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Pyrimido[1,2-*b*]-1,2,4,5-tetrazin-6-ones as HCMV Protease Inhibitors: A New Class of Heterocycles with Flavin-Like Redox Properties

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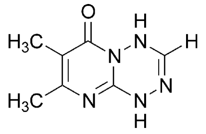
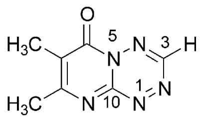
Abstract—The synthesis and biological activity of pyrimidotetrazin-6-ones against HCMV protease is described. The mechanism of action for these inhibitors is the oxidation of several cysteine residues to generate cross-linked enzyme.

Human cytomegalovirus (HCMV), a betaherpesvirus, is an opportunistic pathogen that commonly afflicts immunocompromised (HIV) or immunosuppressed (organ/bone marrow transplant) persons.[1] Unfortunately for these patients, current treatments (ganciclovir, foscarnet sodium, cidofovir, and fomivirsen sodium) suffer from cross-resistance, dose-limiting toxic side effects and are only moderately effectual.[2] As a result, in an effort to identify inhibitors with novel mechansims of action, a number of laboratories have sought inhibitors of the essential protease encoded by the HCMV UL80[3] gene as potentially effective antiviral chemotherapeutic agents. Thus far, several mechanism- and peptide-based inhibitors of this enzyme have been disclosed.[4]

During the course of our search for novel small molecule heterocycles with interesting biological properties, a screening program identified dihydrotetrazine **1a** as a potent inhibitor of the HCMV UL80 protease.[5] However, careful examination of this 'lead' revealed the enzyme inhibition was in fact due to a minor impurity later identified as tetrazine **2a**, the product of air oxidation of **1a** and as such, we became interested in developing an analogue program to probe the biological activity of congeners of this compound. Preliminary SAR data as well as synthetic considerations suggested the modification of C-3 substituent of **2a** as a useful starting point and consequently, we required a general route to these derivatives. In this report the synthesis of these analogues and their in vitro inhibitory activity against the HCMV UL80 protease is disclosed.[6]



1a



2a

Previously, the cyclocondensation of pyrimidinone **3**[7] with commercially available orthoesters or acetals of DMF to afford 1,2-dihydropyrimido-1,2,4,5-tetrazin-6-ones **1** and 1,2,4-triazolopyrimidines **4** was reported (eq 1; X = OMe or NMe_2; R = H, CH_3).[8] Despite the convenience of this route, the lack of regiochemical control and the limited commercial availability of orthoesters severely restrict the utility of this protocol. We anticipated that regiochemical control could be accomplished with the appropriate choice of R groups. In particular, we reasoned that, as R increased in size, steric interactions between this group and the C-5 methyl group would drive the reaction toward the sole

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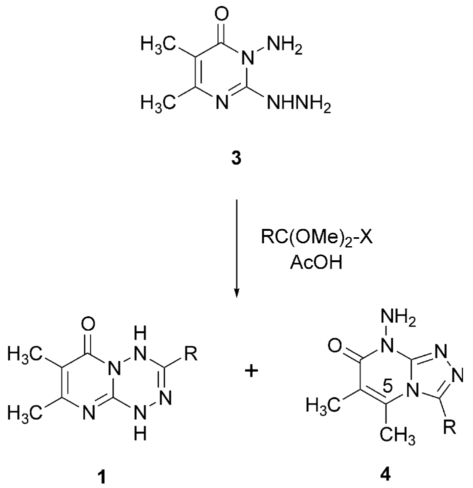
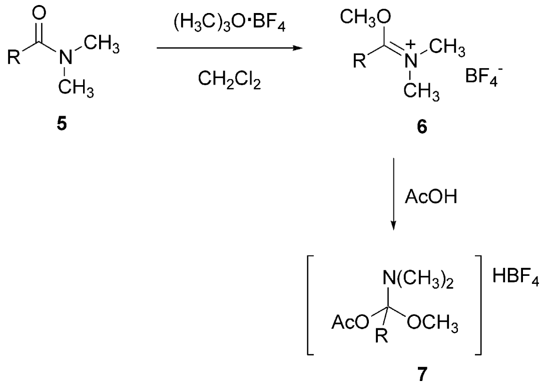
formation of the tetrazine product **1**. Indeed, this turned out to be the case: whereas treatment of **3** with triethyl orthoformate in neat acetic acid afforded a 1:4.5 mixture of **1**:**4** (R = H) in 82% total yield, treatment of **1** with trimethyl orthobenzoate under similar conditions yielded only **1** (R = Ph) in 63%.

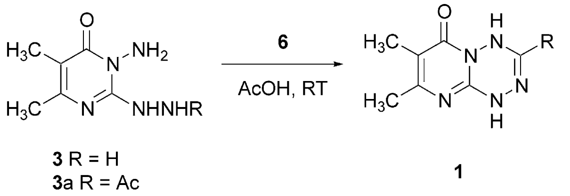


(1)

Next, to circumvent the limited availability of orthoesters,[9] we anticipated that salts **6**, available from the reaction of dimethylamides **5** with trimethyloxonium tetrafluoroborate (eq 2), could function as an orthoester equivalent to afford the desired tetrazine products. To that end, the reaction of **6** (R = Ph) with pyrimidinone **3** in neat acetic acid at rt overnight effected the desired transformation, though the reaction was relatively sluggish and yielded two main products, compound **1** (R = Ph) and an acetylated product tentatively identified as **3a** (eq 3).[10] The latter presumably arises from an in situ generated acetylating species such as **7** (eq 2), derived from nucleophilic addition of acetic acid to salt **6**.[11] This problem was conveniently avoided by replacing acetic acid with methanol as the reaction solvent. In all probability this modification affords in situ generation of the desired dimethylacetal of the dimethylamide and also liberates a mole of HBF_4 which functions as the acid catalyst.



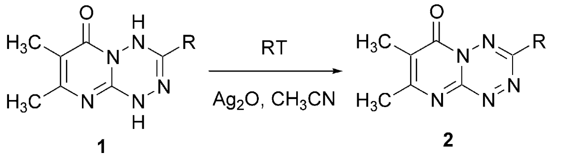
(2)



(3)

Premixing salt **6** (R = Ph) with methanol for 0.25 h, followed by the addition of pyrimidinone **3** deposited the desired product as a bright yellow solid in 50% yield after stirring at 25 °C for 12 h. Bringing the solution to reflux increased the reaction rate so that product formation was ordinarily complete within 1 h.[12] Our results, summarized in the Table 1, demonstrate the generality of this method. This procedure, which tolerates a variety of R groups, rivals the original protocol in simplicity and ease of isolation. In addition, single crystal X-ray analysis of compound **1d** confirmed the 1,4-dihydro arrangement about the tetrazine ring (Fig. 1).[13]

The final step in the synthesis of our target molecules was to oxidize compound **1** to tetrazine **2** (eq 4). This was accomplished with excess Ag_2O (4 equivalents) in acetonitrile overnight at rt.[14] Filtration through Celite, concentration in vacuo and recrystallization from either hexanes or toluene/hexanes gave analytically pure **2** as highly colored powders (see Table 1).



(4)

With the synthesis of these compounds in hand, screening was performed using a scintillation proximity assay as described previously.[15] The results of this assay are presented in Table 2. Overall, there is little SAR but on the basis of this limited data set, there is a marginal preference for hydrogen, smaller alkyl groups or electron rich aromatics at position 3 (entries 1, 2, 7, 8). Sterics (entry 5) and electron deficient substituents (entry 9) are not well tolerated.

Table 1. Summary of yield data for the synthesis of **1** and **2**

R	Compd	% Yield **1**[a]	Compd	% Yield **2**[b]
–H	**1a**	— (20)	**2a**	80
–Me	**1b**	— (32)	**2b**	75
–Bu	**1c**	— (30)	**2c**	72
–*t*Bu	**1d**	49	**2d**	89
–CH(Ph)$_2$	**1e**	42	**2e**	93
Ph	**1f**	52 (63)	**2f**	79
4-MePh	**1g**	41	**2g**	77
4-*t*BuPh	**1h**	25	**2h**	68
4-OMePh	**1i**	22	**2i**	75
4-NO$_2$Ph	**1j**	34	**2j**	83

[a] Yields are not optimized and those in parentheses are for the reaction with orthoesters.
[b] Yields are for recrystallized products.

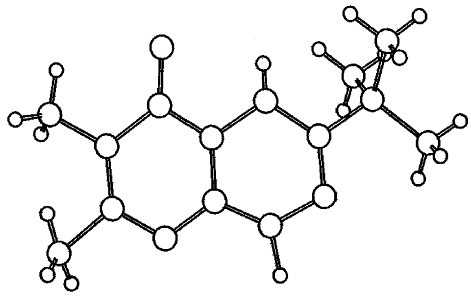
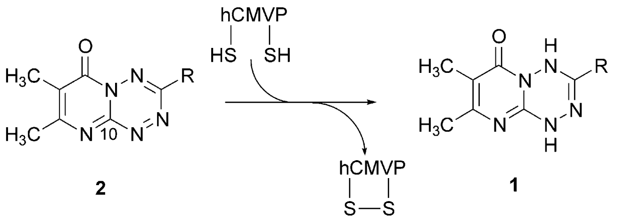


Figure 1. ORTEP representation of the X-ray crystal structure of compound **1d**.

Table 2. Summary of the biological activity of tetrazines **2** against the HCMV UL80 protease

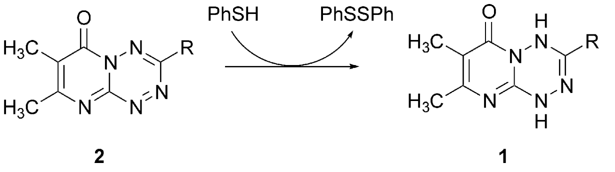
Entry	Compd	R	IC_{50} (μM)
1	**2a**	–H	0.1
2	**2b**	$-CH_3$	0.3
3	**2c**	–*n*Bu	0.4
4	**2d**	–*t*Bu	0.6
5	**2e**	$-CH(Ph)_2$	1.7
6	**2f**	Ph	0.4
7	**2g**	4-MePh	0.3
8	**2i**	4-MeOPh	0.5
9	**2j**	$4-NO_2Ph$	1.7

With several potent inhibitors of HCMV UL80 protease in hand, we next examined their mode of action and discovered tetrazines **2** undergo redox chemistry via a mechanism reminiscent of flavins. In particular, mass spectrometry determined that exposure of HCMV protease to compound **2a** afforded a chemically modified enzyme in which two pairs of Cys residues (Cys84–Cys87 and Cys138–Cys161) were oxidized to disulfides (eq 5).[5] On the basis of these results and molecular orbital calculations, we propose the following mechanism. The nucleophilic sulfur of a Cys residue attacks at C-10 of **2**, pushing electrons into the highly conjugated π system. Subsequent attack by a neighboring Cys sulfur generates a disulfide, releasing the dihydrotetrazine.



(5)

That this redox mechanism is plausible is supported by several pieces of evidence. Firstly, dihydrotetrazines **1** are completely inactive in the scintillation proximity assay. Secondly, riboflavin and flavin mononucleotide are equally potent inhibitors of this protease[5] and finally, the reduction of tetrazines **2** to dihydrotetrazine **1** was easily accomplished with thiophenol. The addition of PhSH to a $CDCl_3$ solution of **2f** (R = Ph) resulted in a slow discoloration with concomitant precipitation of **1f** (eq 6).[16]



(6)

In this report, we have described a modified synthesis of tetrazines **1** and **2**, their usefulness as CMV protease inhibitors, and proposed a mechanism for their antiviral properties.

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6. These compounds demonstrated selectivity for HCMV protease over other known serine proteases—data not shown.

7. Prepared by condensing 1,3-diaminoguanidine with ethyl 2-methylacetoacetate; see: Hlavka, J. J.; Bitha, P.; Lin, Y.; Strohmeyer, T. *J. Heterocyclic Chem.* **1984**, *21*, 1537.

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9. For a review of orthoester syntheses, see: DeWolfe, R. H. *Synthesis* **1974**, 153.

10. That acetylation occurs at this position is presumed based on previously reported results with other electrophilic reagents; see ref 8.

11. The product distribution for this reaction was dependent on the nature of R; salt **6** becomes very reactive toward AcOH as R becomes more electron withdrawing. For instance, when R = 2,4,6-F_3–C_6H_2–, **Ac-3a** and the dimethyl amide precursor **5** were the only observable products under these conditions.

12. General procedure: methanol (25 mL) was added to a flask charged with 2.2 g pyrimidinone **3** (1.3 mmol) and 2.99 g salt **6** (R = *t*Bu; 1.3 mmol) and the reaction was heated to reflux. After 30 min, the reaction was cooled to rt and filtered. The filtrate was washed with ether and recrystallized from MeCN/Et_2O with a trace of AcOH to give analytically pure **1d** (1.5 g; 49%).

13. Crystallographic data for **1d**: $C_{11}H_{17}N_5O$, fw = 235.29, triclinic, space group P-1(#2), colorless plate, a = 11.477 (2) Å, b = 12.144 (4) Å, c = 10.359 (3) Å, α = 112.46 (2)°, β = 107.84 (2)°, γ = 92.96 (8)°, V = 1246.6 (8) $Å^3$, Z = 4, D_{calc} = 1.248 g cm^{-3}, μ (CuK_α) = 6.70 cm^{-1}, Rigaku AFC5R diffractometer, 3704 reflections (unique), R = 0.084.

14. Several other oxidants including air and DDQ were tried as well and although all were moderately successful, Ag_2O was the reagent of choice, offering higher yields, easier workups and cleaner reactions.

15. HCMV protease (2 μM) was preincubated with potential inhibitors for 2 h at 25 °C in 10 mM Tris–Cl, pH 7.5, 50 mM NaCl. Substrate was added and incubation continued for 2 h. The reaction was terminated with pH 5.5 stop buffer containing streptavidin-coated SPA beads followed by scintillation counting. For more details, see ref 5.

16. This is easy to monitor visually—the tetrazines **2** are orange to red and are soluble whereas the dihydro analogues **1** are yellow and insoluble in chloroform.