofuran (50ml). The solution was cooled to -78 °C, added with n-BuLi hexane solution(1.6M, 4.0ml), stirred for reaction for an hour, and added with the dry tetrahydrofuran solution(50ml) of the solid product (1.51mmol). The cooling temperature was gradually increased to 0 °C to continue the reaction for 3 hours. After completion of the reaction, a saturated NH_4Cl solution (10ml) was carefully added dropwise, and the solution was subsequently extracted with ethyl acetate (2×100ml). The organic layer was washed with saline twice (2×75ml), finally dried over anhydrous $MgSO_4$, and evaporated under a reduced pressure to remove the solvent. The residue produced was dissolved into anhydrous tetrahydrofuran (40ml), cooled to -78 °C, and slowly added dropwise with the hexane solution of n-BuLi (1.6M, 20ml). After the solution was stirred for reaction for 2 hours, the reaction was terminated by carefully adding a saturated NH_4Cl solution (20ml). The organic layer was washed with saturated salt water, dried over anhydrous MgSO4, and evaporated under a reduced pressure to remove the solvent. Finally the residue was separated and purified by rapid silica gel column to obtain the compound i (yield=48.5%, ESI-MS: 255[M+H]).

[0066] The synthesis of compound j: The compound i (10mmol) was added into the diluted hydrochloric acid solution (0.2%, 50ml) and stirred for reaction for 6 hours at a room temperature. After completion of the reaction, the solution was neutralized by solid $NaHCO_3$ to pH=7-8 and then filtered. The filtrate was evaporated under a reduced pressure to remove the solvent and the residue was dissolved with dichloromethane and dried over anhydrous Na_2SO_4, and then filtered and evaporated to remove the solvent, so as to obtain the compound j (yield=99.0%, ESI-MS: 215[M+H]).

[0067] The synthesis of compound k: The compound j (5mmol) was dissolved in pyridine (20ml), and the solution was added dropwise with BzCl (5mmol) for reaction at a room temperature for 6 hours. After completion of the reaction, the solvent was removed through evaporation and the residue was purified to obtain the compound k (yield= 85.3%, ESI-MS: 319[M+H]).

[0068] The synthesis of compound 1: The compound k (59.3mmol) and 2,2,6,6-tetramethyl piperidine-N-oxide (0.059mmol) were dissolved in dichloromethane(99ml), the solution was cooled to 0 °C and added with the mixed solution of sodium hypochlorite solution (33.6ml., 8.5~13.5% active chlorine), $NaHCO_3$ (11.2g) and water (190ml) for reaction at 0 °C. After 30 minutes, the reaction solution was added with isopropanol (1.95ml) and stirred for reaction at a room temperature for 10 minutes. The dichloromethane layer separated was washed with water twice, dried over anhydrous $MgSO_4$, filtered, and then evaporated to remove the solvent. The residue obtained was recystalized to obtain the crystalline compound 1 (yield= 87.8%, ESI-MS: 317[M+H]).

[0069] The synthesis of compound m: The compound 1 ((0.05mol) was dissolved in the mixed solution of absolute ethanol (60ml) and ethyl acetate (30ml); the solution was cooled to 0 °C, and then added with sodium borohydride (23.3g, 612mmol) in batches, being stirred for reaction at 0 °C for an hour. After completion of the reaction, the reaction solution was neutralized with dilute acetic acid solution to pH=7-8, filtered, and the filtrate was evaporated. The residue was dissolved in dichloromethane and washed with water. The dichloromethane layer was dried over anhydrous $MgSO_4$, filtered, and evaporated to remove the dichloromethane under a reduced pressure. The residue was recrystallized by the mixed solvent of dichloromethane and petroleum ether to produce a solid product. Then the solid product was dissolved in pyridine. BzCl (50mmol) was added dropwise at 0°C, and after that, the reaction goes on at a room temperature for 3 hours. After completion of the reaction, the solvent was removed through evaporation and the residue was extracted with ethyl acetate, washed with saturated $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, filtered, evaporated to remove the solvent, so that the compound m was obtained (yield=89.7%, ESI-MS: 423[M+H]).

[0070] The synthesis of compound n: The compound m (4mmol) was dissolved in methanol (22ml), and then the solution was added with the mixed solution (4.0M, 20ml) of concentrated hydrochloric acid/dioxane for reaction at a room temperature for 10 hours. After completion of the reaction, the reaction solution was neutralized with $NaHCO_3$ to pH=7-8 and filtered. The filtrate was distilled under a reduced pressure to remove the solvent, and the residue was dissolved in an appropriate amount of dichloromethane, dried over anhydrous Na_2SO_4 for overnight, then filtered, evaporated to dryness, and finally separated and purified by column chromatography to obtain the compound n (yield=89.5%, ESI-MS: 397[M+H]).

[0071] The synthesis of compound o: The compound n (1.76mmol) was dissolved in dichloromethane, and the solution was added with DAST (0.4ml., 3.03mmol) under a room temperature and stirred for 12 hours. After completion of the reaction, the solution was poured into 10 ml saturated $NaHCO_3$ solution and the organic layer was separated. The organic layer was washed with saturated $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, and separated and purified by column chromatography to obtain the compound o (yield=11.5%, ESI-MS: 399[M+H]).

[0072] The synthesis of compound p: The compound o (10mmol) was added in a 20 ml formic acid (80%) solution for reaction and the solution was stirred at a room temperature for 12 hours. After completion of the reaction, the solution was evaporated under a reduced pressure to remove the solvent. The residue and toluene were evaporated together to obtain a solid product. The obtained solid product was dissolved in the solution of dry pyridine and acetic anhydride for reaction at a room temperature for 4 hours. After the reaction was completed, the reaction solution was concentrated and then evaporated together with toluene to obtain the compound p (90.3%, ESI-MS: 427[M+H]). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.07 (s, 3H), 2.85 (s, 1H), 4.62-4.77 (m, 2H), 4.89-5.07 (m, 3H), 7.32-8.17 (m, 10H).

[0073] The synthesis of compound q: The compound p (4.76mmol) was dissolved in anhydrous dichloromethane (40ml) and cooled to 0°C. The HCl gas was slowly introduced into the solution to saturation level for about 3 hours, and the solvent was removed through vacuum evaporation at a room temperature. The residue was dried in vacuum for 2 hours to obtain the compound q (yield=87.1%) which is directly applied in the next reaction.

[0074] The synthesis of compound r: With the existence of $(NH_4)_2SO_4$(50mg), 2,6-diamino-purine (4.78mmol)and Hexamethyldisilazane (HMDS, 9ml) were heated and refluxed to obtain transparent solution (in about 5 hours).The solvent was removed under vacuum to obtain a white solid product, and the solid product was dissolved in 1,2-dichloroethane(35 ml). Then, the 1,2-dichloroethane solution of compound q (4.56mmol, 30ml) and 0.4nm molecular sieve (2.6g) were added, and the solution was stirred for reaction at a room temperature for 6 days under nitrogen protection. After the reaction was detected by TLC as complete, the reaction solution was added with dichloromethane (80 ml) and filtered by diatomite. The filtrate was washed with saturated Na_2SO_4 and saline respectively. The organic layer was dried over anhydrous Na_2SO_4 overnight and the solvent was removed through evaporation to obtain the residue. The residue was separated under a reduced pressure by silica gel column, so as to obtain the compound r (yield=78.5%, ESI-MS: 517[M+H]). ^{1}H NMR($CDCl_3$, 300 MHz)δ: 2.85 (s, 1H), 4.67-4.79 (m, 2H), 4.92-5.13 (m, 3H), 5.79 (br, 1H), 7.32-8.17 (m, 9H), 8.35 (s, 1H).

[0075] The synthesis of compound **25:** The compound r (3.60mmol) was dissolved in the saturated NH_3-CH_3OH solution (30ml), and the solution was stirred for reaction at a room temperature for 15 hours. After completion of the reaction, the solvent was removed through evaporation and the residue was separated and purified by column chromatography to obtain the compound 25(yield=78.9%, ESI-MS: 309[M+H]). ^{1}H NMR($CDCl_3$, 300 MHz)δ: 2.85 (s, 1H), 3.69-3.79 (m, 2H), 4.79-5.07 (m, 3H), 5.60 (br, 1H), 5.79 (br, 1H), 7.32-8.17 m, 4H), 8.35 (s, 1H).

[0076] **In Vitro test on the anti-HIV activity of the present invention:** The anti-HIV activities of the compounds 1, 9, 15, 19, 36, 39, 46, 50 and 52 in the cell culture.

[0077] **Drugs to be tested:** The compounds 1, 9, 15, 19, 36, 39, 46, 50 and 52, no batch number, water-insoluble, DMSO-soluble. During the test, the solution was dissolved by DMSO to suitable concentration, diluted by culture medium, and immediately added into cell culture.

Positive control drugs:

[0078] Zidovudine (AZT), a known HIV-1 nucleoside reverse transcriptase inhibitors that has been put into clinical application, in the form of white powder, purchased from Shanghai Desano Chemical & Pharmaceutical Co., Ltd., with batch number: 040201b;

[0079] Nevirapine (NVP), one of known non-nucleoside reverse transcriptase inhibitors in clinical application, purchased from Nanjing Zezhong Medical & Chemical Information Research Center (Batch No: 0301001).

[0080] **HIV-1IIIB Virus strain for test** was donated by Dr.Jiang Jiandong, Mount Sinai Medical Center, USA, amplified in Cell H9 and under cryopreservation at -196°C.

[0081] **Human T-lymphocyte subculture MT-4,** donated by Professor Zhang Xingquan, Medical Research Center, University of Colorado (USA), subcultured in our laboratory, under cryopreservation at -196 °C. Cell culture medium: RPMI Medium 1640 culture medium containing 10% fetal bovine serum, 100IU/ml penicillin, 100μg/ml streptomycin and kanamycin, and L-glutamine. The cells would be cultured at 37°C, in 5% CO_2 incubator, passaging once every three days.

[0082] **Principal reagents:** RPMI Medium 1640 culture medium, GIBCO(US); Fetal Bovine Serum(FBS), Tianjin Chuanye Biochemical Products Co., Ltd.; penicillin and streptomycin, North China Pharmaceutical Company; kanamycin, Shanghai Xudong Haipu Pharmaceutical Co., Ltd; tetrazolium (Thiazolyl blue, MTT) and citric acid, Sigma(U.S.) ; Triton X-100, KEBO AB STOCKHOLM (Sweden); N, N-dimethyl-Formamide, Beijing Chemical Plant; HIV-1 P24 antigen kit, BioMerieux (Netherlands).

[0083] **Main instruments:** Emax™ (Enzyme-labeled meter), MolICular Devices Inc. (United States);

Method:

[0084] With a 96-well culture plate, a test was conducted on the drug and the positive control drug group of drug-inhibited virus. Each well was added with 100ul DMSO solution of different concentrations of the drugs or the positive control drugs AZT and NVP or oral drugs for rats, different doses of different groups, and then at different time blood

sampling, and making different diluted group, 100ul per well. At the same time, a test was conducted on cell control group, virus control group, positive drugs AZT and NVP control group. MT-4 cells were infected by 100$TCID_{50}$ HIV-1 IIIB for 1.5 hours, washed with medium once, and prepared to solution of 2×10^5 cells/ml that were inoculated in 96-well culture plates of the test groups of drug-inhibited virus, positive control drug group and virus control group. Cell control groups were added in equivalent medium and cultured for 4 days. Cytopathic changes were observed by microscopy, and toxicities were measured by MTT staining method. The P24 antigens in supernatant were measured according to the kits' instructions. Accordingly, the inhibition ratios, the median toxic concentrations (CC_{50}) and the median effective concentrations (IC_{50}) and SI were calculated respectively.

The inhibition effects of the compounds 1, 9, 15, 19, 36, 39, 46, 50 and 52 on HIV-1 in MT-4 cell culture

[0085] The cytotoxicities and anti-HIV-1 activities of the compounds 1, 9, 15, 19, 36, 39, 46, 50 and 52 were determined by MTT, and their cytotoxicities on CC_{50} and SI were compared by using the HIV-1 infected and uninfected cells, as shown in Table 1.

Table 1 Anti-HIV-1 activities of the compound 1, 9, 15, 19, 36, 39, 46, 50, 52 and positive drugs AZT and NVP, in the MT-4 cell culture

Drug	CC_{50}(μg/ml)*		IC_{50}(μg/ml)*	SI	
	HIV-infected	HIV-uninfected		HIV-infected	HIV-uninfected
AZT	>0.625	>0.625	0.000182	>3434	>3434
NVP	>12.5	>12.5	0.00364	>3434	>3434
1	43.6	40.9	0.101	432	405
9	31.23	30.05	0.162	298	291
15	>100	>100	0.00001	>100000	>100000
19	43.8	39.8	0.09	487	422
36	35.12	37.54	0.127	276	296
39	38.26	36.77	1.49	26	25
46	48.52	55.35	0.131	370	423
50	28.3	27.9	2.30	12	12
52	41.3	38.6	0.133	311	290
*: Cytotoxicity: MTT method					

Table 2. The Pharmacological screening results of the compounds 1, 5 and 19 that were obtained by means of the pharmacological model of wild type HIV-1 replication

Compound	Pharmacological model	cell	Administration Routine	Dose (mol/L)	Inhibition ratio (%)	solvent	Remarks IC_{50} (μM)
1	VSVG/HIV-luc	Cell 293	Proir to infection	$1x10^{-5}$	99.4±0.3	DMSO	
				$1x10^{-6}$	90±0	DMSO	0.5μM
				$1x10^{-7}$	28±0	DMSO	
				$1x10^{-8}$	0±3	DMSO	
15	VSVG/HIV-luc	Cell 293	Proir to infection	$1x10^{-5}$	100	DMSO	
				$1x10^{-6}$	100	DMSO	1.5nM
				$1x10^{-7}$	100	DMSO	
				$1x10^{-8}$	72±6	DMSO	

(continued)

Compound	Pharmacological model	cell	Administration Routine	Dose (mol/L)	Inhibition ratio (%)	solvent	Remarks IC_{50} (μM)
19	VSVG/HIV-luc	Cell 293	Proir to infection	$1x10^{-5}$	92.7±0.9	DMSO	
				$1x10^{-6}$	48±0	DMSO	1.0μM
				$1x10^{-7}$	4±1	DMSO	
				$1x10^{-8}$	0±2	DMSO	
AZT	VSVG/HIV-luc	Cell 293	Proir to infection	$1x10^{-7}$	97.2±1.1	DMSO	
				$1x10^{-8}$	54.1±2.0	DMSO	
3TC	VSVG/HIV-luc	Cell 293	Proir to infection	$1x10^{-6}$	86.3±4.3	DMSO	
				$1x10^{-7}$	50.9±2.4	DMSO	
d4T	VSVG/HIV-luc	Cell 293	Proir to infection	$1x10^{-6}$	92.9±1.6	DMSO	
				$1x10^{-7}$	24.0±1.1	DMSO	
EFV	VSVG/HIV-luc	Cell 293	Proir to infection	$1x10^{-8}$	100	DMSO	
				$1x10^{-9}$	55.2±0.6	DMSO	
NVP	VSVG/HIV-luc	Cell 293	Proir to infection	$1x10^{-7}$	73.1±5.2	DMSO	
				$1x10^{-8}$	18.8±2.8	DMSO	

[0086] As can be known from Table 1 and Table 2, all the new 2'-fluoro-4'- substituted nucleosides have good anti-HIV activities, especially the compounds 1, 15 and 19 have good prospects in terms of application and development. Therefore, the development of such compounds will bring good news to patients with AIDS.

In vitro screening of anti-hepatitis B virus drugs in the present invention

[0087] **Object:** HBV genome transfected Hep G2.2.15 cell is used for screening of anti-hepatitis B virus drugs to provide theoretical and experimental basis in research and development of novel anti-HBV drugs.

[0088] **Method:** Lamivudine, compounds **15, 17** with concentration less than TC_{50} were selected to treat cells. Cell culture supernatant was collected at the time point of 144h and 216h respectively, for fluorescent quantitative-PCR detection of HBV DNA levels.

[0089] **Results:** Both Lamivudine and the compound 15 can significantly reduce the HBV DNA copy number after administration.

[0090] **Conclusion:** The compound 15 can significantly reduce HBV DNA activity in vitro tests, with less toxicity. This experiment provided a basis for further study on anti-HBV effects of the compounds.

[0091] **Cell lines:** Hep G2.2.15 cell lines, the HBV DNA cloning transfected human liver cancer HepG2 developed by Mount Sinai Medical Center (United States) in 1986, characterized by the stable expressions of e antigen and surface antigen, donated by the Wuhan Institute of Virology, Chinese Academy of Sciences. Passage and G418 screening were made by our laboratory in the process of cell culture.

[0092] **Test drugs:** synthetic drugs of compound 15 and 17 in the present invention;
Control drugs: Lamivudine (3TC), GlaxoSmithKline, Suzhou, China.

[0093] **Main reagents and instruments:** DMEM culture medium, Hyclone; FBS, Hangzhou Sijiqing; 24-well plates and culture bottles, Coming (USA); HBV PCR Fluorescence quantitative detection kit, Shenzhen PG Biotech; G418, Introvegen.

In vitro studies on Anti-HBV

[0094] Hep G2.2.15 cell culture: culture cell by DMEM medium containing 10% fetal bovine serum and 500mg / L G418, digestion by 0.25% trypsin and 0.02% EDTA; 1: 3 subculture; one passage every three days.

The studies on the Anti-HBV effects of drugs:

[0095] The cells with good growth conditions were selected, and the cell concentration was adjusted to $2X10^4$ cells/ml. Each well was inoculated by 1ml in a 24-well plate. Then the cells were cultured at 37 °C in 5% CO_2 incubator for 48 hours. The test was started as soon as the cells have shown good adhesive growth. Grouping was made based on different concentrations of the drugs (Table 1). Each concentration was set for two wells. The cell culture control group without drug was set as a blank control. On the 3rd, 6th and 9th days after administration, the drug-containing medium and blank medium in the same concentration were renewed once, and the supernatant of each well was sucked into EP tube and stored at -20 °C for inspection.

Table 1. The concentrations of Lamivudine and the compounds 15 and 17

Drug	concentration (μg/L)				
Lamivudine (3TC)	20,000	2000	200	20	2
Compound **15**	10	2	0.4	0.08	0.016
Compound **17**	1000	500	100		

[0096] Fluorescence quantification detection on the drugs for HBV PCR:

the inhibition ratio of HBV DNA (%)=(HBV DNA copy numbers in the blank group - HBV DNA copy numbers in the test group) / HBV DNA copy numbers in the blank group X 100%.

[0097] **Results:** The inhibition effects of drugs on HBV DNA in Hep G2.2.15 cells supernatant are shown in Tables 2 and 3

Table 2. The effects of Lamivudine on the copy numbers of HBV DNA (copy numbers, $x \times 10^5$) in the cell supernatant

Group	Concentartion(μg/ml)	HBV DNA Level	Inhibition ratio
Normal	/	99.79	/
3TC	20μ /ml	12.15	87.82
	2μg /ml	25.32	74.63
	0.2μg /ml	39.59	60.33
	0.02μg /ml	55.98	43.9
3TC EC_{50} is 38.78μg/L			

Table 3. The effects of the compounds 15 and 17 on the copy numbers of HBV DNA (copy numbers, $x \times 10^5$) in the cell supernatant

Group	Dose	HBV DNA copy number (* 10^5)	Inhibition ratio (%)
Normal	/	32.01	/
3TC	10,000μg/L	5.49	82.8
15	2μg/L	5.21	83.7
	0.4μg/L	10.3	67.8
	0.08μg/L	13.5	57.8
	0.016μg/L	18.9	41.0
17	1000μg/L	10.2	67.9

(continued)

Group	Dose	HBV DNA copy number (* 10^5)	Inhibition ratio (%)
	500μg/L	14.3	52.2
	100μg/L	19.2	40.6
For the compound **15, EC50** is 0.19μg/L.			

Table 4. The cytotoxicity of the compound 15 on Hep G2.2.15 (MTT)

Group	Dose	OD Value	Cell Survival Rate
Normal	/	0.644	/
15	1000μg/ml	0.520	82.6%
	200μg/ml	0.552	85.5%
	40μg/ml	0.606	96.7%
	8μg/ml	0.626	98.7%

In Vitro test on the anti- HCV activity of the present invention

1. Materials and methods

[0098] The HCV replication cells (Avva.5) were cultivated in the Dulbecco's improved Eagle medium containing 10% fetal calf serum and 1mg/ml G418. 293 - Sip - L cells were cultivated in the Dulbecco's improved Eagle medium containing 10% fetal calf serum , 250 μg / ml G418 and 150μg / ml hygromycin B.

1.2 Method for determining HCV infection (RT - PCR method)

[0099] Using Petri culture dish in a diameter of 60mm, the cells were cultivated in a positive culture medium containing 100μl of HCV for 12 hours. Then, the cells were cultivated in the fresh medium containing on HCV and the medium was replaced once everyday. When detecting HCV - RNA 7 days after the cells have been infected with HCV, it is necessary to clean the cells using the Dulbecco's improved Eagle medium in trypsinization and centrifugation methods. The upper layer part cleaned for the second time (used for comparison) and the washed cells were collected for RNA extraction and RT - PCR detection. β-actin mRNA was simultaneously determined for the purpose of comparison.

1.3 Quantitative determination on HCV - RNA

[0100] The quantitative determination on HCV - RNA is performed in automatic PCR Enzyme-linked immunosorbent assay (ELISA). (Edition 2.0, Roche Diagnostics, Branchburg, NJ)

2. Results

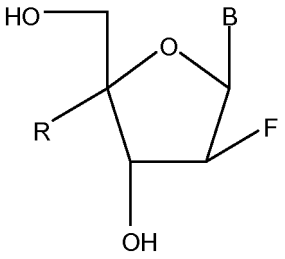
[0101] The compound 17 had inhibition effect when its concentration is 6 μg/ ml, 0.6 μg/ ml or 0.06 μ g/ml (refer to the table below). HCV- RNA level was determined in automatic PCR ELISA.

The concentration of the compound 17 in the cell supernatant	Inhibition effect	
	Test 1	Test 2
0 μg/ml	5.1×10^2	4.4×10^2
0.06 μg/ml	4.9×10^2	4.1×10^2
0.6 μg/ml	0.92×10^2	0.78×10^2
6 μg/ml	1.1×10^2	0.81×10^2

[0102] As shown in this table, the compound 17 had a relatively strong inhibition effect on HCV.

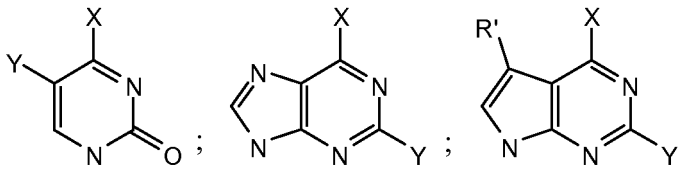
Claims

1. 2'-fluoro-4'- substituted nucleoside analogues, **characterized in that**, the compounds have the structure expressed in the general formula (I):



(I)

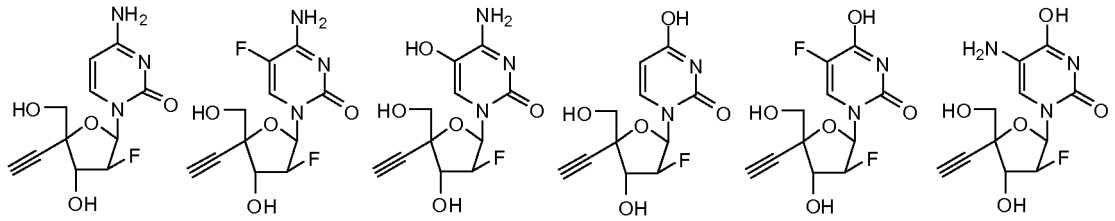
wherein :

B=  ;

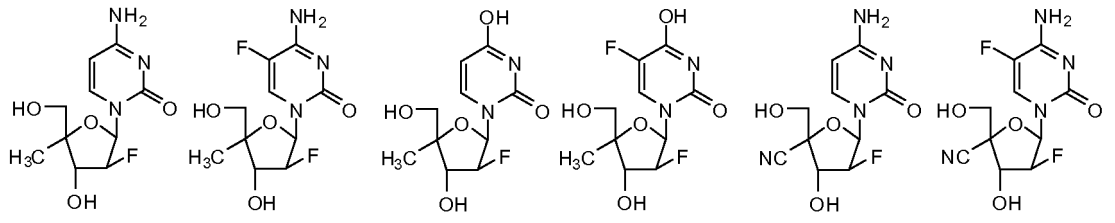
R = CH_3, CN, N_3, C≡CH;
R' = H, F;
X = F, OH, NH_2;
Y = H, CH_3, F, OH, NH_2

2. The 2'-fluoro-4'- substituted nucleoside analogues of claim 1 or pro-drugs or 5'-phosphate esters thereof, **characterized in that**, the compounds may exist in the form of salts, which are generated through the reaction between the active compounds (I) and organic or inorganic acid.

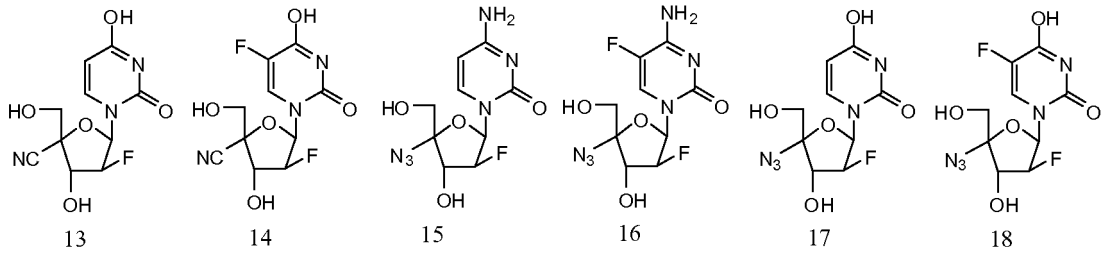
3. The 2'-fluoro-4'- substituted nucleoside analogues of claim 1, **characterized in that**, said compounds are selected from the group consisting of the following compounds:



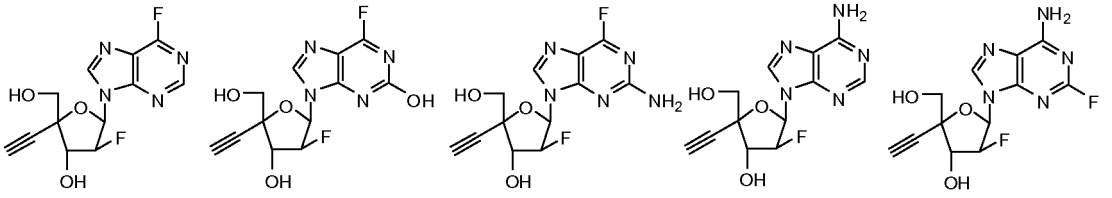
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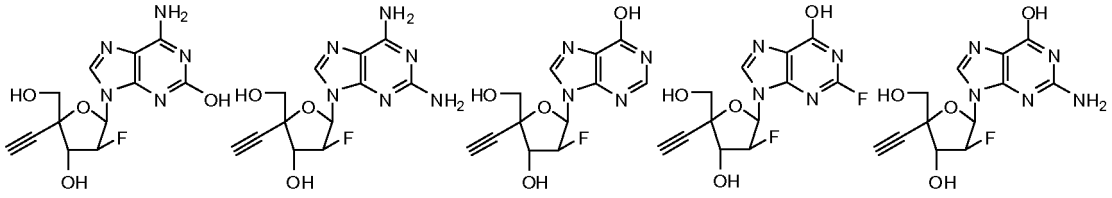
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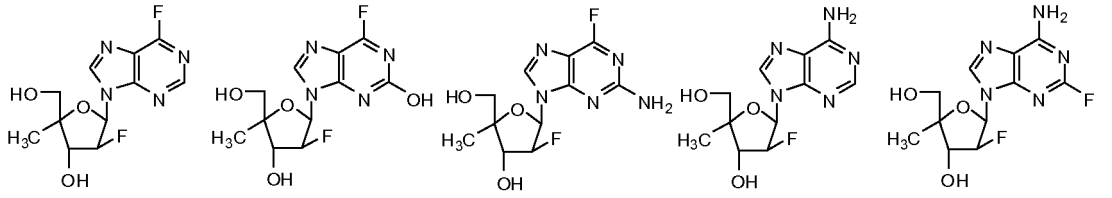
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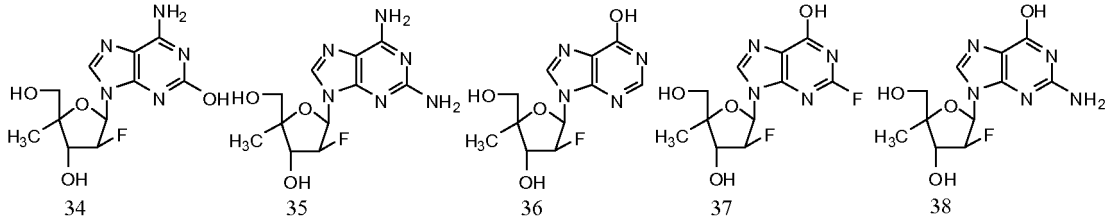
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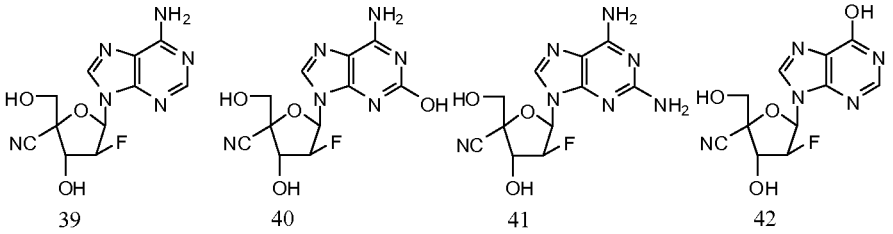
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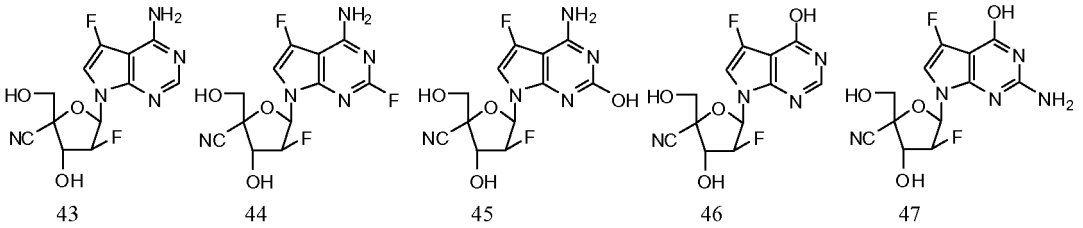
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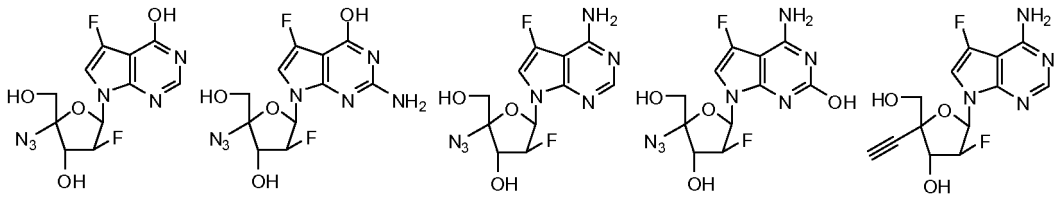


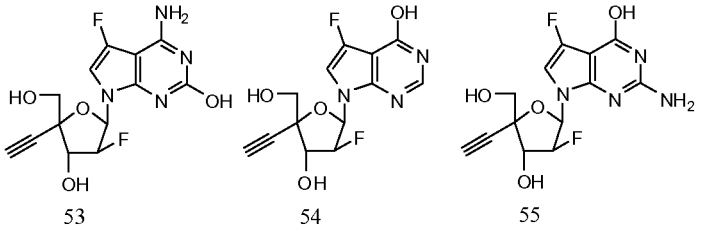
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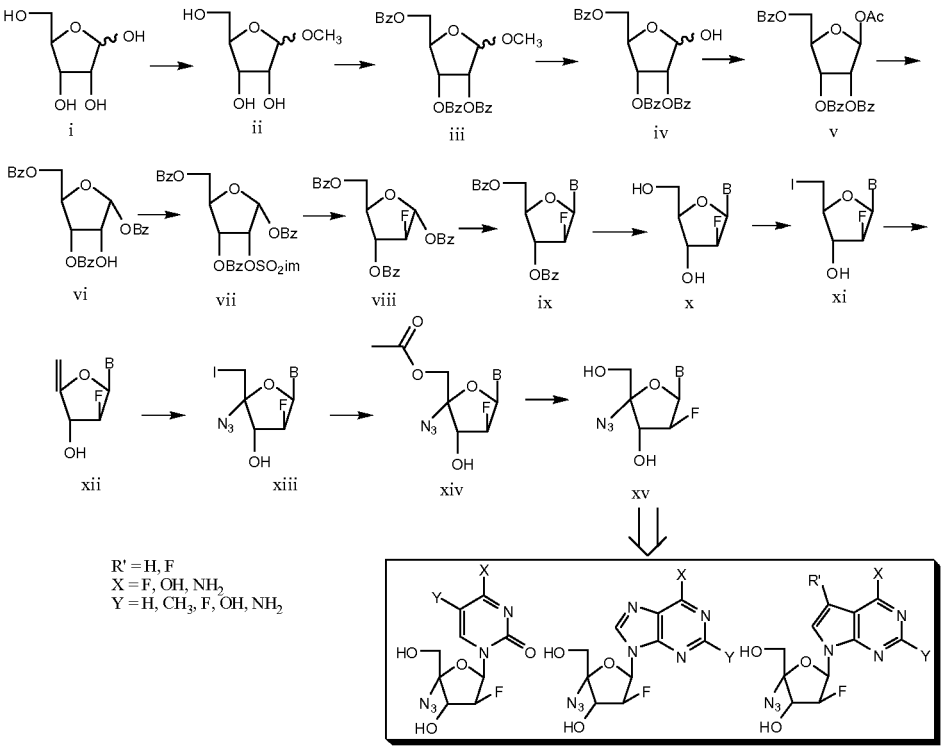
39 40 41 42







4. A method for the preparation of the nucleoside analogues of claim 1, **characterized in that**, said method comprises the steps of:



the synthesis of compound ii: dissolving compound i (D-ribose or L-ribose) in HCl/MeOH, stirring in a water bath under hermetization and warm preservation, adding pyridine into the solution to terminate the reaction, and then drawing it off under a reduced pressure, so as to obtain light yellow syrup-like compound ii;

the synthesis of compound iii: dissolving the compound ii in dry pyridine, slowly adding benzoyl chloride dropwise in an ice-salt bath, stirring for reaction at a room temperature, pouring the reaction solution into ice-water after the reaction is completed, and extracting with chloroform; washing the organic layer sequentially with ice-water, pre-cooled sulfuric acid and saturated sodium bicarbonate until water layer shows weak alkaline, and then washing with ice-water until water layer shows neutral; drying the organic layer over anhydrous sodium sulfate, and then drawing it off under a reduced pressure, so as to obtain light yellow syrup-like compound iii;

the synthesis of compound iv: dissolving benzoyl compound iii in chloroform, adding red phosphorus, slowly adding bromine dropwise while stirring the solution in an ice-water bath, stirring for about 30 minutes, slowly adding ice water dropwise and stirring at a room temperature; then adding broken ice into the solution and stirring the solution until the ice is melted; subsequently pouring the reaction solution into ice water and extracting with chloroform; washing the organic layer with saturated sodium bicarbonate until the water layer shows weak alkaline, washing with ice-water until the water layer shows neutral, drying over anhydrous sodium sulfate, and then drawing it off under a reduced pressure to obtain dark yellow syrup; finally the syrup being separated by silica gel column, so as to obtain white dry syrup compound iv;

the synthesis of compound v: dissolving the compound iv in dry pyridine, slowly adding acetic anhydride dropwise in an ice water bath, and then stirring for about 30 minutes; removing the ice water bath, stirring at a room temperature for about 7 hours, and then the solution being heated up to 40°C and kept for an hour; after adding broken ice and stirring it to be melted, pouring the reaction solution into ice water and extracting with chloroform; washing the organic layer sequentially with ice water, pre-cooled sulfuric acid and saturated sodium bicarbonate until the water layer shows weak alkaline, then washing with ice water until the water layer shows neutral, drying over anhydrous sodium sulfate, and then drawing it off under a reduced pressure, so as to obtain light yellow syrup-like compound v;

the synthesis of compound vi: dissolving the compound v in dry dichloromethane and slowly introducing the dry HCl gas into an ice-water bath, washing with ice-water so that an organic layer is separated; washing the organic layer with saturated sodium bicarbonate until the water layer shows weak alkaline, washing the organic layer with ice-water until the water layer shows neutral, drying over anhydrous sodium sulfate, and drawing it off under a reduced pressure to obtain light yellow syrup; recrystallizing the syrup to obtain white solid compound vi;

the synthesis of compound vii: dissolving the compound vi in dry dichloromethane and dry DMF, slowly adding SO_2Cl_2 into the solution with stirring at about -15 °C for about 30 minutes, naturally warming up to a room temperature for reaction; adding imidazole into the solution for three times at 0°C, and stirring the mixture at a room temperature, diluting the reaction solution by adding CH_2Cl_2 after the completion of the reaction, then washing the solution with ice water; extracting the water layer with CH_2Cl_2; combining the organic layers and then drying over anhydrous sodium sulfate, and drawing it off under a reduced pressure to obtain light yellow syrup; separating and purifying the syrup by silica gel column, so as to obtain white solid compound vii;

the synthesis of compound viii: dissolving the compound vii in ethyl acetate, and adding Et3N • 3HF with stirring; heating up to about 60°C with stirring for about 3h, and then heating up to 70 °C with stirring for 1.5h; adding ice-salt water to terminate the reaction, extracting with dichloromethane; combining the organic layers and then washing sequentially with saline, water and saturated sodium bicarbonate, drying over anhydrous sodium sulfate, and drawing it off under a reduced pressure to obtain dark yellow syrup; purifying the syrup to obtain light yellow syrup, and finally crystallizing the crude product to obtain the white crystalline compound viii;

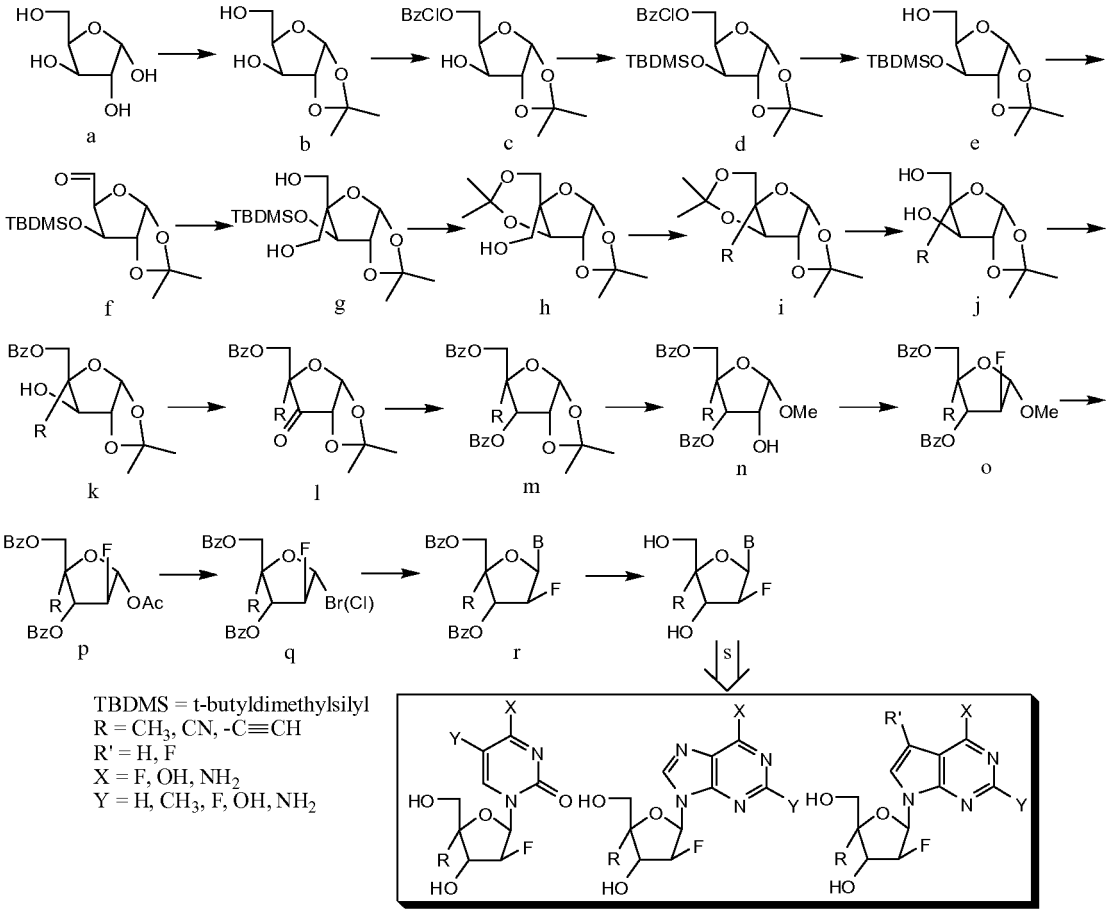
the synthesis of compound ix: dissolving the compound viii in anhydrous dichloromethane , adding a mixed solution of HBr-AcOH, and stirring for reaction at a room temperature; after completion of the reaction, evaporating the mixture to dryness; dissolving the residue with dichloromethane, washing the dichloromethane solution with sodium bicarbonate solution, and removing dichloromethane through evaporation to obtain a syrup-like product; at the same time, the protected cytosine and $(NH_4)_2SO_4$ refluxing under nitrogen protection in the HMDS; after the completion of reaction, evaporating the solvent under a reduced pressure to obtain the silylated cytosine; dissolving the syrup obtained from the above reaction into dichloroethane, which is added into the silylated cytosine, and then refluxing for reaction under N_2 protection; adding ice to terminate the reaction; extracting with dichloromethane; and washing sequentially the dichloromethane layer with saturated sodium bicarbonate and saline, and drying over anhydrous sodium sulfate; evaporating the solvent to obtain white solid, separating and purifying by column chromatography to obtain the compound ix;

the synthesis of compound x: dissolving the compound ix in saturated NH_3-CH_3OH and stirring for reaction at a room temperature; after completion of the reaction, evaporating the solvent to dryness to obtain the residue; and purifying the residue by column chromatography to obtain the compound x;

the synthesis of compound xi: dissolving the compound x, imidazole and triphenylphosphine in tetrahydrofuran, slowly adding iodiferous tetrahydrofuran; stirring for reaction at a room temperature; removing the solvent through

evaporation after completion of the reaction, adding ethyl acetate in the residue and filtering; evaporating ethyl acetate and separating the residue by column chromatography to obtain the compound xi;
the synthesis of compound xii: dissolving the compound ix in tetrahydrofuran and adding DBU; stirring for reaction at about 60°C; evaporating the solvent to dryness after completion of the reaction; separating the residue by column chromatography to obtain the compound xii;
the synthesis of compound xiii: adding the DMF solution dissolved with ICl in the DMF solution dissolved with NaN_3 at 0 °C; stirring at the freezing point for about 10 minutes and slowly adding DMF dissolved with the compound xii to continue the reaction; a after completion of the reaction, adding sodium sulfite in the mixed solution until the color of iodine disappears completely; removing the solvent through evaporation under a reduced pressure; separating and purifying the residue by column chromatography, so as to obtain the compound xiii;
the synthesis of compound xiv: dissolving the compound xiii in DMF, and adding silver acetate with stirring; reacting at a room temperature; filtering after completion of the reaction; removing the solvent under a reduced pressure; purifying the residue by column chromatography, so as to obtain the compound xiv;
the synthesis of compound xv: dissolving the compound xiii in the solution of methanol-triethylamine, stirring for reaction at a room temperature; removing the solvent through evaporation after completion of the reaction, and purifying the residue by column chromatography, so as to obtain the compound xv.

5. A method for preparation of the nucleoside analogues of claim 1, **characterized in that**: said preparation method comprises the following steps:



the synthesis of compound b: adding compound a in acetone, slowly adding the concentrated sulfuric acid while stirring at a room temperature, and stirring for reaction at a room temperature; after the reaction is detected by TLC as complete, adding the concentrated ammonia to adjust pH=7-8, filtering and evaporating under a reduced pressure to remove most of the acetone; then adding 0.4% dilute hydrochloric acid with stirring, and reacting under the acetone reflux condition until the compound a has been completely hydrolyzed into the compound b; neutralizing the reaction solution by solid $NaHCO_3$, filtering, and evaporating to remove the solvent under a reduced pressure; dissolving the residue by dichloromethane, drying over anhydrous Na_2SO_4 for overnight, filtering, and then evaporating to

remove the solvent, so as to obtain the yellow viscous oily compound b;

the synthesis of compound c: dissolving the compound b and triethylamine in dichloromethane; slowly adding dropwise parachlorobenzoyl chloride in an ice salt bath, and mechanically stirring for reaction at a temperature below 0 °C; after completion of the reaction, adding a saturated $NaHCO_3$ solution; washing the dichloromethane layer with water and saturated salt water, drying over anhydrous $MgSO_4$, filtering, and then evaporating under a reduced pressure to remove the solvent; recrystallizing the residue to obtain the white crystalline compound c;

the synthesis of compound d: dissolving the compound c in anhydrous dichloromethane; adding imidazole and tert-butyl-2-methyl chloride silicon (TBDMSCl) under nitrogen protection for the reaction at a room temperature; after completion of the reaction, neutralizing the reaction by hydrochloric acid, and obtaining two layers; washing the organic layer with water and saturated salt water respectively, drying over anhydrous Na_2SO_4, and then evaporating to remove the solvent; separating and purifying the residue by column chromatography, so as to obtain the compound d;

the synthesis of compound e: dissolving the compound xiii in the mixed solution of sodium methoxide and methanol for reaction at a room temperature; after completion of the reaction, neutralizing the solution by dilute acetic acid, filtering, washing with methanol and then evaporating under a reduced pressure to remove the solvent; finally separating and purifying the residue by column chromatography, so as to obtain the compound e;

the synthesis of compound f: adding dropwise DMSO into the dichloromethane solution of oxalyl chloride at about -60 °C; after stirring at the same temperature for about 15 minutes, adding dropwise the dichloromethane solution of the compound e; after stirring for reaction at about -65 °C for 30 minutes, adding triethylamine and stirring for the reaction at a room temperature; after completion of the reaction, adding water into the reaction solution so that an organic layer is separated; drying the organic layer over anhydrous magnesium sulfate, removing the solvent through evaporation; and separating and purifying the residue by column chromatography, so as to obtain the compound xiii;

the synthesis of compound g: adding sodium hydroxide and water in the reaction bottle; after stirring evenly, adding formaldehyde solution, 95% ethanol, and then the compound f, with stirring for reaction at about 35 °C; after completion of the reaction, cooling the reaction bottle with ice water while stirring until the product is completely precipitated; after the suction filtration, washing the solid product with water to neutral, drying, and dissolving it in anhydrous methanol, and then adding sodium borohydride for reflux reaction; after completion of the reaction, neutralizing the reaction solution by dilute hydrochloric acid and then extracting with dichloromethane, drying over anhydrous Na_2SO_4 , and removing the solvent, so as to obtain the compound g;

the synthesis of compound h: dissolving the compound g in methanol, and adding Dowex H^+ (washed with methanol in advance) for reaction at a room temperature; after completion of the reaction, removing the resin from the solution through filtration and repeatedly washing it with methanol, and evaporating the solution to obtain the solid substance; dissolving the obtained solid substance in acetone, then slowly adding concentrated sulfuric acid (a catalytic amount) into the solution for reaction at a room temperature while stirring; adding concentrated ammonia water to adjust pH value, and then filtering and evaporating under a reduced pressure to remove acetone, so as to obtain the compound h;

the synthesis of compound i: adding dropwise DMSO into the dichloromethane solution of oxalyl chloride at about -60 °C, and stirring for about 15 minutes after dripping off; adding dropwise the dichloromethane solution of the compound h, reacting for about 30 minutes at -60 °C after dripping off; adding triethylamine in the solution; after the reaction is carried out at a room temperature for about 30 minutes, adding water to terminate the reaction; separating the organic layer and drying over anhydrous Na_2SO_4; removing the solvent through evaporation and separating and purifying the residue by column chromatography to obtain the solid product; adding $ClCH_2P(C_6H_5)_3Cl$ into anhydrous tetrahydrofuran, cooling to about -78 °C, adding n-BuLi hexane solution, and stirring for reaction for about an hour; then adding the dry tetrahydrofuran solution of the solid product; increasing the temperature of the solution gradually to about 0 °C to continue the reaction; after completion of the reaction, adding a saturated NH_4Cl solution carefully dropwise in the solution, and subsequently extracting with ethyl acetate; washing the organic layer with saline twice, drying over anhydrous $MgSO_4$, and evaporating to remove the solvent; dissolving the residue obtained into anhydrous tetrahydrofuran, cooling to -78 °C, and slowly adding dropwise n-BuLi hexane solution; after stirring the solution for 2 hours, terminating the reaction carefully by using saturated NH_4Cl; washing the organic layer with saturated salt water, drying over anhydrous MgSO4, and

evaporating under a reduced pressure to remove the solvent; finally separating and purifying the residue by rapid silica gel column, so as to obtain the compound i;

the synthesis of compound j: adding the compound i in the diluted hydrochloric acid solution, stirring the solution at a room temperature for reaction; after completion of the reaction, neutralizing the solution with solid $NaHCO_3$, and then filtering; evaporating the filtrate under a reduced pressure to remove the solvent; dissolving the residue in dichloromethane; filtering after drying over anhydrous Na_2SO_4, and evaporating to remove the solvent, so as to obtain the compound j;

the synthesis of compound k: dissolving the compound j in pyridine, adding BzCl dropwise for reaction at a room

temperature; after completion of the reaction, evaporating the solvent to dryness, and purifying the residue to obtain the compound k;

the synthesis of compound 1: dissolving the compound k and 2,2,6,6-tetramethyl piperidine-N-oxide in dichloromethane, cooling to 0 °C and adding the mixed solution of sodium hypochlorite solution, $NaHCO_3$ and water in the reaction solution for reaction at 0 °C for about 30 minutes; adding isopropanol and stirring at a room temperature; washing the separated dichloromethane layer with water twice, drying over anhydrous $MgSO_4$, filtering, and evaporating to remove the solvent; recystalizing the residue to obtain the compound 1;

the synthesis of compound m: dissolving the compound 1 in the mixed solution of absolute ethanol and ethyl acetate and cooling to about 0 °C; adding sodium borohydride in batches and stirring for reaction; after completion of the reaction, neutralizing with dilute acetic acid solution, filtering, and evaporating the filtrate to dryness; dissolving the residue in dichloromethane and then washing with water; drying the dichloromethane layer over anhydrous $MgSO_4$, filtering, and evaporating to remove the dichloromethane under a reduced pressure; recrystallizing the residue by the mixed solvent of dichloromethane and petroleum ether to obtain solid substance; then dissolving the solid product in pyridine, and adding dropwise BzCl at about 0°C; after that, the reaction is carried out at a room temperature; after completion of the reaction, removing the solvent through evaporation and extracting the residue with ethyl acetate, washing with saturated $NaHCO_3$ solution, drying over anhydrous Na_2SO_4, filtering, and evaporating to remove the solvent, so as to obtain the compound m;

the synthesis of compound n: dissolving the compound m in methanol, adding the mixed solution of concentrated hydrochloric acid/dioxane for reaction at a room temperature; after completion of the reaction, neutralizing the reaction solution with $NaHCO_3$, and filtering; removing the solvent from the filtrate by vacuum distillation; dissolving the residue in appropriate amount of dichloromethane, drying over anhydrous Na_2SO_4 for overnight , filtering, and evaporating to dryness; separating and purifying by column chromatography to obtain the compound n;

the synthesis of compound o: dissolving the compound n in dichloromethane, and adding DAST under a room temperature and stirring for reaction; after completion of the reaction, pouring the mixture into the saturated $NaHCO_3$ solution and separating the organic layer; washing the organic layer with the saturated $NaHCO_3$ solution, drying over anhydrous Na_2SO_4, and separating and purifying by column chromatography to obtain the compound o;

the synthesis of compound p: dissolving the compound o in formic acid solution, and stirring at a room temperature for reaction; after completion of the reaction, removing the solvent under a reduced pressure; evaporating the residue and toluene together to obtain the solid product; dissolving the obtained a solid product in the solution of dry pyridine and acetic anhydride for reaction at a room temperature; after the reaction is completed, concentrating the reaction solution and then evaporating together with toluene, so as to obtain the compound p;

the synthesis of compound q: dissolving the compound p in anhydrous dichloromethane and cooling to 0°C; slowly introducing the HCl gas into the solution to a saturation level and removing the solvent by vacuum evaporation at a room temperature; drying the residue in vacuum to obtain the compound q;

the synthesis of compound r: with the existence of $(N_4)_2SO_4$, refluxing 2,6-diamino-purine and Hexamethyldisilazane (HMDS) to be a transparent solution; removing the solvent under vacuum to obtain a white solid product, and dissolving the solid product in 1,2-dichloroethane; adding 1,2-dichloroethane solution of compound q and molecular sieve , and stirring for reaction under nitrogen protection at a room temperature; after the reaction is detected by TLC as complete, adding dichloromethane in the reaction solution and filtering by diatomite; washing the filtrate with saturated Na_2SO_4 and saline respectively; drying the organic layer over anhydrous Na_2SO_4 overnight and removing the solvent through evaporation to obtain the residue; separating the residue under a reduced pressure through silica gel column, so as to obtain the compound r;

the synthesis of compound s: dissolving the compound r in a saturated NH_3-CH_3OH solution, and stirring for reaction at a room temperature; after completion of the reaction, removing the solvent through evaporation and separating and purifying the residue by column chromatography, so as to obtain the compound s.

6. The uses of 2'-fluoro-4'- substituted nucleoside analogues of claim 1, 2, 3 in pharmaceutical preparation, **characterized in that**, said compounds are applied in the preparation of anti-HBV, anti-HCV or anti-HIV drugs.

7. The uses of 2'-fluoro-4'- substituted nucleoside analogue of claim 6 in pharmaceutical preparation, **characterized in that**, HBV antiviral drugs are anti-HBV drugs; HIV antiviral drugs are anti-HIV drugs; and HCV antiviral drugs are anti-HCV drugs.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2008/001239

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C07H19/-, A61K31/-, A61P31/-, A61P1/-

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, PAJ, CNKI, CNPAT, CA: nucleosides fluoro base purine pyrimidine cytidine hepatitis HIV

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CN1712409A(KAITE CHEMICAL INDUSTRY GENERA [CN]) 28 Dec. 2005 (28.12.2005) description pages 2,4-5,example 2, compound B	1-3,6-7
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Y	WO9943691A1(UNIV EMORY [US] etc..) 02 Sept. 1999 (02.09.1999)	1-3,6-7
A	description pages 11,31,34, compounds 14a,14b,15a,15b,17a,17b,18a, 18b,26a,26b, 27a,27b,28a,28b	4-5
Y	WO2005003147 A2 (PHARMASSET LTD [US]; CLARK JEREMY [US]) 13 Jan 2005 (13.01.2005) description page 17 line 3- page 19 line 26	1-3,6-7
A		4-5

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 Sept. 2008(11.09.2008)	**23 Oct. 2008 (23.10.2008)**
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451	Authorized officer WANG Qingeng Telephone No. (86-10)62414030

Form PCT/ISA /210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2008/001239

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2008/001239

continued to page4 Information on patent family members

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continued to page 3 **A. CLASSIFICATION OF SUBJECT MATTER**

C07H19/06 (2006.01) i
C07H19/14 (2006.01) i
C07H19/16 (2006.01) i
A61K31/7064 (2006.01) i
A61K31/7076 (2006.01) i
A61P31/12 (2006.01) i
A61P1/16 (2006.01) i

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REFERENCES CITED IN THE DESCRIPTION

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4′-Ethynyl Nucleoside Analogs: Potent Inhibitors of Multidrug-Resistant Human Immunodeficiency Virus Variants In Vitro

EI-ICHI KODAMA,[1] SATORU KOHGO,[2] KENJI KITANO,[3] HARUHIKO MACHIDA,[3] HIROYUKI GATANAGA,[4] SHIRO SHIGETA,[5] MASAO MATSUOKA,[1,6] HIROSHI OHRUI,[2] AND HIROAKI MITSUYA[4,6]*

Laboratory of Virus Immunology, Institute for Virus Research, Kyoto University, Kyoto 606-8507,[1] Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Sendai 981-8555,[2] Biochemicals Division, Yamasa Corporation, Chiba 288-0056,[3] Department of Microbiology, Fukushima Medical University School of Medicine, Fukushima 960-1295,[5] and Department of Immunopathophysiology and Internal Medicine II, Kumamoto University School of Medicine, Kumamoto 860-0811,[6] Japan, and Experimental Retrovirology Section, Department of Developmental Therapeutics, Medicine Branch, National Cancer Institute, Bethesda, Maryland 20892[4]

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A series of 4′-ethynyl (4′-*E*) nucleoside analogs were designed, synthesized, and identified as being active against a wide spectrum of human immunodeficiency viruses (HIV), including a variety of laboratory strains of HIV-1, HIV-2, and primary clinical HIV-1 isolates. Among such analogs examined, 4′-*E*-2′-deoxycytidine (4′-*E*-dC), 4′-*E*-2′-deoxyadenosine (4′-*E*-dA), 4′-*E*-2′-deoxyribofuranosyl-2,6-diaminopurine, and 4′-*E*-2′-deoxyguanosine were the most potent and blocked HIV-1 replication with 50% effective concentrations ranging from 0.0003 to 0.01 μM in vitro with favorable cellular toxicity profiles (selectivity indices ranging 458 to 2,600). These 4′-*E* analogs also suppressed replication of various drug-resistant HIV-1 clones, including HIV-$1_{M41L/T215Y}$, HIV-1_{K65R}, HIV-1_{L74V}, HIV-$1_{M41L/T69S\text{-}S\text{-}G/T215Y}$, and HIV-$1_{A62V/V75I/F77L/F116Y/Q151M}$. Moreover, these analogs inhibited the replication of multidrug-resistant clinical HIV-1 strains carrying a variety of drug resistance-related amino acid substitutions isolated from HIV-1-infected individuals for whom 10 or 11 different anti-HIV-1 agents had failed. The 4′-*E* analogs also blocked the replication of a non-nucleoside reverse transcriptase inhibitor-resistant clone, HIV-1_{Y181C}, and showed an HIV-1 inhibition profile similar to that of zidovudine in time-of-drug-addition assays. The antiviral activity of 4′-*E*-thymidine and 4′-*E*-dC was blocked by the addition of thymidine and 2′-deoxycytidine, respectively, while that of 4′-*E*-dA was not affected by 2′-deoxyadenosine, similar to the antiviral activity reversion feature of 2′,3′-dideoxynucleosides, strongly suggesting that 4′-*E* analogs belong to the family of nucleoside reverse transcriptase inhibitors. Further development of 4′-*E* analogs as potential therapeutics for infection with multidrug-resistant HIV-1 is warranted.

Combination chemotherapy or highly active antiretroviral therapy (HAART) using two or more reverse transcriptase (RT) inhibitors (RTIs) and protease inhibitors (PIs) has dramatically improved the quality of life and survival of patients infected with human immunodeficiency virus type 1 (HIV-1) (10, 23). However, the ability to provide effective long-term antiretroviral therapy for HIV-1 infection has become a complex issue, since a number of patients who initially achieved very favorable viral suppression later experience treatment failure (40). Moreover, 30 to 40% of HIV-1-infected individuals who had received no prior antiviral therapy fail to achieve viral suppression to undetectable levels (9, 17). In addition, 10 to 40% of antiviral therapy-naive individuals infected with HIV-1 have persistent viral replication (plasma HIV RNA >500 copies/ml) under HAART (11, 12, 37), possibly due to transmission of drug-resistant HIV-1 variants (40). Thus, the development of novel compounds that are active against drug-resistant HIV-1 variants and that prevent or delay the emergence of resistant HIV-1 variants is urgently needed.

Certain 4′-substituted nucleosides have been described in the literature. Maag et al. (19) reported that 4′-azido-2′-deoxythymidine (4′-AZT), the hydrogen atom at the 4′ position of which was substituted for with an azido group, exerted potent activity against HIV-1 in vitro. Subsequently, Chen and colleagues (4) reported that 4′-AZT was active against HIV-1 through its DNA chain-terminating activity. More recently, Sugimoto et al. have reported that 4′-substituted nucleosides including 4′-ethynylthymidine exhibited potent activity against not only HIV-1, but also herpes simplex virus type 1 (38). We recently designed and synthesized a series of 4′-ethynyl (4′-*E*)-2′-deoxynucleosides and their analogs and identified several highly potent anti-HIV-1 compounds, including 4′-*E*-2′-deoxycytidine (4′-*E*-dC), 4′-*E*-2′-deoxyadenosine (4′-*E*-dA), 4′-*E*-2′-deoxyribofuranosyl-2,6-diaminopurine (4′-*E*-dDAP), and 4′-*E*-2′-deoxyguanosine (4′-*E*-dG). These 4′-*E* compounds, unlike all of the currently available nucleoside RTIs (NRTIs), lack the 2′,3′-dideoxyribose configuration but have a 2′-deoxyribose configuration (Fig. 1 and Table 1).

All of these 4′-*E* compounds blocked the replication of a wide spectrum of laboratory and clinical HIV-1 strains in vitro with low cellular toxicities. These compounds also suppressed the replication of various drug-resistant HIV-1 clones, including HIV-$1_{M41L/T215Y}$, HIV-1_{L74V}, HIV-1_{K65R}, HIV-$1_{M41L/T69S\text{-}S\text{-}G/T215Y}$, HIV-$1_{Y181C}$, and HIV-$1_{A62V/V75I/F77L/F116Y/Q151M}$. These 4′-*E* compounds also

* Corresponding author. Mailing address: Medicine Branch, Building 10, Room 5A11, National Cancer Institute, 9000 Rockville Pike, Bethesda, MD 20892. Phone: (301) 402-3631. Fax: (301) 402-0709. E-mail: hmitsuya@helix.nih.gov.

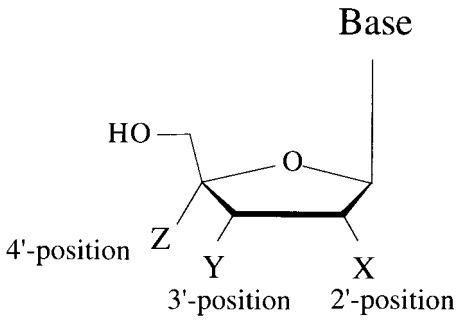


FIG. 1. Structures of 4′-substituted nucleosides. All nucleoside analogs discussed here have substitutions at the 4′ position of the sugar moiety shown here except for the two compounds 4′-*E*-araT and 4′-*E*-araC, which have an arabinofuranosyl sugar moiety. See Table 1.

suppressed various multidrug-resistant clinical HIV-1 variants carrying a variety of drug resistance-related amino acid substitutions, which were isolated from patients for whom virtually all of the currently available antiviral regimens had failed. Furthermore, we demonstrate in this study that these 4′-*E* analogs most likely block HIV-1 by functioning as NRTIs.

MATERIALS AND METHODS

Antiviral agents. 3′-Azido-3′-deoxythymidine (AZT, or zidovudine), 2′,3′-dideoxyinosine (ddI, or didanosine), and 2′,3′-dideoxycytidine (ddC, or zalcitabine) were purchased from Sigma (St. Louis, Mo.). (−)-2′,3′-Dideoxy-3′-thiacytidine (3TC, or lamivudine) was a kind gift from R. F. Schinazi (Atlanta, Ga.). A series of 4′-position-substituted nucleosides were designed and synthesized as described elsewhere (16, 27, 28). Their basic formula is shown in Fig. 1. A non-NRTI (NNRTI), MKC-442, was a gift from Mitsubishi Kasei Corporation (Yokohama, Japan) (2).

Cells. MT-4 and H9 cells were grown in an RPMI 1640-based culture medium, and Cos-7 cells were grown in Dulbecco's modified Eagle medium (DMEM); each of these media was supplemented with 10% fetal calf serum (FCS; HyClone Laboratories, Logan, Utah), 2 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. HeLa-CD4-LTR/β-gal cells (14) were kindly provided by M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (Bethesda, Md.). Prior to use, HeLa-CD4-LTR/β-gal cells were propagated in DMEM supplemented with 10% FCS, 0.1 ng of hygromycin B per ml, and 200 μg of Geneticin per ml. In the anti-HIV assay, cells were cultured in the DMEM-based culture medium with addition of 50 U of penicillin per ml and 50 μg of streptomycin per ml instead of hygromycin B and Geneticin. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy HIV-1-seronegative donors by Ficoll-Hypaque gradient centrifugation and were stimulated for 3 days with phytohemagglutinin M (PHA; 10 μg/ml; Sigma) prior to use.

Viruses and construction of recombinant HIV-1 clones. Three laboratory strains, HIV-1_{LAI}, HIV-2_{ROD}, and HIV-2_{EHO}, were employed. Multidrug-resistant clinical HIV-1 strains were isolated from patients with AIDS who had been treated with 9 to 11 anti-HIV-1 drugs for 39 to 64 months, as previously described (42). These clinical HIV-1 isolates were passaged once or twice in PHA-stimulated PBMCs (PHA-PBMCs), and titers of the culture supernatants obtained were determined for infectivity and stored at −70°C until use.

Recombinant infectious HIV-1 clones carrying various mutations in the *pol* gene were generated as previously described (35, 39, 42). Briefly, the desired mutations were introduced into the *Xma*I-*Nhe*I region (759 bp) of pTZNX1, which encoded Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by the oligonucleotide-based mutagenesis method (42). The *Xma*I-*Nhe*I fragment was inserted into a pHXB2RIP7 (a kind gift from M. Reitz, Jr., National Institutes of Health, Bethesda, Md.)-based plasmid, pSUM9, generating various molecular clones with the desired mutations. Each molecular clone (10 μg/ml as DNA) was transfected into Cos-7 cells (4×10^5 cells/100-mm-diameter dish) by the calcium phosphate method (Promega, Madison, Wis.). After 24 h, MT-2 cells (10^6 cells/dish) were added and cocultured with Cos-7 cells for an additional 24 h. When an extensive cytopathic effect was observed, cell supernatants were harvested,

TABLE 1. Anti-HIV-1 activity of 4′-substituted nucleosides in MT-4 cells[a]

Compound (abbreviation)	Base	Sugar modification at X(2′-) Y(3′-) Z(4′-)	EC_{50} (μM)[b]	CC_{50} (μM)[b]	Selectivity index
4′-Ethynylribosylthymine (4′-*E*-rT)	Thymine	-OH -OH -CCH	>400	>400	ND[c]
4′-Ethynylthymidine (4′-*E*-T)	Thymine	-H -OH -CCH	0.83 ± 0.46	>400	>482
4′-Ethynylarabinofuranosylthymine (4′-*E*-araT)	Thymine	β-OH -OH -CCH	119 ± 22	>400	>4
4′-Ethylthymidine (4′-Et-T)	Thymine	-H -OH $-CH_2CH_3$	>400	>400	ND
4′-Vinylthymidine (4′-V-T)	Thymine	-H -OH $-CHCH_2$	3.9 ± 1.0	>400	>103
4′-Hydroxyethylthymidine (4′-HE-T)	Thymine	-H -OH $-CH_2CH_2OH$	7.0 ± 4.3	>400	>57
4′-Ethynyl-5-iodo-2′-deoxyuridine (4′-*E*-IdU)	5-Iodo-uracil	-H -OH -CCH	0.29 ± 0.063	>400	>1,380
4′-Ethynyl-5-ethyl-2′-deoxyuridine (4′-*E*-EtdU)	5-Ethyl-uracil	-H -OH -CCH	>400	>400	ND
4′-Ethynyl-5-bromovinyl-2′-deoxyuridine (4′-*E*-BVDU)	5-Bromovinyl-uracil	-H -OH -CCH	>15	1.5	ND
3′-Azido-3′-deoxythymidine (AZT)	Thymine	-H $-N_3$ -H	0.032 ± 0.0011	29.4 ± 8.7	9,190
4′-Ethynyl-2′-deoxycytidine (4′-*E*-dC)	Cytosine	-H -OH -CCH	0.0048 ± 0.001	2.2 ± 1.0	458
4′-Ethynylarabinofuranosylcytosine (4′-*E*-araC)	Cytosine	β-OH -OH -CCH	0.043 ± 0.011	2.0 ± 0.4	46.5
4′-Methyl-2′-deoxycytidine (4′-Me-dC)	Cytosine	-H -OH $-CH_3$	0.015 ± 0.063	1.0 ± 0.41	66.7
4′-Fluoromethyl-2′-deoxycytidine (4′-FMe-dC)	Cytosine	-H -OH $-CH_2F$	0.0068 ± 0.0036	0.12 ± 0.02	18
2′,3′-Dideoxy-3′-thiacytidine (3TC)	Cytosine	-H none -H	0.10 ± 0.035	>100	>1,000
4′-Ethyl-N^6-methyladenosine (4′-*E*-NM-A)	6-Methyladenine	-OH -OH -CCH	>400	>400	ND
4′-Ethynyladenosine (4′-*E*-A)	Adenine	-OH -OH -CCH	>400	>400	ND
4′-Ethynyl-2′-deoxyadenosine (4′-*E*-dA)	Adenine	-H -OH -CCH	0.0098 ± 0.0043	16 ± 7.9	1,630
4′-Ethynyl-2′-deoxyribofuranosylpurine (4′-*E*-dP)	Purine	-H -OH -CCH	135 ± 25	>400	>3
4′-Ethynyl-2′-deoxyribofuranosyl-2,6-diaminopurine (4′-*E*-dDAP)	2,6-Diaminopurine	-H -OH -CCH	0.00034 ± 0.00003	0.9 ± 0.17	2,600
4′-Ethynyl-2′-deoxyinosine (4′-*E*-dI)	Hypoxanthine	-H -OH -CCH	0.13 ± 0.035	137 ± 39	1,053
4′-Ethynyl-2′-deoxyguanosine (4′-*E*-dG)	Guanine	-H -OH -CCH	0.0015 ± 0.0003	1.4 ± 0.16	933
4′-Ethynyl-6-chloro-2′-deoxyguanosine (4′-*E*-CldG)	6-Chloroguanine	-OH -OH -CCH	>400	>400	ND

[a] Anti-HIV-1 activity was determined by the MTT method. AZT and 3TC served as controls.
[b] The data shown are mean values with standard deviations derived from the results of three independent experiments.
[c] ND, not determined.

and the virus was further propagated in H9 cells. The culture supernatant was harvested, the titer was determined for infectivity, and the sample was stored at −70°C until use. The presence of intended mutations and the absence of unintended mutations in infectious clones were confirmed by determination of the nucleotide sequence of proviral DNA isolated from the virus-producing H9 cells. HIV-1_{HXB2D} was generated by using pSUM9 (35) and served as a wild-type infectious clone (HIV-1_{wt}).

Determination of drug susceptibility of HIV-1. The inhibitory effects of test compounds on HIV-1 replication were monitored by the inhibition of virally induced cytopathicity in MT-4 cells. Briefly, MT-4 cells were suspended at 10^5 cells/ml and exposed to HIV-1_{LAI} at 100 50% tissue culture infectious doses ($TCID_{50}$s). Immediately after viral exposure, the cell suspension (10^4 cells in 100 μl) was brought into each well of a 96-well flat microtiter culture plate (Costar, Cambridge, Mass.) containing various concentrations of test compounds. After incubation for 5 days, the number of viable cells was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as previously described (15, 30).

The sensitivity of infectious clones to various RTIs was determined in the multinuclear activation of the galactosidase indicator (MAGI) assay (14), with some modifications using the viral preparations titrated as previously described (20). Briefly, target cells (HeLa CD4-LTR/β-gal; 10^4/well) were plated in 96-well flat microtiter culture plates. On the following day, the medium was aspirated, and the cells were inoculated with HIV-1 clones (70 MAGI units/well, which gave 70 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of drug in fresh medium. Forty-eight hours after viral exposure, all blue cells in each well were counted. The cytotoxicity of the compound was determined by the MTT method as previously described (15). All experiments were performed in triplicate.

The drug susceptibility of HIV-1 clinical isolates was determined as previously described (42). Briefly, PHA-PBMCs (10^6 cells/ml) were exposed to each viral preparation at a $TCID_{50}$ of 50 and cultivated in 200 μl of culture medium containing various concentrations of the drug in 10-fold serial dilutions in 96-well culture plates. All assays were performed in triplicate, and the amounts of p24 antigen produced by the cells into the culture medium were determined on day 7 in culture with a commercially available radioimmunoassay kit (Du Pont, Boston, Mass.).

The activity of test compounds is shown as the concentration that blocks HIV-1 replication by 50% (EC_{50}), and cytotoxicity is shown as the concentration that suppresses the viability of HIV-1-unexposed cells by 50% (CC_{50}). The window between CC_{50} and EC_{50} is shown by the CC_{50}/EC_{50} ratios (selectivity indices).

RESULTS

Activity of 4′-substituted nucleosides against HIV-1 in vitro. We designed and synthesized more than 20 novel 4′-substituted nucleoside analogs (16, 27, 28, 38) and tested them for in vitro activity against HIV-1 by using the MTT colorimetric assay employing MT-4 cells. A thymidine analog, 4′-ethynyl (*E*)-T, and a uracil analog, 4′-*E*-IdU, were found to be moderately active against HIV-1_{LAI}, with EC_{50}s of 0.83 and 0.29 μM, respectively (Table 1). Conversion of the 2′-deoxyribose of 4′-*E*-T to ribose, generating 4′-*E*-rT, nullified the antiviral activity of 4′-*E*-T. Other 4′-substituted thymidine and uridine analogs had marginal or no activity. All four 4′-substituted cytidine analogs examined were active, among which 4′-*E*-dC and 4′-fluoromethyl (FMe)-dC were most potent against HIV-1, although the latter was substantially cytotoxic (Table 1). It was noted that 4′-*E*-araC was moderately active.

Among 4′-*E* purine analogs, 4′-*E*-dA, 4′-*E*-dDAP, and 4′-*E*-dG were highly potent against HIV-1, with subnanomolar to nanomolar EC_{50}s (Table 1). 4′-*E*-dI was only moderately active against the virus. It is noteworthy that both 4′-*E*-dDAP and 4′-*E*-dA had a favorable toxicity profile, with selectivity indices of 2,600 and 1,630, respectively (Table 1). All three 4′-*E* purine analogs with a ribose configuration (4′-*E*-NM-A, 4′-*E*-A, and 4′-*E*-CldG) were inactive.

Activity of 4′-substituted nucleosides against HIV-1 variants resistant to various RTIs. We also evaluated whether 4′-substituted nucleosides were active against HIV-1 variants resistant to various nucleoside RT inhibitors (NRTIs) by using the MAGI assay. Four pyrimidine analogs (4′-*E*-dC, 4′-*E*-araC, 4′-Me-dC, and 4′-FMe-dC) that were potent against HIV-1_{LAI} were selected and tested further against various infectious HIV-1 clones carrying resistance-conferring amino acid substitutions (Table 2). It was noteworthy that all four of these cytidine analogs examined suppressed the replication of ddI- and ddC-resistant HIV-1_{K65R} and HIV-1_{L74V}, AZT-resistant HIV-$1_{M41L/T215Y}$, and multi-dideoxynucleoside-resistant (resistant to AZT, ddI, ddC and d4T) variant HIV-$1_{A62V/V75I/F77L/F116Y/Q151M}$ (35, 36). It was noted that among 4′-substituted pyrimidine analogs, only 4′-*E*-dC remained active against 3TC-resistant variants HIV-1_{M184I} and HIV-1_{M184V}, but was less active against the multi-NRTI-resistant (resistant to AZT, ddI, ddC, d4T and 3TC) variant HIV-$1_{M41L/T69S-S-G/T215Y}$ (41), while 4′-FMe-dC remained potent against HIV-$1_{M41L/T69S-S-G/T215Y}$. However, all three 4′-*E* purine compounds (4′-*E*-dA, 4′-*E*-dDAP, and 4′-*E*-dG) were active against the infectious clones tested, including HIV-1_{M184V} and HIV-$1_{M41L/T69S-S-G/T215Y}$. These purine analogs were also active against an NNRTI-resistant infectious clone, HIV-1_{Y181C}. When tested in HeLa-CD4-LTR/β-gal cells, the CC_{50}s of the analogs examined were all >100 or >200 μM, except for that of 4′-*E*-dG.

4′-Ethynyl group is required for activity against HIV-1_{M184V} and HIV-1_{M184I}. To investigate whether and to what extent the 4′-*E* configuration was crucial for activity against HIV-1, three thymidine analogs and two 2′-deoxycytidine analogs substituted at the 4′-position with different groups (vinyl, ethyl, hydroxyethyl, methyl, or fluoromethyl) were synthesized. 4′-ethylthymidine (4′-Et-T) was inert, but 4′-vinylthymidine (4′-V-T) and 4′-hydroxyethylthymidine (4′-HE-T) were active against HIV-1_{LAI}, although they were less so than to 4′-*E*-T (Table 1). These two thymidine analogs were active against the control wild-type infectious clone HIV-1_{HXB2} and a multidideoxynucleoside-resistant infectious clone (HIV-$1_{A62V/V75I/F77L/F116Y/Q151M}$), but were totally inert (>100 μM) against the 3TC-resistant clone HIV-1_{M184V} (Table 2). Two 2′-deoxycytidine analogs, 4′-Me-dC and 4′-FMe-dC, were as potent against HIV-1_{LAI} as 4′-*E*-dC. These analogs were also active against various drug-resistant infectious clones, but were less potent against HIV-1_{M184V} and HIV-1_{M184I} (Table 2). Taken together, although the 4′-*E* substitution is not essential for antiviral activity against HIV-1, the 4′-*E* configuration appears to be required for activity against 3TC-resistant HIV-1_{M184V} and HIV-1_{M184I}.

Activity of selected 4′-substituted nucleosides against HIV-2 strains. We also examined selected 4′-*E* analogs against two HIV-2 strains, HIV-2_{ROD} and HIV-2_{EHO}, in the MAGI assay (Table 3). Although the thymidine analog 4′-*E*-T showed only moderate activity, three cytidine analogs, 4′-*E*-dC, 4′-*E*-araC, and 4′-Me-dC, were highly active against HIV-2, and 4′-*E*-dC was the most potent analog, with 50% inhibitory concentrations (IC_{50}s) of ~0.001 μM (Table 3). Four 4′-*E* purine analogs examined also suppressed the replication of both HIV-2 strains. AZT, tested as a control compound, was active against both HIV-2 strains, as previously described (21). Considering that all of the currently known NNRTIs fail to suppress the

TABLE 2. Antiviral activity of 4′-substituted nucleosides against drug-resistant infectious clones[a]

Compound	EC_{50} (μM)[b]									CC_{50} (μM)[b]
	HXB2	K65R	L74V	M41L/T215Y	M184V	M184I	M41L/T69S-S-G/T215Y	MDR[c]	Y181C	
Pyrimidine analogs										
4′-*E*-T	0.36	0.53	0.68	0.43	0.18	0.14	0.44	0.12	0.13	>200
4′-*E*-IdU	0.37	ND[d]	ND	ND	0.21	ND	ND	0.32	ND	>200
4′-V-T	8.1	ND	ND	ND	>100	ND	ND	9.7	ND	>100
4′-HE-T	15.8	ND	ND	ND	>100	ND	ND	20.7	ND	>100
4′-Et-T	>100	ND	ND	ND	ND	ND	ND	>100	ND	>100
4′-*E*-dC	0.0012	0.0008	0.0013	0.006	0.0024	0.0026	0.015	0.0012	0.0021	>200
4′-*E*-araC	0.0071	0.015	0.026	0.026	0.71	0.48	0.17	0.0079	0.016	>200
4′-Me-dC	0.0058	0.0071	0.0062	ND	0.2	0.74	ND	0.0033	ND	>200
4′-FMe-dC	0.0046	0.065	0.0019	0.0035	2.0	ND	0.066	0.0039	ND	>200
AZT	0.022	0.02	0.02	0.3	0.01	0.017	1.6	15.3	0.014	>100
3TC	0.71	ND	ND	ND	>100	>100	9.9	1.1	ND	>100
ddC	0.2	3.0	1.5	ND	2.2	ND	1.3	5.5	ND	>100
Purine analogs										
4′-*E*-dA	0.008	0.033	0.004	0.012	0.047	0.022	0.065	0.0062	0.011	>200
4′-*E*-dDAP	0.0014	0.00035	0.0007	0.0017	0.0059	0.0027	0.0041	0.001	0.0008	>200
4′-*E*-dI	0.81	0.25	0.61	1.3	16.6	1.5	2.2	0.51	ND	>200
4′-*E*-dG	0.007	0.001	0.0012	0.019	0.008	0.0041	0.0068	0.0048	0.01	52
ddI	3.9	12.7	19.5	3.6	10.1	ND	12.2	25	ND	>100

[a] Anti-HIV activity was determined with the MAGI assay. The abbreviations are defined in Table 1.
[b] The data shown are mean values derived from the results of three independent experiments.
[c] Multidideoxynucleoside-resistant HIV-1 that contains mutations in the *pol* region: an Ala-62 to Val substitution (A62V), V751, F77L, F116Y, and Q151M.
[d] ND, not determined.

replication of HIV-2 (6), it appears that 4′-substituted nucleosides do not belong to NNRTIs.

Activity of 4′-ethynyl-2′-deoxynucleosides against HIV-1 isolated from heavily drug-experienced patients. 4′-*E* analogs were then evaluated for their activity against multidrug resistant clinical HIV-1 isolates in vitro. In this study, we chose three most potent 4′-*E* compounds, 4′-*E*-dC, 4′-*E*-dA, and 4′-*E*-dDAP. Four clinical HIV-1 strains were isolated from patients who received a variety of anti-HIV-1 agents for 39 to 64 months and had failed to respond to any existing regimens of antiviral therapy (42). As shown in Table 4, all four clinical HIV-1 strains contained a variety of drug resistance-conferring amino acid substitutions in the RT- and protease-encoding regions of HIV-1 gene. All three 4′-*E* compounds suppressed the replication of these highly drug-resistant clinical strains as effectively as that of the wild-type clinical strain HIV-$1_{ERS104pre}$ (36) and two HIV-1 clinical strains (HIV-1_{IVR205} and HIV-1_{IVR207}) isolated from drug-naive AIDS patients. It was noted, however, that 4′-*E*-dC was moderately active against HIV-1_{Pt6} and HIV-1_{Pt9}. There was no apparent association of the observed reduced antiviral activity with amino acid substitutions identified in these clinical isolates (Table 4).

TABLE 3. Anti-HIV-2 activity of 4′-substituted nucleosides in MAGI cells[a]

Compound	EC_{50} (μM)	
	HIV-2_{ROD}	HIV-2_{EHO}
4′-*E*-T	0.34	0.29
4′-*E*-dC	0.001	0.0009
4′-*E*-araC	0.012	0.003
4′-Me-dC	0.002	0.0013
4′-*E*-dA	0.0019	0.001
4′-*E*-dI	0.29	0.37
4′-*E*-dDAP	0.001	0.0008
4′-*E*-dG	0.026	0.0042
AZT	0.0061	0.013

[a] Anti-HIV-2 activity was determined with the MAGI assay. The abbreviations are defined in Table 1. The data shown are mean values derived from the results of three independent experiments.

Testing 4′-ethynyl nucleosides in the time-of-drug-addition assay. The triphosphate form of 4′-*E* nucleosides is not presently available, and how 4′-*E* nucleosides block the replication of HIV-1 and HIV-2 remains to be clarified. The time-of-drug-addition assays have been used to approximately determine what stage of the replication cycle of HIV-1 the compound in question blocks (29). We therefore tested two potent 4′-*E* compounds, 4′-*E*-dC and 4′-*E*-araC, in the assay together with three controls: a surface reactant, dextran sulfate 5000 (DS5000); NRTI AZT; and NNRTI MKC-442.

The concentrations of compounds tested were all fixed at their EC_{90}s. At indicated time points, compounds were added to the HIV-1_{LAI}-exposed MAGI cells. As shown in Fig. 2, DS5000, which like DS8000 (24) inhibits the absorption of HIV and formation of syncytia, showed anti-HIV-1 activity only when it was added early after viral exposure. AZT, which requires triphosphorylation before it becomes active (23), exerted its antiviral activity when its addition was delayed for up to 4 h. MKC-442, which does not require activation, was effective when its addition was delayed for up to 6 h. The profiles of anti-HIV-1 activity of 4′-*E*-dC and 4′-*E*-araC were quite similar to that of AZT (Fig. 2). These results suggest that 4′-*E* nucleosides suppress the replication of HIV at or around the step of reverse transcription.

Reversal of antiviral activity of 4′-ethynyl nucleosides by natural 2′-deoxynucleosides. It has been shown that biological effects such as antitumor or antiviral activity of nucleoside analogs are reversed by the addition of natural nucleosides (5, 8, 26). We first tested whether thymidine and 2′-deoxycytidine

FIG. 2. Antiviral activity of 4′-ethynyl nucleosides in the time-of-drug-addition assays. At indicated time points, 4′-*E*-dC (open circles) and 4′-*E*-araC (closed circles) were added to the HIV-1_{LAI}-exposed MAGI (HeLa CD4/LTR-β-gal) cells, and the blue cells produced were counted at the completion of the 48-h period of incubation. DS5000 (open diamonds), AZT (open squares), and MKC-442 (open triangles) served as controls.

reversed the antiviral activity of 4′-*E*-T and 4′-*E*-dC, respectively (Fig. 3). The concentrations of compounds tested were all fixed at their EC_{90}s. Antiviral activity of 4′-*E*-T (2 μM) was suppressed by the addition of thymidine in a dose-dependent manner, and the antiviral activity of AZT (100 nM) was also reversed by the addition of thymidine, in agreement with our previous published data (26), although 100 μM thymidine caused cytotoxicity (Fig. 3A). Activity of 4′-*E*-dC (8 nM) and 4′-*E*-dG (10 nM) was similarly reversed by the addition of 2′-deoxycytidine and 2′-deoxyguanosine, respectively (Fig. 3B and D). Likewise, the activity of ddC (2 μM) and ddG (10 μM) was reversed by the addition of dC and dG, respectively, in agreement with our previous data (25). In contrast, the antiviral activity of 4′-*E*-dA was not reversed by the addition of dA, a similar profile of the activity of ddI versus dA (25) (Fig. 3C).

Acid stability of selected 4′-ethynyl nucleosides. Certain nucleoside analogs such as ddI are acid labile and require antacid upon oral administration, generating adverse reactions, such as abdominal discomfort and diarrhea (33). We therefore examined the acid sensitivity of three selected 4′-*E* analogs (4′-*E*-dC, 4′-*E*-dA, and 4′-*E*-dDAP). These compounds were exposed to 1 N HCl for up to 20 min and then neutralized with 1 N NaOH and added to target cells exposed to HIV-1. As shown in Fig. 4, ddI lost all of its antiviral activity following acid exposure for 20 min; however, 4′-*E*-dC, 4′-*E*-dA, 4′-*E*-dDAP, and the control acid-resistant AZT did not lose their antiviral activity.

DISCUSSION

All of the currently available NRTIs have a 2′,3′-dideoxyribofuranose configuration, and only after anabolic intracellular phosphorylation do they exert their antiviral activity against HIV by functioning as viral DNA chain terminators (22). In this study, we identified 4′-*E* nucleoside analogs potent against a variety of HIV strains, including laboratory strains of HIV-1

TABLE 4. Antiviral activity of 4′-substituted nucleosides against clinical isolates

Strain	Amino acid substitution(s)		EC_{50} (μM)[a]			
	RT region	Protease region	4′-*E*-dC	4′-*E*-dA	4′-*E*-dDAP	AZT
ERS_{104pre}	None	None	0.0012	0.013	0.00083	0.0056
IVR205	None	K20M, M36I, D60E	0.0023	0.0034	0.0001	0.0015
IVR207	G190Q	V77I	0.0046	0.0022	<0.0001	0.0036
Pt 1	T69G, K70R, L74V, A98G, K103N, V179D, M184V, T215F, K219F	L10I, L33I, M36I, M46I, L63P, A71, G73S, V82A, L90M	0.00064 (0.5 fold)	0.00054 (0.4 fold)	0.0011 (1.3 fold)	0.029 (52 fold)
Pt 6	M41L, D67N, M184V, L210W, T215Y	L10I, K20R, L24I, M36I, M46L, I54V, L63P, V82A, L89M	0.013 (11 fold)	0.040 (3 fold)	0.0001 (0.1 fold)	0.28 (50 fold)
Pt7	M41L, D67N, T69D, M184V, T215F	L10I, K45R, I54V, L63P, A71V, V82T, L90M	0.0016 (1.3 fold)	0.009 (0.7 fold)	0.0005 (0.6 fold)	1.9 (340 fold)
Pt 9	M41L, M184V, T215Y	M46I, L63P, A71V, V77I, I84V, N88D, L90M	0.023 (19 fold)	0.029 (2.2 fold)	0.0031 (3.7 fold)	0.97 (170 fold)

[a] EC_{50} were determined with PHA-PBMC. All assays were conducted in triplicate. Numbers in parentheses represent fold changes of EC_{50}s against each HIV-1 isolate compared to those against the wild-type clinical HIV-1 strain, ERS_{104pre}. The abbreviations are defined in Table 1.

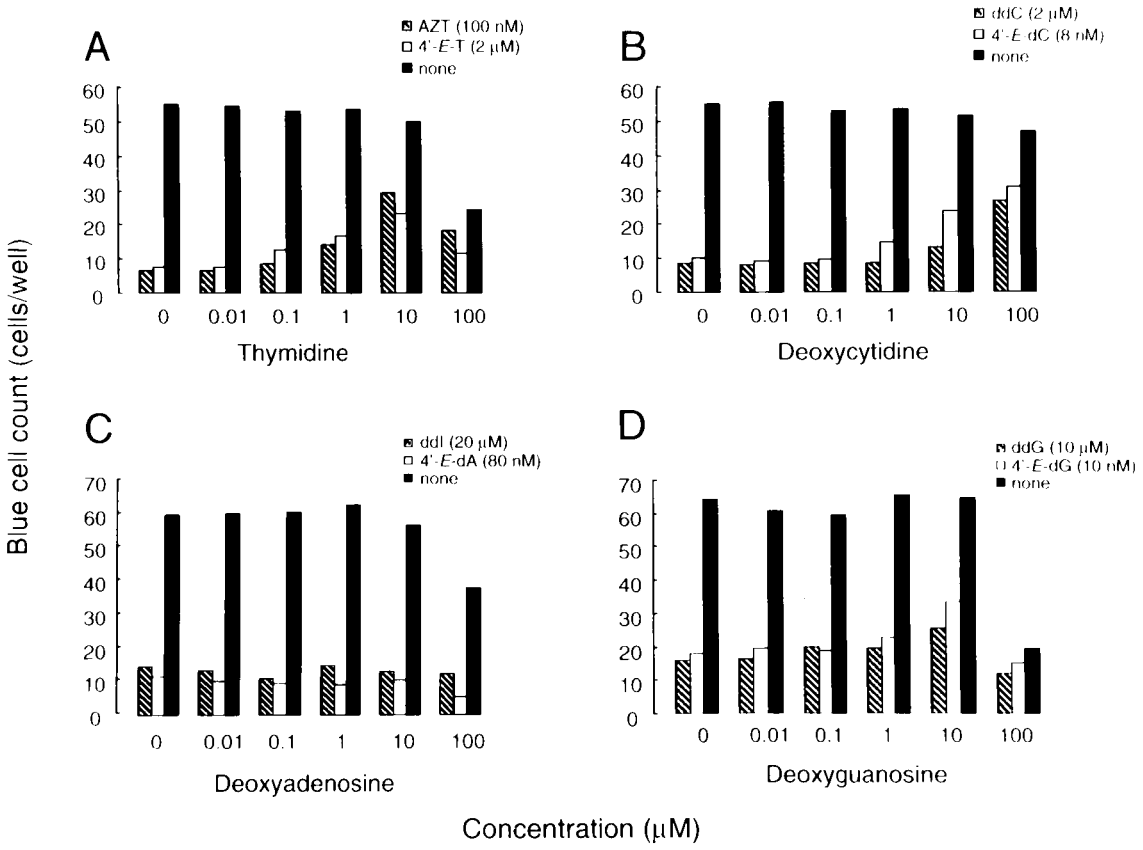


FIG. 3. Reversal of anti-HIV activity of 4′-ethynyl-2′-deoxynucleosides by 2′-deoxynucleosides. Antiviral activities of 4′-*E*-thymidine (4′-*E*-T; 2 μM [open bars]) and AZT (100 nM [hatched bars]) were reversed by the addition of their physiologic counterpart (thymidine) in a dose-dependent manner (A). In the culture without antiviral agents (solid bars), the activities of 4′-*E*-2′-deoxycytidine (4′-*E*-dC; 8 nM) and ddC (2 μM [hatched]) were similarly reversed by the addition of 2′-deoxycytidine (dC) (B). In contrast, the anti-HIV-1 activities of 4′-*E*-2′-deoxyadenosine (4′-*E*-dA; 80 nM [open bars]) and ddI (10 μM [hatched bars]) were not suppressed by the addition of 2′-deoxyadenosine (C). The activities of 4′-*E*-2′-deoxyguanosine (4′-*E*-dG; 10 nM [open bars]) and ddG (10 μM [hatched bars]) were moderately reversed by the addition of 2′-deoxyguanosine (dG) (D).

and HIV-2, infectious clones, clinical HIV-1 isolates, and multidrug-resistant clinical strains.

As of this writing, no 5′-triphosphate forms of the 4′-substituted nucleoside analogs examined in this study have been available, and enzymatic analyses with wild-type and mutant RT to elucidate the exact mechanism of antiretroviral activity of the 4′-substituted nucleoside analogs have not yet been carried out. Nevertheless, it is likely that these 4′-substituted nucleoside analogs serve as NRTIs but not as NNRTIs. First, the 4′-substituted nucleoside analogs were active against the virus with any pyrimidines or purines, although their antiviral potency varied (Table 1), a different profile from that of HEPT and its analogs, which are active only with thymine as its base component (1). Second, although all of the currently available NNRTIs are incapable of inhibiting HIV-2, and this property has been one of the salient features of NNRTIs, the selected 4′-substituted nucleoside analogs examined were active against both HIV-1 and HIV-2 (Tables 1 and 3). Moreover, 4′-*E*-dC, 4′-*E*-dA, and 4′-*E*-dDAP inhibited an NNRTI-resistant clone, HIV-1_{Y181C} (Table 2). Third, in the time-of-drug-addition assays (7), the profiles of anti-HIV-1 activity of two 4′-*E* nucleosides (4′-*E*-dC and 4′-*E*-araC) were quite similar to that of AZT (Fig. 2), suggesting that 4′-*E* nucleosides suppress the replication of HIV-1 at or around the step of reverse transcription in the replication cycle. Fourth, antiviral activity of 4′-*E*-T, 4′-*E*-dC, and 4′-*E*-dG was reversed by the addition of their corresponding physiologic 2′-deoxynucleosides (Fig. 3A, B, and D), strongly suggesting that 4′-*E* nucleosides serve as substrates for RT. In contrast, the activity of 4′-*E*-dA was not reversed by its corresponding physiologic nucleoside 2′-deoxyadenosine (dA)(Fig. 3C). In this respect, the activity of ddA (and ddI) against HIV-1 is not reversed by dA (26), which is possibly due to there being no intracellular change in dATP levels regardless of extracellular dA levels, since the ubiquitous adenosine deaminase (ADA) in the target cells immediately converts dA to 2′-deoxyinosine (3, 34). In fact, the addition of dA in the presence of an ADA inhibitor (2′-deoxycoformycin) reversed the activity of 4′-*E*-dA against HIV-1 (data not shown). Taken together, like the currently available NRTIs, 4′-substituted nucleosides most likely block the replication of HIV by functioning competitive inhibitors for RT.

It is noteworthy that there are a few previously reported 4′-substituted nucleoside analogs active against HIV. Maag et al. synthesized 4′-azidothymidine (4′-AZT), 4′-azido-2′-deoxy-

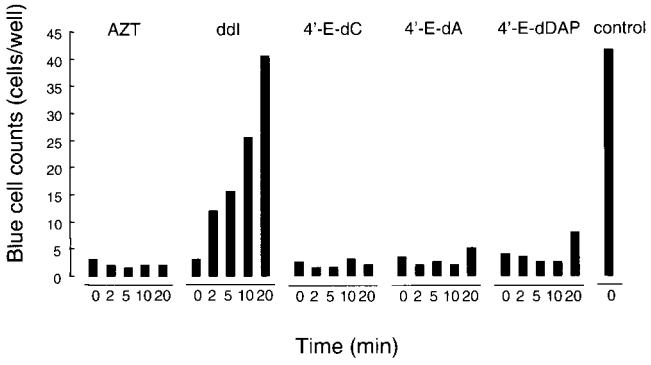


FIG. 4. Acid stability of 4′-ethynyl nucleosides. AZT, ddI, 4′-*E*-dC, 4′-*E*-dA, and 4′-*E*-dDAP were exposed to 1 N HCl for the indicated periods of time, neutralized with 1 N NaOH, and added to the culture in the MAGI assay at final concentrations of 100 nM, 20 μM, 8 nM, 80 nM, and 8 nM, respectively, which approximately represent the EC_{90} of each compound under the conditions used.

guanosine (4′-AZG), and 4′-azido-5-chloro-2′-deoxyuridine (4′-AZU) and found that these compounds were active against HIV-1 (18, 19). With respect to their specific anti-HIV activity, Chen et al. reported that following intracellular anabolic phosphorylation of 4′-AZT, HIV RT efficiently incorporates two consecutive 4′-AZT molecules preventing RT from further elongating the DNA chain (4). Incorporation of two 4′-AZT-monophosphate (MP) molecules separated by one 2′-deoxynucleoside 5′-MP (dAMP, dCMP, or dGMP) also abolishes DNA chain elongation by HIV RT. In contrast, cellular DNA polymerases α and β incorporate a single 4′-AZT-MP molecule into nascent DNA at a very slow rate, but do not incorporate a second consecutive 4′-AZT-MP and continue to elongate cellular DNA (4).

It should be noted that 4′-*E*-dC, 4′-*E*-dA, and 4′-*E*-dDAP suppressed all infectious HIV-1 clones examined which were resistant to all the currently available NRTIs, including two multidrug-resistant HIV-1 variants, HIV-$1_{M41L/T69S\text{-}S\text{-}G/T215Y}$ (41) and HIV-$1_{A62V/V75I/F77L/F116Y/Q151M}$ (35, 36) (Table 2). It is also of note that 4′-*E*-araC, 4′-Me-dC, and 4′-FMe-dC were less potent against two 3TC-resistant HIV-1 variants, HIV-1_{M184I} and HIV-1_{M184V} (Table 2). This difference in drug resistance profiles between the former three 4′-*E* nucleosides and the latter two analogs should be intriguing from a structure-activity relationship point of view. Recently, the extensive structural analysis of RT has shed light on the mechanism of viral resistance to NRTIs (13, 31, 32). Substitutions of amino acids by mutations, which confer drug resistance, are accumulated around the deoxynucleoside triphosphate (dNTP) binding sites. Considering that all of the currently available NRTIs contain 3′-position modifications, HIV-1 is thought to alter the putative dNTP binding site to develop drug resistance. Indeed, codons K65, L74, Q151, and M184, which are associated with resistance to NRTIs, are located in the neighborhood of the enzyme sites. These sites interact with incoming nucleotides and are thought to be critical for the binding of the active site of RT to NRTIs, which prevents the NRTIs from being incorporated into the growing proviral DNA chain (32). However, 4′-*E* nucleosides examined in this study retain 3′-OH moiety like natural substrates, which may enable 4′-*E* nucleosides to interact with the mutated 3′-OH binding site of various types of drug-resistant HIV. Isolation and characterization of resistant mutants against 4′-*E* nucleosides should provide more insights into nucleoside-enzyme interactions. Further development of 4′-*E* analogs as potential therapeutics for infection with multidrug-resistant HIV-1 is warranted.

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D3

THE CHEMICAL RECORD

2′-Deoxy-4′-*C*-Ethynyl-2-Fluoroadenosine, a Nucleoside Reverse Transcriptase Inhibitor, Is Highly Potent Against All Human Immunodeficiency Viruses Type 1 and Has Low Toxicity

HIROSHI OHRUI
Yokohama College of Pharmacy, 601 Matanocho, Tozukaku, Yokohama 245-0066, Japan

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ABSTRACT: An idea to use 4′-*C*-substituted-2′-deoxynucleoside derivatives was proposed based on a working hypothesis to solve the problems of existing acquired immune deficiency syndrome chemotherapy (highly active antiretroviral therapy). Subsequent studies have successfully proved the validity of the idea and resulted in the development of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine, a nucleoside reverse transcriptase inhibitor, which is highly potent to all human immunodeficiency viruses type 1 (HIV-1s) including multidrug-resistant HIV-1 and has a low toxicity. © 2006 The Japan Chemical Journal Forum and Wiley Periodicals, Inc. Chem Rec 6: 133–143; 2006: Published online in Wiley InterScience (www.interscience.wiley.com) DOI 10.1002/tcr.20078

Key words: AIDS; anti-HIV agent; reverse transcriptase; nucleoside reverse transcriptase inhibitor; highly active antiretroviral therapy; drug-resistant HIV; 4′-*C*-substituted-2′-deoxynucleoside

Introduction

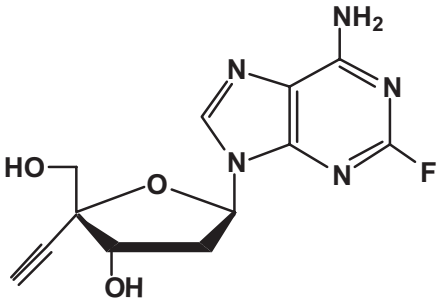
A highly active antiretroviral therapy (HAART) using two or more nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors has dramatically improved the quality of life and prognosis of patients infected by human immunodeficiency virus type 1 (HIV-1).[1,2] However, the existing HAART has several critical problems that remain to be solved. These problems include: (i) the emergence of new drug-resistant HIV-1 mutants, (ii) the need to take large dosages, and (iii) drug side effects. Therefore, the development of new, highly potent anti-human immunodeficiency virus (HIV) drugs that prevent the emergence of drug-resistant mutants and have few side effects is urgently needed.

These problems prompted me to speculate on the reason for the emergence of HIV-1 mutants resistant to clinical NRTIs, which belong to the family of 2′,3′-dideoxynucleoside (ddN). I proposed a working hypothesis for the chemical structure of a new, highly potent NRTI that might prevent the emergence of drug-resistant mutants and which has a low toxicity. The design of 4′-*C*-substituted-2′-deoxynucleoside (4′SdN) was based on the working hypothesis. Studies on the synthesis and biological evaluation of 4′SdNs have proved the validity of the working hypothesis and resulted in the development of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine (4′Ed2FA) (Fig. 1). This substance is highly potent against all HIV-1s and is expected to prevent the emergence of drug-resistant mutants, has a low toxicity, and is stable to enzymatic catabolism. Hence, 4′Ed2FA is an important key for its further development of potential therapeutics to solve the problems being encountered by the present HAART.

▸ *Correspondence to*: Hiroshi Ohrui; e-mail: dhhdb500@ybb.ne.jp

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4′Ed2FA

Fig. 1. Structure of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine (4′Ed2FA).

Working Hypothesis of the Study

Design of the Nucleoside Derivatives That Might Be Effective for Drug-Resistant HIV-Mutants and Possibly Prevent the Emergence of New Drug-Resistant HIV-1 Mutants

The structures of the clinical NRTIs are shown in Figure 2. All belong to the ddN family. The ddN structure is considered essential for the nucleoside derivatives as the chain terminator of the reverse transcriptase (RT)-catalyzed biosynthesis of proviral DNA. However, HIV-1 mutants resistant to all NRTIs have emerged.

The emergence of HIV-1 mutants resistant to ddNs indicates that HIV-1 can acquire the ability to discriminate ddNs from physiological 2′-deoxynucleosides (dNs) and does not accept ddNs into the active center of RT and/or cuts off the incorporated ddNs from the proviral DNA terminus.

Therefore, the anti-HIV nucleoside that might prevent the emergence of drug-resistant HIV-1 mutants must satisfy the following two conditions:

1. To prevent discrimination by HIV-1, the nucleoside must have a structure very much like those of dNs. Because the striking difference of the ddN and dN structures is whether they have 3′-OH, the nucleoside must have 3′-OH.
2. In spite of having 3′-OH, the nucleoside must be the chain terminator of RT-catalyzed biosynthesis of proviral DNA.

Based on the following hypothesis, 4′SdN (Fig. 2) was designed as a nucleoside that could satisfy the mentioned conditions:

1. It would be difficult for HIV-1 to discriminate 4′SdN from dN because 4′SdN has all the functional groups of dN.
2. The introduction of a substituent at the 4′-position makes the 3′-OH into a very unreactive neopentyl-type secondary alcohol. Thus, the 3′-OH of 4′SdN will be used for HIV to mistake 4′SdN for dN, but is too unreactive to be used for the elongation of proviral DNA by RT. Therefore, 4′SdN could be the chain terminators of proviral DNA biosynthesis.
3. Steric hindrance between 3′-OH and 4′-substituent changes the conformation of the furanose ring of 4′SdN preferably to the 3′-endo conformation (*N*-type). This results in 4′SdN being less susceptible to both acidic and enzymatic degradation than that of dN and ddN.
4. Further, the electron-withdrawing 3′-OH makes 4′SdN more acid stable than does ddN even with purines. Thus, various purine derivatives can be made in this way.



Hiroshi Ohrui was born in 1942 in Tokyo, Japan. He received his B.S. (1965) and Ph.D. degrees (1971) from The University of Tokyo under the direction of Professor Masanao Matsui. He joined the Institute for Physical and Chemical Research (RIKEN) in 1966 as a researcher. In 1981, he moved to Tohoku University as Associate Professor in the Department of Food Chemistry, Faculty of Agriculture. In 1997, he was promoted to Full Professor, Graduate School of Life Sciences. In 2006, he moved to Yokohama College of Pharmacy as Professor. He worked for Dr. J. J. Fox at Sloan-Kettering Institute for Cancer Research (1972–1973) as Research Associate, and for Dr. J. G. Moffatt at Syntex Research (1973–1974) as a postdoctoral fellow. He has received several awards including The Agricultural Chemical Society of Japan Award for Young Scientists (1974), Inoue Prize for Science (2001), Japan Prize of Agricultural Science (2004), and The Japan Society for Analytical Chemistry Award (2004). His research interests cover organic synthesis, chiral discrimination, and medicinal chemistry. ■

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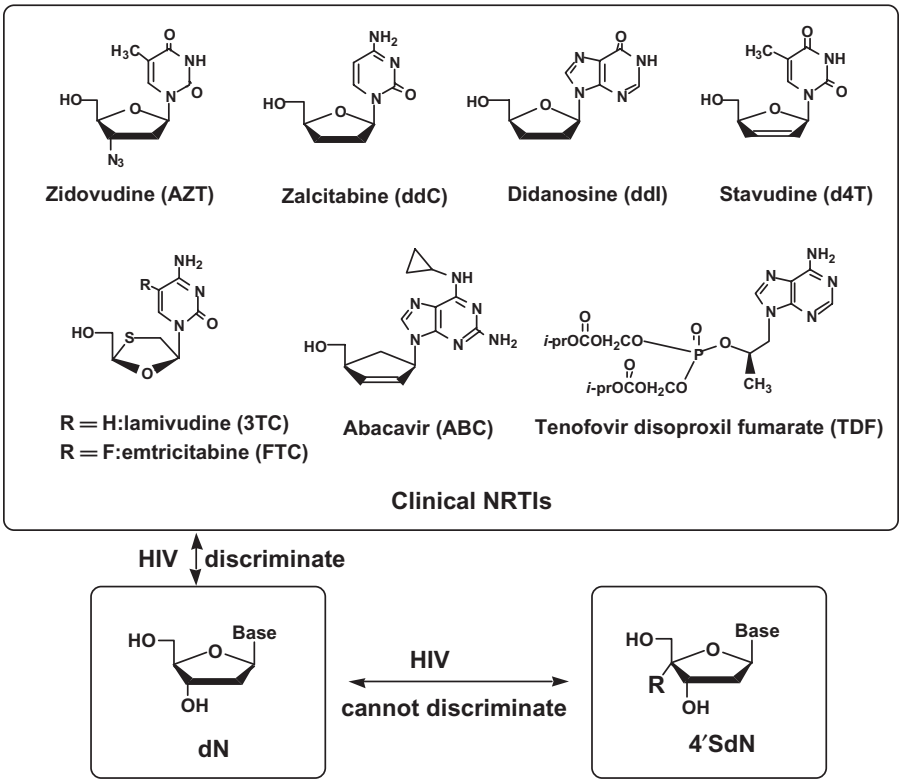


Fig. 2. Structures of clinical nucleoside reverse transcriptase inhibitors (NRTIs), physiologic 2′-deoxynucleoside (dN), and 4′-*C*-substituted-2′-deoxynucleoside (4′SdN). HIV = human immunodeficiency virus; dN = 2′-deoxynucleoside; 4′SdN = 4′-*C*-substituted-2′-deoxynucleoside.

5. The lipophilic substituent at the 4′-position imparts more lipophilicity to 4′SdNs, thus enabling them to penetrate the cell membrane efficiently. This may possibly enhance their bioavailability.

Method of Decreasing the Toxicity of Nucleoside Drugs Based on My Past Findings of the Relationship Between the Biological Activity and Structure of Nucleoside Derivatives

If DNA polymerases also mistake 4′SdN for dN, 4′SdN should be highly toxic. However, ddNs have been used as anti-HIV-1 drugs, meaning that DNA polymerases hardly accept ddNs as their substrates. Thus, the ability of DNA polymerases to distinguish substrates is superior to that of RT.

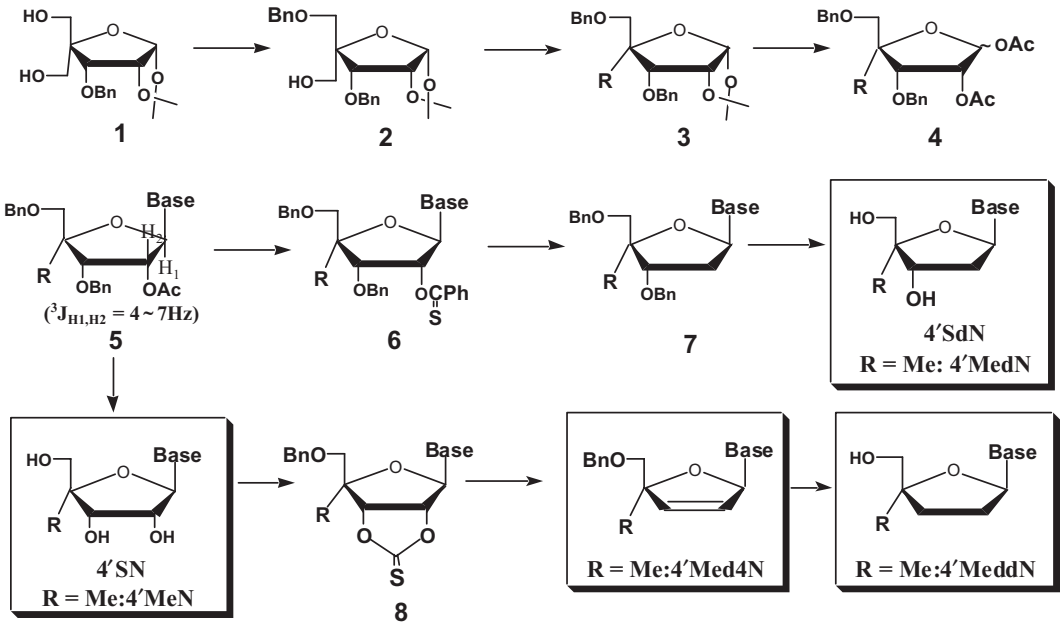
In the 1960s and 1970s, many organic chemists synthesized nucleoside derivatives modified at two or more positions of physiologic nucleosides expecting to get nucleoside derivatives having new and/or higher biological activity. However, none of these modified nucleosides exhibited antimicrobial activity. These results indicate that intracellular enzymes do not recognize the nucleoside derivatives modified at two or more positions of physiologic nucleosides as substrates. Thus, there is a chance of decreasing the toxicity of 4′SdNs further by additional modification.

Results and Discussion

Examination of the Validity of the Working Hypothesis With 4′-*C*-Methyl Nucleosides

At first, to examine the validity of the working hypothesis, 4′-*C*-methylnucleosides (4′MeNs), 4′-*C*-methyl-2′-deoxynucleosides (4′MedNs), 4′-*C*-methyl-2′,3′-dideoxynucleosides (4′MeddNs), and 4′-*C*-methyl-2′,3′-didehydrodideoxynucleosides (4′Med4Ns) were synthesized and evaluated for their biological activity.[3,4] They were synthesized by glycosidation of 1,2-di-*O*-acetyl-3,5-di-*O*-benzyl-4-*C*-methyl-D-ribofuranose (**4**), prepared from D-glucose through 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-α-D-ribofuranose (**1**),[5] and nucleobases and then deoxygenation of the hydroxy groups of 4′MeN (Scheme 1). It should be noted that **4** took a longer reaction time to complete the glycosidation reaction than that of normal *ribo*-furanose derivatives, indicating the lower reactivity of **4**. In addition, the $^{3}J_{1',2'}$ value of 4′-*C*-substituted β-*ribo*nucleoside (**5**) was larger (4 ~ 7 Hz) than that of the normal

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Scheme 1. General synthetic scheme of 4′-*C*-substituted-2′-deoxynucleoside (4′SdNs) from D-glucose. 4′SdN = 4′-*C*-substituted-2′-deoxynucleoside; 4′MedN = 4′-*C*-methyl-2′-deoxynucleoside; 4′SN = 4′-*C*-substituted nucleoside; 4′MeN = 4′-*C*-methylnucleoside; 4′Med4N = 4′-*C*-methyl-2′,3′-didehydrodideoxynucleoside; 4′MeddN = 4′-*C*-methyl-2′,3′-dideoxynucleoside.

β-*ribo*nucleoside (0 ~ 1 Hz), indicating the change of the conformation of the furanose ring of **5**.

4′MedN showed remarkable biological activity (both anti-HIV-1 activity and toxicity), but 4′MeddN and 4′Med4N did not show any notable biological activity (Table 1). These results indicate the importance of the 3′-OH for biological activity. Further, we demonstrated that the 5-*O*-triphosphate of 4′-*C*-methyl-2′-deoxycytidine (4′MedCTP) was the chain terminator of the DNA polymerases.[6] The result indicates that 4′SdN was an NRTI, although further study of 4′MedCTP with RT was not performed.

Structure–Activity Relationship (SAR) of 4′SdNs

Next, to study the SAR of 4′SdNs and develop 4′SdNs having more potent anti-HIV activity and less toxicity than 4′MedNs, 4′SdNs having various kinds of 4′-*C*-substituents and nucleobases were synthesized and evaluated for their biological activity.[7–14] While we were working on our project, the anti-HIV-1 activity of several 4′SdNs was reported by the Syntex group[15–19] and others.[20–22] Therefore, the anti-HIV activities of 4′SdNs that we studied together with those reported by other groups are listed in Table 2. The SARs of 4′-*C*-substituted nucleosides against HIV-1 are summarized as follows:

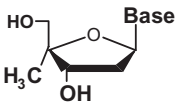
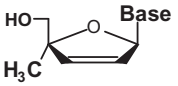
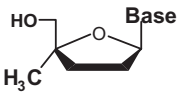
1. The estimated relative order of anti-HIV-1 activity is as follows: CN ≥ C≡CH > N_3 > CH═CH_2 > Me═Et > C≡C—CH_3. Interestingly, the order is the reverse of the $-\Delta G^0$ values between equatorial and axial substituents on a cyclohexane ring: CN < F < C≡CH < CH═CH_2 < Me ≤ Et < ^{t}Bu. Thus, these results indicate that the sterically less demanding substituent at the 4′-position gives more potent anti-HIV activity.
2. Purine analogs are generally less toxic than are pyrimidine analogs; the former generally have greater selectivity indices (SIs). Although 2′-deoxy-4′-*C*-ethynyl-5-fluorocytidine, which is a nucleoside derivative modified at two positions of physiologic 2′-deoxycytidine, gave a very acceptable SI with MT-4 cells, it was toxic with other cells (Kohgo, Yamasa Corporation, private communication).
3. *Arabino* analogs are less active and less toxic compared with their corresponding 2′-deoxy counterparts.
4. Both ddNs and 2′,3′-didehydrodideoxynucleosides (d4Ns), with some exceptions, do not show anti-HIV activity.
5. The *L*-isomers of 4′SdN have no anti-HIV activity,[12,22] although it is known that the *L*-enantiomer of 2′,3′-dideoxy-3′-thia-*L*-cyrtidine (3TC) is as active as the *D*-enantiomer and less toxic than the *D*-enantiomer.[23]

Synthesis of Purine Derivatives of 4′-*C*-Cyano-2′-Deoxynucleoside (4′CNdNs) and 4′-*C*-Ethynyl-2′-Deoxynucleoside (4′EdNs) and Their Anti-HIV Activity

The mentioned results led us to study the biological activity of purine derivatives of 4′CNdN and 4′EdN.[24] Although

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Table 1. Anti-human immunodeficiency virus activity of 4′-*C*-methyl-2′-deoxynucleosides.

Structure	Base	EC_{50} (μM)	CC_{50} (μM)	SI (CC_{50}/EC_{50})
	Ad	2.6	2.6	1.0
	Th	7.2	104	
	Cy	0.072	0.13	1.8
4′MedN	Pu	1.9	>200	>100
	Ad	>500	>500	−1
	Th	21	330	16
4′Med4N	Cy	350	350	
	Ad	30	400	13
	Th	>500	>500	−1
4′MeddN	Cy	27	27	1
AZT		0.001	>20	>2020
ddA		47	>500	>11
d4T		4.1	>500	>120

EC_{50} = 50% effective concentration; CC_{50} = 50% cytotoxic concentration; SI = selectivity index; 4′MedN = 4′-*C*-methyl-2′-deoxynucleoside; 4′Med4N = 4′-*C*-methyl-2′,3′-didehydrodideoxynucleoside; 4′MeddN = 4′-*C*-methyl-2′,3′-dideoxynucleoside; AZT = 3′-azido-3′-deoxythymidine; ddA = 2′,3′-dideoxyadenosine; d4T = 2′,3′-didehydro-3′-didehydro-3′-deoxythymidine.

4′SdNs in our project had been synthesized by the glycosidation of 4-*C*-substituted-D-*ribo*furanose derivatives and nucleobases (Scheme 1), this synthetic route incurred some problems, as follows:

1. Preparation of 4-*C*-substituted-D-*ribo*furanoses and their conversion to the desired 4′SdNs require multistep reactions, and their total yields are low.
2. 4-*C*-Substituted-D-*ribo*furanose derivatives have low reactivity in glycosidation reactions, especially when the substituent is an electron-withdrawing group such as a cyano group.

(In contrast, the low reactivity of the anomeric position of 4-*C*-substituted furanose derivatives indicates that 4′SdN will be more stable to acidic hydrolysis than dN, ddN, and d4N will.)

These problems prompted us to develop another method of preparing purine derivatives of 4′SdN, which starts from dNs (Scheme 2).[24]

The biological activities of the purine derivatives of 4′CNdN and 4′EdN are summarized as follows (Table 3):

1. Some of the purine derivatives of 4′CNdN have high anti-HIV activity, but none of them gives an acceptable SI.
2. All the purine derivatives of 4′EdNs have both high anti-HIV activity and an acceptable SI.

Anti-HIV Activity of 4′SdNs Against Drug-Resistant HIV-1 Mutants[13,14,24]

Many 4′SdNs showed very high anti-HIV activity against wild-type HIV-1. However, the most important point of our study is whether they are active against drug-resistant HIV-1 mutants. The anti-HIV activity of selected 4′SdNs against HIV-1 mutants resistant to various NRTIs is listed in Table 4.

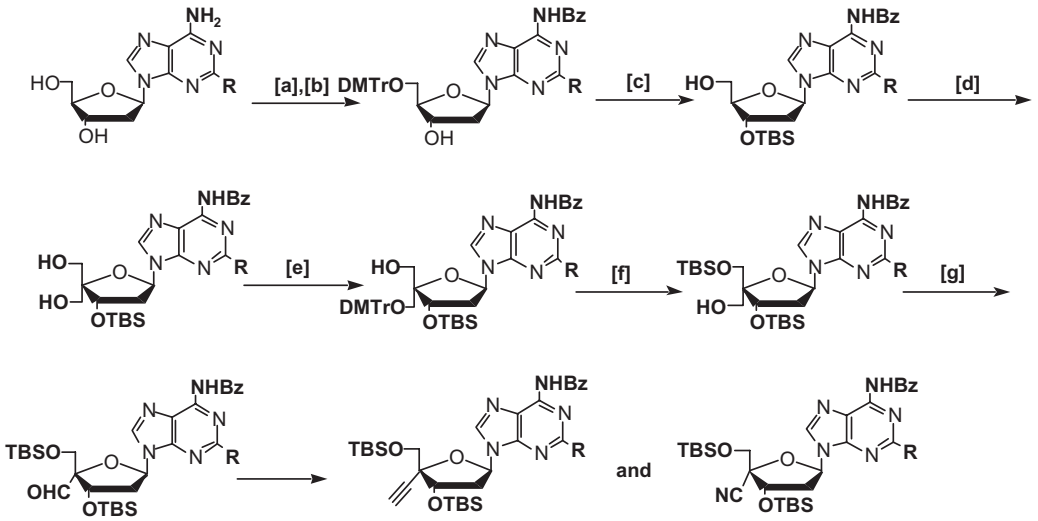
It is noteworthy that the three cytidine derivatives maintained their activity against the drug-resistant HIV-1 mutants, although the activity of 4-*C*-ethynyl-*D*-arabinofuranosylcytosine (4′EaraC) and 4′MedC decreased significantly against M184V, M184I, and 41/69/125/SG. The three purine derivatives, 2′-deoxy-4′-*C*-ethynyladenosine (4′EdA), 2′-deoxy-4′-*C*-ethynyl-2-aminoadenosine (4′Ed2AA), and 2′-deoxy-4′-*C*-ethynylguanosine (4′EdG) except for 2′-deoxy-4′-*C*-ethynylinosine (4′EdI) were highly potent against all drug-resistant HIV-mutants (4′EdI was much less active than the former three derivatives, especially against M184V). Additionally, the three were also active against a non-nucleoside reverse transcriptase inhibitor-resistant Y181C. Further, the three purine derivatives were highly potent against the HIV-1s isolated from seven heavily drug-experienced patients with acquired immune deficiency syndrome (AIDS) as efficiently as against wild-type HIV-1.[13,14,24] Thus, 4′EdA, 4′Ed2AA, and 4′EdG were highly potent against all the existing HIV-1s.

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Table 2. Anti-human immunodeficiency virus activity of 4′-*C*-substituted-2′-deoxynucleosides.

Compound	EC_{50} (μM)[a]	CC_{50} (μM)	SI
4′-*C*-Cyanothymidine	0.002	1	500
4′-*C*-azidothymidine	0.01	8	300
4′-*C*-ethynylthymidine	0.83	>400	>482
4′-*C*-ethynylarabinofuranosylthymine	119	>400	>3.4
4′-*C*-azidomethylthymidine	2.1	333	159
4′-*C*-methylthymidine	7.2	104	14
4′-*C*-ethylthymidine	>400	400	ND
4′-*C*-methoxythymidine	8.49	200	24
4′-*C*-vinylthymidine	>400	>400	ND
4′-*C*-hydroxymethylthymidine	7.0	>400	>57
4′-*C*-propylthymidine	>100	>100	ND
4′-*C*-Cyano-2′-deoxycytidine	0.0012	0.17	142
4′-*C*-azido-2′-deoxycytidine	0.004	0.21	52
4′-*C*-ethyny-2′-deoxycytidine	0.0048	2.2	482
L-4′-*C*-ethynyl-2′-deoxycytidine	>400	>400	ND
4′-*C*-ethynyl-2′-deoxy-5-fluorocytidine	0.030	>100	>3333
4′-*C*-ethynylarabinofuranosylcytidine	0.043	2.0	46.5
4′-*C*-methyl-2′-deoxycytidine	0.015	1.0	66.7
4′-*C*-fluoromethyl-2′-deoxycytidine	0.0068	0.12	18
4′-*C*-methyl-2′-deoxyadenosine	2.6	2.6	1
4′-*C*-azido-2′-deoxyadenosine	0.13	50	385
4′-*C*-ethyny-2′-deoxyadenosine	0.098	16	1630
2′,3′-dideoxy-3′-thia-*L*-cyrtidine (3TC)	0.10	>100	>1000
3′-azido-3′-deoxythymidine (AZT)	0.0032	29.4	9190

[a]Anti-HIV activity was determined by MTT assay. MT-4 cells and HIV-1 LAI were employed. EC_{50} = 50% effective concentration; CC_{50} = 50% cytotoxic concentration; SI = selectivity index; ND = not determined; MTT = 3-(4′5-dimethylthazol-2-yl)-2-5-diphenyl tetrazolium bromide.



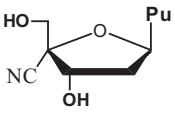
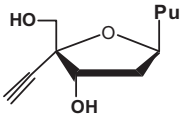
[a] 1.TMSCl, py, 0°C; 2. BzCl, py, 0°C; 3. NH_4OH, H_2O, 0°C. [b] DMtrCl, py, rt. [c] 1.TBSCl, imidazole, DMF, rt; 2.TsOH, H_2O, MeOH, $CHCl_3$, 0°C. [d] 1.EDC·HCl, py, TFA, toluene, DMSO, rt; 2. aq. CH_2O, 1N NaOH, dioxane, rt; 3. $NaBH_4$, EtOH, 0°C, [e]: DMTrCl, Et_3N, CH_2Cl_2, 0°C. [f] 1.TBSCl, imidazole, DMF, rt; 2. TsOH· H_2O, MeOH, $CHCl_3$, rt, 0°C. [g] EDC· HCl, py, TFA, toluene, DMSO, rt.

Scheme 2. Synthetic scheme of purine derivatives of 4′-*C*-cyano-2′-deoxynucleoside and 4′-*C*-ethynyl-2′-deoxynucleoside from 2′-deoxynucleosides. TFA = trifluoroacetic acid; DMF = dimethylformamide; rt = room temperature.

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These results let us suppose that the three purine 4′EdNs could even prevent the emergence of drug-resistant HIV-1s. It should be noted that 4′EdG showed toxicity to *HeLa* cells at 52 μM; thus, 4′EdG will be very toxic.

Table 3. Anti-human immunodeficiency virus activity of purine derivatives of 4′-*C*-cyano-2′-deoxy (4′CNd) and 4′-*C*-ethynyl-2′-deoxynucleoside.

Structure	Base	EC_{50} (μM)[a]	CC_{50} (μM)	SI
	A	0.051	12	235
	I	0.051	23	451
	2AA	0.00079	0.034	43
	G	0.000188	0.034	181
	A	0.098	16	1630
	I	0.15	216	1440
	2AA	0.0003	0.82	2733
	G	0.0014	1.5	975
AZT		0.0032	29.4	9190

[a]Anti-HIV activity was determined by MTT assay. MT-4 cells and HIV-1 LAI were employed.
EC_{50} = 50% effective concentration; CC_{50} = 50% cytotoxic concentration; SI = selectivity index; 2AA = 2-aminoadenine; AZT = 3′-azido-3′-deoxythymidine.

Mouse Toxicity of Purine 4′EdNs

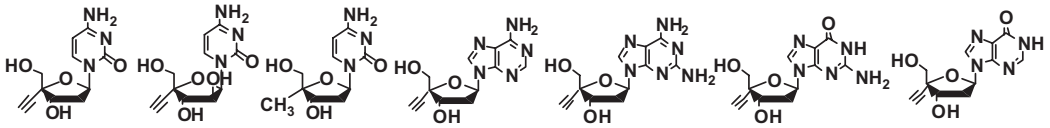
Because the three purine derivatives of 4′EdNs showed high activity against all HIV-1s and acceptable SIs, the mouse toxicity of these 4′EdNs was next examined (Table 5).[22,24]

All eight mice survived after a single dosage of 3 ~ 100 mg kg^{-1} of 4′EdA and 4′EdI by both intravenous and oral administrations, but all mice died after a single dosage of 3 mg kg^{-1} of 4′Ed2AA and 4′EdG irrespective of the administration method (Fig. 3). Thus, it seemed that 4′EdA and 4′EdI were not toxic, but 4′Ed2AA and 4′EdG were highly toxic. However, in mice, it was found that 4′EdA and 4′Ed2AA were easily converted to 4′EdI and 4′EdG, respectively, by adenosine deaminase.[24,25] These results showed that the actual toxicity of 4′EdA and 4′Ed2AA to animals is hard to estimate.

Anti-HIV Activity of 4′EdA Derivatives Stable to Adenosine Deaminase

The fact that both 4′EdA and 4′Ed2AA are deaminated by adenosine deaminase prompted us to prepare 4′EdA derivatives stable to the enzyme. It has been known that the adenine derivatives having a halogen atom at the 2-position of the base are stable to adenosine deaminase.[26,27] Therefore, at first, 4′Ed2FA (Fig. 1) was synthesized and evaluated for anti-HIV

Table 4. Anti-human immunodeficiency virus activity of selected 4′-*C*-substituted-2′-deoxynucleoside.



	EC_{50} (μM)[a]									
Compound	HXB2	KH65R	L74V	41/215	M184V	M184I	41/69/125/SG	MDR	Y181C	CC_{50} (μM)
4′EdC	0.0012	0.0008	0.0013	0.006	0.0024	0.0026	0.015	0.0012	0.0021	>200
4′EaraC	0.0071	0.015	0.026	0.026	0.71	0.48	0.17	0.0079	0.016	>200
4′MedC	0.0058	0.0071	0.0052	ND	0.2	0.74	ND	0.0033	ND	>200
4′EdA	0.008	0.0033	0.004	0.012	0.047	0.022	0.065	0.0062	0.011	>200
4′Ed2AA	0.0014	0.00035	0.0007	0.0017	0.0059	0.0027	0.0041	0.001	0.0008	>200
4′EdG	0.007	0.001	0.0012	0.019	0.008	0.0041	0.0068	0.0048	0.01	52
4′EdI	0.81	0.25	0.61	1.3	1.6	1.5	2.2	0.51	ND	>200
AZT	0.022	0.02	0.02	0.3	0.01	0.017	1.6	15.3	0.014	>100
3TC	0.71	ND	ND	ND	>100	>100	9.9	1.1	ND	>100
ddC	0.2	3.0	1.5	ND	2.2	ND	1.3	5.5	ND	>100
ddI	3.9	12.7	19.5	3.6	10.1	ND	12.2	25	ND	>100

[a]Anti-HIV activity was determined with MAGI assay.
EC_{50} = 50% effective concentration; CC_{50} = 50% cytotoxic concentration; AZT = 3′-azido-3′-deoxythymidine; ND = not determined.

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Table 5. Toxicity of purine derivatives of 4′-*C*-ethynyl-2′-deoxynucleosides to mice. [a]

	Intravenous administration		Oral administration	
	Dose ($mg\,Kg^{-1}$)	Mortality (%)	Dose ($mg\,Kg^{-1}$)	Mortality (%)
4′EdA	100	0	100	0
and	10	0	10	0
4′EdI	3	0	3	0
	1	0	1	0
4′Ed2AA	100	100 (1 day) [b]	100	100 (1 day)
	10	100 (2 days)	10	100 (2 days)
	3	0	3	100 (2 days)
	1	0	1	0
4′EdG	100	100 (1 day)	100	100 (1 day)
	10	100 (2 days)	10	100 (4 days)
	3	100 (4 days)	3	100 (4 days)
	1	0	1	0

[a]Six-week-old ICR male mice were employed.
[b]Numbers in parentheses represent survival days of mice after administration.

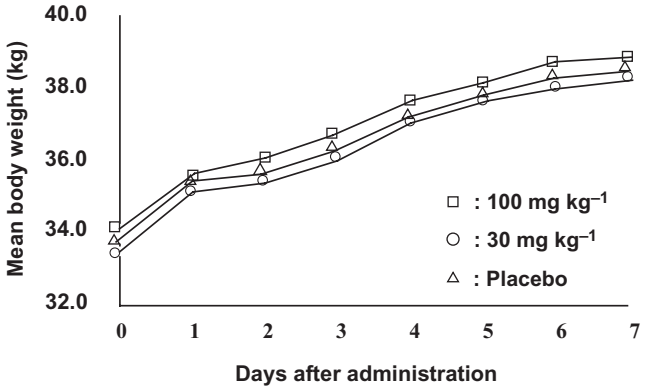


Fig. 3. Body weight change of mice after a single dosage of 2′-deoxy-4′-*C*-ethynyl-2-fluioroadenosine, administrated orally or intravenously to ICR mice.

activity[25,28] and stability to both adenosine deaminase and acidic conditions.

Because 4′Ed2FA is a nucleoside derivative modified at two positions (4′-position and 2-position) of physiologic 2′-deoxyadenosine, the toxicity of 4′Ed2FA is expected to be lower than that of 4′EdA. As shown in Table 6, 4′Ed2FA is highly potent against all HIV-1s including multidrug-resistant and M184V HIV-1 mutants and has an acceptable SI (110,000).[25,28]

Expectedly, 4′Ed2FA was completely stable to adenosine deaminase under the conditions where 4′EdA was completely deaminated in 60 min and, further, fairly stable under acidic conditions. Thus, in 120 min only a small part (3%) of 4′Ed2FA was decomposed under the acidic conditions of gastric juices (pH 1.06) at 24°C, while 2′,3′-dideoxyadenosine (ddA) was completely decomposed in 5 min.

Appearance of Two Papers Claiming that 3′-OH of 4′EdNs Is the Cause of the Toxicity of 4′SdNs

While we were working on our project, two papers on the role of 3′-OH of 4′SdNs appeared in which the authors claimed that 3′-OH was the cause of the toxicity of 4′SdNs. Tanaka and coworkers reported[29] that 4′-*C*-ethynyl-2′,3′-didehydro-3′-deoxythymidine (4′Ed4T) was more potent against wild-type HIV-1 and less toxic than 2′,3′-didehydro-3′-deoxythymidine (d4T). Further, they claimed that 3′-OH of 4′SdNs was the cause of the toxicity. However, they did not note the activity of 4′Ed4T against drug-resistant HIV-1s, the most important point of the chemotherapeutic agent in overcoming the problems of the existing HAART.

In contrast, Marquez and coworkers reported[30] that 3′-OH of 4′SdNs played an important role in the phosphorylation of 5′-OH by cellular kinases, but was the cause of the toxicity of 4′SdNs. This determination was based on their results that 4′-*C*-ethynyl-2′,3′-dideoxycytidine was inactive against HIV-1 in cellular systems, but its 5′-*O*-triphosphate (4′EddCTP) was more active than 3′-azido-3′-deoxythymidine (AZT)-5′-*O*-triphosphate (AZTTP) against the RT of wild-type HIV. They also reported that 4′EddCTP was much less active against the RT of the M184V mutant than against the RT of wild-type HIV-1. In addition, the *L*-isomer of 4′EddCTP was not active against the RT of the M184V mutant.

Anti-HIV Activity of 2′,3′-Dineoxy (dd-) and 2′,3′-Didehydrodideoxy (d4) Analogs of 4′Ed2FA

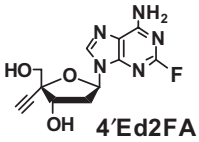
The two papers previously cited caused us to synthesize the dd- and d4-analogs of 4′Ed2FA and evaluate their anti-HIV activity.[25] The anti-HIV activities of 2′,3′-dideoxy-4′-*C*-ethynyl-2-fluoroadenosine (4′Edd2FA) and 2′,3′-didehydro-dideoxy-4′-*C*-ethynyl-2-fluoroadenosine (4′Ed42FA) together with that of 2′-deoxy-4-*C*-ethynyl-2′-chloroadenosine (4′Ed2ClA) are listed in Table 7. Although both 4′Ed42FA and 4′Edd2FA showed some activity against wild-type HIV-1, they significantly lost any activity against drug-resistant HIV-1s. 4′Ed2ClA is active against all HIV-1s, but its activity is lower than that of 4′Ed2FA.

These results indicated that the 3′-OH played important roles not only for the phosphorylation of 5′-OH, but also for the activity against drug-resistant HIV-1s.

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Table 6. Anti-human immunodeficiency virus activity of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine (4′Ed2FA).

HIV-1	Compound	EC_{50} (nM)	CC_{50} (nM)	SI
Wild type	4′ Ed2AA	0.34	900	2600
	4′ Ed2FA	0.068	7500	110,000
	AZT	3.2	29,400	9190
M184V	4′Ed2FA	3.1		
	AZT	16.0		
MDR	4′Ed2FA	0.15		
	AZT	15,300.00		

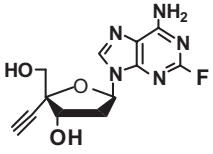


4′Ed2FA

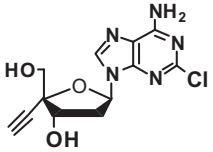
m.p. (decomp.)
224.4–224.6°C
[α] D + 4.64
(σ = 0.5, DMSO)

HIV-1 = human immunodeficiency virus type 1; EC_{50} = 50% effective concentration; CC_{50} = 50% cytotoxic concentration; SI = selectivity index.

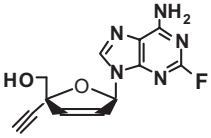
Table 7. Anti-human immunodeficiency virus activity of 4′-*C*-substituted-2′-deoxy-2-haloadenosines.



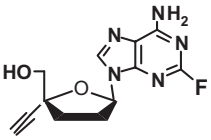
4′Ed2FA



4′Ed2ClA



4′Ed42FA



4′Edd2FA

Compound	Anti-HIV activity (MAGI assay, μg)		
	$HIV\text{-}1_{wild}$	$HIV\text{-}1_{MDR}$	$HIV\text{-}1_{M184V}$
4′Ed2FA	0.00020	0.00014	0.0031
4′Ed2ClA	0.0019	0.0084	0.01
4′Ed42FA	0.80	0.15	1.8
4′Edd2FA	0.94	8.7	97
AZT	0.022	15.3	0.01
3TC	0.71	1.1	>100

MAGI = multinuclear activation of galactosidase indicator; HIV-1 = human immunodeficiency virus type 1; AZT = 3′-azido-3′-deoxythymidine; 3TC = 2′,3′-dideoxy-3′-thia-*L*-cyrtidine.

Toxicity of 4′Ed2FA to Mice and Inhibition of DNA Polymerases

Because 4′Ed2FA is stable to adenosine deaminase, its mouse toxicity was examined.[25,28] 4′Ed2FA did not show any acute toxicity to mice by either oral or intravenous administration up to 100 mg kg^{-1} (Figure 3; Table 8).

It is known that the toxicity of NRTIs to animals is caused by their inhibition of mitochondrial DNA polymerase γ. The 50% effective concentration (EC_{50}) of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine-5-*O*-triphosphate (4′Ed2FATP) to inhibit the incorporation of 2′-deoxyadenosine-5-*O*-triphosphate (dATP) mediated by human mitochondrion DNA polymerase was 10 μM, which was significantly higher than the 0.2 μM of 2′,3′-dideoxyadenosine-5-*O*-triphosphate (ddATP).[31] The EC_{50} values of 4′Ed2FATP against DNA polymerase α and β were higher than 200 μM. These results indicate that the DNA polymerases scarcely recognize 4′Ed2FATP, a derivative modified at two positions of physiologic dATP, as their substrate but that RT does.

Intracellular Metabolism of 4′Ed2FA[31]

The amounts of all fractions of intracellular 4′Ed2FA metabolites, (4′Ed2FA–monophosphate, 4′Ed2FA–diphosphate, and 4′Ed2FATP) increased proportionately with an increase in the

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Table 8. Toxicity of 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine after a single dosage to ICR mice.

	Survivors/total	
Dose (mg Kg^{-1})	p.o	i.v.
Placebo	8/8	8/8
1	8/8	8/8
3	8/8	8/8
10	8/8	8/8
30	8/8	8/8
100	8/8	8/8

concentration of intracellular 4′Ed2FA, while compared to AZT diphosphate and AZTTP, only AZT monophosphate markedly increased with an increase in intracellular AZT concentration. The intracellular half-life ($T_{1/2}$) of 4′Ed2FATP was ~18 h in complete expansion media (CEM) cells, MT4 cells, and multinuclear activation of galactosidase indicator (MAGI)–CCR5 cells ($T_{1/2}$ of AZTTP was 3 h). About 50% of the cells were protected against the infection of HIV-1 for 24 h after removal of extracellular 4′Ed2FA in both MT4 cells and MAGI cells cultured in the presence of 0.1 μM of 4′Ed2FA.

These results indicate that 4′Ed2FA, 2′-deoxy-4′-*C*-ethynyl-2-fluoroadenosine-5-*O*-diphosphate (4′Ed2FADP), and 4′Ed2FATP are very stable against intracellular enzymatic catabolism.

Summary

A study of the synthesis and biological evaluation of 4′EdNs was conducted according to a proposed working hypothesis based on the fundamentals of both organic chemistry and biochemistry. Old scientific evidence proved the validity of the working hypothesis and resulted in the development of 4′Ed2FA, which is highly potent against all HIV-1s, is stable to intracellular enzymatic catabolism and acidic degradation, has a very long intracellular $T_{1/2}$, does not greatly inhibit DNA polymerase γ, and does not have acute mouse toxicity.

These results strongly suggest that 4′Ed2FA deserves further study for the development of a highly potent therapeutic agent for HIV-1 infection (AIDS), and which may solve the problems of the existing HAART.

I am looking forward to the results of a further study.

In addition, it should be noted that 4′Ed2FA could be an ideal drug for both HIV and hepatitis B virus (HBV) infections.

HBV is a DNA virus. However, it also belongs to the family of retroviruses because it uses RT when it replicates. It was found that the NRTIs for HIV are also active against HBV, and 3TC and adefovir dipivoxil have been used as anti-HBV drugs.[32,33] However, an HBV resistant to these drugs has emerged.[32,33] Therefore, the development of an anti-HBV drug that prevents the emergence of a drug-resistant HBV is needed. Because 4′Ed2FA is highly active against 3TC-resistant HIV and might prevent the emergence of drug-resistant HIV, it is also expected to be active against drug-resistant HBV.

Thus, 4′Ed2FA could be an ideal drug for use against HBV infection.

The work described in this paper was achieved through the tremendous efforts of the many coworkers whose names appear in the cited references.

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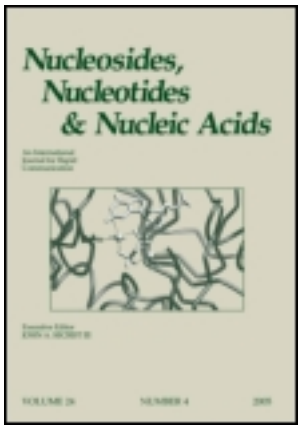
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Phosphoramidate Protides of Carbocyclic 2′,3′-Dideoxy-2′,3′-Didehydro-7-Deazaadenosine with Potent Activity Against HIV and HBV

Kristjan S. Gudmundsson [a], Zhicheng Wang [a], Susan M. Daluge [a], Lance C. Johnson [b], Richard Hazen [b], Lynn D. Condreay [b] & Christopher McGuigan [c]

[a] Division of Chemistry, GlaxoSmithKline, 5 Moore Drive, Research Triangle Park, North Carolina, 27709, USA

[b] Division of Virology, GlaxoSmithKline, 5 Moore Drive, Research Triangle Park, North Carolina, 27709, USA

[c] Welsh School of Pharmacy, Cardiff University, Cardiff, UK

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
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Phosphoramidate Protides of Carbocyclic 2′,3′-Dideoxy-2′,3′-Didehydro-7-Deazaadenosine with Potent Activity Against HIV and HBV

Kristjan S. Gudmundsson,[1,*] Zhicheng Wang,[1] Susan M. Daluge,[1] Lance C. Johnson,[2] Richard Hazen,[2] Lynn D. Condreay,[2] and Christopher McGuigan[3]

[1]Division of Chemistry and [2]Division of Virology, GlaxoSmithKline, Research Triangle Park, North Carolina, USA
[3]Welsh School of Pharmacy, Cardiff University, Cardiff, UK

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ABSTRACT

Synthesis of phosphoramidate protides of carbocyclic D- and L-2′,3′-dideoxy-2′,3′-didehydro-7-deazaadenosine by treatment of the nucleoside with phosphorochloridates in the presence of pyridine and t-BuMgCl is described. Several of these protides showed significantly improved antiviral potency over the parent nucleosides against both HIV and HBV.

Key Words: Carbocyclic 2′3′-dideoxy-2′3′-didehydro-7-deazaadenosine; Phosphoramidate protides; HIV; HBV.

*Correspondence: Kristjan S. Gudmundsson, Division of Chemistry, GlaxoSmithKline, 5 Moore Drive, Research Triangle Park, NC 27709, USA; Fax: (919) 483-6053; E-mail: Kristjan.S.Gudmundsson@gsk.com.

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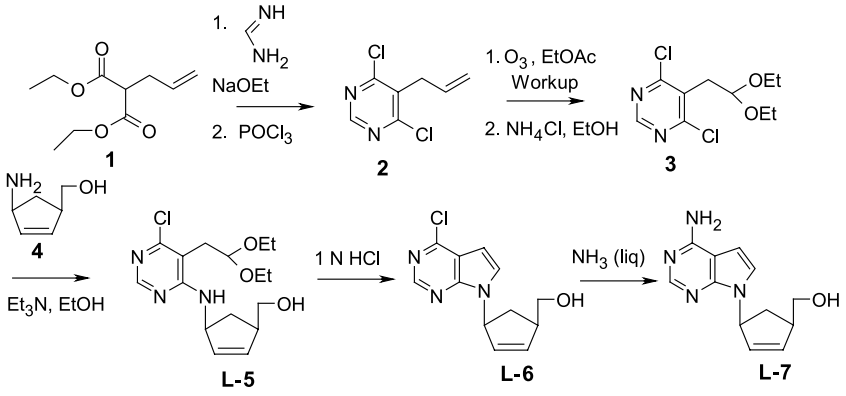
INTRODUCTION

2′,3′-Dideoxyribofuranonucleosides have received considerable attention as this class of nucleosides includes several compounds with potent anti-HIV activity (e.g. ddC, ddI and analogs).[1–3] Besides the synthesis of the natural purine and pyrimidine analogs, several heterocycle-modified 2′,3′-dideoxyribofuranonucleosides, such as pyrrolo[2,3-d]pyrimidines (7-deazaadenine) have been synthesized.[4–6] Several of these have demonstrated antiviral activity. The preclinical research leading to the selection of the carbocyclic nucleoside abacavir (Ziagen) involved the synthesis and evaluation of several racemic carbocyclic analogs, including carbocyclic 2′,3′-dideoxy-2′,3′-didehydro-7-deazaadenosine. As the racemic carbocyclic 2′,3′-dideoxy-2′,3′-didehydro-7-deazaadenosine showed modest HBV activity, we became interested in the synthesis of prodrug derivatives that could deliver its monophosphate intracellularly. Several prodrug strategies have been described in the literature, these include phosphoramidate derivatives,[7–14] cycloSal derivatives[15] and bis(SATE)phosphotriester derivatives.[16,17] All of these have been shown to significantly improve the potency in vitro of a variety of nucleosides against HIV and/or HBV.

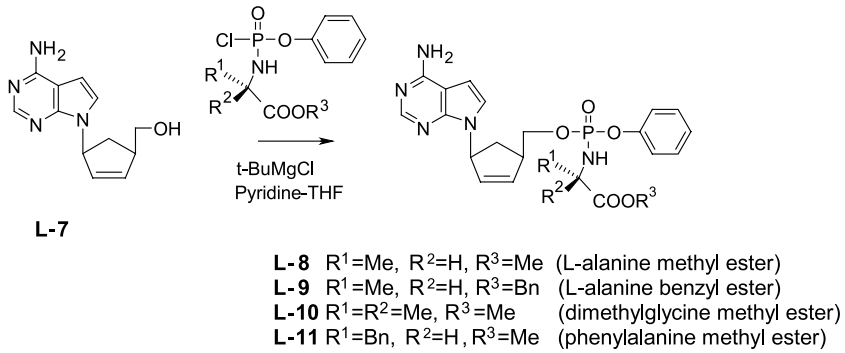
Herein we describe the synthesis of several phosphoramidate prodrugs of carbocyclic 2′,3′-dideoxy-2′,3′-didehydro-7-deazaadenosine along with their anti-HIV and HBV activity.

RESULTS AND DISCUSSION

Several different prodrug strategies have been designed to deliver monophosphates intracellularly. We decided to use the phosphoramidates as this approach is well validated and we have demonstrated that it improves the HBV activity of 7-(2′,3′-dideoxy-3′-fluoro-β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine-4-amine.[18] An additional advantage of the phosphoramidates is that the nucleoside can be converted to the phosphoramidate prodrug in one chemical step. The D- and L-nucleosides (**7**) were



Scheme 1. Synthesis of carbocyclic L-2′,3′-dideoxy-2′,3′-didehydro-7-deazaadenosine (**L-7**, D-analogs were synthesized in a similar fashion).



Scheme 2. Synthesis of phosphoramidates.

synthesized as outlined in Scheme 1 using a synthetic strategy similar to the one previously described by Legraverend et al.[19,20] and Montgomery et al.[21]

We chose to synthesize four different phosphoramidate derivatives, the analogs of which had previously been reported to give good improvement in activity against HIV and HBV for other nucleosides.[7–14] Treatment of 2′,3′-dideoxy-2′,3′-didehydro-7-deazaadenosine with the phosphorochloridate reagents[7–14] in pyridine in the presence of t-BuMgCl (as outlined in Scheme 2) gave good yields of protides **8–11**.

The activity of the protide derivatives **8–11** along with the activity of the parent nucleosides (**L-7** and **D-7**) against HIV and HBV is shown in Tables 1 and 2, respectively.

Phosphoramidate delivery improves the anti-HIV activity of the L-nucleoside significantly, up to 3000 fold for the L-alanine methyl ester derivative **L-8**. Activity of the D-nucleoside against HIV was not improved by protide delivery. Significant

Table 1. Anti-HIV-1 (3B) activity and cytotoxicity in MT-4 lymphocytes[22] of carbocyclic 2′, 3′-dideoxy-2′,3′-didehydro-7-deazaadenosine derivatives and their corresponding phosphoramidates.

Compound number[a]	IC_{50} (μM)[b]	CC_{50} (μM)[b]	Selectivity index[b]
L-7 (nucleoside)	280	>500	>2
L-8	0.091	26	>285
L-9	0.175	>10	>57
L-10	0.3	>10	>33
L-11	0.3	31.5	105
D-7 (nucleoside)	>200	>200	–
D-8	>10	>25	~1
D-9	>78	78	~1
D-10	>490	490	~1
D-11	29	42	~1

[a]All protides were tested as 1:1 mixtures of diastereomers at phosphorus.
[b]IC_{50} is 50% inhibitory concentration, CC_{50} is 50% cytotoxic concentration, selectivity index is CC_{50}/IC_{50}, results are averages of two or more experiments, each in duplicate.

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Table 2. Anti-HBV activity and cytotoxicity in HepG2-2.2.15 cells[23] of carbocyclic 2′, 3′-dideoxy-2′,3′-didehydro-7-deazaadenosine derivatives and the corresponding phosphoramidates.

Compound number[a]	IC_{50} (μM)[b]	CC_{50} (μM)[b]	Selectivity index[b]
L-7 (nucleoside)	2	50	25
L-8	0.09	230	>2500
L-9	0.1	>11	>110
L-10	0.05	>2	>40
L-11	0.055	>36	>650
D-7 (nucleoside)	>200	>200	–
D-8	54	81	~1
D-9	11	18	~1
D-10	73	64	~1
D-11	20	51	~2

[a]All protides were tested as 1:1 mixtures of diastereomers at phosphorus.
[b]IC_{50} is 50% inhibitory concentration, CC_{50} is 50% cytotoxic concentration, selectivity index is CC_{50}/IC_{50}, results are averages of two or more experiments, each in duplicate.

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increases in HIV potency (above 3 orders of magnitude) for the L-isomers suggest that formation of the monophosphate is limiting for carbocyclic L-2′,3′-dideoxy-2′,3′-didehydro-7-deazaadenosine.

Similarly, the activity of the L-enantiomer (**L-7**) against HBV was significantly improved by protide delivery. Thus, all four protides improved activity against HBV about 20-fold. Again the D-isomers proved much less active than the corresponding L-isomers.

The improvements in potency are significantly greater for HIV than for HBV. The SAR for the protide enhancement in activity with variation of the amino acid and ester appear to differ between the HIV (L-alanine methyl ester phosphoramidate gave the best potency) and HBV assay (dimethylglycine methyl ester phosphoramidate gave the best potency), suggesting that different anabolic pathways predominate in liver versus lymphocytes and that the optimal protide structure will vary with the target cell type.

In conclusion, protides of L-2′,3′-dideoxy-2′,3′-7-deazaadenosine significantly improved activity against HIV-1 (about 3 orders of magnitude) and against HBV (about 1–2 orders of magnitude). This was not the case for the corresponding D-analogs. We continue to investigate improvement in activity of nucleosides by protide delivery.

EXPERIMENTAL

Chemistry

Nuclear magnetic resonance (NMR) spectra were obtained at 300 MHz on Varian Unity Plus NMR spectrophotometer. The chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane as an internal standard. Elemental analysis

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were performed by Atlantic Microlab Inc. Flash column chromatography was performed using Merck Silica gel 60 (230–400 mesh), and the stated solvent system under pressure. Mass spectra were obtained on Micromass Platform mass spectrometers from Micromass Ltd. Altrincham, UK, using Electrospray Ionization.

(+)-(1R,4S)-4-[4-Chloro-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (L-6): (+)-(1S, 4R)-4-amino-2-cyclopentene-1-methanol hydrochloride (**4**, 3.96 mmol), triethylamine (1.20 g, 11.8 mmol), and 4,6-dichloro-5-(2,2-diethoxy-ethyl)pyrimidine (**3**, 1.05 g, 3.96 mmol) were refluxed in absolute ethanol (5 mL) for 4 hours. The solution was cooled, 1 N NaOH (8 mL) added, and volatiles removed under reduced pressure. The residue was chromatographed on silica gel with 10% methanol-chloroform. Product-containing fractions (**L-5**) were combined and concentrated to an oil. This oil (**L-5**) was dissolved in dioxane (10 mL)—1 N hydrochloric acid (3 mL) and the solution stirred at ambient temperature for 2 days, neutralized with ammonium hydroxide, and volatiles evaporated under reduced pressure. The residue was chromatographed on silica gel and title compound (**L-6**) eluted with 5–10% methanol-chloroform as a light tan oil (0.60 g, 60%); ^{1}H-NMR (DMSO-d_6) δ: 8.67 (s, 1 H), 7.70 and 6.69 (two d, J = 3.65 Hz, 1 H each), 6.10 (m, 1 H), 6.0–5.8 (m, 2 H), 4.77 (m, 1 H), 3.5 (m, 2 H), 2.9 (m, 1 H), 2.7 (m, 1 H), 1.6 (m, 1 H); MS (API$^+$): 252(35), 250(100), 156(24), 154(73). $[\alpha]_{589}$+ 43.6° (c 0.0045, methanol).

(+)-(1R,4S)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (L-7): (+)-4-[4-Chloro-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (**L-6**, 600 mg, 2.36 mmol) was heated in liquid ammonia (50 mL) in a Parr bomb at 65°C for 3 days. Volatiles were evaporated with 1 N sodium hydroxide (2.3 mL) and the residue chromatographed on silica gel. Title compound was eluted with 10% methanol-chloroform as a colorless oil. Evaporation of an acetonitrile solution gave title compound (**L-7**) as white powder which was washed with hexanes (291 mg, 52%); m.p. 165–168°C; ^{1}H-NMR (DMSO-d_6) δ: 8.07 (s, 1H), 7.06 and 6.56 (two d, J = 3.5 Hz, 1 H each), 7.0 (br s, 2 H), 6.10 (m, 1 H), 5.80 (m, 2 H), 4.75 (m, 1 H), 3.45 (m, 2 H), 2.85 (m, 1 H), 2.6 (m, 1 H), 1.45 (m, 1 H) ; MS (CI): M + 1, 231(100), 163(37), 135(45). Anal. Calcd. for $C_{12}H_{14}N_4O \times 1/2\ H_2O$: C, 60.24; H, 6.32; N, 23.41. Found: C, 60.54; H, 5.96; N, 23.73. $[\alpha]_{589}$ + 42.9° (c 0.0028, methanol).

(1R,4S)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol-*O*-[phenyl (methoxy L-alaninyl)]phosphoramidate (L-8): To (+)-(1R, 4S)-4-[4-amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (**L-7**, 0.19 g, 0.8 mmoles) was added anhydrous pyridine (4 mL) and anhydrous tetrahydrofuran (3 mL). Subsequently, tert-butylmagnesiumchloride (0.9 mL, 1 M solution in tetrahydrofuran) was added and the reaction stirred under nitrogen for 10 minutes at room temperature. A solution of phenyl methoxy-L-alaninyl phosphochloridate (1.2 mmol in anhydrous tetrahydrofuran) was added and the reaction stirred at room temperature for 12 hours. The resulting mixture was concentrated to a syrup under reduced pressure. This syrup was dissolved in dichloromethane (60 mL) and the organic phase washed with water (2 × 30 mL), dried (magnesium sulfate), filtered and concentrated to a foam. This foam was purified by flash column chromatography on silica gel. The title compound (**L-8**) was eluted with 5 % methanol in chloroform to give, after removal of volatiles,

290 mg (77%) of a white foam: ^{1}H-NMR ($CDCl_3$) δ: 8.23 (s, 1 H), 7.22 (m, 2 H), 7.10 (m, 3 H), 6.88 (2 × d, 1 H), 6.27 (d, 1 H), 5.98 (m, 1 H), 5.87 (m, 1 H), 5.80 (m, 1 H), 5.35 (bs, 2 H), 3.85–4.10 (m, 4 H), 3.61 (s, 3 H), 3.06 (m, 1 H), 2.7 (m, 1 H), 1.50 (m, 1 H), 1.28 (m, 3 H); ^{31}P-NMR ($CDCl_3$): 2.99 and 2.80; MS (EI): M + 23 (Na), 494. Anal. Calcd. for $C_{22}H_{26}N_5O_4P \times 3/2\ H_2O$: C, 54.77; H, 6.06; N, 14.52; Found: C, 54.32; H, 5.79; N, 14.22.

(1R,4S)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol-*O*-[phenyl (benzyloxy L-alaninyl)]phosphoramidate (L-9). (+)-(1R, 4S)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (**L-7**, 0.20 g, 0.9 mmoles) was treated with phenyl benzyloxy-L-alaninyl phosphochloridate (2.5 mL of 1 M solution in tetrahydrofuran, 2.5 mmol) as described above to give after silica gel chromatography with 5% methanol in chloroform 330 mg (69%) of the title compound **L-9** as a white foam: ^{1}H-NMR ($CDCl_3$) δ:8.34 (s, 1 H), 6.9–7.4 (m, 10 H), 6.95(2 × d, 1 H), 6.35 (m, 1 H), 5.8 –6.1 (3 × m, 3 H), 5.45 (broad s, 2 H), 5.13 (m, 2 H), 4.0–4.2 (m, 4 H), 3.1 (m, 1 H), 2.75 (m, 1 H), 1.58 (m, 1 H), 1.4 (m, 3 H); ^{31}P-NMR ($CDCl_3$):3.2 and 3.0; MS (EI): M + 1, 548; Anal. Calcd. for $C_{28}H_{30}N_5O_5P \times 1/3\ H_2O$: C, 60.75; H, 5.58; N, 12.65; Found: C, 60.93; H, 5.65; N, 12.58.

(1R,4S)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol-*O*-[phenyl (methoxy L-dimethylglycinyl)]phosphoramidate (L-10): (+)-(1R, 4S)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (**L-7**, 0.20 g, 0.9 mmoles) was treated with phenyl methoxy-L-dimethylglycinyl phosphochloridate (2.5 mL of 1 M solution in tetrahydrofuran, 2.5 mmoles) as described for preparation of **L-8** to give, after silica gel chromatography with 5% methanol in chloroform, 310 mg (73%) of the title compound **L-10** as a white foam: ^{1}H-NMR ($CDCl_3$) δ:8.35 (s, 1 H), 7.1–7.4 (m, 5 H), 6.97 (2 × d, 1 H), 6.38 (m, 1 H), 6.08 (m, 1 H), 5.97 (m, 1 H), 5.90 (m, 1 H), 5.40 (broad s, 2 H), 4.00–4.20 (m, 3 H), 3.73 (s, 3 H), 3.15 (m, 1 H), 2.8 (m, 1 H), 2.25 (s, 1 H), 1.55 (m, 6 H); ^{31}P-NMR ($CDCl_3$):1.73; MS (EI): M + 1, 486; Anal. Calcd. for $C_{23}H_{28}N_5O_5P \times 1/2\ H_2O$: C, 55.87; H, 5.91; N, 14.16; Found: C, 55.93; H, 5.91; N, 14.16.

(1R,4S)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol-*O*-[phenyl (methoxy L-phenylalaninyl)]phosphoramidate (L-11): (+)-(1R, 4S)-4-[4-amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (**L-7**, 0.20 g, 0.9 mmoles) was treated with phenyl methoxy-L-phenylalaninyl phosphochloridate (2.5 mL of 1 M solution in tetrahydrofuran, 2.5 mmoles) as described above to give after silica gel chromatography with 5% methanol in chloroform 320 mg (67%) of the title compound **L-11** as a white foam: ^{1}H-NMR ($CDCl_3$) δ: 8.22 (s, 1 H), 6.9–7.3 (m, 10 H), 6.85(m, 1 H), 6.25 (2 × d, 1 H), 5.7–6.0 (m, 3 H), 5.35 (broad s, 2 H), 4.15 (m, 1 H), 3.55–4.0 (m, 2 H), 3.55 (2 × s, 3 H), 2.6–2.9 (m, 5 H), 1.4 (m, 1 H); ^{31}P-NMR ($CDCl_3$):3.0; MS (EI): M + 1, 548; Anal. Calcd. for $C_{28}H_{30}N_5O_5P \times 0.7$ MeOH: C, 60.48; H, 5.80; N, 12.29; Found: C, 60.49; H, 5.51; N, 11.98.

(−)-(1S,4R)-4-[4-Chloro-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (D-6): Spectral data identical to **L-6**; $[\alpha]_{589} - 44°$ (c 0.05, methanol).

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(-)-(1S,4R)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (D-7): (−)-4-[4-Chloro-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (**D-6**, 4 g, 16 mmol) was treated as described above for **L-7** to give 2.5 g, 68% of **D-7** as a white solid: Spectral data identical to **L-7**. Anal. Calcd. for $C_{12}H_{14}N_4O$: C, 62.59; H, 6.13; N, 24.33. Found: C, 62.70; H, 6.25; N, 24.52. $[\alpha]_{589}$ − 43.2° (c 0.004, methanol).

(1S,4R)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol-*O*-[phenyl (methoxy L-alaninyl)]phosphoramidate (D-8): To (−)-(1R, 4S)-4-[4-amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (**D-7**, 0.5 g, 2.2 mmoles) was added anhydrous pyridine (2 mL) and anhydrous tetrahydrofuran (10 mL). Subsequently, tert-butylmagnesiumchloride (2.8 mL, 1 M solution in tetrahydrofuran) was added and the reaction stirred under nitrogen for 10 minutes at room temperature. A solution of phenyl methoxy-L-alaninyl phosphochloridate (4.3 mmol in anhydrous tetrahydrofuran) was added and the reaction stirred at room temperature for 12 hours. Workup as described above gave 450 mg (44%) of **D-8** as a white foam: Spetral data identical to **L-8**. Anal. Calcd. for $C_{22}H_{26}N_5O_4P \times 3/2\ H_2O$: C, 54.77; H, 6.06; N, 14.52; Found: C, 54.62; H, 5.99; N, 14.13.

(1S,4R)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol-*O*-[phenyl (benzyloxy L-alaninyl)]phosphoramidate (D-9): (−)-(1R, 4S)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (**D-7**, 0.28 g, 1.2 mmoles) was treated with phenyl benzyloxy-L-alaninyl phosphochloridate (1 M solution in tetrahydrofuran, 2.43 mmol) as described above to give after silica gel chromatography with 5% methanol in chloroform 539 mg (81%) of **D-9** as a white foam: Spectral data identical to **L-9**. Anal. Calcd. for $C_{28}H_{30}N_5O_5P \times H_2O$: C, 59.46; H, 5.70; N, 12.38; Found: C, 59.18; H, 5.41; N, 12.37.

(1S,4R)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol-*O*-[phenyl (methoxy L-dimethylglycinyl)]phosphoramidate (D-10): (−)-(1R, 4S)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (**D-7**, 0.28 g, 1.2 mmoles) was treated with phenyl methoxy-L-dimethylglycinyl phosphochloridate (1 M solution in tetrahydrofuran, 2.43 mmoles) as described above to give, after silica gel chromatography with 5% methanol in chloroform, 400 mg of **D-10** as a white foam: Spectral data identical to **L-10**. Anal. Calcd. for $C_{23}H_{28}N_5O_5P \times 1/2\ H_2O$: C, 55.87; H, 5.91; N, 14.16; Found: C, 55.45; H, 5.75; N, 14.39.

(1S,4R)-4-[4-Amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol-*O*-[phenyl (methoxy L-phenylalaninyl)]phosphoramidate (D-11): (−)-(1R, 4S)-4-[4-amino-7H-pyrrolo(2,3-d)pyrimidin-7-yl]-2-cyclopentene-1-methanol (**D-7**, 0.41 g, 1.8 mmoles) was treated with phenyl methoxy-L-phenylalaninyl phosphochloridate (1 M solution in tetrahydrofuran, 3.58 mmoles) as described above to give after silica gel chromatography with 5% methanol in chloroform 350 mg (36%) of **D-11** as a white foam: Spectral data identical to **L-11**. Anal. Calcd. for $C_{28}H_{30}N_5O_5P \times H_2O$ C, 59.46; H, 5.70; N, 12.38; Found: C, 59.42; H, 5.36; N, 12.09.

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ANTIVIRAL ASSAYS

HBV

Antiviral potency and growth inhibition potential of compounds was determined using the assay developed by Jansen et al.[a] Briefly, HepG2-2.2.15 cells constitutively producing HBV[24] were seeded into 96 well microtiter plates at a density of 5×10^3 per well and growth medium containing drug was replaced every other day for 9 days. Supernatants were then collected and analyzed for HBV content. Samples were tested in conjunction with both positive (0.448 fg/ul plasmid DNA) and negative (RPMI medium supplemented with 2mM L-glutamine and 10% fetal calf serum) controls. Data was normalized to non-drug treated cells, and expressed as a percent of control for analysis.

Evaluation of toxicity (i.e. growth inhibition) was made by fixing monolayers with 70% ethanol, and staining with bisbenzimide H33342 for 1 hour at 37°C. Fluorescence values of drug treated cells were compared to non-drug treated cells and expressed as a percentage of control. HBV detection (and hence efficacy determination) was performed by ''capturing'' virus from supernatants on anti-HBsAg coated plates, washing, denaturing to release HBV DNA, performing PCR with biotinylated primers, streptavidin capture of biotinylated PCR products with concomitant probe hybridization, addition of substrate, and reading optical densities of the colorimetric reaction. Dilutions of a standardized HBV-containing supernatant were included on every plate, and HBV DNA concentrations of test wells were calculated from this HBV standard curve.

HIV

Anti-HIV-1 (3B) activity and cytotoxicity in MT-4 lymphocytes was determined as previously described by Averett et al.[b]

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[a]Compounds were tested for anti-HBV activity according to the method described by: From Ref. [23].

[b]Compounds were assayed for HIV activity in MT-4 cells according to the method described by: [From Ref. 22].

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Effects of Substitutions at the 4′ and 2 Positions on the Bioactivity of 4′-Ethynyl-2-Fluoro-2′-Deoxyadenosine

Karen A. Kirby, Eleftherios Michailidis, Tracy L. Fetterly, Musetta A. Steinbach, Kamalendra Singh, Bruno Marchand, Maxwell D. Leslie, Ariel N. Hagedorn, Eiichi N. Kodama, Victor E. Marquez, Stephen H. Hughes, Hiroaki Mitsuya, Michael A. Parniak and Stefan G. Sarafianos

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Effects of Substitutions at the 4′ and 2 Positions on the Bioactivity of 4′-Ethynyl-2-Fluoro-2′-Deoxyadenosine

Karen A. Kirby,[a,b] Eleftherios Michailidis,[a,b] Tracy L. Fetterly,[a,b] Musetta A. Steinbach,[a,b] Kamalendra Singh,[a,b] Bruno Marchand,[a,b] Maxwell D. Leslie,[a,b] Ariel N. Hagedorn,[a,b] Eiichi N. Kodama,[c] Victor E. Marquez,[d] Stephen H. Hughes,[e] Hiroaki Mitsuya,[f,g] Michael A. Parniak,[h] Stefan G. Sarafianos[a,b,i]

Christopher Bond Life Sciences Center, University of Missouri, Columbia, Missouri, USA[a]; Department of Molecular Microbiology & Immunology, University of Missouri School of Medicine, Columbia, Missouri, USA[b]; Division of Emerging Infectious Diseases, Tohoku University School of Medicine, Sendai, Japan[c]; Chemical Biology Laboratory, Center for Cancer Research, National Cancer Institute-Frederick, Frederick, Maryland, USA[d]; HIV Drug Resistance Program, National Cancer Institute-Frederick, Frederick, Maryland, USA[e]; Department of Internal Medicine, Kumamoto University School of Medicine, Kumamoto Japan[f]; Experimental Retrovirology Section, HIV/AIDS Malignancy Branch, National Institutes of Health, Bethesda, Maryland, USA[g]; Department of Microbiology & Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA[h]; Department of Biochemistry, University of Missouri, Columbia, Missouri, USA[i]

Nucleos(t)ide reverse transcriptase inhibitors (NRTIs) form the backbone of most anti-HIV therapies. We have shown that 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) is a highly effective NRTI; however, the reasons for the potent antiviral activity of EFdA are not well understood. Here, we use a combination of structural, computational, and biochemical approaches to examine how substitutions in the sugar or adenine rings affect the incorporation of dA-based NRTIs like EFdA into DNA by HIV RT and their susceptibility to deamination by adenosine deaminase (ADA). Nuclear magnetic resonance (NMR) spectroscopy studies of 4′-substituted NRTIs show that ethynyl or cyano groups stabilize the sugar ring in the C-2′-exo/C-3′-endo (north) conformation. Steady-state kinetic analysis of the incorporation of 4′-substituted NRTIs by RT reveals a correlation between the north conformation of the NRTI sugar ring and efficiency of incorporation into the nascent DNA strand. Structural analysis and the kinetics of deamination by ADA demonstrate that 4′-ethynyl and cyano substitutions decrease the susceptibility of adenosine-based compounds to ADA through steric interactions at the active site. However, the major determinant for decreased susceptibility to ADA is the 2-halo substitution, which alters the pK_a of N1 on the adenine base. These results provide insight into how NRTI structural attributes affect their antiviral activities through their interactions with the RT and ADA active sites.

There are 10 nucleos(t)ide reverse transcriptase inhibitors (NRTIs) that are currently approved for the treatment of human immunodeficiency virus type 1 (HIV-1) infections (1–5). Several more nucleoside analog drugs are approved or being studied for the treatment of viruses, such as herpes simplex virus (HSV), hepatitis C virus (HCV), and hepatitis B virus (HBV), or as anticancer agents (6–10). NRTIs are among the most effective anti-HIV drugs. All approved anti-HIV NRTIs lack a 3′-hydroxyl moiety and, thus, act as chain terminators following their incorporation by the viral reverse transcriptase (RT) into the nascent DNA chain. However, the absence of a 3′-OH, while essential for the inhibition of DNA synthesis, also imparts detrimental properties to these inhibitors, including reduced intracellular phosphorylation to the active triphosphate form and reduced RT binding affinity (11). Prolonged exposure to NRTI-based treatments causes mitochondrial toxicity (12–14) and leads to the development of NRTI resistance mutations (15–18), giving rise to complications in the treatment of HIV-infected patients.

Ideally, an NRTI should have a strong binding affinity for the RT target, a high barrier for the development of resistance, and low toxicity. We have reported that a series of 4′-substituted nucleosides in which the 3′-OH is retained has exceptional inhibitory activity against HIV-1 RT (19). Among these compounds, 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) is a highly active RT inhibitor that prevents translocation of the nucleic acid from the nucleotide-binding or pretranslocation site to the primer-binding or posttranslocation site on RT following its incorporation at the 3′ primer terminus (20). Depending on the DNA template sequence, EFdA can also act (albeit less frequently) as a delayed chain terminator, incorporating one incoming deoxynucleoside triphosphate (dNTP) before DNA synthesis is blocked (20). We previously showed that EFdA inhibits HIV replication in peripheral blood mononuclear cells (PBMCs) with a 50% effective concentration (EC_{50}) of 0.05 nM (20). Additionally, EFdA effectively inhibits the replication of many common drug-resistant strains of HIV, including strains that carry a substitution of R for K at position 65 (K65R) (a 0.2-fold change in EC_{50}) (21, 22), N348I (a 0.9-fold change in 50% inhibitory concentration [IC_{50}]) (23), the excision-enhancing mutations M41L and T215Y (a 1.5-fold change in EC_{50}) (21), and multidrug resistance Q151M complex mutations (A62V/V75I/F77L/F116Y/Q151M) (a 0.7-fold change in EC_{50}) (21). EFdA is also able to inhibit HIV containing the M184V drug resistance mutation (with an EC_{50} of 8.3 nM, or a 7.5-fold change) (21, 24). EFdA is generally a poor substrate for human DNA polymerases in *in vitro* experiments (IC_{50}s of >100 μM for polymerase α and β and 10 μM for polymerase γ; EFdA-triphosphate [EFdA-TP] is incorporated by polymerase γ 4,300-

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Address correspondence to Stefan G. Sarafianos, sarafianoss@missouri.edu.
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fold less efficiently than dATP) and, thus, has a low potential for cytotoxicity (25, 26). Because the 50% cytotoxic concentration (CC_{50}) for EFdA is more than 10 μM in MT4 cells or PBMCs, the selectivity of the compound is high. Moreover, in studies with simian immunodeficiency virus (SIV)-infected macaques, no signs of clinical or pathological drug toxicity were observed after 6 months of continuous EFdA monotherapy (24). These data suggest that EFdA is a strong candidate for further development as a therapeutic agent.

The EC_{50s} of NRTIs are also affected by cellular uptake, activation to the active metabolite by host kinases, and catabolism by cellular enzymes. Previous studies have shown that EFdA has a favorable cellular uptake profile and is efficiently phosphorylated to EFdA-TP (25). Additional reported data strongly suggest that, in cells, EFdA is phosphorylated to EFdA-monophosphate (EFdA-MP) by deoxycytidine kinase (dCK) (21). The molecular details of the efficiency and specificity of this process are being investigated in separate studies. The enzyme adenosine deaminase (ADA), which is present at high concentrations in human serum, is involved in the catabolism of dA and its analogs (27, 28). ADA is responsible for the deamination of adenosine or deoxyadenosine analogs to inosine- or deoxyinosine-based products (27, 29–33) and can therefore affect the activation pathway and intracellular concentration of dA-based compounds. For example, ADA converts dideoxyadenosine (ddA) to ddI, leading to a different and indirect activation pathway for ddA (28, 34, 35). Thus, it is important to study the interaction of dA-based compounds, such as EFdA, with ADA to gain insights into their catabolism and subsequent activation pathways.

We have also previously reported that small changes in the chemical composition of dA analogs can have a pronounced effect on their antiviral potency. These changes can be complex, because they can have different and at times opposing effects on the recognition of NRTIs by the RT target and the various enzymes involved in activation and catabolism. For example, the presence of fluorine or another halogen at the 2 position on the adenine base of 4′-substituted dA analogs significantly enhanced their antiviral activity (21). Similarly, the nature of the 4′ substitutions on the deoxyribose ring also affected the EC_{50}s of dA-based compounds against both wild-type (WT) and NRTI-resistant HIV-1 variants (19).

Additionally, changes in the conformation of the sugar ring and in the nature of the nucleobase can affect the biological activity of NRTIs. In solution, the structure of the deoxyribose ring of nucleosides exists in a dynamic equilibrium between the C-2′-exo/C-3′-endo (north) and C-2′-endo/C-3′-exo (south) conformations. We have previously shown that EFdA exists primarily in the north conformation (36) and that HIV-1 RT prefers the NRTI or incoming dNTP sugar ring in the north conformation for optimal binding (37–40). In contrast, host cell kinases appear to prefer the sugar ring in the south conformation; compounds that favor the north conformation are generally phosphorylated inefficiently (41, 42). Sugar ring conformation has also been reported to play an important role in ADA recognition; the enzyme has been reported to deaminate nucleosides biased toward the north conformation up to 65-fold faster than those biased toward the south conformation (43–46). Additionally, 2-fluoro-2′-deoxyadenosine (FdA) analogs are somewhat resistant to ADA-mediated catabolism due to the electron withdrawing properties of the 2-fluoro group (47, 48), and we have previously reported that EFdA is poorly deaminated by ADA because of the 2-fluoro group (21). However, the effects of specific 4′ substitutions on sugar ring conformation and how these substitutions would affect the recognition of the compounds by HIV RT and ADA have not been determined.

In the present work, we carried out a series of structural, computational, and biochemical studies using a variety of 2- and 4′-substituted dA and dT analogs (Fig. 1) to understand the specific effects of these different substitutions on sugar ring conformation, recognition by HIV RT, and deamination by ADA. Our data show how the 2-fluoro and 4′-ethynyl moieties affect the antiviral potential of NRTIs and help determine the basis for the favorable inhibitory profile of EFdA.

MATERIALS AND METHODS

Chemicals. Compounds dA, FdA, and dT (compound abbreviations are defined and their numbers specified in Results) were purchased from Sigma-Aldrich (St. Louis, MO). Triphosphate forms of dA and dT were purchased from Fermentas (Glen Burnie, MD). Compounds 13 to 14 and the triphosphate forms of compounds 10, 11, 13, and 14 were synthesized by Marquez and colleagues as previously described (49, 50). ADA (bovine intestine, EC 3.5.4.4) was purchased from Calbiochem (San Diego, CA). Deuterated dimethyl sulfoxide (DMSO-D_6) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

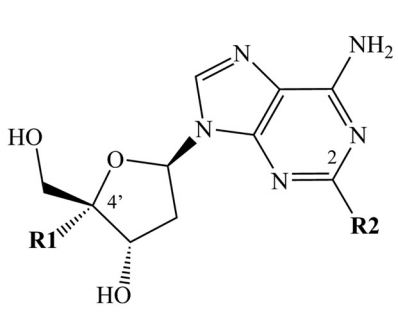
Nucleic acid substrates. DNA oligomers were synthesized by Integrated DNA Technologies (Coralville, IA). In steady-state kinetics assays of dATP and dATP analogs, a 26-nucleotide-long DNA template (T_{d26A}; CCA TAG ATA GCA TTG GTG CTC GAA CA) was used. In steady-state kinetics assays of dTTP and dTTP analogs, a 26-nucleotide-long template (T_{d26T}; CCA TAG TAA GCA TTG GTG CTC GAA CA) was used. An 18-nucleotide-long 5′-fluorescently labeled DNA primer (P_{d18}; TGT TCG AGC ACC AAT GCT) was annealed to both T_{d26A} and T_{d26T}.

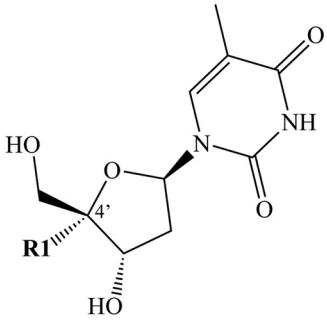
NMR spectroscopy. One-dimensional ^{1}H nuclear magnetic resonance (NMR) spectra were collected in 10°C increments from 20 to 50°C on a Bruker Avance DRX500 operating at 500 MHz and equipped with a 5-mm HCN cryoprobe, as previously described (36). Samples were dissolved in DMSO-D_6 to give concentrations of 2 to 4 mM. Spectra were processed with resolution enhancement using negative line broadening (lb = −0.3). Coupling constants were analyzed by spectral simulation using SpinWorks 3.0 (Kirk Marat, University of Manitoba, Winnipeg, Canada). Simulations were performed for the spin systems on the deoxyribose ring only. The root mean square (RMS) deviations of calculated and experimental coupling constants for all compounds were <0.15 Hz.

Pseudorotational analysis. The sugar ring conformations of the nucleoside analogs were determined using the program PSEUROT 6.3 (DOS version, purchased from Cornelis Altona, University of Leiden, Leiden, The Netherlands) as previously described (36). PSEUROT assumes a two-state approximation (equilibrium between north and south conformations) and utilizes a generalized Karplus equation combined with a nonlinear Newton-Raphson minimization process to examine the conformational flexibility of five-membered rings (51). An iterative methodology was used to determine the optimal pseudorotational parameters P_N and P_S (pseudorotational phase angle [P] for the north [N]or south [S] conformation) and $\phi_m(N)$ and $\phi_m(S)$ (degree of maximum ring pucker [ϕ_m] for the north or south conformation), in which some of these parameters were fixed during refinement in order to constrain the values within the range commonly observed for nucleosides (average observed, $\phi_m = 39°$).

Molecular orbital calculations. In order to determine the role of molecular orbitals in the stability of the north and south sugar ring conformations of EFdA, we carried out molecular orbital calculations using semiempirical quantum chemical methods. All quantum chemical calculations were performed by the density functional theory (DFT) method using the B3LYP approach with 6-31G** basis sets. The quantum chem-

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Deoxyadenosine Analogs

Sugar R1	Base R2	Compound Abbreviation
−H	−H	dA (**1**)
−H	−F	FdA (**2**)
−C≡CH	−H	EdA (**3**)
−C≡CH	$-NH_2$	EdDAP (**4**)
−C≡CH	−F	EFdA (**5**)
−C≡CH	−Cl	ECldA (**6**)
−C≡N	−H	CNdA (**7**)
−C≡N	$-NH_2$	CNdDAP (**8**)
−C≡N	−F	CNFdA (**9**)
$-CH_3$	−H	MedA (**10**)*
$-CH_2CH_3$	−H	EtdA (**11**)*

Thymidine Analogs

Sugar R1	Compound Abbreviation
−H	dT (**12**)
$-CH_3$	MedT (**13**)
$-CH_2CH_3$	EtdT (**14**)

FIG 1 Deoxyadenosine and thymidine analogs used in the study. *, only the triphosphate forms of these compounds were used in this study.

istry program Jaguar (Schrödinger, Inc., NY) was used for all computations. The structures of north and south conformations of EFdA were completely optimized by restricting the position of the 3′-C either in the north or south conformation. To obtain insight into the interaction of the lone pairs of the O4 and 3′-O atoms with the antibonding (π^*) orbitals of the 4′-ethynyl group of EFdA, the natural bond orbitals (NBOs), natural (semi-)localized molecular orbitals (NLMOs), and canonical molecular orbitals (CMOs) were computed using the program NBO (version 5.0) (52, 53).

HIV-1 RT steady-state kinetic assays. The steady-state kinetic parameters, K_m and k_{cat}, for incorporation of dNTPs and dNTP analogs were determined using single-nucleotide incorporation in gel-based assays under saturating substrate conditions. The reactions were carried out in 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 6 mM $MgCl_2$, 100 nM T_{d26A}/P_{d18} for dATP analogs, 100 nM T_{d26T}/P_{d18} for dTTP analogs, and 10 nM RT in a final volume of 20 μl and stopped at the reaction times of 1.5 to 3 min. The products were resolved on 15% polyacrylamide 7 M urea gels, and the gels were scanned with a Typhoon FLA 9000 phosphorimager (GE Healthcare). The bands were quantified using Multi Gauge software (FujiFilm). K_m and k_{cat} values were determined graphically using the Michaelis-Menten equation in GraphPad Prism 4 (GraphPad Software, Inc.).

ADA kinetic assays. The deamination rates of dA (compound 1), EdA (compound 3), and CNdA (compound 7) were determined by following the disappearance of the substrate on a BioTek Synergy HT spectrophotometer at 265 nm in 100-μl solutions at 25°C with 0.1 M phosphate, pH 7.4. A change in molar extinction coefficients for all of the deaminated products was assumed to be $\Delta\varepsilon = -7{,}900$ for all dA analogs (45). For each assay, the substrate concentrations varied between 15 μM and 240 μM. Absorbance was measured every 20 s. Three determinations were made for each analog. K_m and k_{cat} for each compound were determined using Michaelis-Menten plots (GraphPad Prism 4).

Because the reactions of ADA with FdA (compound 2), EFdA (compound 5), ECldA (compound 6), and CNFdA (compound 9) were very slow under these conditions, a high-pressure liquid chromatography (HPLC) method was used to observe the hydrolytic deamination of these compounds (54). In order to separate the substrate and product of the reaction, an elution gradient was developed using various combinations of 0.1 M phosphate, pH 7.4, and methanol. The following gradient was run on an Agilent 1100 series HPLC system: 7% methanol for 3 min, 14% methanol for 2 min, 21% methanol for 2 min, 28% methanol for 2 min, 35% methanol for 5 min, and 7% methanol for the final 2 min, returning the conditions to the original state. The gradient ran for a total of 16 min at a flow rate of 1 ml/min and an injection volume of 60 μl. The method ran at room temperature, and the absorbance was measured at a wavelength of 260 nm. The solvents used for the HPLC gradient were vacuum filtered prior to use.

Kinetic measurements were made using dA (compound 1), FdA (compound 2), EdA (compound 3), EFdA (compound 5), ECldA (compound 6), CNdA (compound 7), and CNFdA (compound 9) as ADA substrates. The reactions were carried out in 0.1 M phosphate buffer, pH 7.4. The FdA (compound 2) and EFdA (compound 5) analogs were tested using concentrations ranging from 10 μM to 9 mM. Compounds dA (compound 1), EdA (compound 3), ECldA (compound 6), CNdA (compound 7), and CNFdA (compound 9) were tested at 500 μM. Reactions were started by the addition of 2.4 mU ADA. The reactions were run for 45 min at 25°C before being terminated by the addition of perchlorate at 0.3

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TABLE 1 Summary of pseudorotational analysis of the sugar ring of deoxyadenosine and thymidine analogs

Compound (no.)	P_N (°)	P_S (°)	$\phi_m(N)$ (°)	$\phi_m(S)$ (°)	% North	RMS error (Hz)[a]
Deoxyadenosine analogs						
dA (1)[b]	18.7	169.1	39.0[c]	31.1	23	0.03
FdA (2)	3.4	169.1	38.7	29.2	24	0.01
EdA (3)	37.3	152.1	39.0[c]	39.0[c]	64	0.40
EdDAP (4)	39.7	152.1	39.0[c]	39.0[c]	63	0.49
EFdA (5)[b]	38.7	146.5	39.0[c]	39.0[c]	75	0.20
EClda (6)	38.4	148.0	39.0[c]	39.0[c]	73	0.21
CNdA (7)	47.8	152.8	38.0[c]	38.0[c]	61	0.12
CNdDAP (8)	45.0	154.0[c]	37.2	38.0[c]	56	0.12
CNFdA (9)	41.6	154.2	38.0[c]	38.0[c]	59	0.23
Thymidine analogs						
MedT (13)	24.3	132.5	38.0[c]	31.5	43	0.05
EtdT (14)	23.3	150.3	38.0[c]	30.2	39	0.18

[a] Root mean square deviation between experimental and calculated coupling constants.
[b] Values previously reported (36).
[c] Value fixed during calculations.

M final concentration. Denatured protein was removed from the reaction mixture by using centrifugation at 10,000 rpm for 5 min to pellet the protein. The clear supernatant was then removed, and the final pH was adjusted to 7.4 by adding Na_2CO_3 to reach a final concentration of 2.5 mM (54).

Structural analysis of predicted EFdA interactions at the active site of ADA. Structural analysis of EFdA interactions at the ADA active site were carried out using the coordinates of human ADA in complex with dA obtained from the RCSB Protein Data Bank (PDB ID 3IAR). EFdA was drawn and minimized using the ChemBioDraw Ultra 13.0 and ChemBio3D Ultra 13.0 software from the ChemBioOffice Suite (PerkinElmer, Waltham, MA). Using the Coot model building software package (55), the structure of EFdA was superposed to the dA molecule, followed by the removal of dA.

RESULTS

Effect of 4′ substitutions on the structure of dA and dT analogs. To evaluate the structural effects of the 4′ substitutions on the sugar ring conformations of the dA and dT analogs, we collected one-dimensional ^{1}H NMR spectra over a range of physiologically relevant temperatures for the following compounds: 2′-deoxyadenosine (dA, compound 1), 2-fluoro-2′-deoxyadenosine (FdA, compound 2), 4′-ethynyl-2′-deoxyadenosine (EdA, compound 3), 4′-ethynyl-2′-deoxyribofuranosyl-2,6-diaminopurine (EdDAP, also known as ENdA, compound 4), 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA, compound 5), 4′-ethynyl-2-chloro-2′-deoxyadenosine (EClda, compound 6), 4′-cyano-2′-deoxyadenosine (CNdA, compound 7), 4′-cyano-2′-deoxyribofuranosyl-2,6-diaminopurine (CNdDAP, compound 8), 4′-cyano-2-fluoro-2′-deoxyadenosine (CNFdA, compound 9), thymidine (dT, compound 12), 4′-methyl-thymidine (MedT, compound 13), and 4′-ethyl-thymidine (EtdT, compound 14) (Fig. 1) (36). Resolving the coupling constants between most of the hydrogen atoms on the deoxyribose ring required spectral simulation (see Table S1 in the supplemental material). The coupling constants determined from each spectrum were used to calculate the structural parameters of the deoxyribose ring of each compound. The changes in the value of each coupling constant with temperature were used to calculate the pseudorotational phase angle, P, and the degree of maximum ring pucker, ϕ_m, for the C-2′-exo/C-3′-endo (north or *N*) and C-2′-endo/C-3′-exo (south or *S*) sugar ring conformations (see Fig. S1 in the supplemental material) of the nucleoside analogs in this study.

The pseudorotational parameters listed in Table 1 were calculated using PSEUROT 6.3 as described in Materials and Methods and our previous report (36). The value of P_N for each of the 4′-ethynyl- or cyano-substituted deoxyadenosine compounds 3 to 9 (P_N = 37.3 to 47.8°) lies in the northern hemisphere of the pseudorotational pathway (P_N = 0 to 90°), although these values are higher than what is typically observed for most nucleosides (P_N = 0 to 36°) (see Fig. S1 in the supplemental material). These values correspond to a conformation between $_4T^3$ (P_N = 36°) and $_4$E or 4′-exo (P_N = 54°) in the pseudorotational pathway (see Fig. S1) (56). A similar conformation was determined by Siddiqui et al. from the crystal structure of 4′-ethynyl-2′,3′-dideoxycytidine (P_N = 43.5°) (57). The P_N values of the 4′-cyano-substituted compounds 7 to 9 (P_N = 41.6 to 47.8°) are slightly higher than those observed for the 4′-ethynyl-substituted compounds 3 to 6 (P_N = 37.3 to 39.7°). The values of P_N for the 4′-methyl- and 4′-ethyl-substituted thymidine compounds (P_N = 24.3° and 23.3°, respectively) were also in the northern hemisphere but were much lower than those observed for the 4′-ethynyl and 4′-cyano analogs. These values correspond to a conformation between ^{3}E or 3′-endo (P_N = 18°) and $_4T^3$ (P_N = 36°) in the pseudorotational pathway (see Fig. S1) (56).

The pseudorotational values determined for dA (compound 1) are in agreement with previous reports, demonstrating that dA strongly prefers the south conformation (36, 45). Comparison of the conformational parameters of dA (compound 1) versus EdA (compound 3) (23% versus 64% north) and FdA (compound 2) versus EFdA (compound 5) (24% versus 75% north) showed that the 4′-ethynyl strongly favors the north conformation of the sugar (Table 1 and Fig. 2). Comparison of dA (compound 1) versus CNdA (compound 7) (23% versus 61% north) and FdA (compound 2) versus CNFdA (compound 9) (24% versus 59% north) showed that the 4′-cyano also has a large effect on sugar conformation, albeit smaller than that of the 4′-ethynyl group (% north in EdA [compound 3] > % north in CNdA [compound 7] and % north in EFdA [compound 5] > % north in CNFdA [compound 9]) (Table 1).

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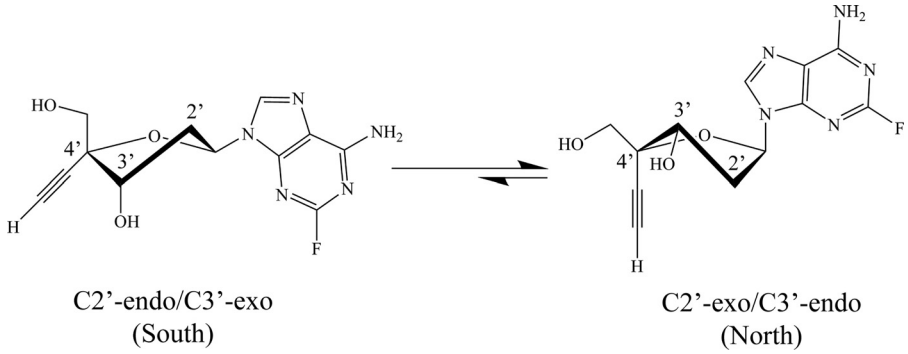


FIG 2 Dynamic equilibrium between the south and north sugar ring conformations of 4′-ethynyl-substituted compounds in solution (EFdA is shown). Natural deoxynucleoside substrates favor the south conformation.

Evaluation of the conformational parameters of the thymidine analogs dT (compound 12), MedT (compound 13), and EtdT (compound 14) demonstrated that 4′-methyl and ethyl substitutions have smaller effects on the sugar ring conformation than 4′-ethynyl and cyano groups. Although we were unable to accurately determine the coupling constants for dT (compound 12) due to overlap of one of the 2′-H signals with the DMSO solvent peak, it has previously been determined that the deoxyribose ring of dT favors the south conformation, which is similar to dA (56, 58). 4′-Methyl and 4′-ethyl substitutions shift the equilibrium toward the north conformation relative to the equilibrium of thymidine (43% and 39% north, respectively), but this effect is smaller than that of a 4′-ethynyl or 4′-cyano substitution in dA analogs (64% and 61% north compared to 23% in dA) (Table 1).

Hence, the 4′-ethynyl substitution imparts the largest effect on the conformation of the deoxyribose ring, followed by the 4′-cyano, the 4′-methyl, and the 4′-ethyl groups.

Effect of 2 position substitutions on the structure of dA analogs. Using the same method, we also assessed the effect of substitutions at the 2 position of the adenine base on the structure of dA analogs. Evaluation of the conformation parameters of dA (compound 1) versus FdA (compound 2) showed that substitutions at the 2 position of the adenine base have little to no effect on sugar ring conformation. Similarly, comparisons of the 4′-ethynyl-substituted EdA (compound 3), EdDAP (compound 4), EFdA (compound 5), and ECldA (compound 6) showed that the 2-fluoro and 2-chloro substitutions have only a modest effect on the sugar ring conformation (Table 1). Finally, comparisons of CNdA (compound 7), CNdDAP (compound 8), and CNFdA (compound 9) also confirmed that 2-fluoro and 2-amino substitutions on the adenine ring have no significant impact on the sugar ring conformation (61%, 56%, and 59% north, respectively) (Table 1).

Molecular orbital calculations. Potential stabilizing orbital interactions can occur between a lone pair of electrons in a p-like fully occupied orbital and an unoccupied antibonding orbital, allowing the delocalization of electrons and, thus, increasing molecular stability. To determine whether there are any favorable orbital interactions in the north or south sugar ring conformations of EFdA that could stabilize one conformation versus the other, we carried out quantum chemical computations to assess the orbital interactions of the lone pairs of the O4 and 3′-O atoms with the antibonding (π^*) orbitals of the 4′-ethynyl moiety. We first determined the electronic composition of natural bond orbitals (NBOs) and their contribution in the formation of natural (semi-)localized molecular orbitals (NLMOs). The NBOs are localized orbitals that describe the Lewis-like molecular bonding pattern of electron pairs (59). The NLMOs have been described as semilocalized alternatives to canonical molecular orbitals (CMOs) for representing the electron pairs of MO-type wave functions. Each NLMO consists of a parent NBO (strictly localized) and associated delocalizations needed to describe the density of a full electron pair (59). The NBOs were used to compute CMOs. Both the north and south sugar ring conformations of EFdA have 375 NBOs (which include bonding orbitals, core orbitals, lone pairs, antibonding orbitals, and Rydberg orbitals), 76 NLMOs, and 375 CMOs. To assess the orbital interaction involving the lone pairs of O4 and 3′-O and the antibonding orbitals (π^*) of the 4′-ethynyl group, we examined the second-order perturbative estimates of donor-acceptor (bond-antibond) NBO interactions for both north and south conformations. These estimates provide the measure of delocalization due to the interaction of the O4 or 3′-O lone pair with the π^* antibonding orbital of the 4′-ethynyl group. Table 2 summarizes the energy of the donor-acceptor NBO interactions. These results suggest that the interaction of the lone pairs of the O4 and 3′-O atoms with the antibonding orbitals of the 4′-ethynyl moiety contributes to the greater stabilization energy (~2-fold) of the north conformation. Further NBO analyses show the reduced occupancy of the O4 and 3′-O lone pairs (10% and 6%, respectively) and the remote interaction of both lone pairs with the antibonding NBOs of the 4′-C≡C in the north conformation. In the south conformation, the O4 and 3′-O lone pairs have a reduced occupancy of 7% and 4%, respectively, and only the O4 lone pair has a remote interaction with the antibonding NBOs of the 4′-C≡C. The NLMO analyses of the north conformation showed that the lone pairs of both O4 and 3′-O

TABLE 2 Summary of molecular orbital calculations[a]

NBO donor (LP)	NBO acceptor (π^*)	Interaction energy (kcal/mol) for EFdA conformation	
		North	South
O4	4′-C≡C	1.63	1.25
3′-O	4′-C≡C	0.71	0.0
Total energy		2.34	1.25

[a] LP, lone pair; π^*, antibonding orbital.

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TABLE 3 Steady-state kinetic parameters for incorporation of the triphosphate forms of the 4′-substituted compounds by HIV-1 RT

dNTP	K_m (μM)[a]	k_{cat} (min^{-1})[a]	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)	Fold change in incorporation efficiency[b]
dATP	2.35 ± 0.06	0.66 ± 0.13	0.3	1
EFdA-TP	0.27 ± 0.01	3.47 ± 0.04	12.9	45.9
EdDAP-TP	0.15 ± 0.01	2.55 ± 0.10	17.0	60.7
MedA-TP	0.29 ± 0.01	1.24 ± 0.05	4.3	15.3
EtdA-TP	0.88 ± 0.19	3.41 ± 0.12	3.9	13.9
dTTP	0.60 ± 0.01	1.04 ± 0.12	1.7	1
MedT-TP	0.68 ± 0.22	1.58 ± 0.15	2.3	1.3
EtdT-TP	2.00 ± 0.22	1.44 ± 0.20	0.7	0.4

[a] Values are means ± standard deviations of two independent experiments and were determined from the Michaelis-Menten equation using GraphPad Prism 4.
[b] Fold change in incorporation efficiency is the ratio of the incorporation efficiency (k_{cat}/K_m) of NRTI-TP over that of dATP or dTTP [$(k_{cat}/K_m)_{NRTI\text{-}TP}/(k_{cat}/K_m)_{dATP}$ or $(k_{cat}/K_m)_{NRTI\text{-}TP}/(k_{cat}/K_m)_{dTTP}$].

atoms are delocalized into the vicinal region (C-5′ and 4′-C≡C, ~5% and 2%, respectively). The delocalization of the O4 lone pair in the vicinal C-5′ and 4′-C≡C groups for the south conformation is significantly less than ~1%, and there is almost no delocalization of the 3′-O lone pair in the C-5′ and 4′-C≡C vicinity. Taken together, these data suggest (i) that the north sugar ring conformation is more stable than the south and (ii) that the north conformation is stabilized by the interactions of the O4 and 3′-O lone pairs with the antibonding orbitals of the 4′-ethynyl group.

Effect of 4′ substitutions on the ability of dA and dT analogs to block reverse transcription *in vitro*. In order to assess the effect of various 4′ substitutions on the ability of dA and dT analogs to block reverse transcription *in vitro*, we tested the triphosphate forms of compounds 1, 4, 5, and 10 to 14 for their ability to block DNA synthesis by HIV-1 RT (Table 3). Additional previously published *in vitro* and cell-based inhibition data for dA and dT analogs are summarized in Table S2 in the supplemental material. Remarkably, almost all 4′-substituted analogs are excellent substrates of HIV-1 RT and are incorporated even more efficiently than the canonical substrate (higher k_{cat}/K_m). Specifically, the 4′-ethynyl substitution resulted in the highest efficiencies of inhibitor incorporation (EdDAP-TP and EFdA-TP have 60- and 45-fold higher k_{cat}/K_m than dATP). The higher efficiency appears to be the result of both increased catalytic turnover rate (k_{cat}) and decreased K_m (Table 3). Notably, the enhancement in incorporation efficiency *in vitro* appears to be more pronounced for the dA than for the dT analogs (Table 3). Moreover, 4′-ethynyl dA analogs are 3 to 4 times more efficiently incorporated by HIV-1 RT than 4′-methyl compounds. Inhibitors containing a 4′-ethyl modification are the least efficiently incorporated by HIV-1 RT.

Effect of 4′ substitutions on susceptibility to ADA. Bovine ADA was chosen to study the hydrolytic deamination of compounds 1 to 7 and 9 because it is readily available and because it is highly similar to human ADA (91% overall and 100% active-site sequence identity) (60, 61), allowing us to use the biochemical data to infer what would happen with the human enzyme. We found that 4′ substitutions decreased the ability of ADA to use these nucleosides as substrates, primarily due to an increase in K_m (Table 4). The 4′-ethynyl and 4′-cyano groups have similar effects on ADA catabolism.

TABLE 4 Summary of steady-state kinetic data for the deamination of dA, EdA, and CNdA by ADA[a]

Compound (no.)	Mean K_m ± SD (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
dA (1)	42.6 ± 15.0	13.5	0.32
EdA (3)	145.9 ± 37.6	11.3	0.08
CNdA (7)	156.9 ± 84.2	8.9	0.06

[a] Data were determined spectrophotometrically.

Effect of 2 position substitutions on susceptibility to ADA. Because the 2 position-substituted compounds were refractory to hydrolytic deamination by ADA, we were unable to obtain reliable kinetic measurements using the spectrophotometric assay. In order to at least obtain a relative order of susceptibility to ADA, we used an HPLC-based assay (54), which allowed long incubation times and a determination of the amount of product from the slow hydrolytic deamination of compounds 2, 5, 6, and 9 by ADA. These experiments showed that incubation of 500 μM substrate for 45 min resulted in 15% conversion of FdA (compound 2), 1.3% of EFdA (compound 5), 2.8% of ECldA (compound 6), and <1% of CNFdA (compound 9) (Fig. 3). These data suggest that the 2-halo combined with 4′ substitutions resulted in significantly decreased susceptibility of the dA compounds to ADA and that the decreasing order of stability was CNFdA (compound 9) > EFdA (compound 5) > ECldA (compound 6) > FdA (compound 2). This is consistent with our previous report that EFdA is not efficiently deaminated by ADA (21).

Structural analysis of predicted EFdA interactions at the ADA active site. Using the crystal structure coordinates of human ADA, we looked for potential interactions between the active-site residues and the modeled EFdA that could lead to decreased susceptibility of this inhibitor to ADA. As shown in Fig. 4, the placement of EFdA at the expected position of a substrate would result in unfavorable steric interactions between the 4′-ethynyl group of EFdA and the sulfur and ε-carbon of Met155 (d = 2.2 and 2.0 Å, respectively). These potential steric effects could contribute to a lower binding affinity for EdA than for dA, consistent with an ~3.5-fold difference in the corresponding K_m values ($K_{m\text{-EdA}}$ = 146 μM versus $K_{m\text{-dA}}$ = 43 μM), which is the primary contributing factor in the observed decrease in the catalytic efficiency of ADA when EdA is the substrate.

DISCUSSION

We have previously demonstrated that EFdA is more potent against WT and drug-resistant HIV strains than other approved NRTIs (19–24) in both biochemical and virological assays and that it has low cytotoxicity (24–26). This suggests that EFdA not only has a high affinity toward HIV-1 RT but is also likely to be efficiently activated by host kinases (21, 25) and is relatively stable in cells (21). In fact, a favorable NRTI EC_{50} depends on multiple desirable attributes that include (i) a strong binding affinity of the active metabolite for the RT target, (ii) efficient cellular uptake and activation by host kinases, (iii) an ability to withstand deactivation by cellular enzymes, (iv) a high barrier to resistance, and (v) low cytotoxicity. The present study investigates how different substitutions on the sugar ring and adenine base contribute to the structural features of NRTIs and the effects of these features on the first three of the factors listed above.

Sugar puckering affects several aspects of the activity of EFdA

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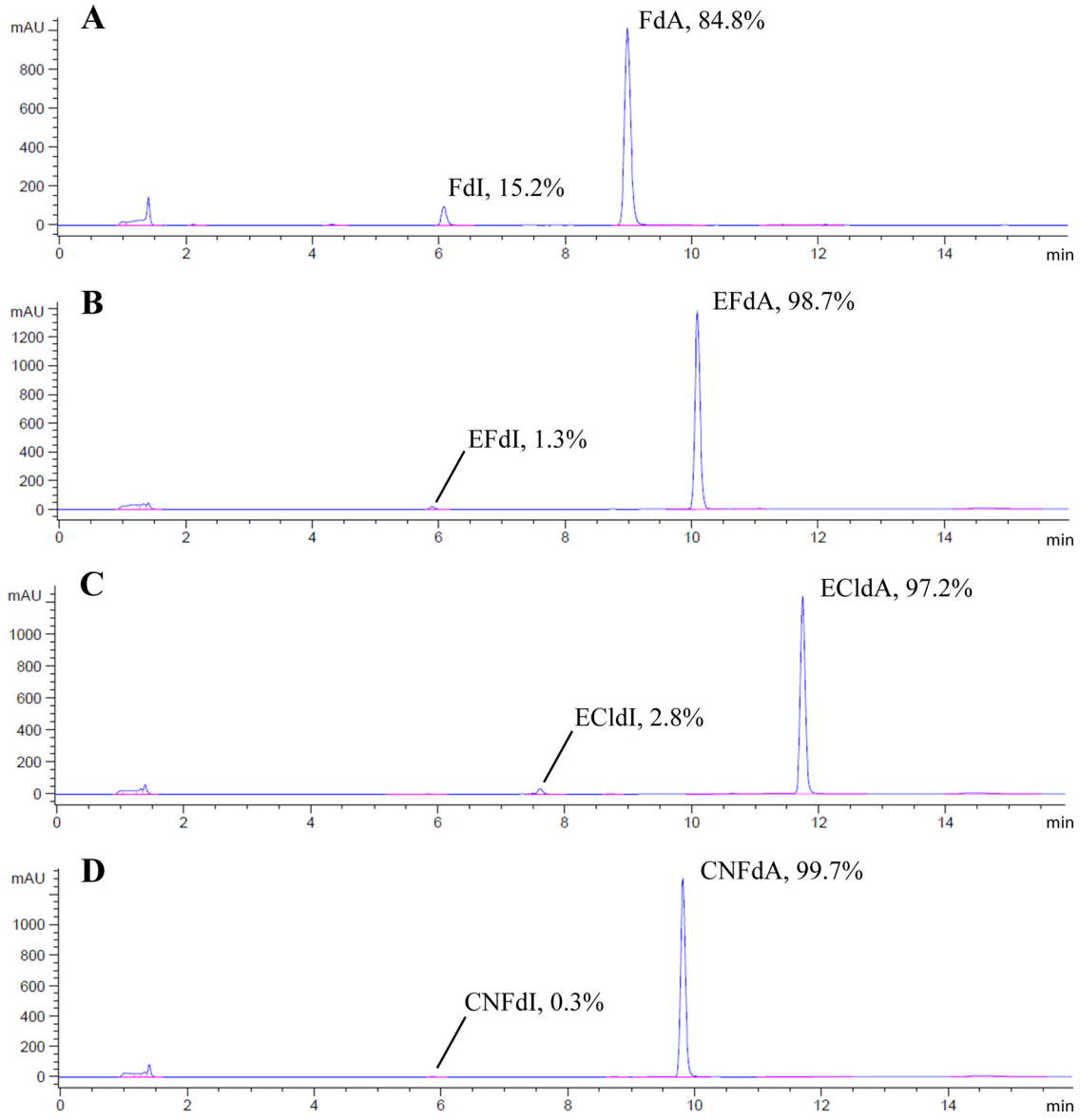


FIG 3 HPLC data taken after reaction of dA analogs (500 μM) with ADA for 45 min (260-nm signal is shown in blue). FdA (A), EFdA (B), ECldA (C), and CNFdA (D) were converted to the corresponding deoxyinosine (dI) forms to different extents. Percentages of dA-based reactant and dI-based product were calculated based on peak areas.

by influencing its interactions with HIV RT, dCK, and ADA. We have previously proposed that the extent to which an NRTI can exist in the north conformation affects its potency. In that respect, we have shown (Marquez and colleagues) that HIV-1 RT prefers as a substrate a version of zidovudine-triphosphate (AZT-TP) that is locked in the north rather than in the south conformation (38), suggesting that the HIV-1 RT active site prefers nucleosides or their analogs in the north conformation. Our NMR data and molecular orbital calculations demonstrate that the 4′-ethynyl substitution causes the sugar ring conformation of EFdA to be primarily in the north conformation. This causes it (and presumably the structure of its active metabolite EFdA-TP) to reside in a favorable conformation for binding at the polymerase active site of HIV-1 RT (36). This hypothesis is further supported by our steady-state kinetic assays and a previous report of *in vitro* HIV RT inhibition by EFdA-TP (20).

For a nucleoside analog to be a potent inhibitor of HIV replication, it must be converted to the triphosphate form in cells. The nucleoside substrate type or sugar ring conformation can affect the ability of cellular kinases to activate nucleoside analogs. The type of nucleoside substrate can determine which kinase will carry out the initial phosphorylation step. For example, dCK preferentially phosphorylates dC, dA, and dG analogs (62, 63), whereas thymidine kinase 1 phosphorylates dT analogs (64). Hence, the differences in phosphorylation efficiency of various NRTIs likely depend, at least in part, on the fact that different kinases are used to convert the various analogs to the active metabolite (65). Moreover, the activation of NRTIs involves multiple enzymes whose specificity may also impact the efficiency of activation. For example, AZT-MP is not phosphorylated efficiently to AZT-diphosphate (AZT-DP) by thymidylate kinase, and this is the rate-limiting step in AZT-TP formation (25, 66).

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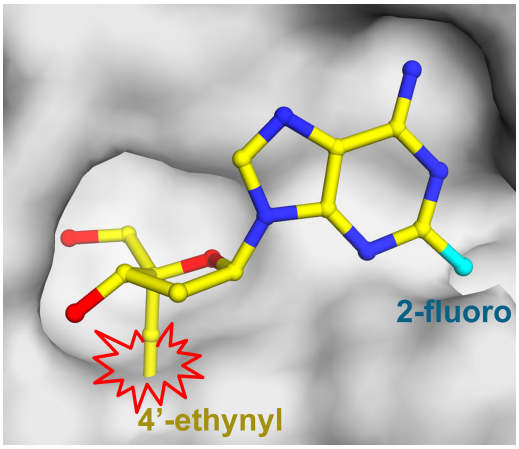


FIG 4 Structural analysis of the predicted effect of the 4′-ethynyl group of EFdA (yellow sticks) on interactions with the binding pocket of ADA (gray surface). When EFdA binds in a manner similar to that of other substrates, the 4′-ethynyl group may clash with the sulfur and ε-carbon atoms of Met155 in ADA. The figure was made using the PyMOL molecular graphics software package (http://www.pymol.org/).

The sugar ring conformation can also affect the ability of host kinases to activate nucleoside analogs. For example, dCK is known to bind its natural nucleoside substrates in the south conformation (41, 42). Interestingly, although the EFdA sugar ring favors the north conformation, it appears to be phosphorylated efficiently by dCK in cells (25). This suggests that other structural features of EFdA may contribute to its efficient phosphorylation, such as the 4′-ethynyl, the 2-fluoro, or both. In fact, it has been previously reported that a 2-fluoro substitution on dA decreases the K_m value 33-fold compared to that of dA, making FdA a very efficient substrate for dCK (63). Ongoing structural studies of EFdA in complex with dCK should reveal the basis of efficient activation of EFdA by dCK.

The EC_{50} of NRTIs can also be affected by catabolizing enzymes. For example, ADA can deaminate dA-based nucleosides and change the pathway and efficiency of their activation. The activity of ADA is affected by the structural determinants of its substrates. While we have previously demonstrated (Marquez and colleagues) that this enzyme efficiently deaminates nucleoside analogs in the north conformation (43–46), the kinetic analysis of ADA that we report here shows that the presence of a substituent at the 2 position of the adenine base makes these derivatives poor substrates for ADA. These results agree with previous studies reporting that 2-halo-substituted compounds are not efficiently deaminated by ADA (29, 31–33).

The mechanism by which 2-halo substitutions affect ADA activity does not appear to involve steric interactions in the ADA binding pocket (Fig. 4). This suggests that they decrease susceptibility through electronic rather than steric effects. The electronic effect of 2-fluoro can be explained in terms of changes in pK_a and charge distribution on the adenine base. The deamination mechanism by ADA involves an addition-elimination reaction on the substrate to form an inosine-based product (Fig. 5A). The first step involves electrophilic attack to protonate the N1 position of the purine ring, followed by nucleophilic attack at the C-6 position of the ring to form a tetrahedral intermediate (67–70). The introduction of an electronegative fluorine atom at the 2 position has been reported to dramatically lower the pK_a of the N1 atom of adenosine-based compounds (more than 100-fold) (Fig. 5B) (71). This is the result of the highly electronegative fluorine atom increasing the positive charge at the C-2 position (C-2 ∂^+, F ∂^-), which would raise the energy barrier of N1 protonation. Hence, the N1 protonation step becomes the rate-limiting step of the hydrolytic deamination reaction, thus reducing the yield of the 2-fluoro deoxyinosine product (Fig. 3). The presence of a 2-amino instead of a 2-halo substitution has a different effect on the mechanism and efficiency of the deamination reaction. In the case of EdDAP, a resonance effect caused by the 2-amino substitution would distribute the positive charge of the protonated N1 to all neighboring nitrogen atoms. Hence, differences in the deamina-

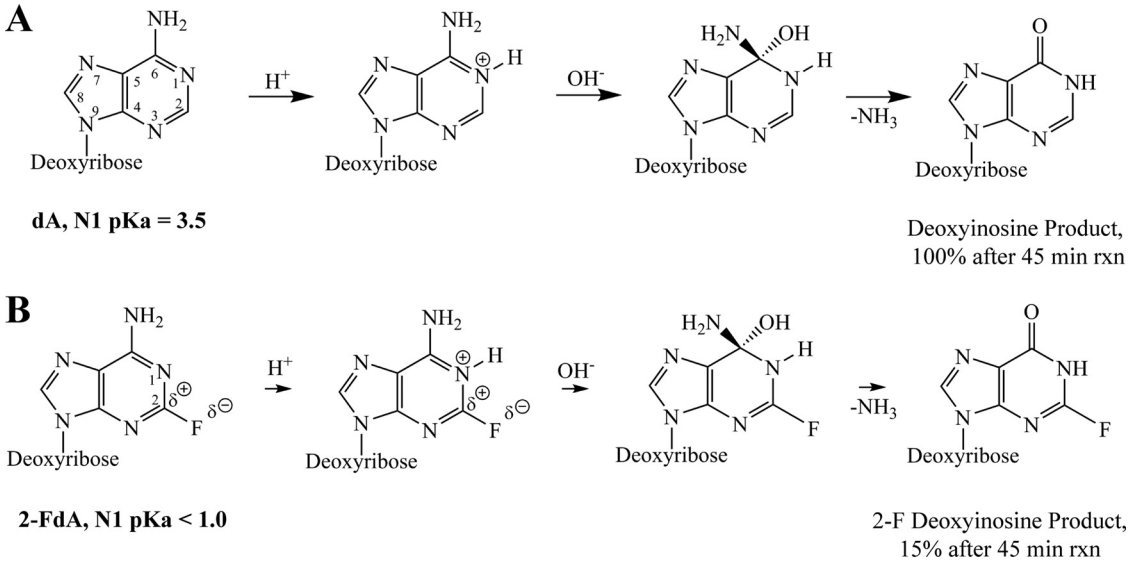


FIG 5 Reaction mechanism of hydrolytic deamination of dA (A) and FdA (B) by adenosine deaminase (67–70). The electronegative 2-fluoro group causes a dramatic decrease in pK_a at the N1 position (71), resulting in a high-energy barrier for the N1 protonation step of the reaction. rxn, reaction.

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tion efficiency of EdDAP and EFdA can be accounted for in terms of different charge distribution effects on the adenine base. This explanation is consistent with experimental results that demonstrated that EdDAP was catabolized more efficiently than EFdA but less efficiently than either dA or EdA, suggesting that a 2-amino substitution has a smaller impact on deamination by ADA than a 2-fluoro substitution (E. N. Kodama, personal communication).

In addition to 2 position substitutions, the presence of a 4′-ethynyl or cyano group also reduces deamination by ADA. For example, ADA deaminates EdA or CNdA less efficiently than dA (Table 4) and EFdA or CNFdA less efficiently than FdA (Fig. 3). Our structural analysis of the predicted interactions of EFdA at the ADA active site suggests that the 4′-ethynyl group may sterically interact with the flexible Met155 side chain (Fig. 4), resulting in a moderate decrease in the binding and reactivity of EFdA compared to the binding and reactivity of dA (Table 4). These data suggest that the effect of 4′-ethynyl on ADA activity is caused primarily by steric interactions. Hence, the substrate specificity of ADA is affected by both electronic and steric interactions from different structural determinants of dA-based NRTIs (2-fluoro and 4′-ethynyl, respectively).

In conclusion, strategic substitutions have a pronounced and complex effect on the ability of dA-based inhibitors to block HIV-1 replication. In particular, substitutions at the 4′ position of the deoxyribose ring and the 2 position of the adenine base of EFdA have separate effects that enhance its antiviral activity by favoring structural conformations that improve its interactions with the RT target and by decreasing its susceptibility to ADA. Because EFdA is a poor substrate for ADA, it is likely to remain intact for longer periods of time, thus reducing the dosage required to achieve an antiviral effect.

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FORM 26
THE PATENTS ACT, 1970
(39 of 1970)
&
The Patent Rules, 2003
FORM OF AUTHORISATION OF A PATENT AGENT/OR ANY PERSON IN A MATTER OR PROCEEDING UNDER THE ACT
[See sections 127 and 132 and rule 135]

We, **SANKALP REHABILITATION TRUST**, having its office at, S. S Bengali Municipal School, First Floor, Thakurdwar Road, Charni Road East, Mumbai – 400002; hereby authorize Rajeshwari H., Gopalan Deepak Sriniwas, Sweety Sharma, Pragya Singh Thakur and Lata Tiwari, all Indian citizens, Advocates / Patent Agents of RAJESHWARI & ASSOCIATES, A – 202, FIRST FLOOR, SHIVALIK ENCLAVE, MALVIYA NAGAR, NEW DELHI - 110017, INDIA, jointly or severally to act on our behalf for filing an opposition and/or representation by the way of opposition against an invention entitled:**"4 SUBSTITUTED NUCLEOSIDE-DERIVATIVES AS HIV REVERSE TRANSCRIPTASE INHIBITORS" Indian Application No: 201617031899 filed on 19th September, 2016** by **MERCK SHARP & DOHME CORP.** is a National Phase of PCT Application No. PCT/US2015/022621 dated 26.03.2015 under the above mentioned Act and in all matters and proceedings relating to the patent applications before the Controller of Patents or the Government of India in connection therewith or incidental thereto and in general to do all acts or things including filing of representation, statements, replies, extensions, fees, evidence and any or all documents or pleadings, attending hearings and appointment of a substitute or substitutes as the said Agent(s) may deem necessary or expedient and request that all notices, requisitions and communication relating thereto may be sent to such Agent(s) at Rajeshwari & Associates, India.

We hereby revoke all previous authorization, if any made, in respect of same matter or proceeding.

We hereby assent to the action already taken by the said person in the above matter.

Dated this 1st day of December, 2020

Signature:



To
The Controller of Patents
The Patent Office, Delhi