

C-NUCLEOSIDES: ASPECTS OF CHEMISTRY AND MODE OF ACTION

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ABSTRACT

Among the relatively few naturally occurring nucleosides, which contain ribose bound to a carbon atom of the heterocyclic aglycone, some—the formycins, oxazinomycin, pyrazomycin, and showdomycin—appear to act as *antagonists* to essential metabolites, whereas others—pseudo-uridine, also formycin A—appear to resemble their respective counterparts—uridine, adenosine—to such degree as to be capable of functioning as metabolic *substitutes*. Recent studies have focused on the effects of C-nucleosides on ribonucleotide reductase and purine ribonucleoside phosphorylase, on the mode of action of C-nucleosides and on their biosynthesis. The properties of polymers and co-polymers of formycin have been interpreted in terms of the relative contributions of the 'syn-' and 'anti-' conformations of this C-nucleoside (Ward, Reich).

The observation of specific reversal of anti-viral activity of pyrazomycin by uridine alone points to pyrimidine biosynthetic pathways as the possible vulnerable site for growth inhibition. Support for this notion derives from experiments with orotidylic decarboxylase of rat liver, 50 per cent inhibition of activity being observed at levels of 5×10^{-8} M pyrazomycin-5'-phosphate. The antimicrobial activity of oxazinomycin (Haneishi *et al.*, Sankyo Ltd., Tokyo) was efficiently reversed only by deoxycytidine, perhaps suggesting nucleotide reductase as site of action. The synthesis of showdomycin and of oxoformycin has recently been accomplished (F. Sorm *et al.*, Prague), while 'pseudo-azauridine' has been obtained by conversion of pseudo-uridine (Sorm).

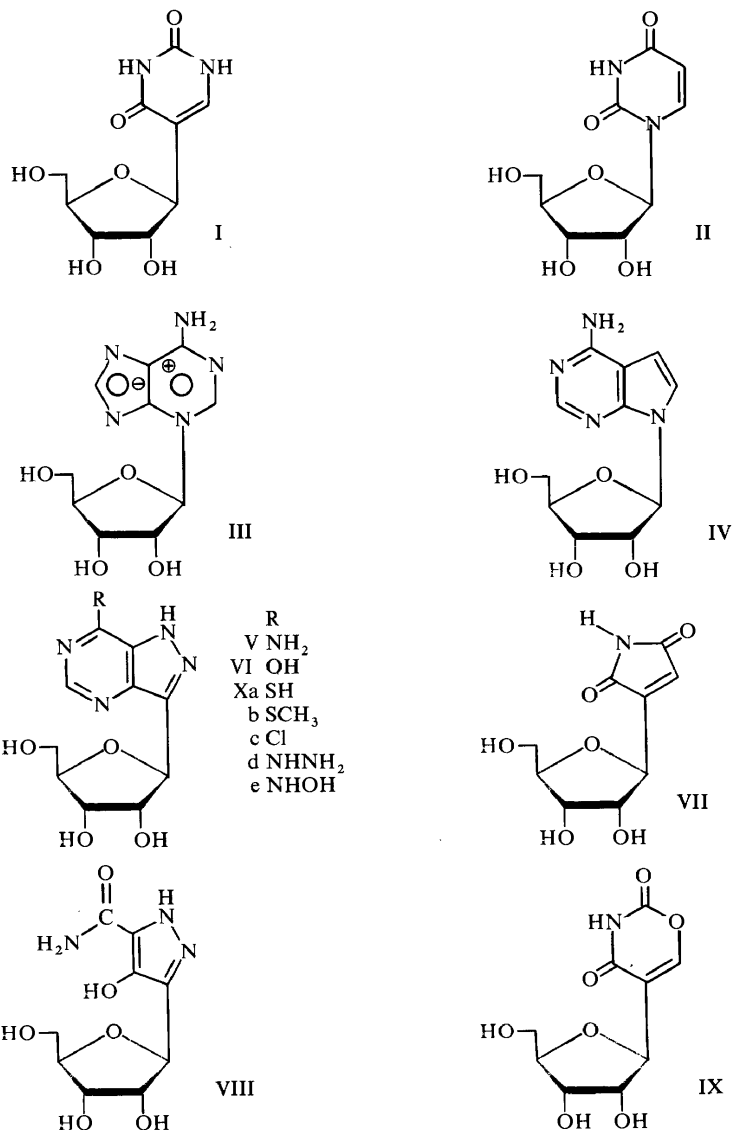
The agricultural potential of formycin B against rice plant disease has been noted but no medical usefulness has thus far been demonstrated for members of the C-nucleoside class. The preparation of additional C-nucleosides by synthesis or by modification of naturally available materials, together with a detailed understanding of structure-activity relationships, hopefully will assist in the realization of the therapeutic potential of this versatile class of agents.

The first carbon-linked nucleoside, pseudo-uridine (I)^{1,2}, discovered in 1959, resembles uridine (II), in its shape and in the disposition of its functional hydrogen bonding sites, to such a degree that it can efficiently substitute for this metabolite in enzymic conversion at the nucleotide³ as well as at the polynucleotide level⁴. Its failure to support the growth of the uracil-*E.coli* mutant W 3362⁵ is presumably due to stability of its ribose-to-carbon linkage and represents a rare instance in which pseudo-uridine is not capable of replacing uridine.

When reflecting on the comparison and resemblance of molecules, the

near-identity of the pair uridine-pseudouridine in biochemical conversions can be envisaged, but not necessarily predicted, from an inspection of their structural formulae.

On the other hand, 3-iso-adenosine (III)⁶ and 7-deaza-adenosine (tuber-cidin) (IV)⁷, nucleosides with structures closely resembling that of adenosine,



are cytotoxic toward mammalian cells⁶ and thus in this system appear to act as antagonists rather than as substitutes.

Among C-nucleoside antibiotics⁸⁻¹⁴, formycin A⁸ (V) and B⁹ (VI), show-

domycin¹⁰(VII), pyrazomycin¹¹(VIII) and oxazinomycin¹²(IX), modification products¹³(X) and synthetic analogues¹⁴, the type of biological activity encountered varies from near-perfect substitution (formycin A for adenosine¹⁵) to powerful antagonism (inhibition of murine tumours by formycin⁸). The pattern of inhibition shown by showdomycin (VII) is complex; this antibiotic acts both as a uridine antagonist¹⁷ and as a powerful SH-reactor^{17, 18}.

Detection of molecular resemblance between metabolite and inhibitor at the enzyme site has been approached in our laboratories by a study of reversal of anti-viral activity *in vitro*. Replicating virus growing in tissue culture presents an efficient, informative (*in vivo*) system which avoids the complexities—e.g. absorption and metabolism—of animal studies and is more meaningful than *in vitro* enzyme studies alone.

In this manner we have studied viral inhibition-reversal patterns of the fermentation product mycophenolic acid¹⁹(XI), an antibiotic of low toxicity recently shown to inhibit growth of several murine tumours²⁰, of the C-nucleoside pyrazomycin (VIII), and of a small number of known anti-metabolites with established mode of action.

Recent surveys²¹ dealing with C-nucleoside research include a penetrating discussion of the relationship between nucleoside conformation and biological activity by Ward and Reich^{21a}. Suhadolnik has thoroughly reviewed the pertinent literature up to 1970^{21b}. Mention will be made, below, of reports that have appeared since that date.

VIRAL INHIBITION AND REVERSAL BY METABOLITES

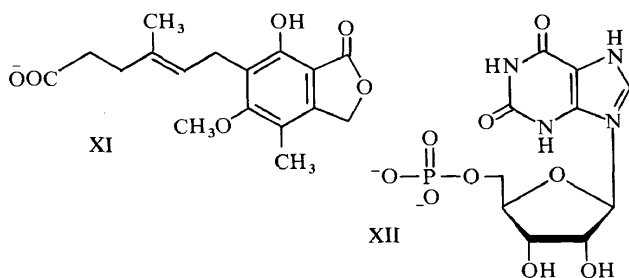
Briefly, the agar-diffusion method²² used in this work consists of a monolayer of BS-C-1 cells infected with virus and an overlay of agar on which small filter paper discs impregnated with the inhibitor substances are applied. Following incubation at 37° for 72 hours, the virus is inactivated, the surviving cells fixed to the glass and stained. The cytopathological and cytotoxic effects are estimated by visual and microscopic inspection. Plaque reduction, virus multiplication and cellular competence can also be estimated.

For reversal studies the inhibitory substance is incorporated in the agar overlay at concentrations affording essentially complete protection. Solutions of metabolites applied to paper discs reveal reversal of anti-viral action as zones of virus break-through.

Reduction of vaccinia virus plaque count was noted with a fermentation broth subsequently shown to contain mycophenolic acid²². The cytoprotective effect of 10 µg/ml of mycophenolic acid was effectively reversed by guanosine, guanylic acid, and deoxyguanylic acid (Ref. 22, Table 1, Figures 2 and 3). Guanine, due perhaps to its low solubility, was less effective, while xanthosine, inosine, and other purine or pyrimidine nucleosides were ineffective as reversal agents.

This surprising observation of reversal by guanine species raises the intriguing notion of a close molecular resemblance of the carboxylic acid (XI), probably as the anion, to a guanine related nucleotide, e.g. xanthylic acid (XII). Such resemblance, although not readily predicted on the basis

of structural formulae (XI and XII), is conceivable, nevertheless, in terms of similarity of fit of the three-dimensional molecules to a receptor site.



Guided by the observation of reversal by guanosine, guanylic synthetase, isolated from susceptible murine tumours, was in fact inhibited by mycophenolic acid²³. Investigators elsewhere independently showed that inosinic dehydrogenase, also, is inhibited by XI²⁴.

Though these findings strongly support the existence of a direct metabolite-antagonist relationship for the pair XI-XII, the reality of such a relationship and of the underlying molecular resemblance remains to be proved. If correct, perhaps XI itself could be viewed as a C-nucleoside type of anti-metabolite? A molecular resemblance between the anti-fungal agent griseofulvin and adenosine has been mentioned previously as a basis for inhibition and reversal phenomena observed²⁵.

To the cancer chemotherapist the notion of a purine antagonist (XI) which itself is not a purine derivative is an attractive one. A carboxylic acid rather than a phosphate, conversion of mycophenolic acid (XI) to a triphosphate stage and incorporation into nucleic acid, a mode of action often noted with nucleoside antagonists²⁶, is constitutionally precluded.

For these and other reasons the search for and the design of molecules which, in spite of a lack of evident similarity, behave as antagonist (as does XI) to a critical metabolite, present a challenge of considerable importance.

PYRAZOMYCIN-5'-PHOSPHATE—OROTIDYLIC ACID PAIR

In the course of screening fermentation broths for anti-viral activity, significant growth inhibition was noted by a culture of *Streptomyces candidus*. Inhibition of vaccinia virus¹⁶ by the active component, pyrazomycin¹¹, a crystalline, water-soluble compound of composition C₉H₁₃N₃O₆ was found to be reversed specifically by uridine (II) and by uridylic acid but not by cytidine. A structure related to uridine was a logical proposal. Among the limited number of possible structures that of the C-nucleoside, 3(5)-β-D-ribofuranosyl-4-hydroxy-pyrazole-5(3)-carboxamide (VIII), was favoured. This structure was confirmed by physical studies (H₁ occurs at 5 p.p.m. in the n.m.r. spectrum) and by chemical degradation¹¹.

Using the method described above, reversal studies with a small number of known anti-metabolites (see Table 1) demonstrate that the respective

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Table 1. Specificity of reversal of *in vitro* anti-viral activity

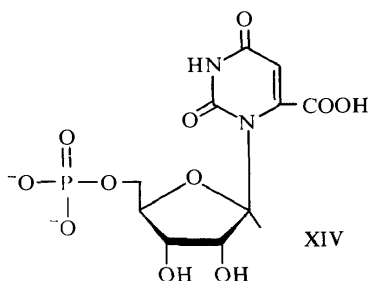
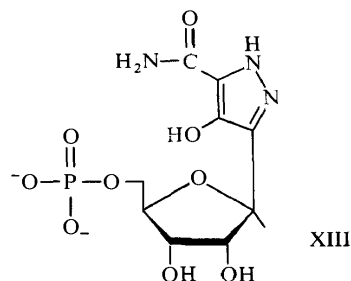
Virus inhibitor	Reversing agent
Iododeoxyuridine ²⁸	Thymidine
Cytosine arabinoside ²⁹	Deoxycytidine
6-Azauridine ³⁰	Uridine, cytidine
Mycophenolic acid	Guanine species
Pyrazomycin	Uridine

reversing agents are compatible in each case with the known mode of action²⁷ of the inhibitor²⁸⁻³⁰.

These findings (Table 1) testify to the accuracy and dependability of *in vitro* reversal studies as an aid in structure determination as well as in pinpointing probable sites of enzymatic vulnerability.

In this manner, uridine reversal of pyrazomycin activity points to orotidylic decarboxylase inhibition as a probable mode of action. Pyrazomycin-5'-phosphate (XIII), prepared from pyrazomycin and *p*-nitro-phenylphosphate with the aid of carrot nucleoside phosphotransferase³¹, was shown to be a powerful inhibitor of this decarboxylase, 50 per cent reduction of ¹⁴CO₂ release being effected by a concentration of 5×10^{-8} M¹⁶. 6-Azauridylic acid produces a similar inhibition only at twenty-fold higher levels, while pyrazomycin (VIII) itself is essentially without activity.

Presumably, pyrazomycin-5'-phosphate (XIII) and orotidylic acid (XIV) share chemical, physical and spatial characteristics to such an extent that resemblance of molecular architecture is realized for the decarboxylase, a resemblance perhaps envisaged more readily by the medicinal chemist *a posteriori* than recognized *a priori*.



REPORTED OBSERVATIONS ON MODE OF ACTION

Published articles^{17a,32} report that the inhibition of mammalian cell growth by showdomycin (VII) can be reversed by various nucleosides as well as by thiol-containing compounds. A clear resemblance to a particular metabolite remains difficult to define.

An analogue of adenosine, formycin A (V) in the form of its mono-, di-, or tri-phosphate can, on the one hand, efficiently replace adenine-nucleotides

in several enzymatic conversions, including polynucleotide phosphorylase (of *E. coli*)³³ and tRNA-CCA pyrophosphorylase³³. The latter reaction produces functional tRNA in which formycin replaces the terminal adenosine unit.

On the other hand, formycin-5'-phosphate is a potent inhibitor of adenylate synthesis by adenine phosphoribosyltransferase³⁴ of Ehrlich ascites cells. In mammalian cells formycin suppresses synthesis of DNA, RNA, and protein³⁵. Formycin also inhibits certain murine tumours including the leukaemia L1210, influenza A₁ virus and *Xanthomonas oryzae*, the pathogenic bacterium causing disease of rice plants^{8, 36}.

This dualistic behaviour and other characteristics of formycin, especially as a unit of homo- and hetero-polynucleotides, have been related to its unusual conformational features^{21a, 37}. Due to increased bond length of the C—C glycosidic linkage in formycin, compared to the C—N bond in purine nucleosides, the rotational barrier about this linkage is lowered. Consequently, the *syn*-conformation (V) for formycin is favoured to a degree which depends on the nature of the polynucleotide: *syn* (V) in neutral polyformycin and a mixture of *syn* (V) and *anti* (XV) in single-stranded co-polymers^{21a, 37}.



Returning to the theme of this paper, namely the recognition of actual molecular resemblance in biochemical interaction, attention is directed toward a comparison (Ref. 38, *Figure 3*) in which a formycin-3'-phosphate unit (of poly-F) in the *syn*-conformation is superimposed on a 3'-cytidylic unit (of poly-C) in the *anti*-conformation. The near-perfect overlap of functional hydrogen-bonding sites (N⁷ and N¹ of F with N⁴ and N³ of C) signifies a close resemblance between the 'purine'- and the pyrimidine nucleotide. This resemblance serves to explain³⁸ the surprising susceptibility of polyformycin to the transphosphorylation step (yielding 2',3'-cyclic-FMP) of bovine pancreatic ribonuclease A, an enzyme well known for its rigorous specificity for pyrimidine nucleotides (hydrolysed to the 3'-phosphate).

In this instance, the adenosine-analogue formycin A, due to its C-nucleoside structure, reacts as, and resembles, a pyrimidine nucleoside.

Now, while slowly learning certain principles of molecular resemblance from these occasional examples acquired through biochemical or biological observations, the chemotherapist strongly feels the need of learning to predict and design such resemblance of molecules and to express their molecular architecture in terms allowing facile, effective comparison. [An example of unsuspected resemblance mentioned in recent publications is that of the pair

erythromycin and lincomycin^{39,40}]. An inosine analogue, the C-nucleoside formycin B⁹ (VI) is produced from formycin A by the action of adenosine deaminase⁴¹. Presumably acting at the nucleoside level, formycin B inhibits *X. oryzae* by preventing exogenously supplied nucleosides from entering the cell⁴².

The inhibition of *S. aureus* 209B (minimum inhibitory concentration 1.6 µg/ml) by oxazinomycin (IX), is effectively reversed by deoxycytidine and by other pyrimidine- but not by purine-nucleosides¹². Oxazinomycin also inhibits Ehrlich ascites in mice¹². Enzymatic information and mechanistic studies have not been reported.

In summary, C-nucleosides represent an important class of antibiotics due to their close analogy to the normal nucleoside metabolites. The varied effects of C-nucleosides on numerous enzymatic conversions render their further investigation as potential chemotherapeutic agents a worthwhile undertaking.

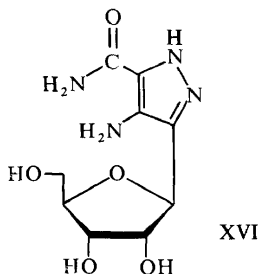
SYNTHESIS AND BIOSYNTHESIS

In a further extension of their nucleoside investigations Šorm, Farkaš and their associates in Prague have achieved a first total synthesis of showdomycin (VII), ozonolysis of suitably protected 1-(β-D-ribofuranosyl)-2,4,6-trimethoxybenzene representing a critical step in producing the methyl ribosyl-glyoxalate required for the construction of the heterocyclic ring^{14c}. The synthesis of 'pseudo-6-azauridine', involving ozonolysis of natural pseudo-uridine, has been reported earlier by Šorm and co-workers^{14d}. In another approach, ribosyl-diazomethane, obtained from 1-cyano-ribose, was allowed to react with diethyl acetylenedicarboxylate yielding a substituted 3-ribosylpyrazole which was converted in four steps to oxoformycin^{14b}, identical to the product obtained by metabolic oxidation of formycin A (V) or B (VI) *in vivo*⁴³.

Several substituted 7-substituted 3-(β-D-ribofuranosylpyrazolo)-[4,3-d]pyrimidines (X) have been prepared from the 7-chloro-analogue (Xc), itself obtained from formycin B (VI)⁴⁴. The 7-hydrazino-(Xd) and the 7-hydroxylamino-(Xe) members of this group differ in their physical properties (u.v. spectra) from these same compounds prepared earlier^{45,46} from the 7-thiol compound (Xa). The interpretation of the biological data of these agents therefore must wait clarification.

Conversion of formycin B to 4-amino-3-(β-D-ribofuranosyl)-pyrazole-5-carboxamide (XVI) by Townsend and his co-workers⁴⁷ makes this amino-analogue of pyrazomycin available for biological testing. The evident significance of this agent stems from its analogy to the purine precursor, aminoimidazole-carboxamide-ribonucleotide (AICAR).

In order to study biosynthetic pathways leading to C-nucleosides, Suhadolnik has begun the investigation of showdomycin (VII) formation by cultures of *S. showdoensis* using ribose-1-¹⁴C and acetate-1- and -2-¹⁴C^{48,49}. Ribose is utilized *in toto*. Acetate serves to form two or perhaps all four carbon atoms of the maleimide-moiety without evidence for the participation of C₄-dicarboxylic acid intermediate of the tricarboxylic acid cycle. Fluoroacetate does not inhibit incorporation of acetate into the maleimide ring system^{48,49}.



No information on the early steps of biosynthesis of the pyrazole-containing C-nucleosides has been published. The terminal steps of formycin biosynthesis by *N. interforma* involve conversion of formycin B-5'-phosphate via 5'-FMP to formycin A⁵⁰.

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