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NOVEL, SELECTIVE MECHANISM-BASED INHIBITORS OF THE HERPES PROTEASES

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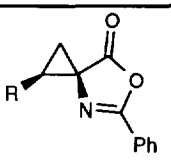
Abstract: A novel class of inhibitor of the herpes proteases acting upon the catalytic apparatus by forming covalent complexes are described. Two new families of inhibitor, the spirocyclopropyl oxazolones and the benzylidine N-sulphonyloxyimidazolones, have been shown to be submicromolar inhibitors of HSV-2 and HCMV proteases which are selective relative to a panel of standard serine proteases.

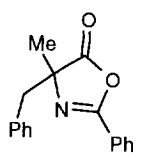
Viruses of the *Herpesviridae* family such as herpes simplex types 1 and 2 (HSV), human cytomegalovirus (HCMV), varicella zoster virus (VZV) and Epstein-Barr virus (EBV) are responsible for a variety of diseases states from sub-clinical infections to fatal diseases in the immunocompromised. Current therapy relies on nucleoside analogue viral DNA polymerase inhibitors such as acyclovir and famciclovir, which are principally used against HSV-1, HSV-2 and VZV infections. The only available agents against HCMV are ganciclovir and forscarnet which are toxic and associated with undesired side effects.[1] There is a medical need for new anti-viral agents that address the drawbacks of current therapies particularly for cytomegalovirus infections. The recent discovery of protease activity in the protein product of the gene UL80 of HCMV, an enzyme essential for virion assembly, represents a novel opportunity to intervene in the life cycle of the herpes virus.[2,3] This protease is well conserved throughout the herpes family and its proteolytic activity is required for cleaving a scaffold protein involved in maturation of the capsid into which viral DNA is packed prior to envelopment and release from infected cells.[4,5] The protease is self-processing and contains two cleavage sites, the C-terminal maturation site (M site) which it shares with the scaffold protein , and the release site (R site) which results in the release in the N-terminal catalytic domain. The proteolytic activity of the herpes proteases has been classified as belonging to the serine protease family, however there is no sequence homology with any known protease.[6]



	R	IC_{50} (μM) HSV-2 pr.	IC_{50} (μM) HCMV pr.	Inhibition at 100μM or IC_{50} Elastase	Trypsin	Chymotrypsin
1	Ph	0.7	1.8	42%	63%	92% (22 μM)
2	4-MeOPh	0.5	0.2	35%	64%	92%
3	4-AcOPh	1.2	1.1	-	-	-
4	N-Ac-3-indolyl	0.14	0.16	-	-	-

Table 1

Directed mechanism-based screening targeted to compounds that could afford a stable acyl-enzyme adduct of the active site serine identified a novel class of serine protease inhibitor, the spirocyclopropyl oxazolones represented by **1** - **4** as submicromolar inhibitors of HSV-2 and HCMV proteases (Table 1).[7] When **1** and **2** were assayed for inhibition against a panel of standard serine proteases from the chymotrypsin superfamily at a concentration of 100 μM, only modest inhibition of elastase and trypsin was observed. Inhibition of chymotrypsin was somewhat higher, as might be anticipated from the affinity of this enzyme for aromatic groups, but the IC_{50} of **1** against chymotrypsin was only 22 μM. Both **1** and **2** thus demonstrated good selectivity for the herpes proteases.



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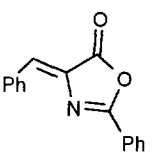

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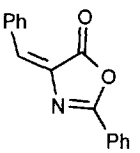

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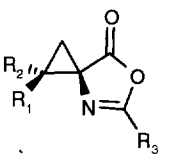
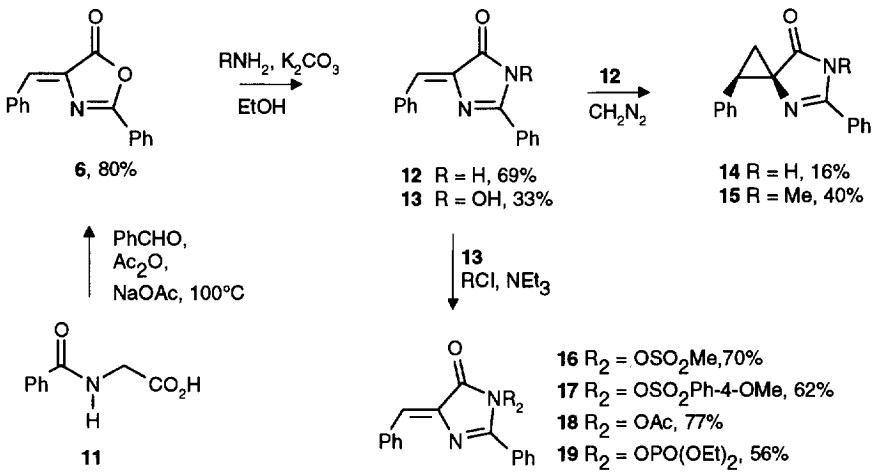


8 R_1 = H, R_2 = Ph, R_3 = Ph
9 R_1 = H, R_2 = H, R_3 = Ph
10 R_1 = Ph, R_2 = H, R_3 = Me

In order to elucidate the mechanism of inhibition, HSV-2 protease was incubated with compound **1** in a 1:1 ratio and then examined for covalent complex formation by electrospray mass spectroscopy.[8] An adduct of the protease was identified with a mass increment of 263 indicating the addition of a single inhibitor molecule to the enzyme. To determine the site of addition on the enzyme, the complex was digested with trypsin (50:1, complex:enzyme for 3 hrs at 37°C) followed by LC/ESMS of the fragments. This led to the identification of the peptide LLYLITNYLPSVSLSTK (putative active site serine underlined) as the fragment containing covalently bound **1**. Post source decay MS/MS of this tryptic peptide confirmed that the binding site resides

within the SLSTK region of the sequence, consistent with **1** forming an acyl-enzyme complex with the active site serine.

The structural features necessary for activity in **1** were determined by synthesis of a defined series of analogues. The role of the cyclopropyl group was investigated by preparing the acyclic derivative **5** as well as replacing it with a double bond as with **6** and **7** using literature methodology (Scheme 1).[9-11] These changes caused a loss of activity suggesting the cyclopropyl ring plays an important role, possibly through the orientation of the aryl ring. The cyclopropyl compound **8** with the alternative geometry for the aryl ring also led to a loss of potency for HSV-2 protease, while removal of the aryl ring altogether as in **9** resulted in a loss of potency for the HCMV enzyme. These results confirm the observations from modification of the cyclopropyl ring that the presence and orientation of this aryl moiety is important for herpes protease inhibition. The 2-phenyl substituent on the oxazolone ring was less sensitive to change in that replacement by a methyl group, as in **10,** retained good potency against both enzymes (Table 2).



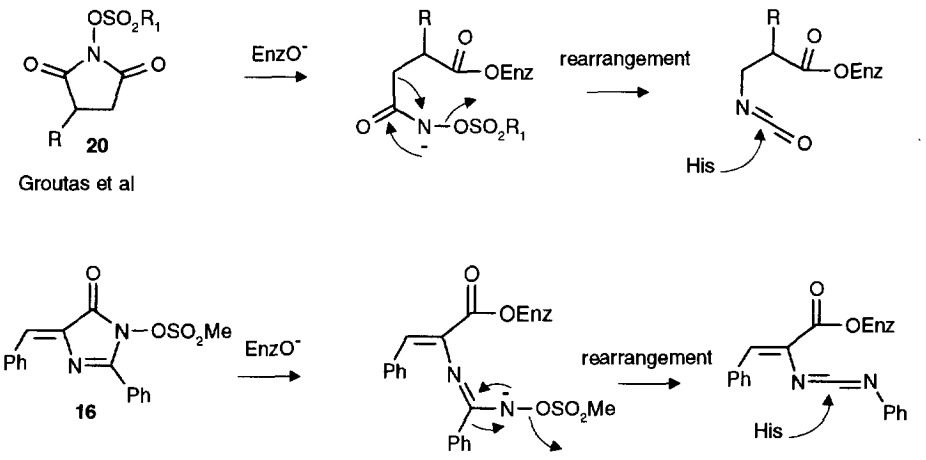
Scheme 1

Modification of the oxazolone ring was investigated in order to enhance hydrolytic stability. To this end, the imidazolones **14** and **15** were prepared from the oxazolone **5** by treatment with ammonia followed by cyclopropanation with diazomethane which also led to partial methylation of the nitrogen (Scheme 1).[12] Both compounds were devoid of activity possibly due to the large increase in the hydrolytic stability of the imidazolone ring. Reaction of **5** with hydroxylamine led to the benzylidine adduct **13** which upon sulfonylation provided **16** and **17**, while acylation yielded **18** and phosphorylation **19**.[13]

	HSV-2 protease		HCMV protease	
	$IC_{50}(\mu M)$	Inh at 10μM	$IC_{50}(\mu M)$	Inh at 10μM
1	0.7		1.8	
8		24%		85%
9		71%		16%
10		82%		88%
16		33%	0.4	
17		20%	2.3	
18		14%		35%
19		5%		20%

Table 2

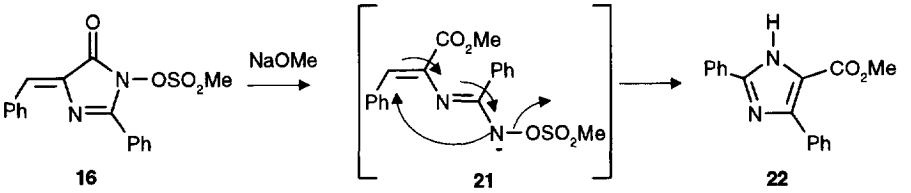
The sulphonates **16** and **17** were good inhibitors of HCMV protease, with **16** displaying a sub-micromolar IC_{50} value (Table 2). This series of compounds was selective for HCMV protease displaying little inhibition of the HSV-2 enzyme. The corresponding cyclopropyl analogues, however, were inactive as were the hydroxy (**13**), acyloxy (**18**) and phosphoryloxy (**19**) analogues. Compound **16** was also selective with respect to the standard serine proteases with no inhibition of elastase or trypsin at 100 μM and an IC_{50} of 18 μM for chymotrypsin.



Scheme 2

The fact that compounds **17-19** demonstrated reduced potency relative to **16** is consistent with elimination of the sulfonyloxy group playing a role in enzyme inhibition. In support of this, mass spectral studies of the complex generated from **16** and HCMV protease showed the presence of an M+247 adduct indicating formation of a 1:1 complex of the protease with **16** accompanied by loss of the methanesulfonyloxy

moiety. A potential model for the mechanism of loss of the sulfonyloxy group is provided by chymotrypsin inhibitors such as **20** described by Groutas *et al* (Scheme 2).[14] Ring opening of **20** by the active site serine of chymotrypsin is followed by elimination of the sulfonyloxy moiety and Lössen rearrangement of the resulting nitrene. When an N-sulfonyloxy-succimide of general structure **20** was treated with sodium methoxide the Lössen products were reportedly formed supporting the chemical integrity of the proposed mechanism of inhibition.[15] However, when compound **16** was treated with sodium methoxide in methanol, no rearrangement products were detected, and the only product isolated was the imidazole **22** in 57% yield (Scheme 3). Compound **22** is presumably formed from the acyclic intermediate **21** and it would appear probable that the nitrene is not produced in this instance. We therefore believe that the inhibition of HCMV protease by **16** is unlikely to proceed by the Lössen rearrangement mechanism shown in Scheme 2. On the basis of the mass spectroscopy and the model methanolysis study, we tentatively suggest that **16** may inhibit HCMV protease by formation of the acyl-enzyme analogue of **22**.



16 **21** **22**

Scheme 3

In conclusion a variety of cyclopropyloxazolones have been shown to be potent and selective inhibitors of HCMV and HSV-2 proteases that act by acylation of the catalytic serine. A second class of inhibitor based on benzylidine N- sulfonyloxy imidazolones proved to be HCMV selective. Both classes of compound are unique serine protease inhibitors.

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7. The protease assay comprised of pre-incubation of inhibitor with HSV-2 protease catalytic domain[16,17] or HCMV protease catalytic domain for 15 min in 25 uL buffer (50 mM sodium phosphate, 150 mM sodium chloride, 0.001mM EDTA, 0.01% PEG 3400; pH 8.0) then adding 250 μM R-site 14-mer peptide substrate, Ac-HTYLQASEKFKMWG and incubating at 27°C for 60 mins. The reaction was quenched with 40 μL 5% trifluoroacetic acid solution and the extent of proteolysis examined by quantifying the amounts of the substrate peptide and of the N-terminal and C-terminal cleavage fragments by HPLC analysis. The percentage cleavage was calculated and expressed as a fraction of the value obtained for an uninhibited control sample. Compounds **16-19** were assayed in a quenched fluorescence peptidolytic assay (A. Patel, unpublished results).

8. Mass spectra analysis was carried out by reverse phase HPLC electrospray on a Finnigan TSQ700 and post source decay MS/MS on a Micromass TOF Spec E.

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13. Preparation of compound **16**: To a suspension of 0.40g (1.52mmol) **13** in 15ml ether was added 0.23ml (1.67mmol) triethylamine followed by 0.13ml (1.67mmol) methanesulfonyl chloride. The solution was stirred for 18hrs then separated between ethyl acetate and brine. The organics were isolated, dried ($MgSO_4$), and concentrated *in vacuo*. Chromatography using chloroform as eluant provided 0.376g (73%) of **16** as a yellow solid. Mp: 152-153°C. ^1H NMR ($CDCl_3$):- δ 3.5 (s, 3H); 7.3 (s, 1H); 7.5 (m, 6H); 8.2 (dd, J = 7 & 2 Hz, 2H); 8.25 (dd, J = 7 & 2 Hz, 2H).

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