Synthesis and Antitumor Activity of Ribavirin Imidates. A New Facile Synthesis of Ribavirin Amidine (1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamidine Hydrochloride)

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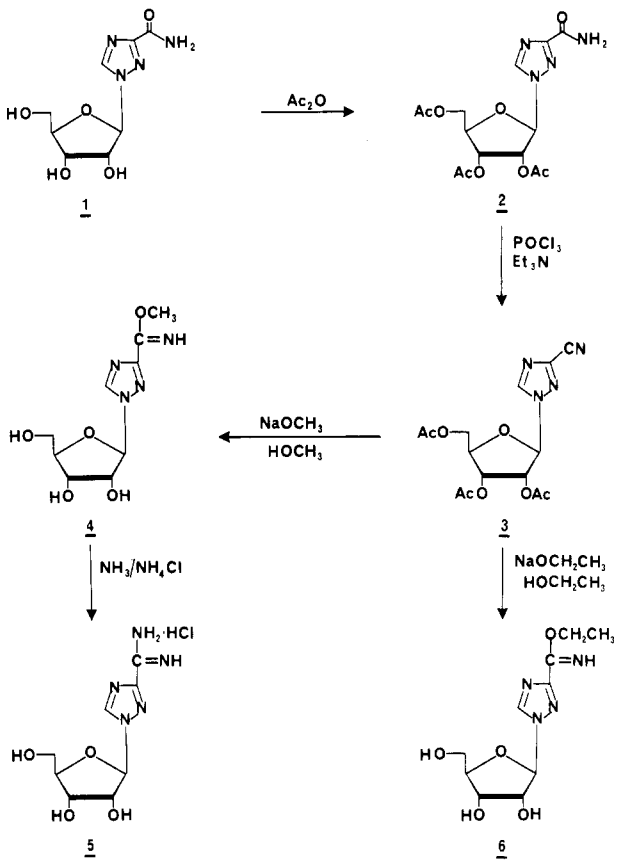
Methyl 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboximidate (4) and ethyl 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboximidate (6) were synthesized and tested for antitumor and antiviral activity. A new facile synthesis of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride (5), starting with imidate 4, was also developed. The imidates 4 and 6 differed greatly in solubility and dosing requirements. Even so, both compounds exhibited significant activity in vivo against murine leukemia L1210. Nontoxic dosing with 4 also significantly diminished Friend leukemia induced splenomegaly. In contrast, neither imidate was active in vitro.

Ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, reported[1] by Robins et al. is a broad-spectrum antiviral[2,3] agent of considerable interest. The antiviral activity of ribavirin and its clinical applications have been reviewed.[4-6] McCormick and co-workers have reported[7] that ribavirin inhibits the replication of HIV in human T lymphocytes at 50 μg/mL. Several derivatives of ribavirin have been synthesized,[8] including the 2′,3′-dideoxyribavirin.[9] Among these, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride (5), also first reported[8] by Robins et al., is of considerable interest as an antiviral agent as well. As part of an ongoing program of the synthesis of nucleosides as potential antiviral and antitumor agents, the hitherto unknown imidate derivatives methyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboximidate (4) and ethyl 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboximidate (6) were synthesized and tested. As discussed in detail in this paper, imidates 4 and 6 were inactive in vitro but were found to exhibit significant antitumor activity in laboratory mice. A new facile synthesis of amidine 5 starting with imidate 4 was also developed and is discussed in this paper.

Chemistry

The reaction sequence is outlined in Scheme I. Ribavirin (1) was acetylated with acetic anhydride and pyridine in quantitative yields. The tri-O-acetylated carboxamide 2 was subsequently dehydrated with phosphorus oxychloride and triethylamine in chloroform solution, leading to the nitrile 3-cyano-1-β-D-ribofuranosyl-1,2,4-triazole (3), in yields in excess of 90%. Treatment of nitrile 3 with sodium methoxide in methanol resulted in imidate 4. Treatment of 4 with methanolic ammonia in the presence of ammonium chloride gave amidine 5. This is the first known instance of the synthesis of amidine 5 via the corresponding imidate, 4. We have found this method to be extremely facile and useful for the synthesis of amidine 5. Earlier synthesis of this compound was accomplished[8] by the glycosylation of 3-cyano-1,2,4-triazole with 1,2,3,5-tetra-*O*-acetyl-D-ribofuranose followed by separation of the isomers by chromatography. In similar fashion, upon treatment of 3 with sodium ethoxide in ethanol, imidate 6 was obtained in 64% yield after chromatography.

Scheme I



Anticancer Activity and Host Toxicity

The solubilities of 4 and 6 were greatly dissimilar. As a result, the maximum dosage of 6 that could be delivered as a single bolus using our volumetric scheme (see the Experimental Section) of dosing was 3704 mg/kg while that of 4 was only 480 mg/kg. Administered qd (once daily) day 1 at those dosages, 6 was lethally toxic for L1210-inoculated mice (Table I) and 4 was not (Table II).

With the qd day 1 schedule of delivery, the maximum nonlethal dosage of 6 (1333 mg/kg) produced a T/C of 149,

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Table I. Response of Mice Inoculated with L1210 Leukemia to Ethyl 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboximidate (6)

schedule of administration	dosage,[a] mg/kg	postinoculation life span:[b] %T/C	residual cell population after last treatment:[c] % of control
qd, day 1[d]	3704	48	tox
qd, day 1	2222	92	tox
qd, day 1	1333	149	1.3
qd, day 1	800	159	0.4
qd, day 1	800	144	1.7
qd, day 1	480	149	1.1
qd, day 1	288	114	27.0
qd, day 1–7	800	70	tox
qd, day 1–7	480	134	tox
qd, day 1–7	288	144	1.7

[a] All solutions were delivered ip (0.01 mL/g mouse weight). Control mice were injected with a 0.9% solution of NaCl. [b] Treatment responses (six mice/treatment group) presented as % T/C were calculated according to the equation: (mean life span of treated mice/mean life span of control mice) × 100. The data presented were derived from three different studies in which 10 control mice lived 6.50 ± 0.53, 6.44 ± 0.53, and 6.60 ± 0.89 days. A T/C ≥ 125 is considered biologically significant. Tox indicates that one or more mice were killed by treatment. [c] Calculations of residual leukemic cell populations were made with inoculum–response data indicating the relationship between inoculum size and resultant postinoculation life span. [d] qd = once a day.

which, as gauged by inoculum response data (see the Experimental Section), reflected a leukemic cell kill of 98.7%. Similar results were produced by qd day 1 treatment with 800 or 480 mg/kg of **6** but at the 288 mg/kg level therapeutic efficacy was substantially diminished. When administered qd days 1–7, lethal toxicity was produced by lower dosages of **6**. On this schedule the increase in mean life span (T/C 144) produced by the maximum nonlethal dosage of **6** (288 mg/kg) was about the same as that observed when higher dosages were given less frequently. This finding indicates the anti-L1210 activity of **6** to be more dosage than schedule dependent.

The maximum soluble dosage of **4** (480 mg/kg) produced repeated T/C values of ~115 when administered qd day 1. Because of limited solubility relative to its biological activity, **4** was administered multiple times a day to determine its therapeutic potential. With qid day 1 delivery, the drug (480 mg/kg per injection) was lethally toxic but tid day 1 administration produced a T/C of 153, indicating a leukemic cell kill of >99%. Additional scheduling trials did not identify more effective therapy, suggesting that the anti-L1210 activity of **4** as that of **6** may be more dosage than schedule dependent. Antileukemic activity was not detected following oral administration of **4**.

Friend leukemia related splenomegaly was significantly diminished by nontoxic dosing with **4** but not with **6** (Table III). It should be noted that this erythroleukemia is of viral origin and its proliferation is virus dependent. It is not presently clear, therefore, whether the effects produced by **4** resulted from cytotoxic or antiviral actions of the drug.

It appears noteworthy that neither **4** nor **6** was cytotoxic for neoplastic cells growing in vitro. Both compounds were tested (for experimental details, see ref 10) for their activity against L1210 murine lymphocytic leukemia, WI-L2 human B lymphoblastic leukemia, and CCRF-CEM human T lymphoblastic leukemia in vitro. Under culture conditions, neither compound was significantly growth inhibitory for these cell lines. Similarly, both **4** and **6** were inactive when tested (for experimental details, see ref 11) against parainfluenza type 3, adeno type 2, influenza A, rhino A, semliki forest, visna, and herpes simplex type 2 viruses in culture.

Table II. Response of Mice Inoculated with L1210 Leukemia to Methyl 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboximidate (4)

schedule of administration[a]	postinoculation life span:[b] %T/C	residual cell population after last treatment:[c] % of control
qd, day 1[d]	113	32
qd, day 1	114	30
qd, day 1	116	21
bid, day 1[d]	125	9
tid, day 1[d]	153	0.6
qid, day 1[d]	60	tox
qd, day 1, 4, 7	119	73
qd, day 1, 3, 5, 7	121	46
bid, day 1, 3, 5, 7	162	12
tid, day 1, 3, 5, 7	76	tox
qd, day 1–7	162	12
qd, day 1–7	142	7
qd, day 1–7 (po)	97	139
bid, day 1–7	101	tox
tid, day 1–7	61	tox

[a] On every schedule, the dosage of **4** was 480 mg/kg per injection. Except where otherwise indicated, all solutions were delivered ip (0.01 mL/g mouse weight). Control mice were injected with a 0.9% solution of NaCl. [b] Treatment responses (six mice/treatment group) presented as % T/C were calculated according to the equation: (mean life span of treated mice/mean life span of control mice) × 100. The data presented were derived from three different studies in which 10 control mice lived 6.50 ± 0.53, 6.60 ± 0.89, and 6.44 ± 0.53 days. A T/C ≥ 125 is considered biologically significant. Tox indicated that one or more mice were killed by treatment. [c] Calculations of residual leukemic cell populations were made using inoculum–response data indicating the relationship between inoculum size and resultant postinoculation life span. [d] qd = once a day; bid = twice a day; tid = three times a day; qid = four times a day.

Imidates **4** and **6** were not specifically synthesized as prodrugs of ribavirin, and mechanism of action studies to clarify that possibility have not been performed. The imidates were, instead, synthesized as part of a program to identify nucleosides with better antiviral or anticancer activity than ribavirin and, to some extent, that seems to have been accomplished. In the present studies, both compounds were more effective in the treatment of L1210 leukemia than ribavirin was previously reported to be.[12] Imidate **4** was active against Friend erythroleukemia as was ribavirin in an earlier study[13] while **6** did not produce significant effects in this experimental model. This discrepant activity and the seeming requirement of imidates **4** and **6** for activation in vivo constitute fairly significant leads toward the design of effective new anticancer agents.

Experimental Section

Chemistry. NMR data were obtained on an IBM NR-300 spectrometer in $(CD_3)_2SO$ or $CDCl_3$ solvents using the residual proton as internal reference. Melting points were obtained in open capillaries with a Haake-Buchler apparatus and are uncorrected. Combustion analyses were performed by Robertson Laboratories, Florham Park, NJ.

Therapeutic and Toxicity Determinations in Vivo. In vivo assessments of therapeutic efficacy and host toxicity with murine leukemia L1210 were made as previously detailed.[14] Briefly, BDF_1

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Table III. Effect of Treatment with Methyl 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboximidate (4) or Ethyl 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboximidate (6) on Leukemia-Related Splenomegaly in DBA_2 Mice

drug administered[a]	schedule of administration	dosage, mg/kg	spleen weight,[b] mg
control wo/tumor	qd, day 1, 3, 5, 7, 9, 11[c]		82 ± 11
control w/tumor	qd, day 1, 3, 5, 7, 9, 11		1538 ± 561
ethyl (6)	qd, day 1, 3, 5, 7, 9, 11	1333	tox
	qd, day 1, 3, 5, 7, 9, 11	800	tox
	qd, day 1, 3, 5, 7, 9, 11	480	1257 ± 185
methyl (4)	qd, day 1, 3, 5, 7, 9, 11	480	216 ± 60 ($P < 0.01$)
	bid, day 1, 3, 5, 7, 9, 11[c]	480	tox
	qd, day 1, 3, 5, 7, 9, 11	288	599 ± 133 ($P < 0.02$)
	bid, day 1, 3, 5, 7, 9, 11	288	150 ± 53 ($P < 0.005$)

[a] All solutions were delivered ip (0.01 mL/g mouse weight). Control mice were injected with a 0.9% solution of NaCl. [b] Spleens were collected and weighed ~24 h after the last treatment. Statistical inference was by the Student's t test. [c] qd = once a day; bid = twice a day.

female mice (~18 g) purchased from the Charles River Co. were inoculated ip on day 0 with 1×10^6 L1210 cells and treatment by ip bolus injection was initiated 24 h later. Friend leukemia evaluations were performed similarly except female DBA_2 mice were inoculated with 0.2 mL of a 1:9 spleen homogenate formed by mincing spleen fragments in TC199. Drugs, solubilized in water immediately before use, were delivered in uniform volumes of 0.01 mL/g mouse weight. Control mice received equivalent volumes of a 0.9% solution of NaCl.

The incidence of drug- or leukemia-related deaths, the post-inoculation life span of mice that died, and the drug modulation of leukemia-induced splenomegaly were the end points by which responses to treatment were gauged. Temporal patterns of death and observations at necropsy examination were the major criteria for assigning deaths to leukemia or drug toxicity. Inoculum response data were used to calculate the body burdens of leukemia cells that survived treatment.

2′,3′,5′-Tri-*O*-acetyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (2). A suspension of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (28.4 g, 116.4 mmol) in acetic anhydride (200 mL) and pyridine (50 mL) was stirred at room temperature overnight. The resulting clear solution was concentrated in vacuo to yield a clear foam (43.1 g, quantitative). This foam was homogeneous on TLC and used directly for the next step without purification. A small amount was purified by flash chromatography to yield an analytical sample; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.01, 2.08, 2.09 (3 s, 9 H, $COCH_3$), 4.10 (m, 1 H), 3.52 (m, 2 H), 5.58 (t, 1 H), 5.66 (m, 1 H), 6.33 (d, 1 H, J = 3.0 Hz, C_1H), 7.73, 7.92 (2 s, 2 H, $CONH_2$), 8.86 (s, 1 H, C_5H triazole). Anal. ($C_{14}H_{18}N_4O_8$) C, H, N.

3-Cyano-2′,3′,5′-tri-*O*-acetyl-1-β-D-ribofuranosyl-1,2,4-triazole (3). To a solution of **2** (43.1 g, 116.4 mmol) in chloroform (500 mL) was added triethylamine (244 mL) and the mixture cooled to 0 °C in an ice–salt bath. Phosphorus oxychloride (30.7 mL, 330 mmol) was added dropwise with stirring and the solution allowed to warm to room temperature. After the mixture was stirred at room temperature for 1 h, TLC (hexane/acetone 3:1) indicated complete disappearance of starting material. The brown reaction mixture was concentrated to dryness in vacuo and the residue dissolved in chloroform (500 mL). This organic solution was washed with saturated aqueous sodium bicarbonate (3 × 200 mL), dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was chromatographed over silica gel (flash chromatography) with 20% acetone in hexane to yield 33.14 g (81% from ribavirin) of pure **3** as an amorphous solid. This solid was identical in all respects with an authentic sample:[8] mp 101–103 °C; IR (potassium bromide) ν 2250 (CN), 1750 (C=O), cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.04, 2.06, 2.07 (3 s, 9 H, acetyl methyls), 4.15 (dd, 1 H), 4.40 (m, 1 H), 5.47 (t, 1 H), 5.63 (dd, 1 H), 5.95 (d, 1 H, J = 3.2 Hz, C_1H), 8.34 (s, 1 H, C_5H triazole).

Methyl 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboximidate (4). To a suspension of **3** (8.7 g, 24.7 mmol) in methanol (100 mL) was added a molar solution of freshly prepared methanolic sodium methoxide (25 mL) and the mixture stirred at room temperature overnight. The clear colorless solution was treated with methanol washed Dowex H^+ resin till the pH of the solution was 4. The resin was filtered, and the filtrate was adsorbed on silica gel. It was then loaded on a silica gel column and chromatographed (flash chromatography) with 5% methanol in dichloromethane as eluent to yield pure **4** (4.0 g, 63.2%) as an amorphous solid: mp 150–153 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.82 (s, 3 H, OCH_3), 5.8 (d, 1 H, J = 3.87 Hz, C_1H), 8.82 (s, 1 H, C_5H triazole), 8.93 (s, 1 H, imidate NH), and other sugar protons. Anal. ($C_9H_{14}N_4O_5$) C, H, N.

1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamidine Hydrochloride (5). To a suspension of **3** (4.0 g, 11.4 mmol) in methanol (100 mL) was added a molar solution of methanolic sodium methoxide (12 mL) and the mixture stirred at room temperature overnight. The solution was acidified to pH 4 with methanol washed Dowex H^+ resin, the resin was filtered, and the filtrate was concentrated to dryness in vacuo. The residue was dissolved in a minimum amount of methanol (15 mL) and transferred to a pressure bottle. Ammonium chloride (0.61 g, 11.4 mmol) and a solution of methanol saturated at 0 °C with dry ammonia gas (75 mL) were added, the bottle was sealed, and the solution was stirred at room temperature overnight. The solution was concentrated to dryness in vacuo and the resulting residue crystallized from acetonitrile/ethanol to yield **5** as a crystalline solid (2.95 g, 93%). This sample was identical in all respects with an authentic sample.[8]

Ethyl 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboximidate (6). To a suspension of **3** (10.0 g, 28.4 mmol) in ethanol (100 mL) was added a 0.5 M solution of freshly prepared ethanolic sodium ethoxide (20 mL) and the mixture stirred at room temperature overnight. The clear colorless solution was acidified with ethanol washed Dowex H^+ resin to pH 5. The resin was filtered, and the filtrate was adsorbed on silica gel. It was then loaded on a silica gel column and chromatographed (flash chromatography) with 5% ethanol in dichloromethane as eluent to yield pure **6** (5.0 g, 64.9%) as a foam: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.31 (t, 3 H, CH_2CH_3), 5.8 (d, 1 H, J = 3.87, C_1H), 8.75 (s, 1 H, C_5H triazole), 8.90 (s, 1 H, imidate NH), and other sugar protons. Anal. ($C_{10}H_{16}N_4O_5 \cdot 0.5H_2O$) C, H, N.

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