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A pyrimidine–pyrazolone nucleoside chimera with potent in vitro anti-orthopoxvirus activity

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Abstract—Synthetic hybridization of two privileged drug scaffolds, pyrazolone on the one hand and pyrimidine nucleoside on the other, resulted in the generation of two novel 5-substituted pyrimidine nucleosides with potent in vitro antiviral activity against two representative orthopoxviruses, vaccinia virus and cowpox virus.

In 1983, the World Health Organization (WHO) declared smallpox, a disease responsible for more deaths than all the wars of all time, eradicated.[1,2] By 1983, all known stocks of variola virus were in two World Health Organization (WHO) collaborating centers: the US Centers for Disease Control and Prevention (CDC) in Atlanta and (after a transfer in 1994) the Russian State Research Center of Virology and Biotechnology (the Vektor Institute) in Novosibirsk. The WHO Committee on Orthopoxvirus Infections voted on several occasions to recommend destruction of the stocks, but each time the decision was deferred to permit more research on live variola virus.

Meanwhile, intelligence estimates have suggested the possible existence of clandestine stocks of variola and the possibility of bioengineered strains resistant to the classical vaccination protection. When these concerns are combined with the established fact that the present form of smallpox vaccination is the most dangerous vaccination extant in terms of morbidity and mortality, it is patently obvious that novel antiviral agents for this threat be discovered.[3–14] The goal of the US Government is to provide two FDA approved drugs with two more in the development pipeline. Only one drug, cidofovir, is available for treatment of smallpox vaccination complications.[12–14]

To discover novel antivirals for orthopoxvirus infections, we have employed two different ''privileged'' drug scaffolds;[15,16] that is, those molecular frameworks that have spawned a significant number of drugs and other biologically active agents and can be used to discover molecular 'masterkeys.'[17] The first is the nucleoside scaffold that has yielded many valuable therapeutics for HIV and herpes virus infections. The second scaffold is the pyrazolone ring that is the basis of agents with various biological activities including antihyperglycemic properties,[18] anti-tumor necrosis factor activity,[19,20] non-steroidal anti-inflammatory drugs (NSAIDs),[21] inhibition of human telomerase,[22] and antibacterial activity.[23]

Entry to such chimeric structures centered on the 5-position of pyrimidine nucleosides can be gained most readily through the intermediacy of the previously described 5-formyl-2′-deoxyuridine.[24–27] The aldehyde precursor, 3′,5′-di-*O*-acetyl-5-formyl-2′-deoxyuridine (**3**, Scheme 1), was prepared through the oxidation of 3′,5′-di-*O*-acetylthymidine (**2**, Scheme 1) with potassium peroxysulfate ($K_2S_2O_8$) in the presence of $CuSO_4{\cdot}5H_2O$ and 2,6-lutidine in aqueous acetonitrile. The corresponding deacetylated counterpart, 5-formyl-2′-deoxyuridine (**8**, Scheme 2), was prepared by first protecting

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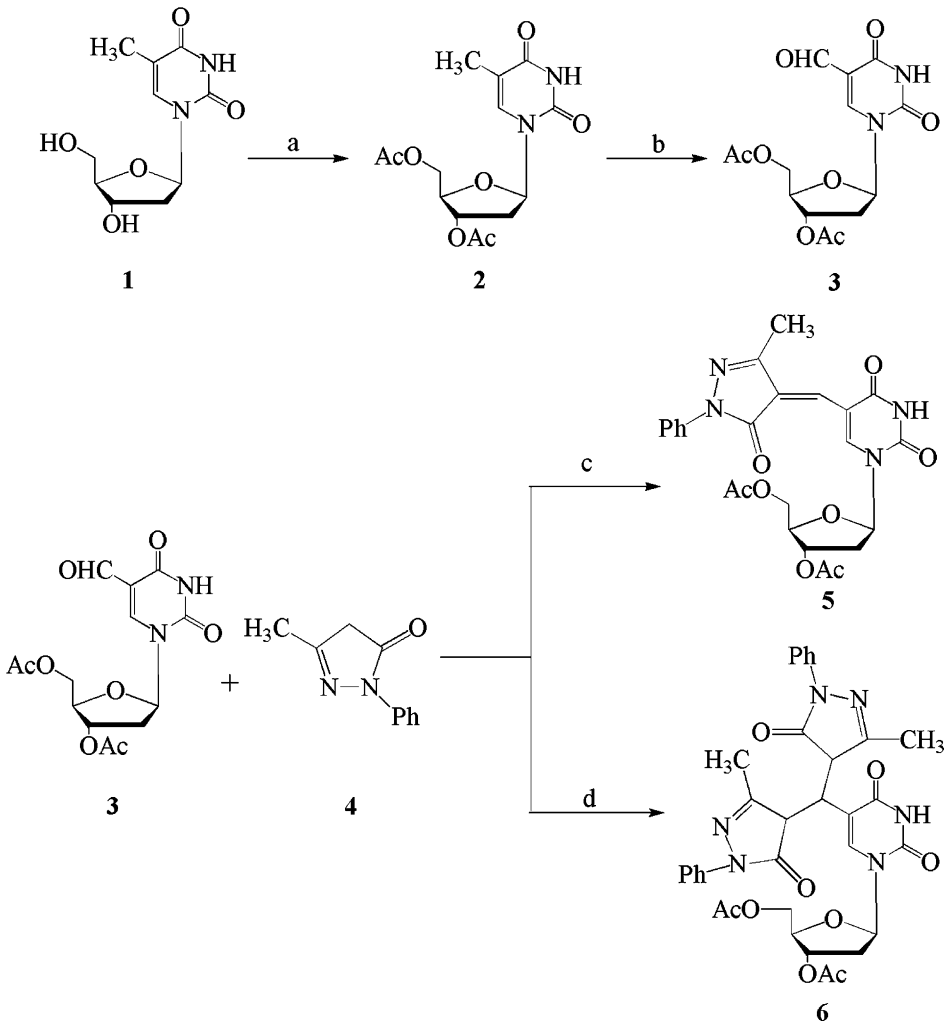
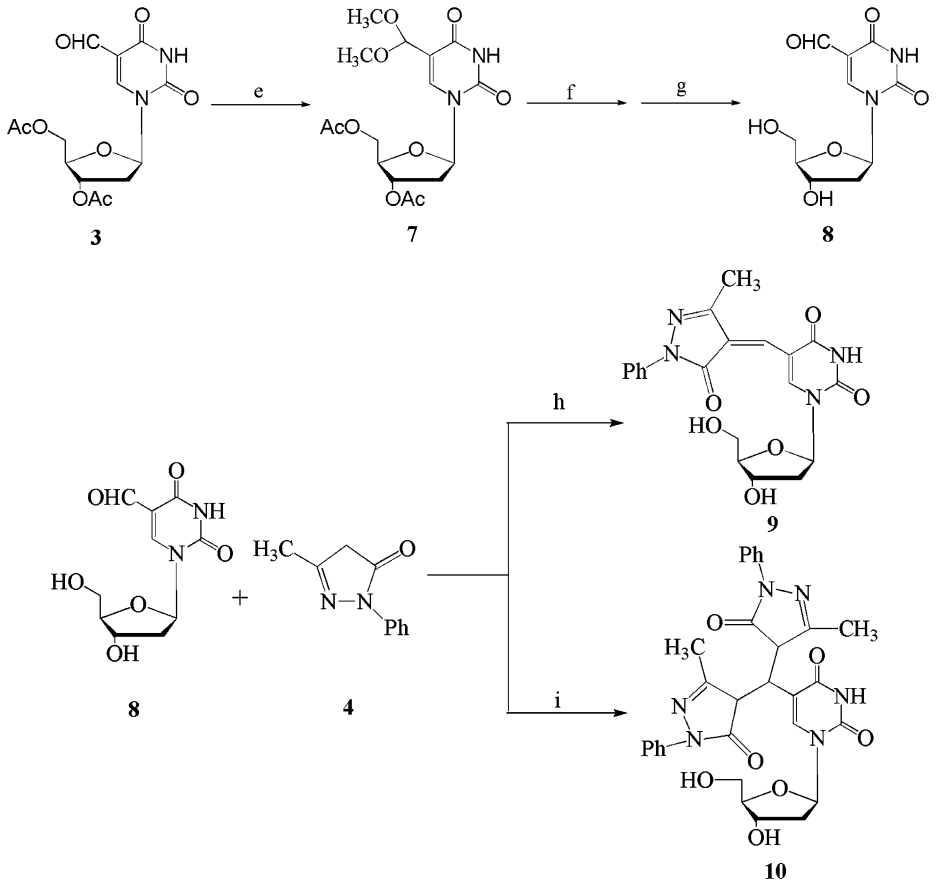


Scheme 1. Reagents and conditions: (a) $(CH_3CO)_2O$, DMAP, rt, overnight, 92%; (b) $K_2S_2O_8$, $CuSO_4{\cdot}5H_2O$, 2,6-lutidine, CH_3CN/H_2O (1:1, v/v), 65 °C, 2 h, 35%; (c) ethanol, rt, overnight, molar ratio of **3** and **4**: 1:1; (d) ethanol, rt, overnight, molar ratio of **3** and **4**: 1:2.5.

the aldehyde group of **3** as the dimethyl acetal (**7**), followed by sequential treatment with NaOMe/MeOH and $AcOH/H_2O$ to give **8**. The pyrazolone derivatives were prepared through the condensation of an appropriate aldehyde (**3**, Scheme 1 or **8**, Scheme 2) with 1-phenyl-3-methyl-2-pyrazolin-5-one (**4**). Condensation product **5**[28] was obtained by stirring a solution of **3** with one equivalent of **4** in ethanol at room temperature overnight (Scheme 1). Compound **6**[28] was obtained from the condensation of **3** with 2.5 equiv of pyrazolone **4** (Scheme 1). The other two pyrazolone derivatives **9**[28] and **10**[28] (Scheme 2) were prepared in a similar manner. Condensation of the pyrimidine aldehyde and the pyrazolone led to a new double bond exocyclic to the pyrazolone ring C2 and in conjugation with the pyrimidine ring. This alkene substructure can exist as two isomers, namely *E* and *Z*. From the ^1H NMR and ^{13}C NMR results, it was clear that only one isomer was formed since there was one signal each for the newly formed vinylic proton and for the pyrimidine H6. The identification of the *Z*-configuration was based on NOE experiments which showed that the vinylic proton exhibited a strong NOE effect with the protons of the pyrazolone 3-methyl group.

In vitro activities (Table 1) were determined according to previously described methodologies.[29] None of the compounds described here had any significant cytopathic effect on uninfected cells under these conditions. The CC_{50}'s all were in excess of 250 μM. These novel agents possessed in vitro antiviral activities that exceeded the activity of cidofovir against VV and CV under these in vitro test conditions. For the pyrimidinylidene monopyrazolone (**9**), the EC_{50}, as defined by the CPE assay, was 0.3 μM. These compounds (**9** and **10**) were somewhat more active against CV than against VV. Since CV is often considered to be a closer model to the smallpox variola virus than is VV, these results have to be considered to be encouraging. The potent antiviral activities of compounds **9** and **10** provide strong leads to novel orthopoxvirus antivirals; moreover, the novelty of these structures implies a new paradigm in the structure–activity driven search for nucleosides with antiviral activity. Such potent activity in the context of pyrimidine nucleoside 5-substituent hypermodification has not been observed previously. Studies are in progress to ascertain the spectrum of activity of **9** and **10**, and to elucidate the mechanism of inhibition of virus replication and the in vivo potential of these novel agents.



Scheme 2. Reagents and conditions: (e) MeOH, Amberlite IR-120 (H^+), reflux, 2 h; (f) NaOMe/MeOH, rt, 3 h; (g) AcOH/H_2O, 50 °C, overnight; (h) ethanol, 60 °C, 2 h; rt, overnight, molar ratio of **8** and **4**: 1:1; (i) ethanol, 60 °C, overnight, molar ratio of **8** and **4**: 1:2.5.

Table 1. Inhibition of orthopoxvirus replication by pyrazolo-pyrimidine nucleosides[a]

Compound	Efficacy EC_{50}[b] (μM)				Toxicity CC_{50}[c] (μM) Neutral red uptake
	Vaccinia[d] CPE	Vaccinia[d] PR[e]	Cowpox[d] CPE	Cowpox[d] PR[e]	
Cidofovir	3.2	20 ± 11	7.1	32 ± 10	>317 ± 0
5	32	56 ± 5.1	33.7	48 ± 20	>252 ± 37
6	43	119 ± 81	37.1	25 ± 1.1	>288 ± 13
9	1.7	6.9 ± 0.9	0.3	5.6 ± 5.2	>286 ± 25
10	20	11 ± 1.0	1.8	9.0 ± 7.0	>292 ± 14

[a] Assays were performed according to the procedures described previously.[29]
[b] EC_{50}, effective concentration to reduce viral cytopathogenicity (CPE) or plaque formation (PR) by 50%. Values without a standard deviation refer to experiments carried out once in triplicate.
[c] CC_{50}, concentration which causes a cytotoxic effect (as ascertained by neutral red uptake) on 50% of uninfected cells.
[d] Virus used for challenge: vaccinia virus (Copenhagen) or cowpox virus (Brighton).
[e] Values are the means ± standard deviation of 2 or more assays.

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trations. Infection with vaccinia virus (VV) Copenhagen or cowpox virus (CV) Brighton at 1000 PFU per well was followed by incubation at 37 °C for 7 days. After incubation, the plates were stained with a crystal violet solution for 4 h, rinsed, allowed to dry for 24 h, and then read on a BioTek Plate Reader at 620 nm. Confirmatory assays using a plaque reduction (PR) assay were performed using HFF cells seeded in 6-well plates 2 days prior to use and infected with VV or CV at 20–30 PFU per well. After a 1 h incubation period, various concentrations of drug were added in triplicate and the plates were incubated at 37 °C for 3 days. The cells were stained with a neutral red in PBS and incubated for 5–6 h. After aspiration of the stain, viral plaques were counted using a stereomicroscope at 10× magnification. The concentration of agent that inhibited viral CPE or plaque formation by 50% was defined as the EC_{50} and was calculated using standard methods. The effect of the potential antiviral agent on uninfected host cell viability was evaluated using HFF cells seeded in 96-well plated incubated with various concentrations of drug for 7 days. After incubation, cell monolayers were stained with a solution of neutral red and the concentration of agent that reduced neutral red uptake by 50% was defined as the CC_{50}.