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TITLE: SUICIDE INHIBITORS OF REVERSE TRANSCRIPTASE IN THE THERAPY OF AIDS AND OTHER RETROVIRUSES

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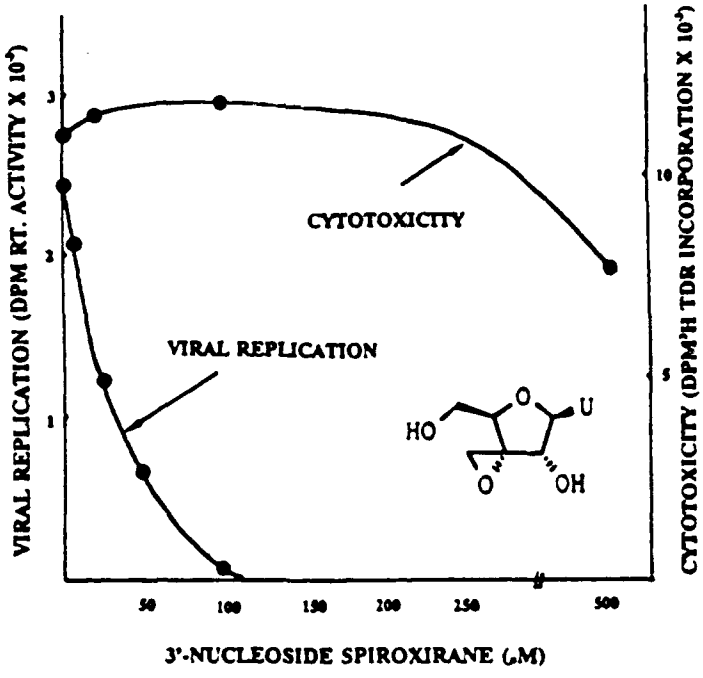
A-1

Summary

The contract period was extended to 7/31/91, without additional funding in order to complete evaluation of the antiviral activity of compounds synthesized and to evaluate the mechanism of action of certain of the synthetic analogs showing antiviral activity against HIV and other viruses in cell culture. These latter studies focused on the compound 3' uridine spiroxirane. This compound was designed as a potential suicide inhibitor of the HIV-1 transcriptase through the oxirane functionality at the 3' position. This compound showed good antiviral activity against HIV in cell culture and also against a second retrovirus (Equine Infectious Anemia Virus). The compound was also selective, the I_{50} for inhibition of viral replication being in the 10-25 μM range whereas the I_{50} for cytotoxicity was greater than 500 μM as shown in the figure below.



In order to determine if the observed antiviral activity was due (as predicted) to inhibition of reverse transcriptase, the kinetic properties of 3' uridine spiroxirane were evaluated against the purified recombinant HIV-reverse transcriptase in vitro. In order to do this the triphosphate derivative of the nucleoside analog (the true putative inhibitor) was synthesized using a newly developed procedures for synthesizing nucleoside triphosphates.

It was found that the 3' uridine spiroxirane triphosphate derivative was an effective inhibitor of the HIV

reverse transcriptase in the 0.1 to 2 micromolar range. The time course of the inhibition was progressive as expected for a suicide type inhibitor. Furthermore, the inhibition was not reversed by addition of excess template thus distinguishing it from a simple chain-terminating inhibitor and confirming that the inhibition resulted in the progressive and permanent inactivation of the enzyme characteristic of a suicide inhibitor. Thus it is concluded that the antiviral activity of the spiroxirane analogs we have synthesized is indeed related to their ability to function as suicide susbstrates for the HIV reverse transcriptase.

SYNTHESIS OF NUCLEOSIDE TRIPHOSPHATE ANALOGS FOR ANTIVIRAL TESTING:

The classical procedures for synthesis of nucleotide tripl osphates require relatively large quantities of the nucleoside precursor and frequently give poor yields.

Ludwig and Eckstein (J. Org. Chem. 1989, 54,631-635) have reported an approach involving condensation of an activated nucleoside derivative with inorganic pyrophosphate. In this synthesis 2-chloro-4H-1,3,20-benzodioxaphosphorin-4-one phosphitylates the 5`-hydroxy group of a nucleoside to form an intermediate 2, which on subsequent reaction with pyrophosphate produces a nucleosidylcyclotriphosphite 3. This intermediate is oxidized with iodine/water to furnish nucleoside 5'-triphosphate. The procedure is suitable for use with low milligram quantities.

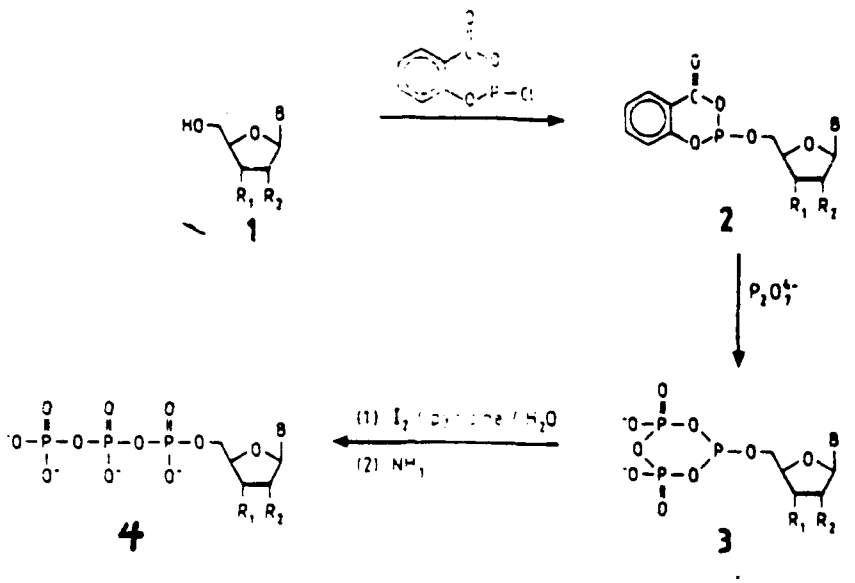
The series of reactions illustrated in Scheme I was successfully applied to microscale preparations of the 5'-triphosphates of both AZT and 3' uridine spiroxirane 1a which was previously synthesized and shown to have antiviral activity.

The formation of intermediate 3 was detected by ^{31}P NMR spectroscopy. The observed chemical shifts are comparable to those reported in the literature. The spectrum is of the ABX type where the AB part corresponds to the two phosphate groups with close but not identical shifts and the X part corresponds to the trivalent phosphorous atom. Oxidation of the intermediate 3 yields the normal triphosphate. In the ^{31}P NMR spectrum, the triplet of the trivalent phosphorous atom of the intermediate compound 3 disappears indicating complete oxidation.

We suspected some nucleotide by-products due to the contamination by a small amount of triphosphate. Also hydrolysis of intermediate 2 could result in phosphorous containing contamination detected by ^{31}P NMR. These by-products must copurify with the unreacted nucleoside on DEAE-cellulose chromatography.

The antiviral activity of the synthesized AZTTP was tested and proved comparable to, and in some cases even more effective than, a sample provided by the Burroughs Wellcome Co., thus validating the procedure. The nucleoside spiroxirane triphosphate may exist in two different isomeric forms which may or may not have identical antiviral activity. We have isolated one isomer that has proved antiviral activity. Isolation and characterization of the other isomer requires further investigation. The validated procedure was then applied to synthesize the 5' triphosphate of 3' uridine spiroxirane.

Scheme I



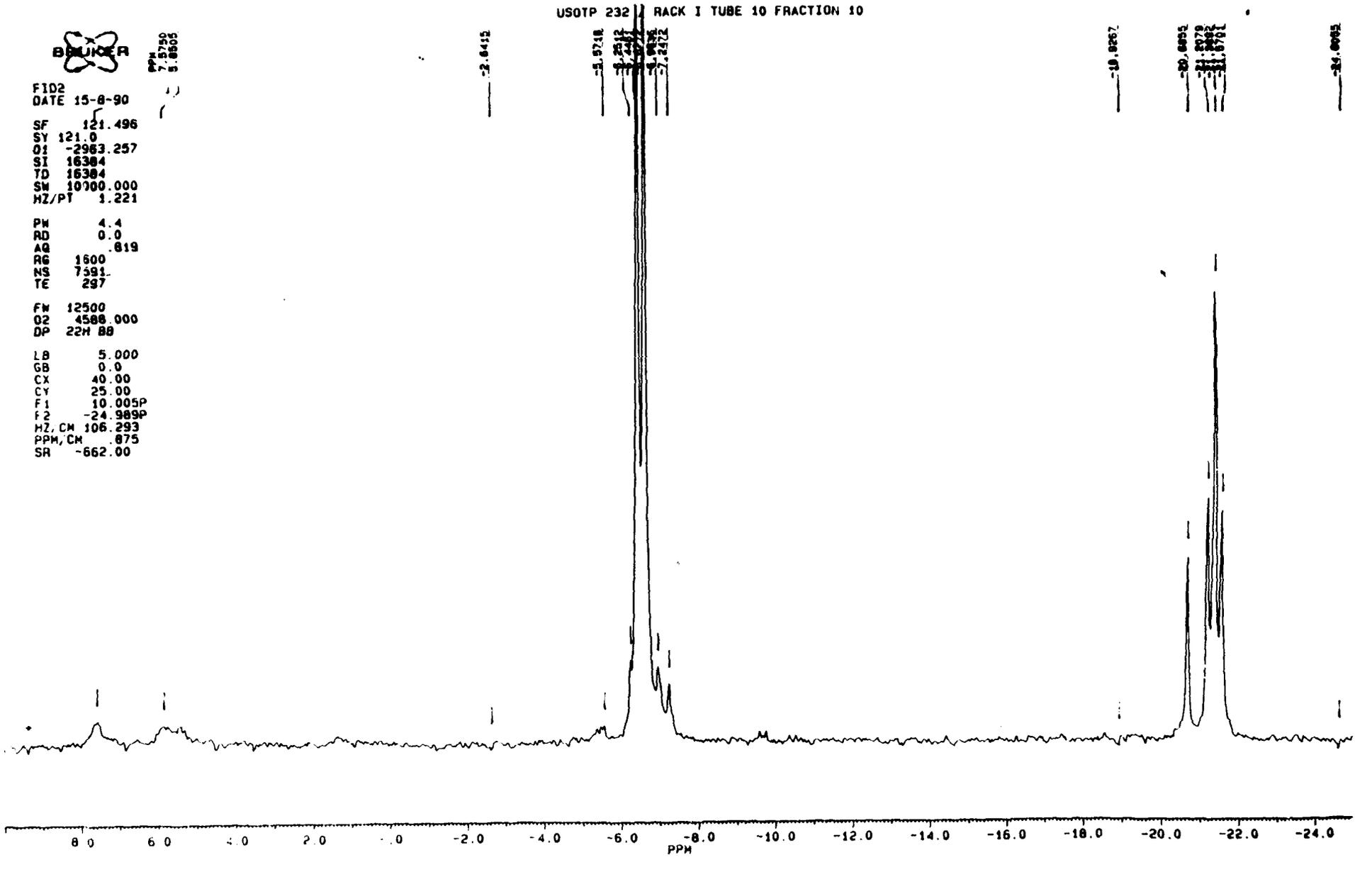
The phosphitylating agent 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one was purchased from Aldrich. Dimethylformamide (DMF) was dried over $MgSO_4$ and distilled under reduced pressure. Anhydrous dioxane was distilled from LAH. Anhydrous pyridine was prepared by fractional distillation of refluxing pyridine with potassium hydroxide.

^{31}P NMR spectra were recorded on a Brucker 300 spectrometer with broad band decoupling. TLC was performed on either silica GHLF (Analtech) developed with isopropanol, ammonia, water (3:1:1) (system 1) or on DEAE-cellulose (Analtech) developed with 0.02 N hydrochloric acid (system 2).

Bis(tri-n-butylammonium) pyrophosphate. Tetrasodium diphosphate decahydrate (2.23 g, 5 mmol) was dissolves in water (50 ml), the solution was applied to a column of Dowex 50WXB in the H^+ form and the column was washed with water. The eluate was directly dropped into a cooled (ice water) and stirred solution of tri-n-butylamine (2.38 ml, 10 mmol) in ethanol (20 ml). The column was washed until the pH of the eluate increased to 5.0 (approximately 70 ml of water). The ethanol/water solution was evaporated to dryness and reevaporated twice with ethanol and finally with anhydrous DMF and diluted to 10 ml. This solution was stored over 4-Å molecular sieves.

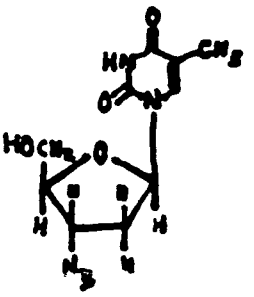
Nucleoside spiroxirane 1a (100 μmol) was dissolved in anhydrous pyridine/DMF, 1/4, V/V and evaporated to dryness in vacuo. The residue was dried further over P_2O_5 under reduced pressure for 2 hours at room temperature. The reaction flask was filled with nitrogen, during all the following manipulations a small positive pressure of nitrogen was maintained in the reaction vessel. Anhydrous pyridine (200 μl) and DMF (800

NMR SPECTRUM OF 3' URIDINE SPIROXIRANE TRIPHOSPHATE DERIVATIVE



μl) were injected through septum. A freshly prepared 1 M solution of 2-chloro-4H-1.3.2-benzodioxaphosphorin-4-one in anhydrous dioxane (110 μl, 110 μl) was then injected into the well-stirred solution of nucleoside. After 15 minutes a well-vortexed mixture of a 0.5 M solution of bis (tri-n-butylammonium) pyrophosphate in anhydrous DMF (300 μl) and tri-n-butylamine (100 μl) was quickly injected and the reaction mixture was stirred for 10 minutes. A solution of 1% iodine in pyridine/water (98/2, V/V) (2 ml, 157 μmol) was then added. After 15 minutes excess iodine was destroyed by adding a few drops of a 5% aqueous solution of $NaHSO_3$ and the reaction solution was evaporated to dryness. The residue was dissolved in water and applied to a DEAE-cellulose column which was eluted with a linear gradient of 800 ml of each 0.05 M and 1 M TEAB. The fractions were characterized by NMR spectroscopy. The presence of the characteristic triphosphate group was confirmed by NMR as indicated in the figure below.

1a



1.b

ENZYME INHIBITION STUDIES WITH 3' URIDINE SPIROXIRANE TRIPHOSPHATE

The recombinant HIV-reverse transcriptase was expressed in the vaccinia virus construct VCP21 as described in previous progress reports. The enzyme was purified from culture fluids by column chromatography on DEAE cellulose and carboxy-methyl cellulose columns. The purified enzyme was used to synthesize DNA using a poly rAdT template and following the enzyme activity by measuring incorporation of ^{3}H labelled dTTP. The synthesized ^{3}H DNA was collected on acid washed filter, and counted in a scintillation counter. It was found that 3' uridine spiroxirane was an excellent inhibitor of the enzyme in this system. The inhibition was time and concentration dependent consistent with the irreversible inhibition associated with a suicide inhibitor. More detailed kinetic studies on reversibility however will be required to establish this and to distinguish the kinetics observed from those of a chain terminating inhibitor.

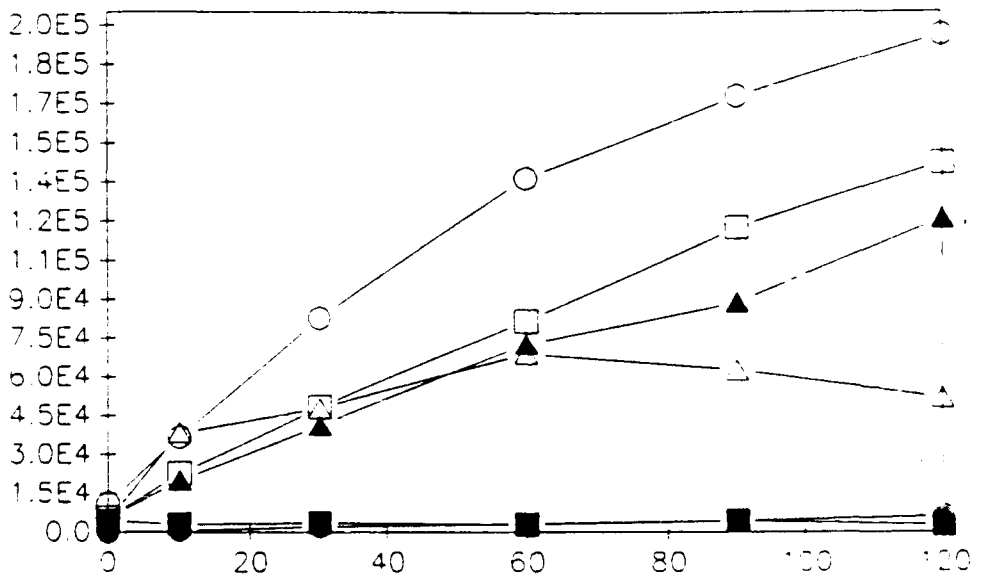


Figure: Activity of HIV-reverse transcriptase and inhibition by synthetic 3' uridine spiroxirane triphosphate

Key ○ control □ 0.05 μM △ .25 μM △ .25 μM ■ 2.5 μM ● Reagent blank

The triphosphate of 3' uridine spiroxirane was more potent as an inhibitor of the HIV reverse transcriptase than the triphosphate of AZT synthesized and tested in this system under the same conditions.

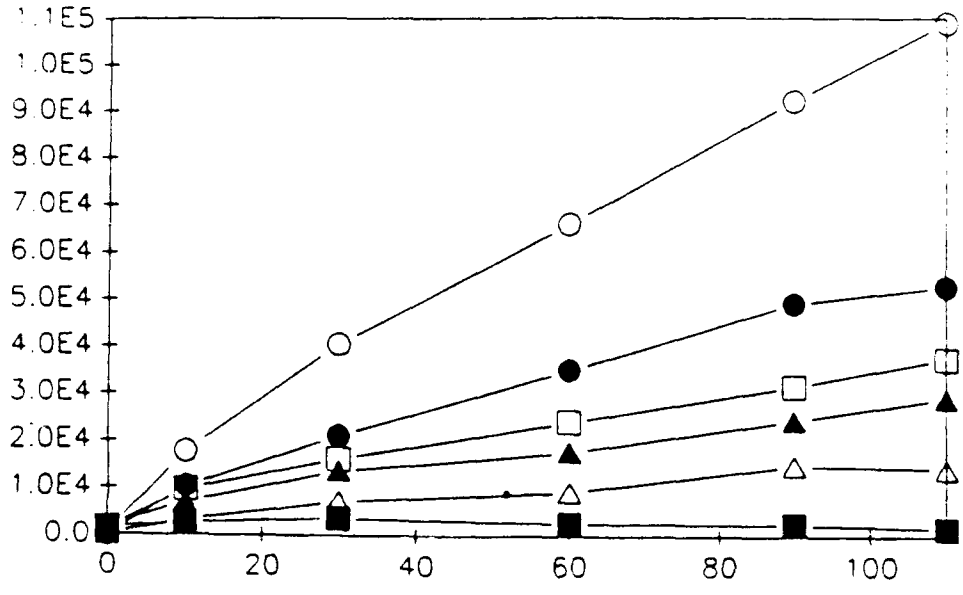


Figure: Inhibition of HIV-reverse transcriptase by synthetic AZT-triphosphate

Key ○ control ● .05 μM □ .25 μM △ .25 μM △ 2.5 μM ■ Reagent blank

Preliminary experiments have been conducted to characterize further the nature of the inhibition by 3' uridine spiroxirane triphosphate. The inhibition is further confirmed as irreversible suicide type by the fact that addition of excess template does not reverse it, thus distinguishing it from that of a chain terminating inhibitor where new template would be expected to overcome the inhibition.

APPENDIX

SUMMARY OF SIGNIFICANT PROJECT ACCOMPLISHMENTS TO DATE AND RECOMMENDATIONS FOR FUTURE EXPLOITATION OF FINDINGS

PROJECT TITLE: SUICIDE INHIBITORS OF VIRAL POLYMERASES AS VIRAL PROPHYLACTICS AND BIOLOGICAL WARFARE ANTIDOTES.

a) Problems to be studied. This project will synthesize new types of antiviral drugs based upon suicide inhibitors of viral polymerases. These compounds will be screened in collaborative studies with the U.S. Army Antiviral Testing Facility for antiviral activity against a spectrum of 10 viruses of interest as military disease hazards and biological warfare agents.

b) Significance and Uniqueness. Suicide and affinity inhibitors of both DNA and RNA viral polymerases will be synthesized. This type of inhibitor contains a latent reactive moiety which selectively and irreversiblv inactivates the viral enzyme. In preliminary studies, about 30 compounds with these potentialities have been synthesized and screened for antiviral activity in tissue culture. A number of active compounds including a new family - the nucleoside spiroxiranes - have been identified. Cytotoxicity assays in cultured T-lymphocytes also indicate favorable therapeutic indices for these types of drugs.

Two compounds, 2'3' sulfinyl cytidine hydrochloride and $2',0^2$ anhydrocytidine hydrochloride, which have proved to be highly effective against vaccinia virus in tissue culture, will be synthesized in larger quantities for further characterization and *in vivo* studies in the U.S. Army Antiviral Facility, Fort Detrick, MD. Congeners of compounds that have shown moderate activity against Punta Toro and yellow fever viruses will also be developed. Test data on selected compounds are given in the appendix.

Preliminary studies have begun on a series of nucleoside 5' oxaphosphorins and dioxaphospholes that are suicide analogs directed against enzymatic displacement reactions at the 5' α phosphate of the nucleotide substrate. The action mechanism and selectivity of the drugs will be characterized against viral and host nucleotide polymerases *in vitro*.

In the continuing studies, the range and selectivity of the nucleoside spiroxiranes will be extended by synthesizing additional members of the family. The suicide nature of their action and sensitivity will be determined in kinetic studies using viral and cellular DNA and RNA polymerases *in vitro*. Samples (75 mg) of each compound will be supplied to USAMRID for *in vitro* testing. Larger samples (2 g) of compounds showing activity *in vitro* will be supplied for further testing *in vivo*.

c) Relevance to USAMRDC mission studies. Suicide inhibitors represent a new class of antiviral drugs potentially capable of great selectivity. Orally administered antivirals could be militarily useful for temporary viral prophylaxis in emergency troop deployments to environments where advance vaccination is not possible, as antidotes following battlefield exposure to vaccinia-based or other biological warfare vectors, and in other unanticipated epidemic situations.

d) Estimated Project Duration and Personnel. Organic chemist (50% time), 1 or 2 graduate students. Duration 3 years.

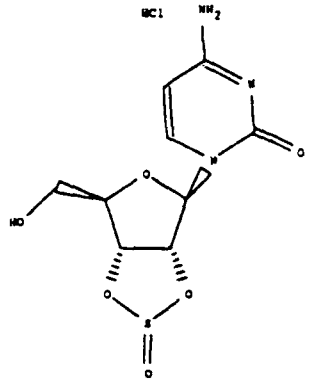
e) Animal use. No animal or human use except USAMRDC in-house testing of antivirals supplied as requested.

USAMRIID

Antiviral Drug Screening Program

08/06/9

STRUCTURE CHIRAL



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HANDLING/STORAGE

SOLUBILITY

STABILITY

ALT NAME
2',3'-O-SULFINYLCYTIDINE HYDROCHLORIDE

COMPOUND NAME
2',3'-O-SULFINYLCYTIDINE HYDROCHLORIDE

SCREEN INSTRUCTION

PRIORITY=PT>VEE>YF>KHF>PIC>JE>SF>VV>AD2>VSV

IN VIVO TOXICITY [mg/kg]

HOST	VH	RTE	LD50	MTC	LAB PR	DATE

IN VITRO SCREEN [ug/ml]

VIR	VR	VR+	ID50	CELL	MTC	TI	TI+	LAB PRT DATE
HIV			NOT ACT	MT2	< .32	0		SO MTT04-APR-90
HIV			NOT ACT	MT2	.31	0		SO MTT24-APR-90
JE			NOT ACT	VERO	60.6	0		SO MTT06-MAR-90
PT			NOT ACT	VERO	93.4	0		SO MTT06-MAR-90
SF			NOT ACT	VERO	91.5	0		SO MTT06-MAR-90
VEE			NOT ACT	VERO	46.6	0		SO MTT09-MAR-90
VV			3.28	VERO	61.1	18.66		SO MTT29-MAR-90
VV			1.72	VERO	24.4	14.12		SO MTT19-APR-90
YF			NOT ACT	VERO	83	0		SO MTT06-MAR-90

IN VIVO SCREEN [Dose = mg/kg]

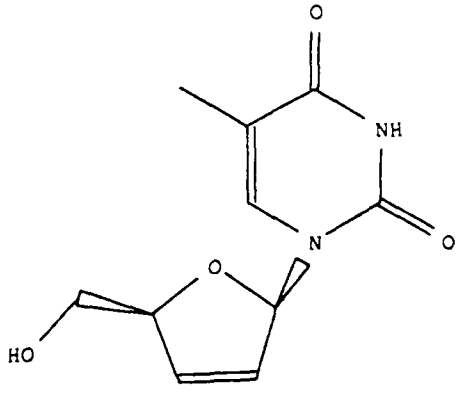
VIR	HST	VR	VR+	DOSE	MTC	VEH	RTE	D TOX	SP	L PR	DATE

USAMRIID

Antiviral Drug Screening Program

08/06/90

STRUCTURE CHIRAL



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HANDLING/STORAGE

SOLUBILITY

STABILITY

ALT NAME
2',3'-DIDEOXYTHYMIDINENE

COMPOUND NAME
2',3'-DIDEOXYTHYMIDINENE

SCREEN INSTRUCTION

PRIORITY=PT>VEE>YF>KHF>PIC>JE>SF>VV>AD2>VSV

IN VIVO TOXICITY [mg/kg]

HOST	VH	RTE	LD50	MTC	LAB PR	DATE

IN VITRO SCREEN [ug/ml]

VIR	VR	VR+	ID50	CELL	MTC	TI	TI+	LAB PRT DATE
HIV			4.99	MT2	66.4	13.29		SO MTT04-APR-90
HIV			.32	CEM	71.1	> 222.13		SO MTT26-APR-90
JE			NOT ACT	VERO	184	0		SO MTT06-MAR-90
PT			NOT ACT	VERO	182	0		SO MTT06-MAR-90
SF			NOT ACT	VERO	171	0		SO MTT06-MAR-90
VEE			NOT ACT	VERO	> 320	0		SO MTT09-MAR-90
YF			NOT ACT	VERO	173	0		SO MTT06-MAR-90

IN VIVO SCREEN [Dose = mg/kg]

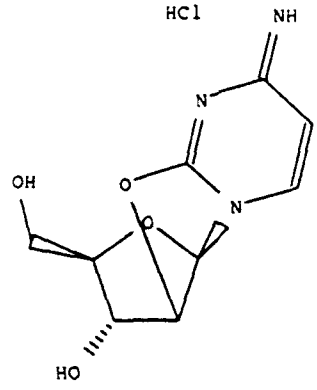
VIR	HST	VR	VR+	DOSE	MTC	VEH	RTE	D TOX	SP	L	PR	DATE

USAMRIID

Antiviral Drug Screening Program

09/06/90

STRUCTURE CHIRAL



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HANDLING/STORAGE

SOLUBILITY

STABILITY

ALT NAME

2',O2-ANHYDROCYTIDINE HYDROCHLORIDE

COMPOUND NAME

2',O2-ANHYDROCYTIDINE HYDROCHLORIDE

SCREEN INSTRUCTION

PRIORITY=PT>VEE>YF>KHF>PIC>JE>SF>VV>AD2>VSV

IN VIVO TOXICITY [mg/kg]

HOST	VH	RTE	LD50	MTC	LAB PR	DATE

IN VITRO SCREEN [ug/ml]

VIR	VR	VR+	ID50	CELL	MTC	TI	TI+	LAB PRT DATE
HIV			NOT ACT	MT2	< .32	0		SO MTT04-APR-90
HIV			NOT ACT	MT2	.09	0		SO MTT24-APR-90
JE			NOT ACT	VERO	3.05	0		SO MTT06-MAR-90
JE			NOT ACT	VERO	1.6	0		SO MTT22-MAR-90
PT			NOT ACT	VERO	> 320	0		SO MTT06-MAR-90
PT			NOT ACT	VERO	.81	0		SO MTT22-MAR-90
SF			NOT ACT	VERO	> 320	0		SO MTT06-MAR-90
SF			NOT ACT	VERO	.91	0		SO MTT22-MAR-90
VEE			NOT ACT	VERO	> 320	0		SO MTT09-MAR-90
VEE			NOT ACT	VERO	10	0		SO MTT23-MAR-90
VV			.18	VERO	1.86	10.53		SO MTT19-APR-90
VV			.25	VERO	2.01	8.03		SO MTT10-MAY-90
YF			NOT ACT	VERO	3.11	0		SO MTT06-MAR-90
YF			NOT ACT	VERO	.82	0		SO MTT22-MAR-90

IN VIVO SCREEN [Dose = mg/kg]

VIR	HST	VR	VR+	DOSE	MTC	VEH	RTE	D	TOX	SP	L	PR	DATE