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Antiviral activities of novel bridgehead C-nucleosides

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As a part of our program to develop chemotherapeutically useful analogs of the naturally occurring purines, with particular reference to the antiviral agents, the synthesis of several new C-nucleosides possessing bridgehead nitrogen has been accomplished. They include 3-β-D-ribofuranosyl-s-triazolo(4,3-a)pyrimidine (I) and 2-β-D-ribofuranosyl-s-triazolo(1,5-a)pyrimidine (II) basic systems which could be potential analogs of the antibiotics formycin A and B and their methylated derivatives (Fig. 1) [1, 2]. A major difference in structure between studied analogs and formycin is located at position 5 where bridgehead nitrogen is introduced; moreover, the NH function in pyrazole ring of analogs is lost, thus excluding the possibility of hydrogen bonding of the Watson–Crick type. Also, the pyrimidine part of the fused triazolo(4,3-a)pyrimidine in I does not possess the appropriate functions resembling formycin. However, it is known that isosteric 5,7-dimethylpyrazolo(1,5-a)pyrimidines act as potential inhibitors of 3′,5′-cyclic-AMP phosphodiesterase [3]. Therefore we suggest that the model compounds I and II might be useful for studying their substrate specificity in the antiviral test systems [4–7]. Furthermore, it has been proposed that the highly selective antiviral properties of some analogs, such as ara-adenine and ribavirin derivatives, can be attributed to their stability in the high anti-conformation [7]. In order to achieve such high anti-conformation with the compounds studied, a bulky group was introduced in I at peri position to the ribose moiety in fused aromatic heterocycle.

In the present report we described the antiviral effect of C-nucleosides with bridgehead nitrogen against some DNA and RNA containing viruses grown in mammalian cell cultures and their effects on the synthesis of cellular nucleic acids. This study was directed toward determination of the selective antiviral effects of analogs in relation to their conformation around the glycosidic bond.

The synthesis of C-nucleosides I and II whose structures are shown in Fig. 1, has been carried out as follows. Compound I (5,7-dimethyl-3-β-D-furanosyl-s-triazolo[4,3-a]pyrimidine) was prepared when 2-chloro-4,6-dimethyl pyrimidine was treated with 5-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl) (2H)-tetrazol in refluxing toluene for 30 hr and the isolated protected nucleoside was deblocked in NaOMe/MeOH solution. 5,7-Dimethyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine (II) was isolated when deblocking procedure was performed in methanolic ammonia. The ^{1}H n.m.r. and Nuclear Overhauser Enhancements (NOE) data for these analogs have been determined. 5,7-Dimethyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine: ^{1}H n.m.r. (deuterium oxide) δ-HMDS (capillary)—2.66 (s, 3, CH_3 [7]), 2.77 (s, 3, CH_3 [5]), 3.93 (m, 2, H2′H H5′), 4.20 (m, 1, H4′), 4.3–4.5 (m, 2, H2′H3′), 5.11 (d, 1, H1′), 7.15 (s, 1, H6) NOE $f_{H1'}$(CH_3 [5] saturated) = 0.

5,7-Dimethyl-3-β-D-ribofuranosyl-s-triazolo[4,3-a]-pyrimidine: ^{1}H n.m.r. (deuterium oxide) δ_{HMDS} (capillary)—2.53 (s, 3, CH_3 [7]), 2.88 (s, 3, CH_3 [5]), 3.68 (m, 2, H5′H5″), 4.16 (m, 1, H4′), 4.36 (dd, 1, H3′), 5.01 (dd, 1, H2′), 5.42 (d, 1, H1′), 6.92 (s, 1, H6) NOE $f_{H1'}$ (CH_3[5] saturated) = 0.17.

7-Methyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine: ^{1}H n.m.r. (deuterium oxide) δ_{TMS} (capillary)—8.39 (d, 1, H5, J_{56} = 7 Hz), 6.85 (d, 1, H6), 4.70 (d, 1, H1′, $J_{1'2'}$ = 5Hz), 3.73–4.08 (m, 3, H2′, H3′, H4′), 3.53 (m, 2, H5′H5″), 2.25 (s, 3, CH_3).

5-Methyl-3-β-D-ribofuranosyl-s-triazolo[4,3-a]pyrimidine: ^{1}H n.m.r. (deuterium oxide) δ_{TMS} (capillary)—2.64 (s, 3, CH_3), 3.36 (m, 2, H5′H5″), 3.84 (dd, 1, H4′), 4.04 (dd, 1, H3′), 4.66 (dd, 1, H2′), 5.07 (d, 1, H1′, $J_{1'2'}$ = 6 Hz), 6.58 (d, 1, H6), 8.06 (d, 1, H7, J_{67} = 4.5 Hz).

Additional ^{13}C n.m.r., NOE, and relaxation data for C-nucleosides synthesized will be published elsewhere.

The experiments reported in this paper were performed

with monolayers of mouse fibroblasts (L_{929} cells), KB cells and HeLa cells. The cells were maintained in Minimum Essential Medium supplemented with 5% (v/v) fetal bovine serum at 37° in 5% CO_2. Herpes simplex virus and mengovirus were grown in KB and L_{929} cells, respectively, and assayed as previously described [8]. The antiviral activity of the drugs was measured by their ability to reduce cytopathogenicity of either virus and was evaluated by the virus rating ("VR") method according to Sidwell and Huffman [9]; the virus rating (VR > 0.9) is usually indicative of definitive antiviral activity, whereas VR of 0.5 to 0.9 indicates moderate or questionable activity.

The effect of a series of C-nucleosides with bridgehead nitrogen on herpes simplex virus-induced cytopathogenicity in KB cells is presented in Table 1. Of the compounds tested only II exerts a certain antiviral activity ("VR" = 0.5) whereas the others show little or no activity as compared to effective ribavirin ("VR" = 1.1). Compound II reduces cytopathogenicity of virus at concentrations of 320–1000 μg/ml which are not toxic for uninfected KB cells, as determined by the cytotoxicity test [9]. The antiviral activity of these compounds against mengovirus is also shown in Table 1. Compound II shows moderate activity ("VR" = 0.70) in contrast to I which is not active ("VR" = 0.1). Other compounds listed in Table 1 can be classified as low-active or inactive in reducing cytopathogenicity of mengovirus. Contrary to the very active ribavirin ("VR" = 1.0) which was used as a reference antiviral substance, its 5-methyl derivate is quite inactive ("VR" = 0.1).

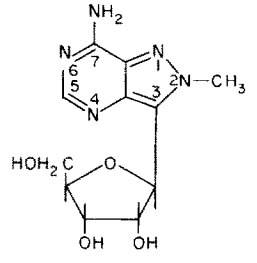
The effect of II and I on the growth of mengovirus is shown in Table 2. Compound II is more effective than I in inhibiting mengovirus growth, and at the concentration of 500 μg/ml it depresses the virus growth by about 90 per cent as compared to untreated control. The data presented in Table 2 show that the growth of herpes virus, a DNA-virus which replicates in the nucleus, is not significantly supressed by 500 μg/ml of II, and is not affected at all by the same concentration of I.

Under these experimental conditions these analogs did not affect the cell multiplication and the synthesis of DNA and RNA of KB or HeLa cells. The synthesis of cellular macromolecules was measured in the cell cultures pretreated with either compound for 24 hr, by labeling with a 30-min pulse of ^{3}H-guanosine (3 μCi/ml; Radiochemical Centre, Amersham, Bucks, U.K.). At a concentration of 1000 μg per ml, however, II inhibits cell growth, but this inhibition becomes apparent only after a 24 hr period of incubation with the drug; unlike II, I did not affect the cell growth even at very high concentrations.

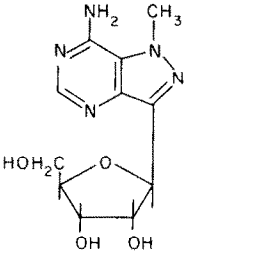
The data presented in this paper indicate that of a series of C-nucleosides with bridgehead nitrogen synthesized, compound II has the most favorable antiviral activity against both herpes simplex and mengovirus infections in cell cultures, a phenomenon which is associated with very low cytotoxicity.

Furthermore, our data suggest that specific conformation of studied analogs is of great importance for their selective antiviral effects. For instance, I shows a great diminuition of the antiviral activity as compared to that of II, which may be attributed to methyl groups at position 5 near bridgehead nitrogen, and in a peri position relative to the ribose moiety; this precludes the rotation around glycosidic bond as seen from the CPI space-filling molecular model. This fact was already determined by ^{1}H and ^{13}C n.m.r. investigation of compounds I and II in solution, and the rigid anti-conformation has been established by NOE (Nuclear Overhauser Enhancements) and ^{1}H and ^{13}C spin–lattice relaxation measurements. In contrast, 5,7-dimethyl-2-β-D-ribofuranosyl-s-triazolo[1.5-a]pyrimidine (II) was deduced as a flexible rotating nucleoside model compound which we think is associated with its moderate antiviral activity.

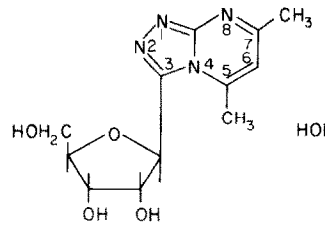
To obtain more information regarding the above-mentioned structural phenomena, the synthesis of monomethyl derivatives has been approached; only 5-methyl-3-β-D-ribofuranosyl-s-triazolo[4.3-a]pyrimidine has been isolated. Since this compound did not show any antiviral activity



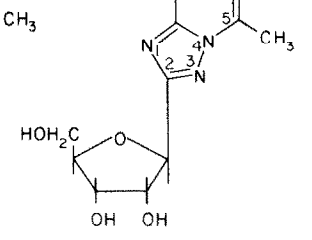
N_2 – methyl formycin A



N_1 – methyl formycin A



5,7-dimethyl-3-β-D-ribofuranosyl -s-triazolo (4,3-a) pyrimidine (I)



5,7-dimethyl-2-β-D-ribofuranosyl -s-triazolo (1,5-a) pyrimidine (II)

Fig. 1. Structures of C-nucleosides with bridgehead nitrogen, analogs of methyl formycin.

Table 1. Comparative antiviral effect of synthetic nucleosides on herpes simplex virus and mengovirus

	Virus rating (VR)	
	Herpes simplex virus‡	Mengo virus§
5,7-Dimethyl-3-β-D-ribofuranosyl-s-triazolo[4,3-a]pyrimidine (I)	0.1	0.1
5-Methyl-3-β-D-ribofuranosyl-s-triazolo[4,3-a]pyrimidine	< 0.1	0
5,7-Dimethyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine (II)	0.5	0.7
7-Methyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine	0.3	0.4
1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin)	1.1	1.0
*5-Methyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide	0.1	0
†3,5-Dimethyl-1-β-D-ribofuranosyl-1,2,4-triazole	0.1	0

*Ref. 11, †Ref. 12.
‡ KB-cells were infected with herpes simplex virus (100 × $CCID_{50}$‖) and exposed to different concentrations of the test compounds followed by incubation for 2 days. At the end of the period of incubation the reduction of CPE was evaluated by the "virus rating" method.
§ L-cells were infected with 100 × $CCID_{50}$‖ mengovirus and exposed to different concentrations of test compounds for 2 days. The reduction of CPE was evaluated by the "virus rating" method.
‖ Cell culture infecting dose 50 (infecting 50 per cent of the cell cultures).

Table 2. Effect of I and II on mengovirus and herpes simplex virus growth

	Mengo virus*	Herpes simplex virus†
	titer (log_{10} PFU/ml)	
Control: 0 hr	3.23	3.48
Control: 24 hr	8.08	8.51
5,7-Dimethyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine (II)		
200 μg/ml	7.40	8.45
500 μg/ml	6.95	8.28
5,7-Dimethyl-3-β-D-ribofuranosyl-s-triazolo[4,3-a]pyrimidine (I)		
200 μg/ml	7.85	8.46
500 μg/ml	7.28	8.43

*L-cells.
† KB-cells.
Cell monolayers (1×10^5 cells per 35-mm Petri dish) were washed with phosphate-buffered saline and incubated with either virus at a multiplicity of 10 PFU per cell. At the end of the period of adsorption the monolayers were washed 3 times with culture medium and one plate was frozen as zero-time control. The other dishes received 2 ml of complete medium containing either II or I as indicated. After 24 hr of incubation all cultures were frozen and thawed 3 times and virus titrations were performed [8].

(VR < 0.1), it gave additional support to the former hypothesis that the flexibility of the glycosidic bond is an important parameter in determining the biological activity of the drug. This compound has the 5-methyl group at the peri position pushing ribose moiety to the anti position as in I. The data for 7-methyl-2-β-D-ribofuranosyl-s-triazolo [1,5-a]pyrimidine, on the other hand, are consistent with previous considerations, since it mimics II, lacking one methyl group and not affecting the rotation about the glycosidic bond.

It was found by Witkowski *et al.* [5], that none of the ribavirin analogs substituted at position 5 did exhibit any favorable antiviral activity as compared to the parent 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin). Theoretical studies [10] ascribe this inactivity to steric reasons, which prohibit the high anti-region required as a biologically important conformation specified by the enzyme IMP dehydrogenase. Therefore, it was of interest to include 5-methyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide [11] as well as 3,5-dimethyl-1-β-D-ribofuranosyl-1,2,4-triazole [12] as reference compounds to our study test. It was concluded that methyl group at position 5 in a triazole ring hindered the rotation of the ribose moiety from ^1H and ^{13}C n.m.r. relaxation and NOE data, which precluded the formation of the active high anti-conformation and is in complete agreement to the theoretical calculations [10]; the antiviral activity of 5-methyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide has been determined as zero.

We conclude that of the compounds synthesized 5,7-dimethyl-2-β-D-ribofuranosyl-s-triazolo[1,5-a]pyrimidine has the most favorable inhibitory effects on both herpes simplex virus and mengovirus. In contrast, 5,7-dimethyl-3-β-D-ribofuranosyl-s-triazolo[4,3-a]pyrimidine is ineffective in either antiviral test system, suggesting that the flexibility of the glycosidic bond is an important parameter in determining the biological activity of the drug.

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High benzo[a]pyrene hydroxylase activity in the marine fish *Stenotomus versicolor*

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Hepatic microsomal cytochrome P-450 systems in fish are generally like those in mammals, with cytochromes P-450 having similar optical and electron paramagnetic resonance (e.p.r.) spectra and catalytic functions [1, 2]. Hepatic mixed-function oxygenase activities in fish are, however, often low when compared to those in mammals [3, 4]. Fish mixed-function oxygenases, notably aryl hydrocarbon (benzo[a]pyrene; BP) hydroxylase, can be experimentally induced by treatment with known organic contaminants [5, 6], and it is also possible for such induction to occur in the environment [6].

In order to define relationships between the chemical environment and cytochrome P-450 systems in fish, it is necessary to understand these systems in untreated or uninduced animals. In some untreated, freshwater brown trout (*Salmo trutta*), for example, the activity of hepatic microsomal BP hydroxylase was found to be much greater than that observed in mammals [7, 8]. However, the trout used in this study were all held for some period in captivity, which could introduce factors that might influence hepatic mixed-function oxygenases.

There have been no reports dealing with such high BP hydroxylase in marine fish. This communication describes some characteristics of hepatic microsomal mixed-function oxygenase activities in *Stenotomus versicolor* (scup or porgy), a marine teleost with high BP hydroxylase activity, and compares these and other features of microsomal electron transport systems to those in the mouse.

BP and 7,8-benzoflavone (7,8-BF) were obtained from the Aldrich Chemical Co, Milwaukee, WI. All other cofactors, coenzymes and substrates were obtained from the Sigma Chemical Co., St. Louis, MO.

Adult male and female scup (*S. versicolor*), about 100–200 g, were collected by angling in Great Harbor, Woods Hole, MA, or outer Hadley Harbor, Gosnold, MA, in June–August, 1975–1978. Fish were either used within 1 hr of capture, or held for periods of 2 weeeks to 8 months in 800-gal tanks at the National Marine Fisheries Service, Woods Hole, MA. Fish held were fed a diet of chopped smelt and clams, *ad lib.*, every 2 days. Tanks were equipped with flow-through water at 19 ± 1°, filtered through gravel and sand. All fish were killed outside the spawning season. Mice (*Mus musculus*) were 90-day-old adult Charles River CD-1 females of 23–26 g, maintained at the animal facilities of the Marine Biological Laboratory, Woods Hole, MA. Mice were fed Charles River diet and Purina Lab Chow, and maintained on Absorb-Dri and Bedda-Chip hardwood bedding at a density of 7/ft^2.

Animals were killed by cervical dislocation (mice) or decapitation (scup) and excised livers were placed immediately in ice-cold 0.1 M phosphate buffer, pH 7.3. Tissues were minced and homogenized in 4 vol. of 0.1 M phosphate buffer, pH 7.3, containing 1.15% KCl and 3 mM $MgCl_2$, using a Potter–Elvehjem tissue grinder with four passes of the pestle at 1350 and four passes at 1900 rev/min. Microsomal preparations were isolated from the 9000 *g* supernatant fraction by centrifuging for 90 min at 40,000 *g* and resuspended in 3 vol. of 0.1 M phosphate buffer, pH 7.3, per g of liver.*

NADPH-cytochrome *c* (cytochrome P-450) reductase (EC 1.6.2.4) was assayed at 25° (scup) or 37° (mouse) by a modification of the method of Phillips and Langdon [9], with a reaction mixture containing 0.175 mM NADPH and 80 μM horse heart cytochrome *c* in 0.2 M potassium phosphate buffer, pH 7.7. NADH-cytochrome *c* (cytochrome b_5) reductase (EC 1.6.2.2) was assayed using the conditions for NADPH-cytochrome *c* reductase, with 0.25 mM NADH replacing 0.175 mM NADPH. Reduction of cytochrome *c* was followed at 550 nm and reference cuvettes in both cases contained reaction mixtures with no enzyme.

BP hydroxylase was assayed using 0.5-ml reaction mixtures containing an NADPH-generating system described

*Cytochrome P-450 was undetectable in the 40,000 *g* supernatant fraction. Relative specific activities of BP hydroxylase in microsomal and 40,000 *g* supernatant fractions were 5.5 and 0.1 respectively. Relative specific activities of AP demethylase were 6.3 and 0.05 respectively.