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Synthesis and Determination of Antiviral Activity of the 2′(3′)-*O*-Methyl Derivatives of Ribavirin (1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide)

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Diazomethane treatment of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) in the presence of $SnCl_2$ as catalyst led to quantitative formation of the 2′-*O*-methyl and 3′-*O*-methyl derivatives of the parent compound. The products were successfully fractionated on a basic ion-exchange column and isolated in crystalline form. Identification was based on the elution sequence from the column and on ^1H NMR spectroscopy. Both derivatives were found to be inactive, relative to the parent compound, against several virus types in cell culture. Unlike ribavirin itself, the 2′(3′)-*O*-methyl derivatives did not suppress cellular DNA synthesis. NMR data showed that the loss of biologic activity upon 2′(3′)-*O*-methylation was not due to a change of conformation of the nucleoside sugar moiety.

Ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide,[1] originally referred to as "virazole", has been described as the first broad-spectrum antiviral agent which is not an interferon inducer.[2] Its spectrum encompasses nearly all major virus groups (adeno-, herpeto-, pox-, picorna-, toga-, orthomyxo-, paramyxo-, rhabdo-, and retroviridae). Particularly interesting are the inhibitory effects of ribavirin on influenza and parainfluenza virus replication,[2-7] both in cell cultures and in animals, including mice, hamsters, and ferrets.[7-10] The drug has been licensed for human use in several countries and is currently progressing through clinical trials.

It has been proposed that ribavirin inhibits virus multiplication via depletion of the GTP pool, although recent findings are at variance with this concept.[6] Ribavirin 5′-phosphate has, in fact, been found to competitively inhibit, in vitro, IMP dehydrogenase (IMP:NAD^+ oxidoreductase, E.C. 1.2.1.14),[11] presumably because of its structural resemblance to IMP and GMP.[12] If the mode of action of ribavirin in vivo does occur at the level of conversion of IMP to XMP, it must first be phosphorylated by the appropriate cellular kinase, probably deoxyadenosine kinase.[13]

With a view to further defining the molecular mechanism(s) involved in the antiviral activity of ribavirin, two new analogues have been prepared and their activities assayed in a number of virus cell systems, viz., 2′-*O*-methylribavirin (**1**) and 3′-*O*-methylribavirin (**2**) (Scheme I), both of which are theoretically capable of phosphorylation by cellular kinase(s).

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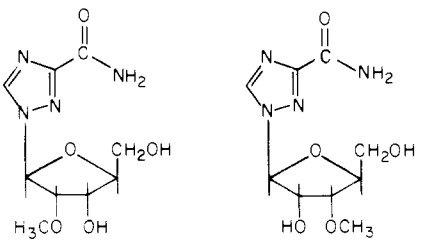
Scheme I



1 **2**

Chemistry. According to Robins et al.,[14] treatment of ribonucleosides with diazomethane in the presence of about 1 mM $SnCl_2 \cdot 2H_2O$ as catalyst leads to a quantitative conversion of the ribonucleosides to their 2′(3′)-*O*-methyl derivatives. The same treatment applied to ribavirin led to the formation of unidentified side products and incomplete etherification of the *cis*-hydroxyls. Subsequent trials demonstrated that, with a concentration of 5 mM catalyst in methanol and a molar ratio of ribavirin to catalyst of 4:1, formation of a mixture of the 2′- and 3′-*O*-methyl derivatives was quantitative. The course of the reaction was followed by TLC on silica gel.

The two monomethylated products **1** and **2** were then fractionated according to Dekker[15] on a Dowex (OH^-) column. Because of the strongly basic nature of this column, and the alkaline lability of the 3-carboxamide substituent, it was essential to achieve fractionation as rapidly as possible. The time required was brought down to 1 h with the use of a small column and a rapid elution rate; under these conditions, hydrolysis of the carboxamide substitutent to carboxyl was about 15%. Use of a larger

Table I. Chemical Shifts (in ppm vs. DSS) in Neutral D_2O Solution of the Triazole Ring H(5) and of the Ribose and Methyl Protons of the 2'(3')-*O*-Methyl Analogues of Ribavirin

Analogue	Chemical shift							
	H(1')	H(2')	H(3')	H(4')	H(5')	H(5'')	CH_3	H(5)
2'-*O*-Methylribavirin	6.16	4.38	4.60	4.22	3.89	3.73	3.51	8.77
3'-*O*-Methylribavirin	6.07	4.81	4.20	4.29	3.90	3.73	3.51	8.75

Table II. Vicinal Proton–Proton Coupling Constants for the Ribose Ring Protons of Ribavirin and Its 2'(3')-*O*-Methyl Derivatives (in D_2O) and the Resulting Calculated Preferred Conformations of the Sugar Rings and the Exocyclic 5'-CH_2OH Groups

Compd	Coupling constant, Hz						C(2')-endo, %	Gauche-gauche, %
	J(1',2')	J(2',3')	J(3',4')	J(4',5')	J(4',5'')	J(5',5'')		
Ribavirin[a]	3.5	5.0	5.5	3.3	5.2	−12.5	39	45
2'-*O*-Methylribavirin	3.5	4.9	5.2	3.5	5.6	−12.8	42	35
3'-*O*-Methylribavirin	3.4	4.2	5.7	3.0	5.1	−13.0	41	50

[a] Data from Dea et al.[18]

Table III. Effect of Ribavirin and Its 2'(3')-*O*-Methylated Derivatives on Virus-Induced Cytopathogenicity in Primary Rabbit Kidney (PRK) and HeLa Cell Cultures

Virus	Cell culture	Min inhibitory concn,[a] μg/mL		
		Ribavirin	2'-*O*-Methyl-ribavirin	3'-*O*-Methyl-ribavirin
Vesicular stomatitis	PRK	70	>200	>200
Herpes simplex 1	PRK	20	>200	>200
Vaccinia	PRK	20	>200	>200
Polio 1	HeLa	40	>200	>200
Coxsackie B4	HeLa	40	>200	>200

[a] Required to inhibit viral cytopathogenicity by 50%. The compounds were added immediately after virus adsorption. Virus input was 100 $CCID_{50}$ (dose infecting 50% of the cell cultures) per culture. Viral cytopathogenicity was recorded as soon as it reached completion in the control cell cultures (at 2 days for vesicular stomatitis, vaccinia, polio, and Coxsackie; at 3 days for Herpes simplex).

column, with accompanying increase in fractionation time, resulted in up to 80% hydrolysis of products during the 6-h period required for fractionation.

The two isolated products were crystallized from absolute ethanol. Both gave satisfactory elemental analyses for partially hydrated monomethyl derivatives of ribavirin. Each exhibited the characteristic UV absorption spectrum of the parent compound, with an absorption maximum at about 210 nm. In accord with the behavior of other 2'-*O*-methyl and 3'-*O*-methyl ribonucleosides, the sequence of elution from the Dekker column served to tentatively identify the two derivatives. Final identification was based on the ^1H NMR spectra, described below.

^1H NMR Analysis. The chemical shifts for the nonexchangeable protons of the two products are listed in Table I. It will be noted that the product with the lower retention time on the column, expected to be less acidic due to etherification of the 2'-OH,[15] exhibits marked shielding of H(2') relative to H(3'), while the reverse holds for the second product.[16] This effect, due to the magnetic anisotropy of the *O'*-methyl substituent in each compound,[17] unequivocally identifies the first as 2'-*O*-methylribavirin (1) and the second as 3'-*O*-methylribavirin (2). The virtually identical values of H(5) of the aglycon in both derivatives also suggest that both have the same conformation about the glycosidic bond, like ribavirin itself.[18]

Table II lists the measured coupling constants of the ribose and exocyclic 5'-CH_2OH protons for both compounds in D_2O, as well as those reported by Dea et al.[18] for the parent ribavirin, also in D_2O. From these values, the preferred conformations of the ribose rings and exocyclic CH_2OH groups were calculated from the Karplus relation as described elsewhere.[19,20] It will be observed from the table that methylation of the 2'- and 3'-hydroxyls affects to only a minor extent the conformations of the ribose ring and the exocyclic group.

Biological Tests. The activities of the two new analogues were assayed in several virus-cell systems previously employed[21] to assess the antiviral potentials of a fluorinated analogue of ribavirin, 5-fluoro-1-β-D-ribofuranosylimidazole-4-carboxamide (FICAR). The results are summarized in Table III. Unlike ribavirin itself, neither of the two methylated derivatives exhibited any appreciable effects on the replication of vesicular stomatitis, Herpes simplex type 1, vaccinia, polio type 1, or Coxsackie type B4 viruses. The new ribavirin analogues were also assayed for their inhibitory effects on host cell DNA synthesis, according to previously established procedures.[21-23] While ribavirin itself inhibited [*methyl*-^3H]thymidine incorporation into DNA of primary rabbit kidney cells at concentrations which coincided quite well with those required to block virus multiplication (ID_{50} or dose inhibiting thymidine incorporation by 50%, ~20 μg/mL), neither 2'-*O*-methylribavirin nor 3'-*O*-methylribavirin exerted any inhibitory effect on DNA synthesis at 100 μg/mL, the highest concentration tested.

The results obtained with the *O'*-methylated derivatives of ribavirin, together with those previously reported for the *O'*-methyl analogues of *ara*-C,[22] *ara*-A,[23] and formycin,[24] indicate that, in general, *O'*-methylation of nucleosides, whether ribosyl or arabinosyl, leads to a marked decrease or abolition of antiviral activity. The inactivity of the 2'-*O*-methyl and 3'-*O*-methyl analogues of ribavirin may be ascribed to their inability to serve as substrates for cellular kinase(s) or to the inability of the 5'-monophosphates to inhibit IMP dehydrogenase. The NMR results (Table II) would tend to exclude major modifi-

cations in conformation as the source of the biologic inactivity of the O′-methylated ribavirin derivatives.

Experimental Section

Melting points (uncorrected) were measured on a Boetius microscope hot stage. TLC made use of Merck GF-254 silica gel plates, with the solvent system $CHCl_3$–EtOH (4:1, v/v). Column chromatography was essentially as described by Dekker,[15] the adsorbent being Bio-Rad 200–400 mesh AG 1 X2 (OH^-). Evaporations were carried out with a Büchi rotatory evaporator under reduced pressure. UV spectra were run on a Zeiss (Jena, GDR) Specord UV–VIS instrument. ^1H NMR spectra were recorded with a Joel-100 instrument on 0.2 M solutions in ^2H_2O (99.9 mol % ^2H, from Merck) with DSS as internal standard.

Methylation of Ribavirin. To a solution of 488 mg (2 mmol) of ribavirin and 111 mg (0.5 mmol) of $SnCl_2 \cdot 2H_2O$ in 100 mL of MeOH was added slowly, with constant stirring, 30 mL of a solution of CH_2N_2 in 1,2-dimethoxyethane (solution B of Robins et al.[14]), and the course of the reaction was followed by TLC. Because of the low UV absorption of ribavirin at 254 nm, spots were detected by spraying the chromatograms with H_2SO_4, followed by heating to 110 °C. Following disappearance of the starting compound (R_f = 0.10), about 2 h, the products (which gave a single spot, R_f = 0.40) were brought to dryness and taken up in 10 mL of 30% aqueous MeOH.

The products were fractionated portionwise. About 1 mL of solution (50 mg) was deposited on a 30 × 0.8 cm column and elution carried out with 30% aqueous MeOH at a rate of 3 mL/min, so that fractionation was complete in about 1 h. With collection of 6-mL fractions, the two expected products were found in fractions 10–13 and 15–30. The fractionated products were then rechromatographed under the same conditions to give two well-separated peaks. Because of the low UV absorption of the products at 254 nm, the eluates were monitored in a spectrophotometer at 235–240 nm. The hydrolysis products formed during fractionation, the 3-carboxyl derivatives of **1** and **2**, are retained on the column under these conditions.

1-(2′-*O*-Methyl-β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide (2′-*O*-Methylribavirin, 1). The combined fractions from the first peak were brought to dryness to yield 150 mg (30% yield). Crystallization from absolute ethanol gave 128 mg in the form of long needles: mp 132–134 °C; λ_{max} (pH 7) 208 nm (ϵ_{max} 2.0 × 10^3). Anal. Calcd for $C_9H_{14}N_4O_5 \cdot 0.5H_2O$: C, 40.45; H, 5.62; N, 20.99. Found: C, 40.80; H, 5.60; N, 20.81. From its NMR spectrum the product was identified as 2′-*O*-methylribavirin.

1-(3′-*O*-Methyl-β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide (3′-*O*-Methylribavirin, 2). The combined fractions from the second peak yielded 200 mg (40%, total yield 70%). Crystallization from absolute ethanol gave 176 mg of long sharp needles: mp 90–93 °C; λ_{max} (pH 7) 208 nm (ϵ_{max} 2.0 × 10^3). Anal. Calcd for $C_9H_{14}N_4O_5 \cdot 0.5H_2O$: C, 40.45; H, 5.62; N, 20.99. Found: C, 40.16; H, 5.24; N, 20.94. Identification as 3′-*O*-methylribavirin was based on the NMR spectrum.

Biological Activity. The procedures for measuring antiviral and antimetabolic activity have been described previously.[22-24]

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