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TITLE: CONDUCT STUDIES TO SYNTHESIZE ANTIVIRAL COMPOUNDS

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13. ABSTRACT (Maximum 200 words)

Ribavirin, a broad spectrum antiviral compound shows promise *in vitro* and *in vivo* against many viral diseases. The specific aim of this project was to synthesize a polymeric drug conjugate of ribavirin to provide time-release doses with enhanced endocytosis. A practical and standard approach was planned to couple ribavirin with carboxy-dextran (MW 46,000) by protecting 2', 3'-hydroxyl groups of ribavirin and then coupling ribavirin with polymer via an ester linkage to the 5'-hydroxyl group. This report describe successful synthesis and characterization of 2', 3'-isopropylidine ribavirin and carboxy-dextran. Conjugation of ribavirin to polymer may provide enhanced solubility and sustained release of drug. Further, a recommendation is also made in this report to achieve successful synthesis of conjugates.

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FOREWORD

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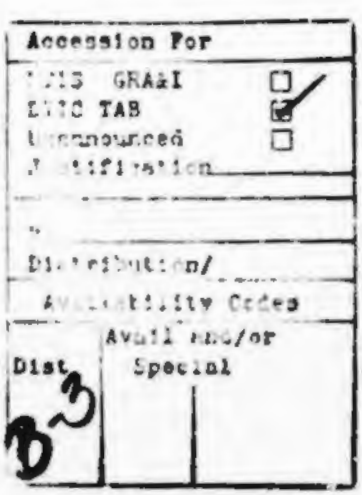
____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Devinder Gill 92/02/18

PI - Signature DATE

Hemant Misra



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Introduction

Nucleosides and nucleoside analogs constitute the major class of compounds which to date exhibit significant *in vitro* and *in vivo* antiviral activity. One of the reasons is that these compounds rapidly cross the plasma membrane of the cell by a facilitated transport mechanism (1), thus gaining rapid entry into the cell. Most nucleoside derivatives are phosphorylated within the cell by a viral or cellular kinase to the 5'-phosphate and then further converted to the 5'-triphosphate, which is an active form of the drug. There is a continuing need for new antiviral agents to be developed or modified, (2) for wider use against viral diseases.

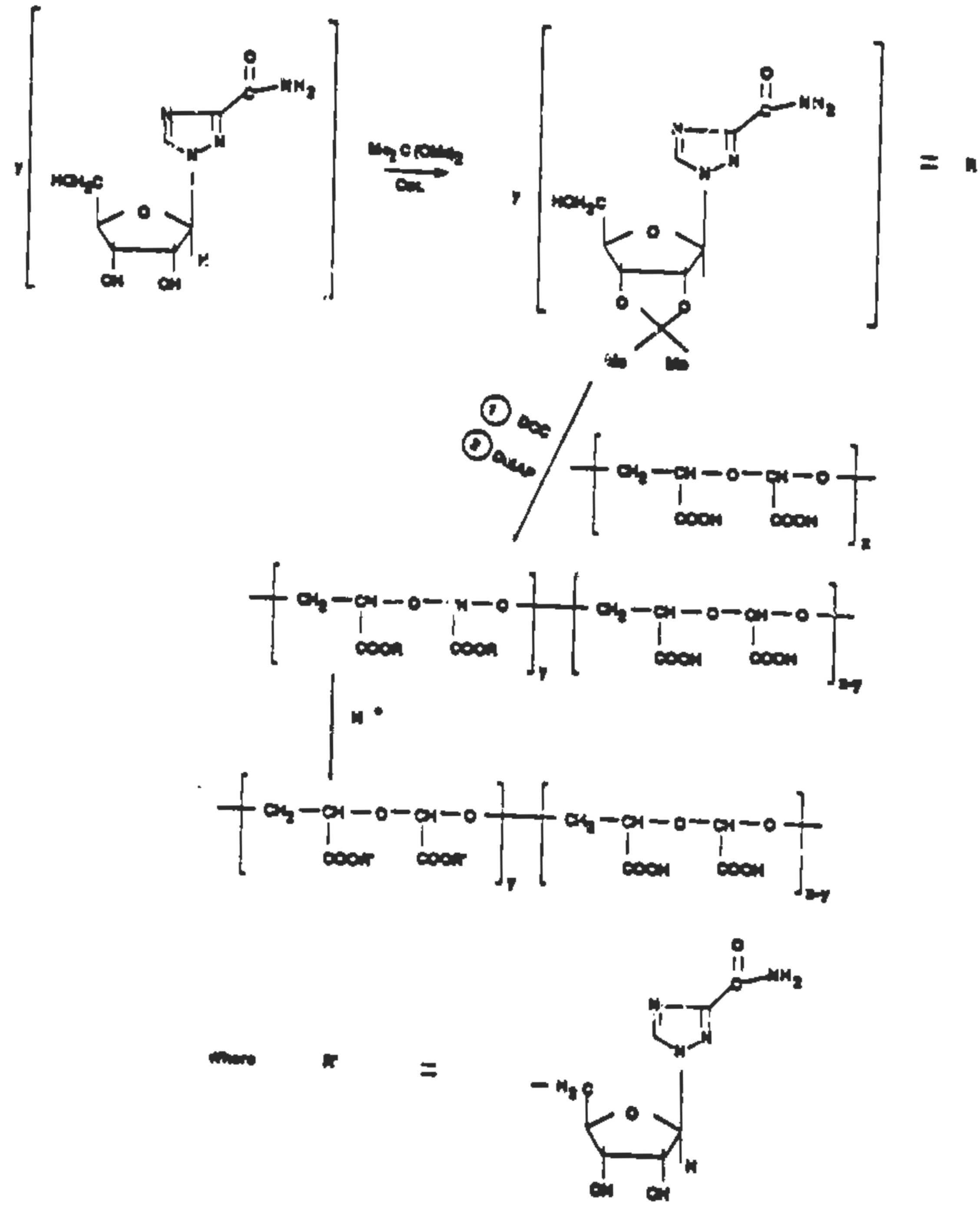
Antiviral chemotherapy is aimed at preventing or curing a viral disease in a human patient. Ribavirin, a triazole nucleoside, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide was synthesized by Witkowski, et al (3) and shown to have a remarkably broad spectrum of antiviral activity in laboratory animals (4). There are numerous reports available in the literature where experimental studies were performed *in vitro* (5-7) in both cell and organ cultures, *in vivo* (8-10) and in plant virus systems. The antiviral and toxicological activities of ribavirin that were seen in experimental systems appear to carry over reasonably well to the human situation, and controlled clinical studies are accumulating data to further substantiate the antiviral efficacy of ribavirin, particularly against hepatitis A, influenza, respiratory syncytial disease and herpes infections. Many studies world-wide of the mechanism of antiviral action completed to date indicate this drug

may have a multi-faceted effect in selectively controlling viral infections.

The specific aim of this project was to synthesize a polymeric drug conjugate of the drug ribavirin to provide enhanced endocytosis and time-release doses suitable for testing in laboratory animals, while not specifically targeting the drug to any particular organ or cell type. Such a polymeric drug conjugate could be useful in the evaluation of both efficacy and toxicity of "enhanced" ribavirin in animal systems, and studies toward the synthesis of a hydrophilic and biodegradable polymeric drug conjugate (e.g. carboxy dextran) are described in this report. Such conjugates should be expected to provide sustained, enhanced delivery and therapeutic activity and maximize water solubility for relatively non-soluble drugs.

Technical Approach

Polymeric Drug Conjugates of Ribavirin: It was originally planned to couple Ribavirin with carboxy dextran (MW 46,000) via an ester linkage to the 5'-hydroxyl group of its 2',3'-isopropylidine derivative, using dicyclohexyl carbodiimide (DCC) and dimethylpyridine (DMAP). (Scheme I). Conjugation of ribavirin to completely oxidized dextran should provide enhanced solubility and endocytosis, increased half-life and slow and sustained release of the drug, but will not target the drug to specific sites. The following syntheses were successfully performed in partial achievement of the objectives of this contract.



It should be noted that the conjugation of ribavirin to the polymer will be in random fashion. The above representation is a simplified version. Reproducible results are obtained under identical conditions in case of P(trans-DACH) carboxyamide complexes.

Scheme 1

Experimental

Coupling of N-t-BOC-glycine to Ribavirin:

In order to couple the conjugate spacer N-t-BOC-Glycine with ribavirin at 5'-OH position, the 2' and 3'-OH groups were protected first with a 2',3'-isopropylidine group. Three different methods were evaluated: (a) dicyclohexyl carbodiiride (DCC) with dimethylpyridine (DMAP); (b) p-toluenesulfonic acid monohydrate; (c) catalytic amounts of concentrated sulfuric acid. Among all these methods, the best one appears to be catalytic amounts of concentrated H_2SO_4.

(A) Synthesis of Tris-(N-t-BOC-Gly)-Ribavirin.

A reaction mixture of ribavirin (100 mg, 0.4 mmol), proton sponge (80 mg), and N-t-BOC-Glycine (200 mg, 1.2 mmol) was dissolved in DMSO (5 ml) under argon at room temperature. Dicyclohexylcarbodiimde (250 mg) was then added in five portions during the reaction period (72 hrs). TLC of the reaction mixture indicated some unreacted ribavirin (AVS), and a product with R_f at 0.52 (positive to anthrone) was generated. The turbid reaction mixture was filtered. The insoluble material proved to be dicyclohexylurea as determined by the comparison of its m.p. with standard (m.p.: 233°C). Filtrate was loaded onto Florisil chromatographic column (50 g, 40 cm x 3.0 cm i.d.) and the column was eluted with chloroform/methanol (10:3). The fractions which gave positive tests to anthrone and ninhydrin reagents, were combined and concentrated to dryness The physical, analytical nd

spectroscopic characteristics of the product (20 mg) are given below:

m.p.: 112-114°C

R_f: 0.92 in chloroform:methanol (10:3; v/v)

IR (KBr) 3378, 3333 (NH), 1706, 1685, 1655 (CO), 1560 (NH) cm^{-1}.

Disappearance of the hydroxyl group of AVS was noted by comparing with spectra of native AVS. (IR spectra are enclosed, see Appendix Page 16 and 18).

^{1}H-NMR in d_6-DMSO

Chemical Shift	Shape of the Signals	Integration	Assignment
7.80	s	2H	NH_2 in AVS
5.30	s	1H	H-1' in ribose
4.05	d	2H	H-2' and H-3'
3.30	d	2H	5'-CH_2
2.60	s	2H	CH_2-Gly
1.50	s	9H	t-BOC

A parallel experiment was also performed with dimethylaminopyridine as a catalyst instead of proton sponge which gave the similar results.

(B) <u>Synthesis of 2',3'-isopropylidene-ribavirin</u>

A rapidly stirred suspension of ribavirin (100 mg; 0.04 mmol) in dry acetone (15 ml) was treated with a catalytic amount of concentrated H_2SO_4 (30 µl) under anhydrous conditions at room temperature. 2',3'-Dimethyoxypropane (0.4 ml) was added dropwise to a rapidly stirred ribavirin suspension. The color of solution gradually turned yellow and ribavirin dissolved gradually. After

30 minutes all ribavirin dissolved completely and stirring was continued for one hour. The progress of the reaction was monitored by thin layer chromatography (TLC). The reaction was quenched with powdered sodium bicarbonate (0.5 g) and the mixture was stirred for another 20 minutes, filtered and the filtrate was dried in vacuo. The filtrate was dissolved in hot water and lyophilized. The white powder obtained was dissolved in ethyl acetate:acetone (1:1) and precipitated by adding ether. The white precipitate was filtered and dried in a vacuum oven. The physical and spectroscopic characteristics of the product are given below.

m.p. = 194-198°C

Yield = 70 mg; 60%

R_f = larger than ribavirin in Acetone:methanol 20:1

NMR = showed the presence of isopropylidine group (the presence of two methyl groups showed as two singlets at δ 1.5 and δ 1.3 ppm). Furthermore, H-1' of sugar ring exhibited a downfield shift from parent compound at δ 6.2 ppm. The 2'-OH and 3'-OH absorption disappeared while a sharp triplet due to 5'-OH is present at δ 4.95 ppm. H-2', H-3' and H-4' protons of sugar ring also showed a downfield shift, while H-5' and H-5'' protons did not exhibit any shifting compared to parent ribavirin molecule. A copy of the original spectrum is enclosed (see Appendix P. 1.-14).

(C) Synthesis of 5'-t-BOC-gly-isopropylidine ribavirin

2',3'-Isopropylidine-ribavirin (50 mg) was placed in a round

bottom flask, 10 ml of DMSO/Acetone solvent mixture (1:9) was added and the reaction mixture was stirred under argon gas. To the stirred mixture, t-BOC-gly (50 mg) and DMAP (10 mg) were added carefully. DCC (30 mg) was added to the reaction mixture portionwise and the mixture was refluxed for eight hours. The byproduct dicyclohexylurea was removed by filtration. The acetone-soluble portion was concentrated in vacuo and partially purified by column chromatography to give three fractions. After concentration in vacuo these materials were analyzed by NMR and showed complicated spectra of unknown structures. None of them were characterized to be 5'-t-Boc-gly-isopropylidine ribavirin.

(D) S ynthesis of monosubstituted t-BOC-Gly-Ribavirin.

A reaction mixture of ribavirin (50 mg), 4-dimethylaminopyridine (6.3 mg) and N-t-BOC glycine (52.6 mg, 0.3 mmol) was dissolved in DMF (4 ml) under argon at room temperature. The dicyclohexylcarbodiimide (200 mg) was added in four portions during the reaction period (72 hrs). TLC of the reaction mixture showed it had gone to completion and several products were formed with R_f between 0.67-0.92.

The supernatant of the mixture was separated from the precipitate by filtration and loaded onto Florisil chromatographic column (50 g, 40 x 3.0 cm i.d.). The column was eluted with acetonitrile followed by methanol and the fractions with R_f at 0.85 which were positive to ninhydrin, were pooled and concentrated,

gave a white powder (15 mg). The physical, and spectroscopic characteristics of the product is as follows:

m.p. : [illegible]-155°C

R_f: 0.85 in chloroform:methanol (10:3)

IR spectra showed similarity with IR spectra of trisubstituted product but the presence of hydroxyl peak at 3300 cm^{-1} was observed. (IR spectra is enclosed, see Appendix Page 17).

Synthesis of Carboxydextran (Mw 46000)

a. Synthesis of Dextran-Dialdehyde:

A reaction mixture of dextran (2.0 g; Mw 46,000), sodium periodate (12.0 g) and water (80 ml) was taken in a 250 ml Erlenmeyer flask covered with aluminum foil to protect it from light. It was stirred at 7°C for 20 hours in a refrigerator. Excess sodium periodate and other salts were removed by exhaustive dialysis against water using 6,000 to 8,000 Mw cut-off membrane. The pure product was isolated by freeze-drying. The yield was 1.44 g (90%).

b. Synthesis of Carboxydextran:

A mixture of dextran dialdehyde (1.0 g) and sodium chlorite (6.5 g), in 0.5 M acetic acid (50 ml) was stirred at room temperature in a 250 ml beaker. There was an immediate color change from white to red, and a completion of the reaction was indicated by the disappearance of the red color over a period of four hours. Carboxydextran was isolated by adding the reaction

mixture drop-wise to rapidly stirring ethanol (300 ml) and the precipitate was collected by filtration. The product was purified by dissolving it in water and reprecipitation in ethanol. Final purification involved dialysis against water. The pure carboxy-dextran was isolated by lyophilization in 85% (1.05 gm) yield.

Conclusion

A successful synthesis of polymeric drug conjugate of the ribavirin can be achieved by standard methods of coupling ribavirin with carboxydextran via an ester linkage to the 5'-hydroxyl group of 2',3' protected derivative of ribavirin. In the present contract we successfully achieved the synthesis of 2',3'-isopropylidine-ribavirin. Esterification of ribivirin in our experience needs to be repeated with modified conditions to obtain the target product without hydrolysing the 2',3'-isopropylidine group. Carboxydextran (MW 46,000) was prepared successfully from dextran in two steps in 80% yield. Product was purified by dialysis and finally lyophilized to produce 1.05 gm material from 2.0 gm of dextran. Final coupling of ribavirin derivative with carboxydextran can be achieved by using DCC and DMAP as shown in Scheme I.

If a standard procedure involving the protection of 2',3'-hydroxyl groups of ribavirin, coupling it with polymer, and finally deprotecting it, fails to give final desired product, it might be possible to couple ribavirin directly with carboxydextran without protecting 2',3' hydroxyl groups, as an ester formation may be favored at 5' due to a more reactive primary hydroxyl group. Thus,

the conjugation of unprotected ribavirin to carboxydextran might be achieved by using 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) in water. The success of this reaction would provide a facile route for conjugation. It has been previously shown that in the reaction of nucleotides with proteins, the formation of ester linkages with secondary hydroxyl group is negligible (11). Side reactions with the carboxamide group are not anticipated under the proposed experimental conditions.

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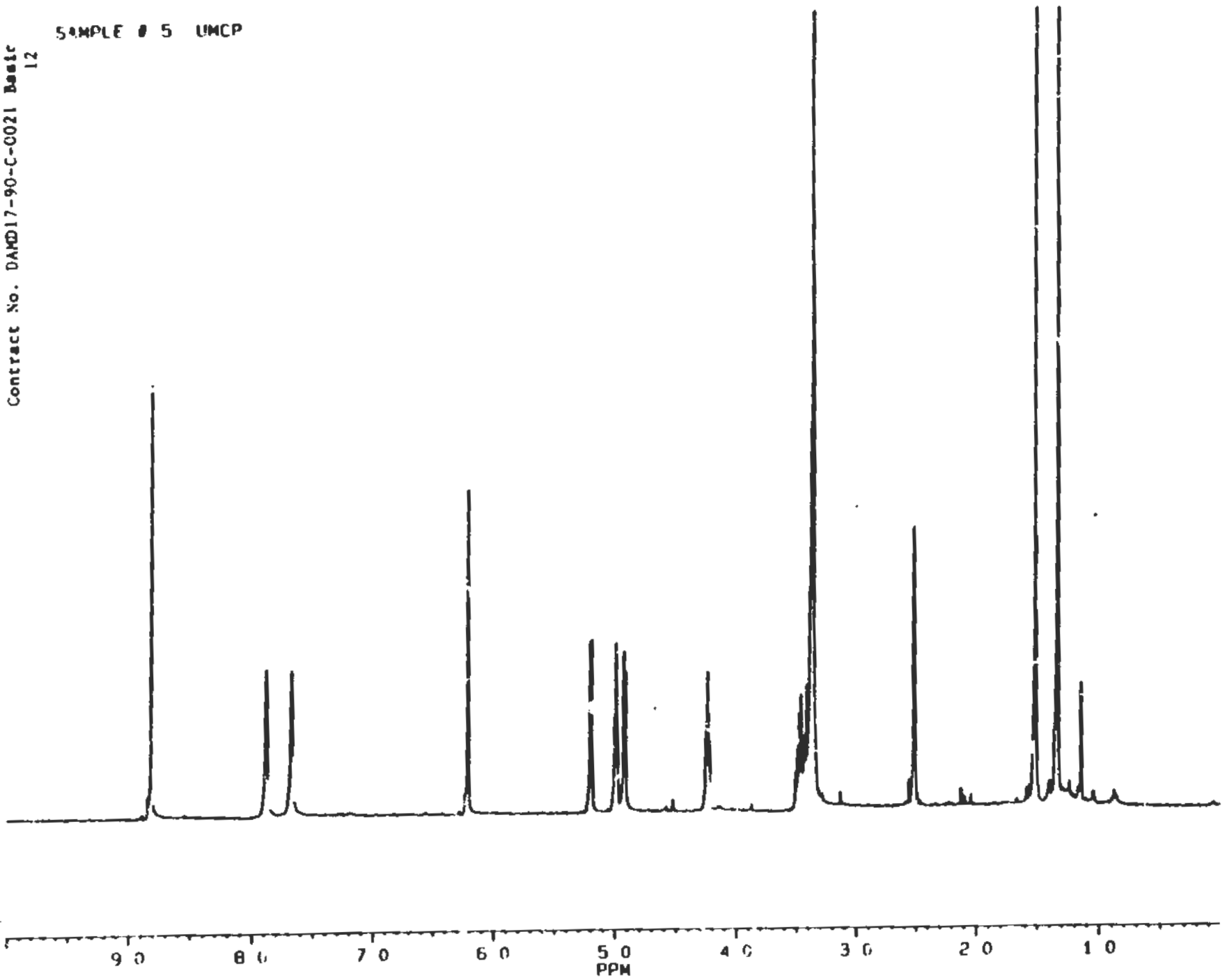
^{1}H-NMR Spectra of 2',3'-isopropylidine ribavirin (400 MHz)



SAMPLE # 5. UMCP.

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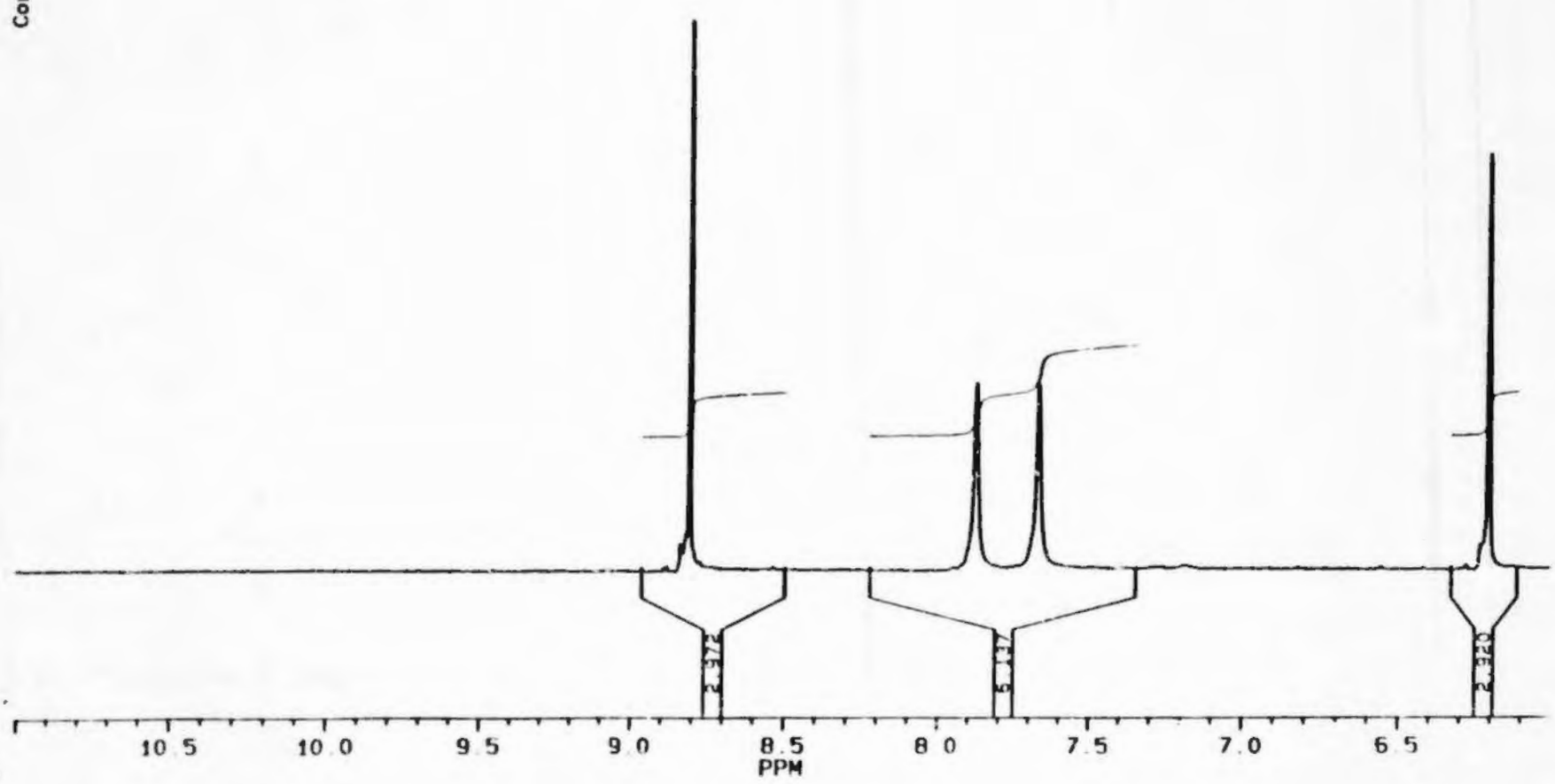

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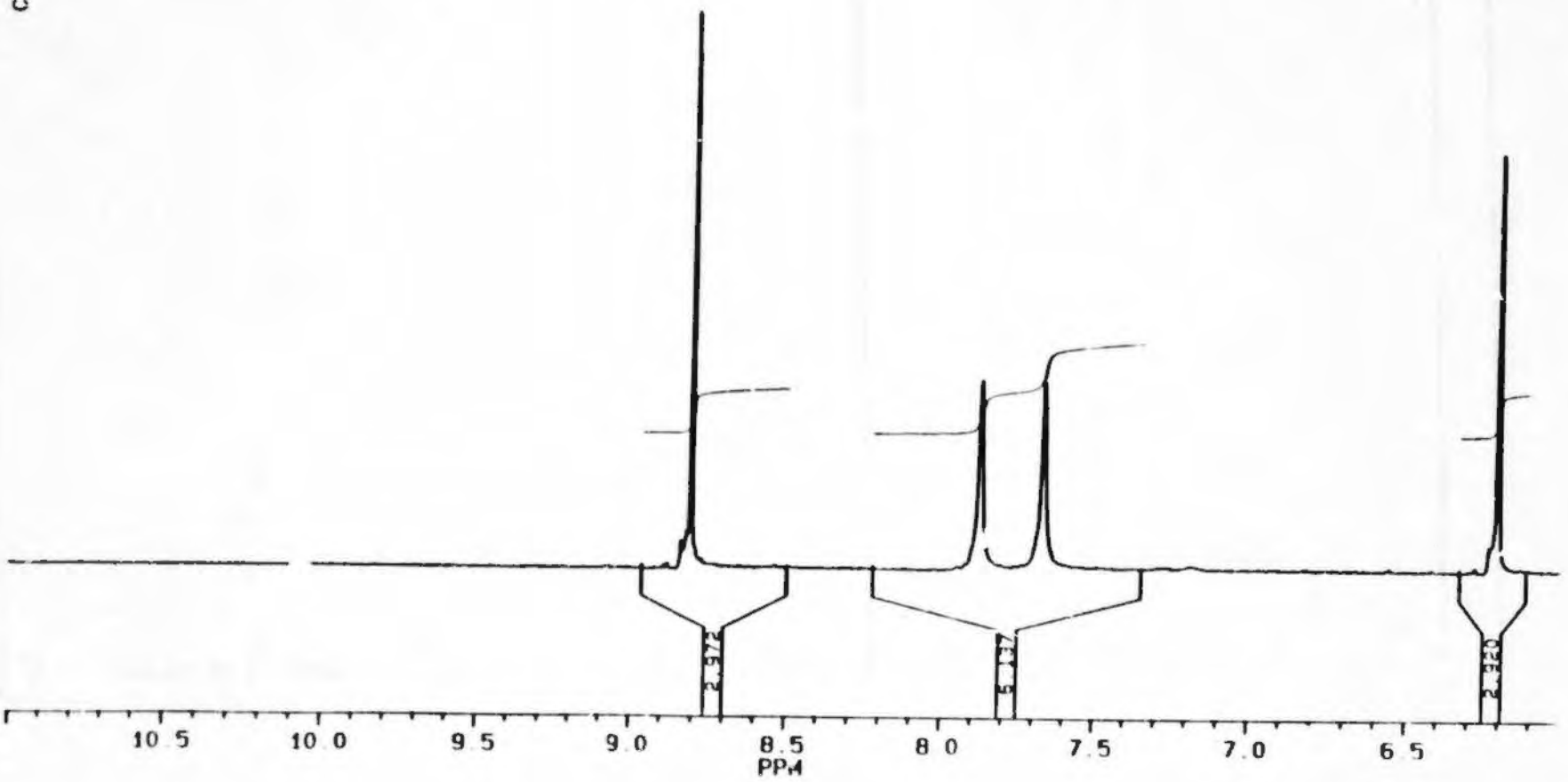

^{1}H-NMR spectra of Ribavirin

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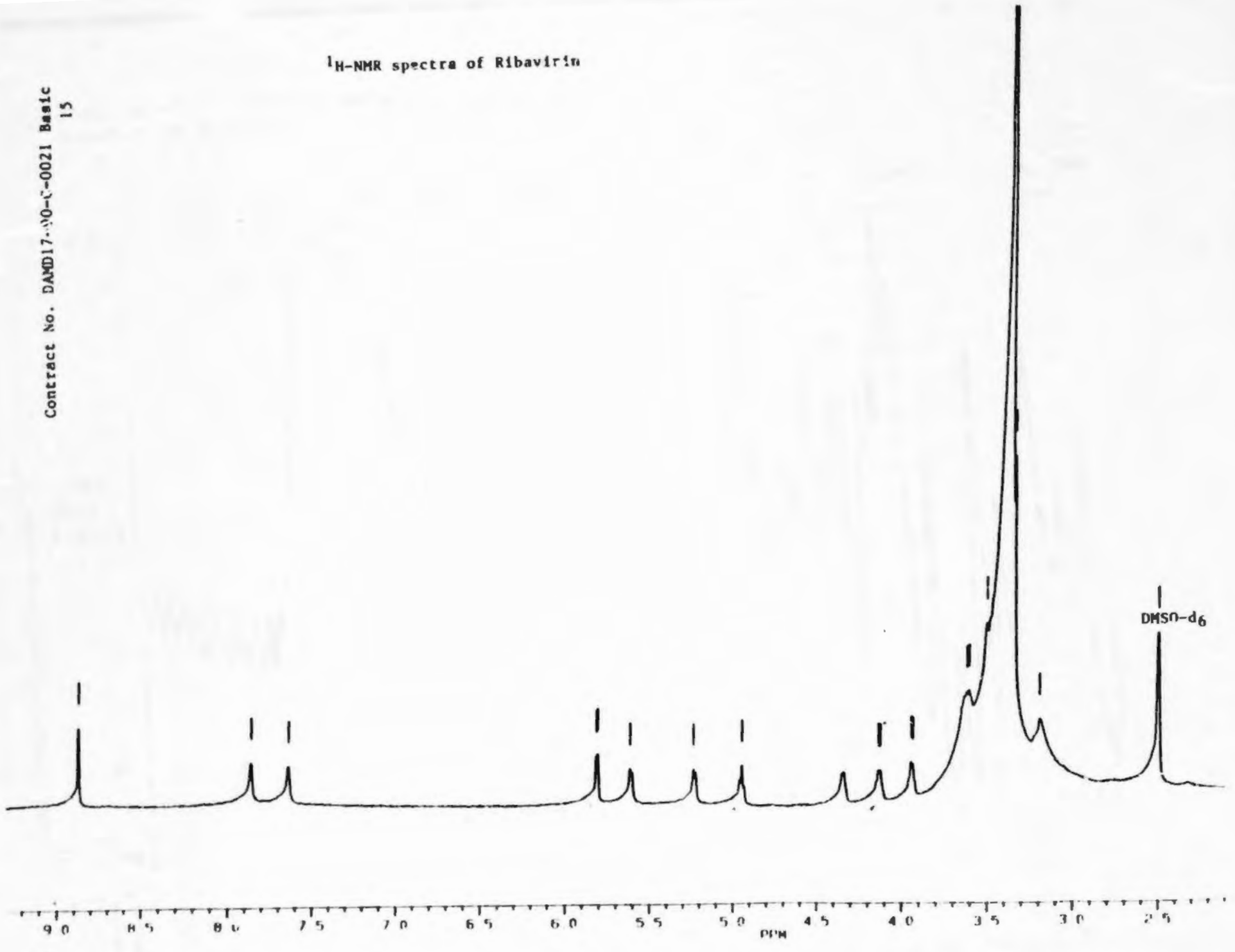

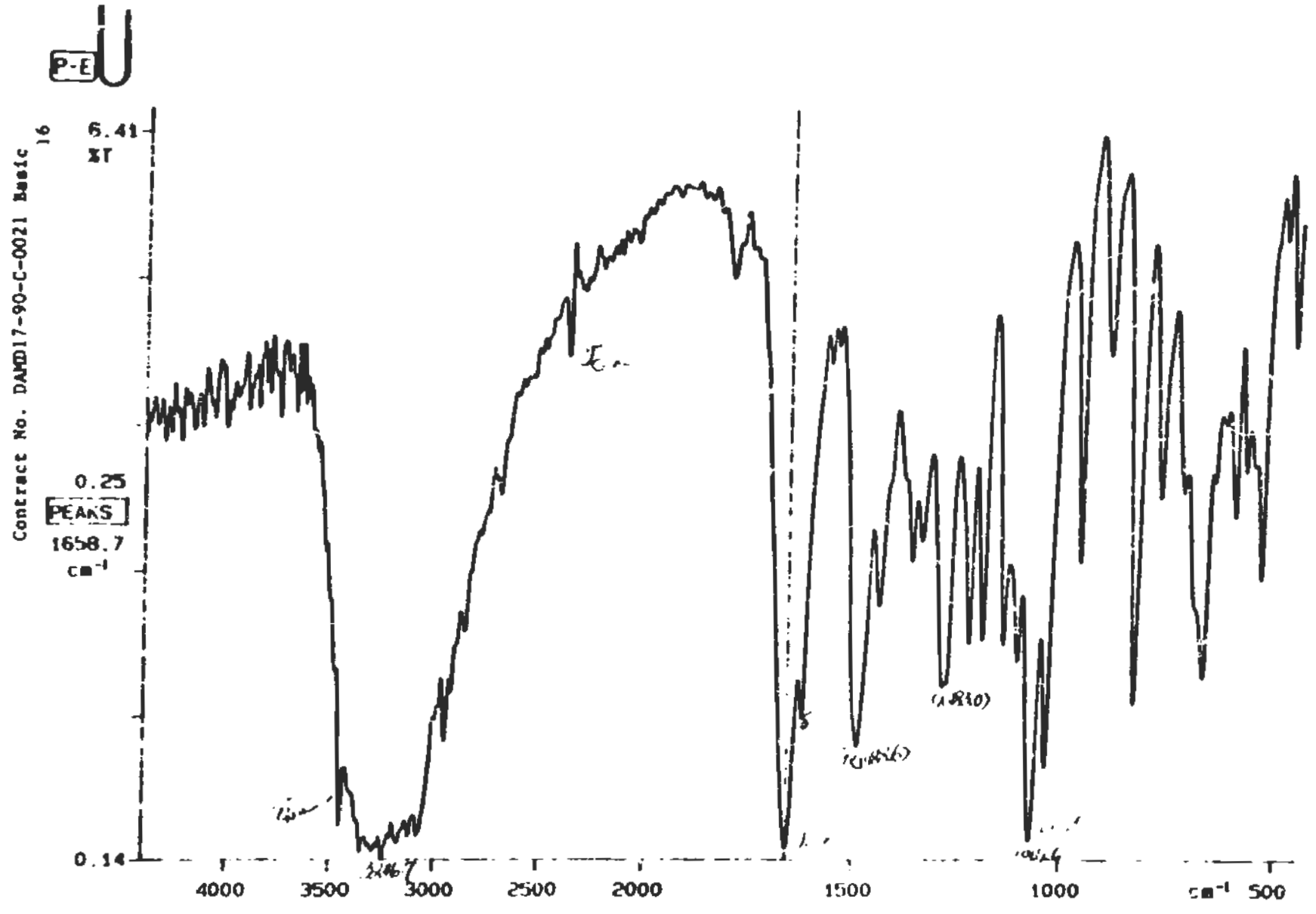
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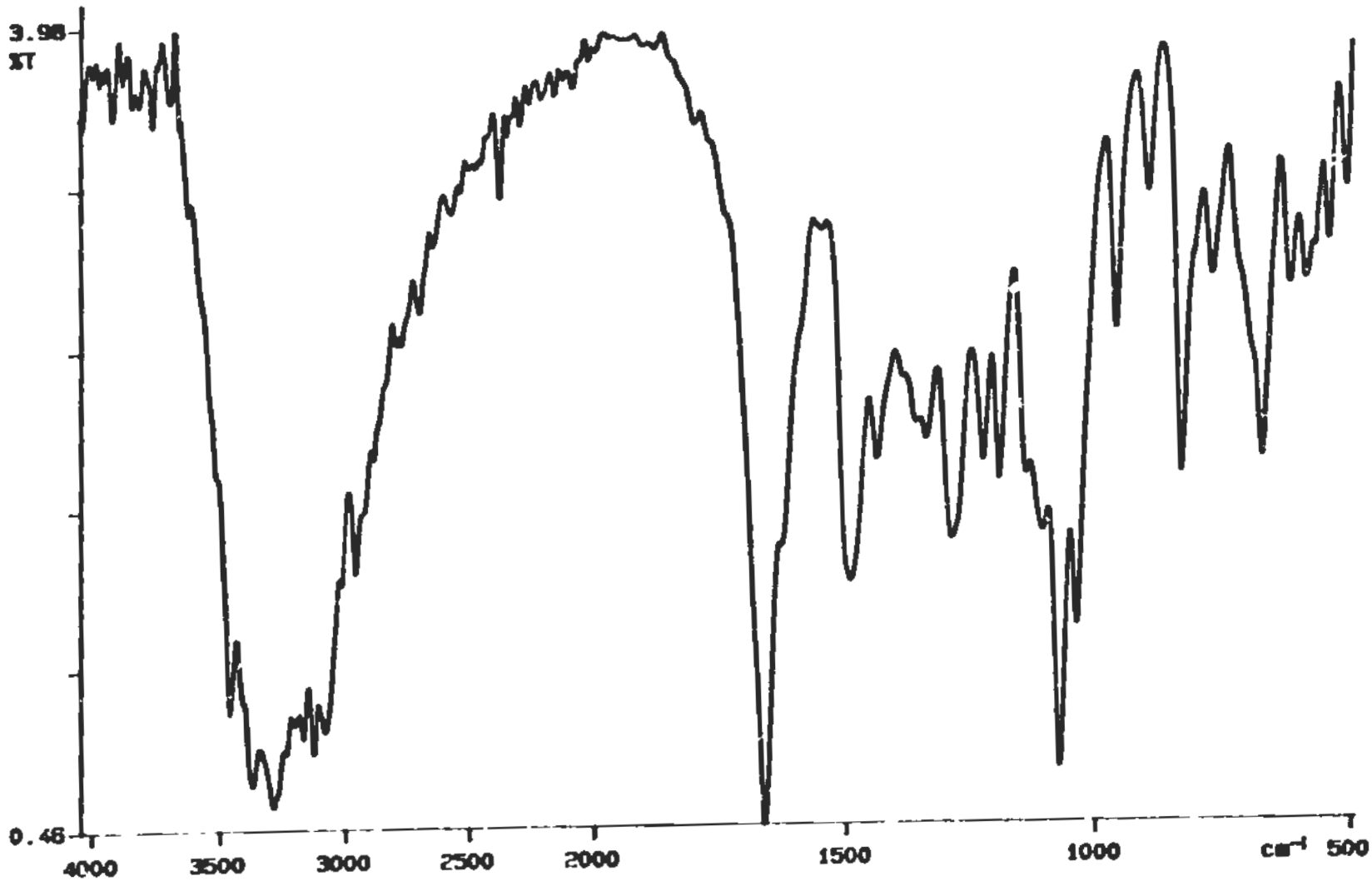
17 P-E



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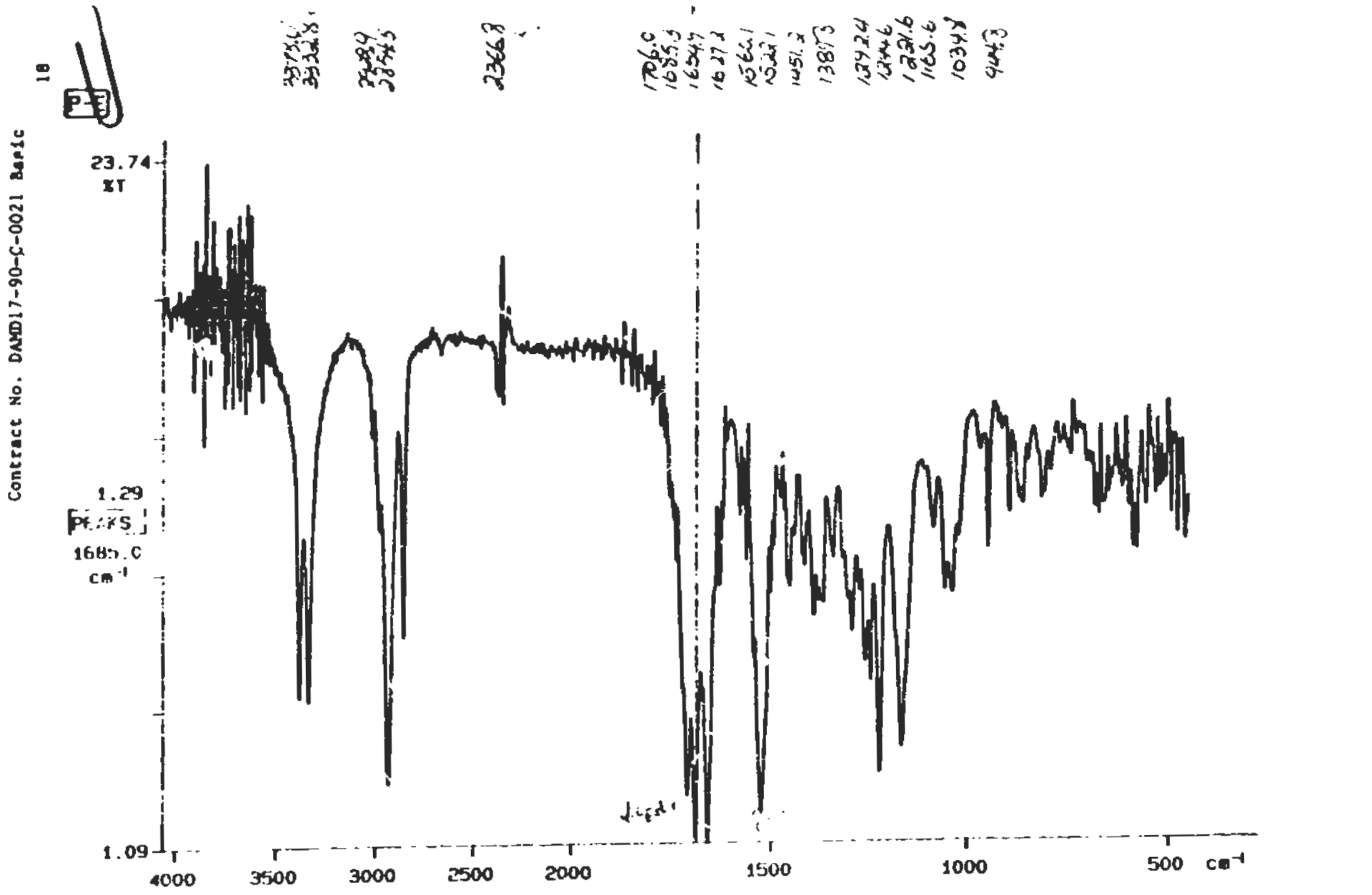
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SCAN: 16 scans, 4.0cm-1

STATEMENT OF PUBLICATIONS, GRADUATE DEGREES AND NAMES OF PERSONNEL RECEIVED PAY

There is no publication, meeting abstract and Graduate degree resulted from this contract support

The following people worked on this contract and got paid:

Devinder Gill
Nala Fernando
Huiro Zhao
Hemant Misra

Contract No. DAMD17-9C-C-0021 Basic 20

INVENTORY SCHEDULE B

(See SF 1426 for Instructions)

INVENTORY SCHEDULE B: [ ] PARTIAL [X] FINAL	TYPE: [ ] TERMINATION [X] NONTERMINATION	TYPE OF CONTRACT: CPFF	DATE: 21JAN92
FORM APPROVED OMB NO. 3090-0120	PAGE NO. 1	NO. OF PAGES 1	

TYPE OF INVENTORY

- [X] RAW MATERIALS (Other than scrap)
- [ ] PURCHASED PARTS
- [ ] FINISHED COMPONENTS
- [ ] FINISHED PRODUCT
- [ ] PLANT EQUIPMENT
- [ ] MISCELLANEOUS

THIS SCHEDULE APPLIES TO (Check one)

- [ ] A PRIME CONTRACT WITH THE GOVERNMENT
- [ ] SUBCONTRACT OR PURCHASE ORDER

PROPERTY CLASSIFICATION: Chemical

GOVERNMENT PRIME CONTRACT NO.: DAMD17-90-C-0021

SUBCONTRACT OR P.O. NO.: N/A

REFERENCE NO.: -

COMPANY PREPARING AND SUBMITTING SCHEDULE: ANDRULIS Research Corp

STREET ADDRESS: 4600 East-West Highway, Suite 900

CITY AND STATE (Include ZIP Code): Bethesda, Maryland 20814

CONTRACTOR WHO SENT NOTICE OF TERMINATION

NAME: N/A

ADDRESS (Include ZIP Code):

LOCATION OF MATERIAL: ANDRULIS Research Corp
11800 Baltimore Avenue (Rt-1), Suite 113
Beltsville, Maryland 20705

PRODUCT COVERED BY CONTRACT OR ORDER: Furnished by Ft. Detrick under DAMD17-90-C-0021

FOR USE OF CONTRACTING AGENCY ONLY	ITEM NO. (a)	ITEM DESCRIPTION (b)	GOVERNMENT PART OR DRAWING NUMBER AND REVISION NUMBER (b1)	TYPE OF PACKING (Bulk, bbls, cases, etc.) (b2)	CONDITION (Use code) (c)	QUANTITY (d)	UNIT OF MEASURE (d1)	COST (for finished product, show contract price instead of cost) UNIT (e)	COST TOTAL (f)	CONTRACTORS OFFER (g)	FOR USE OF CONTRACTING AGENCY ONLY
	1	Ribavirin		small bottle		2.75	grams	unk.*	unk.*	$1.00	

*Unknown- The Government never assigned a value when the chemical was originally supplied to us.

INVENTORY SCHEDULE CERTIFICATION

[illegible] 1 to 1 [illegible] 21 JAN 92 [illegible]

[illegible]

NAME OF CONTRACTOR	BY (Signature of Authorized Official)	TITLE	DATE
ANDRULIS Research Corporation	James T. Parker II	Facilities Manager	21 JAN 92

NAME OF SUPERVISORY ACCOUNTING OFFICIAL	TITLE
Donald L. Horseman	Vice President, Finance

NSN 7540-01-142-9135
1428-101

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