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Studies on the Mechanism of Antiviral Action of 1-(β-D-Ribofuranosyl)-1,2,4-triazole-3-carboxamide (Ribavirin)

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Syntheses are described for the 5′-phosphates of the 2′- and 3′-*O*-methylribavirins (**2a** and **2b**) and the methyl ester of ribavirin 5′-phosphate (**3**). The 5′-phosphate of 1-β-(D-ribofuranosyl)-1,2,4-triazole (**2d**) was obtained via the 3-carboxyl derivative of ribavirin 5′-phosphate (**2c**). Compounds **2a**, **2b**, and **2d** were inactive as inhibitors of IMP dehydrogenase under conditions where the parent ribavirin 5′-phosphate (**2**) was an effective inhibitor. Weak inhibitory activity was exhibited by **3** ($K_i \approx 200$ μM) and **2c** ($K_1 \approx 70$ μM). Under conditions where ribavirin (**1**) is effectively phosphorylated by rat liver nucleoside kinase, the 2′- and 3′-*O*-methylribavirins (**1a** and **1b**), the 3-carboxylate of ribavirin (**1c**), and the riboside of 1,2,4-triazole (**1d**) were totally inactive. The overall results are fully consistent with the lack of antiviral activity of **1a** and **1b**, while the specificity of ribavirin as an antiviral agent is further underlined by the behavior of the methyl ester **3**.

Ribavirin, 1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide, originally designated as "virazole", has been shown to be a broad-spectrum antiviral agent in vitro.[1-3] Particularly striking are the inhibitory effects of this compound on influenza and parainfluenza virus replication in cell cultures and in some animals.[1,4-9] The drug has been licensed for human use in some countries and is currently undergoing clinical trials, although reports on its efficacy are somewhat conflicting.

Ribavirin 5′-phosphate has been found to competitively inhibit IMP dehydrogenase (IMP:NAD^+ oxidoreductase, EC 1.2.1.14),[10] ascribed to its structural resemblance to IMP and GMP.[11] Taken in conjunction with the fact that ribavirin is converted to the 5′-phosphate by the appropriate cellular kinase, presumably adenosine kinase,[12] this led to the proposal that the compound inhibits virus multiplication via depletion of the GTP pool.[10,13,14] Some reservations have been raised with regard to this concept,[15,16] and, at least in the case of influenza virus, the RNA polymerase of which is potently inhibited by ribavirin 5′-triphosphate,[17] it is conceivable that this may represent the major site of action of this antimetabolite, at least in this instance. It is of interest in this regard that ribavirin 5′-triphosphate is inactive in vitro against eukaryotic DNA polymerases α and β, eukaryotic RNA polymerases I and II, and eukaryotic poly(A) polymerase.[14]

Regardless of the eventual decision regarding the clinical utility of ribavirin, its broad spectrum of activity, encompassing nearly all major virus groups, suggests the utility of examining in further detail the mechanism of its in vitro activity. We previously showed that 2′- and 3′-*O*-methyl derivatives of ribavirin were virtually inactive against several viruses in cell culture notwithstanding that, unlike the parent ribavirin, the 2′(3′)-*O*-methyl derivatives did not suppress cellular DNA synthesis.[18] The lack of activity of the 2′(3′)-*O*-methylribavirins could conceivably be due to their inability to undergo phosphorylation by cellular kinase(s) or to the failure of their 5′-phosphates to inhibit IMP dehydrogenase. We have therefore synthesized the appropriate 2′(3′)-*O*-methylribavirin 5′-phosphates and examined the foregoing two possibilities. We have also prepared several additional analogues of ribavirin and its 5′-phosphate with a view to delineate more accurately its specificity of action.

Chemistry. The 2′- and 3′-*O*-methylribavirin 5′-phosphates (**2a** and **2b**) were obtained by phosphorylation of the parent nucleosides[18] according to the procedure of Yoshikawa,[19] with slight modifications.[10] This method, although convenient, is frequently capricious and not always as selective as claimed.[20] Consequently, the isolated nucleotides, following purification on a DEAE-Sephadex A-25 (HCO_3^-) column, were checked by treatment with

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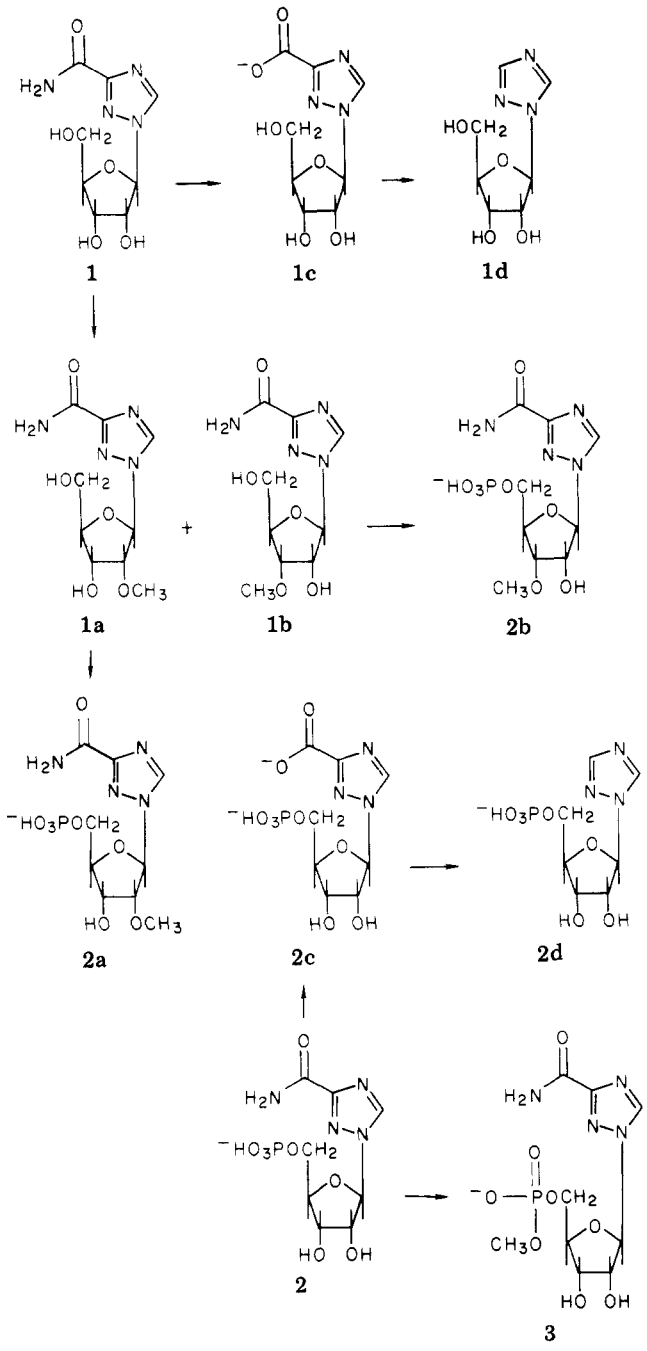
Scheme I



crude snake venom (as a source of 5′-nucleotidase) and with purified 5′-nucleotidase. Under conditions where 2′(3′)-CMP was resistant, the nucleotides **2a** and **2b** were slowly converted quantitatively to the parent nucleosides, the 2′-*O*-methyl derivative being hydrolyzed about three times more rapidly than the 3′-*O*-methyl analogue. This confirms the structure of each nucleotide.

Ribavirin 5′-phosphate was converted to the methyl ester by treatment with methanol in the presence of dicyclohexylcarbodiimide.[21] The product was identified by its resistance to bacterial alkaline phosphatase and by its hydrolysis by purified phosphodiesterase I[22] to ribavirin 5′-phosphate, although the reaction was very slow. Of some interest was the fact that the methyl ester of **2** was very slowly hydrolyzed by purified 5′-nucleotidase to the nucleoside and, probably, methyl phosphate.[22]

The carboxamide group of ribavirin 5′-phosphate was degraded in two steps. Alkaline hydrolysis converted the amide group to give the carboxylate **2c**, and the latter was

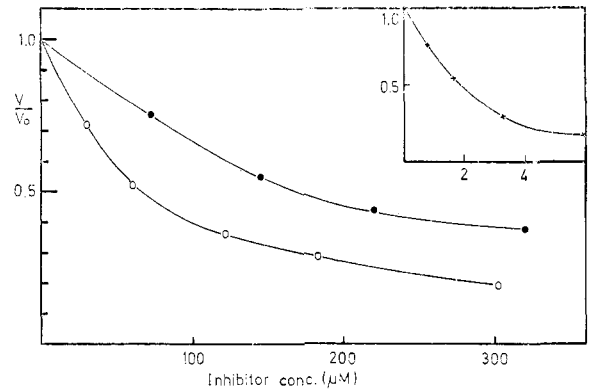


Figure 1. Effect of ribavirin 5′-phosphate analogues on the activity (V) of rat spleen IMP dehydrogenase. Enzyme activity in the absence of inhibitor (V_0) was 0.8 nmol of NAD^+ reduced min^{-1} (mg of protein)$^{-1}$, and the incubation medium contained 0.66 mg of protein/mL: (○) 3-carboxyl derivative of ribavirin 5′-phosphate (**2c**); (●) methyl ester of ribavirin 5′-phosphate (**3**). Insert: (×) ribavirin 5′-phosphate.

decarboxylated by heating to 180–185 °C in Me_2SO to give **2d**. Both products were purified on a DEAE-Sephadex A-25 column, and the carboxyl derivative was obtained as the crystalline potassium salt. Both of these were further identified by hydrolysis with 5′-nucleotidase to the parent nucleosides (Scheme I). It is of interest that the 5′-phosphate of 1-(β-D-ribofuranosyl)-1,2,4-triazole (**2d**) was hydrolyzed by 5′-nucleotidase 50-fold more rapidly than the 3-carboxylate **2c**.

The nucleosides 1-(β-D-ribofuranosyl)-1,2,4-triazole (**1d**) and its 3-carboxylate **1c** were obtained by stepwise degradation of the carboxamide group of ribavirin (**1**). The products were identical with those previously obtained by another route.[1,23] The 3-carboxylates of ribavirin and its 5′-phosphate may also be readily obtained by hydrolysis of each of these in 1 N HCl at 90 °C for about 1 h, with no detectable hydrolysis of the glycosidic bond.

Enzymatic Aspects. (a) Nucleoside Kinase. Of all four analogues examined, i.e., 2′-*O*-methylribavirin (**1a**), 3′-*O*-methylribavirin (**1b**), the 3-carboxylate of ribavirin (**1c**), and the riboside of 1,2,4-triazole (**1d**), none showed any evidence of phosphorylation by nucleoside kinase under conditions where the parent ribavirin, as well as adenosine (used as a control), were readily phosphorylated. As a further check on the validity of these results, it was verified that the 5′-phosphates of all four derivatives were stable to the hydrolysis conditions employed in the final stage of the assay.

(b) IMP Dehydrogenase. Under conditions where the parent ribavirin 5′-phosphate gave 50% inhibition of enzyme activity at a concentration of about 2 μM, no detectable inhibition could be observed with the 5′-phosphates of 2′-*O*-methylribavirin (**2a**), 3′-*O*-methylribavirin (**2b**), and the triazole nucleoside **2d** even at concentrations 100-fold higher, i.e., 200 μM. Furthermore, **2a** and **2b**, at concentrations of 20 μM, did not interfere with inhibition by the parent nucleotide.

The methyl ester of ribavirin 5′-phosphate was a feeble inhibitor of the enzyme, with 50% inhibition at a concentration of 160 μM, but even an increase in concentration to 300 μM gave no more than 60% inhibition. Somewhat more effective inhibitory properties were displayed by the 5′-phosphate of the 3-carboxylate **2c**, which gave 50% inhibition at a concentration of 70 μM (Figure 1).

Discussion

The total lack of substrate properties of the 2′- and 3′-*O*-methyl derivatives of ribavirin toward the rat liver

kinase, under conditions where the parent ribavirin is readily phosphorylated, can explain the loss of the antiviral activity of ribavirin when one of the cis hydroxyls is methylated. Attention has previously been drawn to the fact that such a loss of activity is not due to any modification of nucleoside conformation resulting from O'-methylation.[18] It is, of course, conceivable that the O'-methyl derivatives may turn out to be suitable substrates for virus-induced kinase(s) which, in some instances, have been found to display broader specificities than the corresponding cellular enzymes.[24,25] However, if this proved to be the case, then the failure of the 5'-phosphates of the two O'-methyl derivatives to inhibit IMP dehydrogenase would equally account for lack of antiviral activity.

Relevant to the foregoing are the reported good antiviral activities of 2',3',5'-tri-O-acetylribavirin[8] and some phosphate esters of ribavirin.[28] The high activity of the former is readily accounted for, as suggested, by its intracellular hydrolysis by nonspecific esterases to release active ribavirin. For the latter, the situation is more complex, since the 2'(3')-phosphates and the 2',3'-cyclic phosphate were also active; since nucleotides do not readily penetrate the cell membrane, it appears likely that the phosphate esters underwent hydrolysis to free ribavirin at the cell surface. The lack of antiviral activity of the 2'(3')-O-methylribavirins, by contrast, is presumably due to the fact that these do not undergo enzymatic hydrolysis to free ribavirin. It should be noted that replacement of the 3'-OH by an amino group, which is not hydrolyzed intracellularly, also leads to complete loss of activity.[26]

As regards the specificity of ribavirin 5'-phosphate as an inhibitor of IMP dehydrogenase, this is strikingly underlined by the fact that five very closely related analogues inhibit the enzyme to only a minimal extent, if at all. It follows that modification of the functional groups significantly decreases antiviral activity. While ribavirin 5'-phosphate leads to a decrease in the 5'-GMP pool,[13] this is not necessarily the only pathway for ribavirin inhibition of viruses, as indicated by the fact that the 5'-triphosphate is an effective inhibitor of influenza virus RNA polymerase.[17]

The specificity of the 5'-phosphate of ribavirin as an IMP dehydrogenase inhibitor is further underlined by the fact that the 5'-pyrophosphate and the 5'-triphosphate are 1000- and 200-fold less effective, respectively, as inhibitors.[27] The present observation that the methyl ester of ribavirin 5'-phosphate (**3**) is 100-fold less potent than the parent nucleotide consequently suggests that steric, rather than electrostatic, factors play a dominant role here. The same follows from the behavior of 1-(5,6-dideoxy-β-D-*ribo*-hexofuranosyl-6-phosphonic acid)-1,2,4-triazole-3-carboxamide,[30] where replacement of the 5'-O by a CH_2 group in ribavirin 5'-phosphate results in a 33-fold decrease in inhibitory activity toward IMP dehydrogenase and a simultaneous abolition of antiviral activity. This may be due to resistance to enzymatic hydrolysis of a phosphoester–methylene bond (thus preventing entry of the nucleoside into the cell) or to the decreased inhibition of IMP dehydrogenase. In apparent contradiction with this is the known antiviral activity of the 3',5'-cyclic phosphate,[28] but this is readily explicable by the known ability of such a derivative to undergo cleavage of the cyclic phosphate ring intracellularly, with release of the 5'-phosphate. It would, nonetheless, be of interest to examine the inhibitory properties of the cyclic phosphate derivative toward IMP dehydrogenase.

As regards the heterocyclic moiety of ribavirin, the presence of the carboxamide group appears to be a prerequisite for antiviral activity.[29] Analogues with only slight modifications of this group, e.g., thiocarboxamide or carboxamidine, are equally active but only against some viruses.[29] More extensive modifications, e.g., carboxyhydroxyamidine, lead to total loss of activity.[29]

If inhibition of viral growth is indeed due mainly to inhibition of IMP dehydrogenase, then the phosphorylation step by kinase(s) would account, in the case of the thiocarboxamide and carboxamidine derivatives, for their selective antiviral activities, on the assumption that they are substrates only for those kinases of the viruses against which they are active. In the case of inactive analogues, the lack of antiviral activity may then be due to their either not being substrates for the kinase (cellular or viral) or their 5'-phosphates not being inhibitors of IMP dehydrogenase. On this basis, one would expect the 1,2,4-triazole derivative **1d** and the 3-carboxy derivative **1c** to be inactive as antiviral agents.

The structures of the two analogues with antiviral activity (thiocarboxamide and carboxamidine), as compared to that of the 3-carboxy derivative **2c** with its low inhibitory activity toward IMP dehydrogenase, render doubtful the validity of the proposal that the inhibition of IMP dehydrogenase by ribavirin 5'-phosphate involves hydrogen bonding between the two via the carboxamide group of the latter.[10] The thiocarboxamide and carboxamidine derivatives are active, notwithstanding that hydrogen bonding via a sulfur atom or a protonated carboxamidine group is relatively weak, while the carboxyl derivative is a weak inhibitor of IMP dehydrogenase (see above) despite the fact that it is capable of strong hydrogen bonding.

Experimental Section

Ribavirin and ribavirin 5'-phosphate were kindly provided by Dr. R. Sidwell of ICN Pharmaceuticals (Irvine, Calif.). Purified snake venom 5'-nucleotidase (EC 3.1.3.5) was a product of Sigma (St. Louis, Mo.). Russell viper venom, used as a source of 5'-nucleotidase, was a gift from Dr. P. Bhargava (Hyderabad, India). Purified phosphodiesterase I (EC 3.1.4.1) was obtained from Worthington (Freehold, N.J.).

Thin-layer chromatography made use of Merck (Darmstadt, GFR) silica gel $60F_{254}$ on aluminum foil with the following solvent systems: (A) acetonitrile–0.1 M NH_4Cl (7:3, v/v); (B) 2-propanol–H_2O–concentrated NH_4OH (7:2:1, v/v). Since ribavirin exhibits relatively low absorption at 254 nm, it is difficult to locate with the aid of a dark UV lamp. Spots were therefore revealed by spraying the chromatograms with ethanol–H_2SO_4–anisaldehyde (18:1:1, v/v), followed by heating to 120 °C.

Column Chromatography of Nucleotide. In all instances, nucleotides were fractionated by chromatography on a 22 × 1.2 cm column of 5 g of DEAE-Sephadex A-25 (HCO_3^-). The solution containing the nucleotide was loaded on the column, and elution was carried out with a linear gradient of 0–0.5 M triethylammonium carbonate at a flow rate of 1.8 mL/min and collection of 9-mL fractions. The eluate was monitored by the absorption at 254 nm with a Uvicord II detector and an LKB recorder.

Snake Venom Hydrolysis. To 1 μmol of nucleotide in a total volume of 70 μL consisting of 50 μL of 0.1 M Tris-HCl buffer (pH 9) and 20 μL of 0.05 M $MgCl_2$ was added 2.5 mg of snake venom in 100 μL of the same buffer solution. Incubation was at 37 °C and the extent of hydrolysis was followed by TLC at 1, 3, and 24 h. A control was run with 5'-AMP as substrate.

Hydrolysis with 5'-Nucleotidase. To 1 μmol of nucleotide in a total volume of 70 μL, consisting of 50 μL of 0.1 M Tris-HCl buffer (pH 9) and 20 μL of 0.05 M $MgCl_2$, was added 1 unit of purified 5'-nucleotidase in 50 μL of the same buffer. The course of hydrolysis was followed by TLC after 3, 24, and 48 h.

Phosphodiesterase Hydrolysis. The incubation mixture consisted of 100 μL of 0.25 M Tris-HCl buffer (pH 9.0) plus 20 μL of 0.01 M $MgCl_2$, 1 μmol of substrate (compound **3**), and 10 μg of purified phosphodiesterase I. Incubation was at 37 °C.

Protein was determined by the method of Lowry et al.[31] with bovine serum albumin as standard.

Enzyme Preparations and Assays. Inosinate dehydrogenase (IMP:NAD^+ oxoreductase, EC 1.2.1.14) from rat spleen was isolated by a modification of the procedure of Jackson et al.[32] Crude extracts were prepared in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.5 mM 2-mercaptoethanol. The precipitate resulting from 30–40% saturation with $(NH_4)_2SO_4$ was taken up in 0.1 M phosphate buffer (pH 7.4), 1 mM EDTA, and 0.5 mM 2-mercaptoethanol, dialyzed vs. the same solution for 4 h, and stored at −70 °C. The specific activity of this preparation was 0.5–0.8 nmol of NAD^+ reduced min^{-1} (mg of protein)$^{-1}$.

Activity was assayed in a medium consisting of 0.1 M phosphate buffer (pH 7.7), 1 mM EDTA, 0.5 mM 2-mercaptoethanol, and 0.2 mM each of NAD and IMP. The latter was added to initiate the reaction, and the decrease in absorbance at 340 nm vs. a blank (no IMP) was monitored at 37 °C in a Cary Model 118 for up to 1 h. The A (mM) at 340 nm was 6.2 cm^{-1}.

Nucleoside kinase was isolated from rat liver in a medium containing 20 mM Tris-HCl (pH 7.3), 1 mM EDTA, and 3 mM 2-mercaptoethanol. All steps were at about 2 °C. The liver was homogenized in a glass–Teflon homogenizer (1 g of liver/3 mL of medium). The homogenate was centrifuged at 10000g for 30 min and the resulting supernatant at 144000g for 1 h. The top lipid layer was removed and the clear supernatant fractionated with solid $(NH_4)_2SO_4$ according to Streeter et al.[33] The precipitate formed between 55 and 90% saturation was collected, dissolved in the isolation medium, dialyzed against the same medium overnight, and stored at −70 °C. The rate of phosphorylation of ribavirin by this preparation, relative to adenosine, was similar to that reported elsewhere.[33]

Activity was measured as described by Dobersen and Greer[25] but with modifications in the reaction medium and in the procedure for precipitation of P_i. The reaction medium included 50 mM Tris-HCl (pH 8.0), 5 μM $MgCl_2$, 25 mM KCl, 0.05–0.1 μCi of 1 mM [γ-^{32}P]ATP, 0.64 unit of pyruvate kinase, 2 mM phosphoenol pyruvate, and substrate and enzyme as given below in 0.1 mL. Incubation was at 37 °C for 10 or 20 min, and the reaction was terminated by heating to 100 °C for 1 min. Addition of 0.8 mL of 0.1 mM K_2HPO_4 favored precipitation of liberated P_i (the original procedure suggests 1 mM K_2HPO_4, which may be a misprint).

Phosphorylation of 2′(3′)-*O*-Methylribavirin. To a solution of 50 mg (~0.2 mmol) of 2′- or 3′-*O*-methylribavirin (**1a** and **1b**) in 1.2 mL of trimethyl phosphate at 0 °C was added, with stirring, 60 μL of $POCl_3$. The reaction mixture was kept on ice for 5 h and then at −20 °C for 2 days. The progress of the reaction was followed by TLC on silica gel with solvent A (R_f of nucleosides 0.64 and of nucleotides 0.23). Following 90% conversion to the nucleotide, the reaction mixture was diluted with 10 mL of water and loaded on a 15-g column of a mixture of charcoal and Celite (2:1, g/g). The column was washed with water until the effluent gave a neutral reaction. The nucleotide was then eluted with ethanol–water–concentrated NH_4OH (10:10:1, v/v) until the absorption of the effluent at 210 nm showed a marked drop in value (total volume of effluent 50 mL). The product was then further purified by chromatography on DEAE-Sephadex as described above. Each of the two nucleotide products eluted at about 0.1 M triethylammonium carbonate, the 2′ isomer in fractions 12–19 and the 3′ isomer in fractions 18–24. The pooled fractions were brought to dryness, and the residue was taken up in absolute ethanol several times and brought to dryness to remove triethylammonium carbonate. The nucleotides were converted to the ammonium salts by percolation of their aqueous solutions through a column of 4 mL of Dowex 50 Wx2 (NH_4^+). The eluates from this column were concentrated to a small volume, and the nucleotides were precipitated with absolute ethanol and dried under vacuum over P_2O_5. Yields ranged from 42 to 46%, free of inorganic phosphate and with UV spectra identical with that of the nucleoside. Treatment of each of the isomeric nucleotides with purified 5′-nucleotidase led to conversion to the corresponding nucleosides, thus confirming their structures.

Methyl Ester of Ribavirin 5′-Phosphate (3). The 5′-phosphate of ribavirin (**2**; 32.5 mg, 0.1 mmol) was converted to the triethylammonium salt by dissolving it in a solution of 4 mL of methanol containing 75 μL of anhydrous triethylamine. To this solution was added 166 mg of dicyclohexylcarbodiimide, and the mixture was maintained at 37 °C overnight. TLC on silica gel with solvent system B demonstrated the disappearance of starting substance (R_f 0.1) and formation of one product (R_f 0.40). To the mixture was added 10 mL of water to hydrolyze unreacted dicyclohexylcarbodiimide and precipitate cyclohexylurea, and the mixture was placed in a cold box for 4 h and then filtered. The precipitate was washed with cold water, and the combined filtrates were concentrated to a volume of several milliliters. This was loaded on a DEAE-Sephadex column, and elution was carried out as above. Fractions 13–17 (0.15 M triethylammonium carbonate), containing the required ester, were pooled, brought to dryness, and coevaporated several times with absolute ethanol. The product was converted to the ammonium salt, precipitated with absolute ethanol, and dried under vacuum over P_2O_5 to yield 26 mg (71%), free of inorganic phosphate. Treatment of this product with snake venom phosphodiesterase I led to its slow conversion to the 5′-phosphate.

1-(β-D-Ribofuranosyl)-1,2,4-triazole-3-carboxylate 5′-Phosphate (2c). A solution of ribavirin 5′-phosphate (**2**; 32.4 mg, 0.1 mmol) in 1 mL of 1 N KOH was heated in a sealed ampule for 20 h at 90 °C. At this point, TLC on silica gel demonstrated almost quantitative conversion of the amide (R_f 0.35) to the carboxylate (R_f 0.20). The reaction mixture was diluted with water to a total volume of 6 mL and loaded on a column of DEAE-Sephadex. The column was washed with water, and elution was then carried out as above. Fractions 35–57 (0.24 M triethylammonium carbonate), containing the triethylammonium salt of the desired product, were pooled and brought to dryness, and the residue was coevaporated with absolute ethanol several times. The resulting syrup was taken up in water and percolated through a 5-mL column of Dowex 50 x2 (K^+). The effluent was brought to dryness, the resulting potassium salt of the product was taken up in a minimal volume of water, and ethanol was added to faint turbidity. The mixture was maintained on ice for 10–20 min. Further storage at room temperature for 2 days led to colorless platelets (10 mg). The mother liquors were concentrated, and the remainder of the product was precipitated with absolute ethanol to give 28 mg (overall yield 89%). Treatment of the product with purified 5′-nucleotidase led to slow conversion to the parent nucleosides.

1-(β-D-Ribofuranosyl)-1,2,4-triazole 5′-Phosphate (2d). To 22 mg (~ 0.07 mmol) of the free acid of the carboxyl derivative **2c** was added 100 μL of Me_2SO, and the mixture was heated for 8 min on an oil bath at 180 °C. At this point, TLC on silica gel demonstrated 80% conversion to a new product, with only traces of byproducts. The mixture was taken up in 2 mL of water and loaded on a DEAE-Sephadex column. Fractions 31–42 (0.14 M triethylammonium carbonate), containing the desired product, were pooled and brought to dryness to yield a dark yellow syrup. The nucleotide was converted to the potassium salt which, after drying, gave 10 mg of a dark yellow, hygroscopic powder (44%), homogeneous on silica gel with solvent A (R_f 0.35; R_f of starting substance 0.20). This product on treatment with 5′-nucleotidase was converted very rapidly to the corresponding nucleoside.

1-(β-D-Ribofuranosyl)-1,2,4-triazole-3-carboxylic Acid (1c). A solution of 488 mg (2 mmol) of ribavirin (**1**) in 5 mL of 1 N KOH was left for 2 days at ambient temperature. TLC with solvent A demonstrated the disappearance of starting substance (R_f 0.75) and formation of one product (R_f 0.40). The reaction mixture was percolated through a column of 20 mL of Dowex 50 Wx2 (H^+). The resin was washed with 100 mL of water, and the combined effluents were concentrated to small volume to induce crystallization. Recrystallization from water gave 455 mg (93%) of thick colorless needles, mp 190 °C dec, as compared to 188–190 °C for the same product by another route.[1]

1-(β-D-Ribofuranosyl)-1,2,4-triazole (1d). To 200 mg (0.82 mmol) of the 3-carboxylate **1c** was added 1 mL of Me_2SO, and the mixture was heated at 180–185 °C on an oil bath.[1] TLC demonstrated virtually total conversion to a new product after 12 min, while the reaction mixture itself turned dark brown. The reaction mixture was diluted threefold with water and loaded on a column of 12 mL of Dowex 50 Wx2 (H^+). The column was washed with water to remove Me_2SO and traces of the unreacted carboxylate starting compound (about 100 mL of water). The product was then eluted with 5% NH_4OH (100 mL). The ammoniacal eluate was brought to dryness, and the residue was taken up several times in absolute ethanol and brought to dryness. The

resulting brownish syrup was dissolved in 5 mL of absolute ethanol and passed through a paper filter to remove the dark brown precipitate. The filtrate was concentrated to small volume, leading, after several hours at room temperature, to a crop of crystals (79 mg, 49%), mp 146–148 °C.[23] Concentration of the mother liquors led to a further crop of crystals (14 mg; overall yield 57%).

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Orally Active Esters of Cephalosporin Antibiotics. 3.[1] Synthesis and Biological Properties of Aminoacyloxymethyl Esters of 7-[D-(-)-Mandelamido]-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-3-cephem-4-carboxylic Acid

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The synthesis of six amino acid acyloxymethyl esters of cefamandole (**1**), a semisynthetic broad-spectrum cephalosporin antibiotic, is described. These esters were examined as potentially useful orally active antibiotic prodrugs. When tested for oral efficacy against *Streptococcus pyogenes* C203 in mouse protection tests, the esters were not notably more active than lithium cefamandole. Further studies demonstrated that significant blood and urine levels of **1** were not obtained after dosing **2a**, **2b**, and **2f** orally at 17 mg/kg in mice. A study of the stability to chemical hydrolysis and the possible relationship of hydrolysis to the lack of oral absorption of these esters is also presented.

Cefamandole (**1**), 7-(D-mandelamido)-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-3-cephem-4-carboxylic acid, a semisynthetic broad-spectrum cephalosporin antibiotic, is active against a wide variety of Gram-positive as well as Gram-negative organisms in vitro.[2] Particularly noteworthy is the activity of cefamandole against indole-positive *Proteus spp.* and *Enterobacter spp.*, organisms which are resistant to most cephalosporins.[3] The in vitro activity of cefamandole has been confirmed in many laboratories, and reports of its clinical efficacy are numerous.[4] Since cefamandole is not efficiently absorbed following oral administration, an investigation of the effect of esterification of the C-4 carboxyl group on oral absorption was undertaken. Acetoxymethyl and pivaloylmethyl esters of cefamandole (esters which increase the oral absorption of ampicillin[5] and cephaloglycin[6]) were only marginally active orally because they lack sufficient solubility in water for efficient absorption from the gastrointestinal tract to occur. However, when formulated with a solubilizing vehicle, solutions of these esters of

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