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INHIBITION OF HSV-1 PROTEASE BY BENZOXAZINONES

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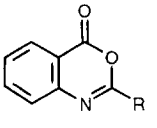
Abstract: Benzoxazinones have been discovered which are mechanism based inhibitors of HSV-1 protease with micromolar IC_{50} values. Formation of a monoadduct consistent with the acyl-enzyme complex was detected by mass spectroscopy. A parallel array synthesis was developed to explore 2-heteroatom substituted SAR.

Members of the human herpesvirus family include herpes simplex types 1 and 2 (HSV-1 and HSV-2), cytomegalovirus (CMV), varicella zoster virus (VZV) and Epstein-Barr virus (EBV) which are responsible for a variety of disease states from sub-clinical infections to fatal diseases in the immunocompromised. The recent discovery that the protein encoded by the UL26 gene of HSV-1 is a protease,[1] has afforded a potential new target for antiviral therapy. This protease plays an essential role in virus capsid maturation, cleaving a scaffold protein which is encoded in-frame with the C-terminal part of the UL26 gene.[2] The protease is also 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 release of the N-terminal catalytic domain. There is sequence conservation of the protease across the herpesvirus family and a highly conserved P_4-P_1' cleavage site with the cleavage occurring between alanine and serine. The herpes proteases do not share sequence homology with known proteases but have been shown to belong to the mechanistic family of serine proteases.[3]

2-Substituted benzoxazinones **1** are known mechanism-based inhibitors of standard serine proteases of the chymotrypsin superfamily[4,5] and inhibit by formation of an acyl-enzyme complex through attack of the active site serine on the carbonyl group. The structure of a resulting acyl-enzyme complex of elastase has been solved by X-ray crystallography.[6]



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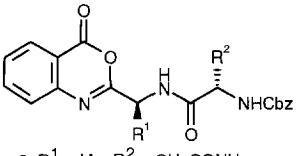
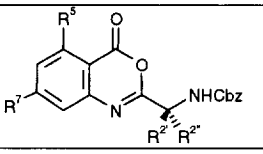


2 R^1 = Me R^2 = CH_2CONH_2
3 R^1 = CH_2CONH_2 R^2 = $CHMe_2$

Benzoxazinones **2** and **3** were targeted as containing elements of the HSV-1 M-site VNA-S cleavage sequence and were synthesised by water-soluble carbodiimide (WSCI) mediated coupling of the corresponding dipeptide to anthranilic acid followed by cyclisation. The compounds were evaluated in an hplc assay

measuring the peptidolytic activity of HSV-1 protease.[7] It was found that both compounds had an IC_{50} of 90 μM. When the HSV-1 protease was incubated with **2** or **3** and then subject to matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectroscopy, monoadducts with mass increments of 440 and 500 respectively were observed. This suggests that these benzoxazinones act as mechanism-based inhibitors of HSV-1 protease by formation of an acyl-enzyme complex.

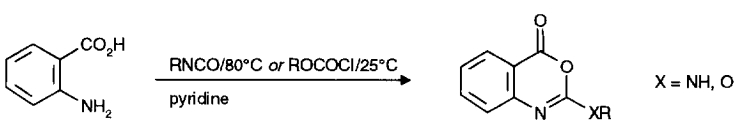
To elucidate structure activity relationships around these lead compounds, the simpler Cbz-alanine derived side chain of **4** was utilised (Table 1). Increasing the size of the 5-substituent through methyl, chloro and ethyl was clearly detrimental to HSV-1 protease inhibition (compounds **4** - **8**). Compound **9**, the (*R*)-enantiomer of **5**, was less inhibitory than the (*S*)-enantiomer indicating some stereoselectivity. Introduction of the 7-Cbz-alaninyl-amino group (**11**) afforded increased potency.



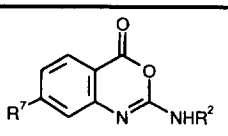
No.	$R^{2'}$	$R^{2''}$	R^5	R^7	IC_{50} (μM)
4[5]	Me	H	H	H	50
5	Me	H	F	H	50
6	Me	H	Me	H	120
7	Me	H	Cl	H	>300
8	Me	H	Et	H	>300
9	H	Me	F	H	200
10	Me	H	H	NH_2	150
11	Me	H	H	Cbz-Ala-NH	12

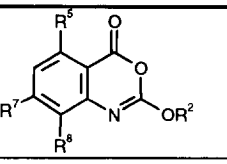
Table 1. Inhibition of HSV-1 protease by 2-Cbz-alanine derived benzoxazinones.

In order to rapidly explore SAR in 2-heteroatom substituted benzoxazinones, a simple one-step procedure for parallel array synthesis was developed. 2-Alkoxy benzoxazinones can be formed by the reaction of anthranilic acids with chloroformates, whilst 2-amino benzoxazinones are usually formed in a two step process by consecutive acylation and cyclisation.[5] We found that treatment of anthranilic acids with 2.2 equivalents of isocyanate in pyridine at 80°C afforded 2-aminobenzoxazinones directly. Use of these conditions with either the isocyanate (for 2-amino substituents) or chloroformate (for 2-alkoxy substituents) followed by precipitation in water allowed the rapid synthesis of a number of benzoxazinones in parallel arrays.[8] The isolated product was usually of acceptable purity although purification by silica gel chromatography could be carried out if necessary (the most significant impurities were the symmetrical ureas or carbonates from isocyanates and chloroformates respectively).



In the 2-amino series, inhibition was associated exclusively with secondary alkylamino groups such as compounds **13**, **18** and **19**, whereas primary alkyl compounds such as **12** and aryl compounds such as **20** were ineffective (Table 2). 7-Acylamino derivatives **15** and **16** were prepared by WSCI mediated acylation of **14**; the use of the additive HOAt[9] was key to the success of this reaction. The switch from HOBt to HOAt resulted in an increase in coupling yield from 4% to 50% with Cbz-Asn(trityl)-OH. Variation of the 7-substituent afforded a ten-fold range of potencies, and as in the 2-carba series, the 7-Cbz-Ala-NH substituent afforded the compound with the best potency (**15**, IC_{50} = 5 μM).





No.	R^2	R^7	IC_{50} (μM)	No.	R^2	R^5	R^8	IC_{50} (μM)
12[5]	Bu	H	300	**21**[4b]	Et	H	H	25
13[5]	iPr	H	25	**22**	Et	F	H	75
14[5]	iPr	NH_2	55	**23**[5]	Et	Me	H	15
15	iPr	Cbz-Ala-NH	5	**24**	Et	Cl	H	7
16	iPr	Cbz-Asn-NH	15	**25**	Et	Et	H	20
17	iPr	$iBuCO_2NH$	50	**26**	Et	Cl	Cl	1.5
18	MeO_2CCMeH	H	25	**27**	allyl	H	H	15
19	(*R*)- PhCMeH	H	60	**28**	Ph	H	H	10
20	3-F-Ph	H	>300	**29**	4-MeO-Ph	H	H	2.5

Table 2. Inhibition of HSV-1 protease by 2-amino and 2-oxy benzoxazinones.

In the 2-alkoxy series, a wider range of 2-substituents afforded protease inhibition, including aryloxy (Table 2). The best 2-substituent identified from the parallel array syntheses was the 4-methoxyphenoxy group of **29**, affording an IC_{50} of 2.5 μM. In contrast to the 2-Cbz-Ala series of compounds, the 5-fluoro was the worst 5-substituent and the 5-chloro was most favoured.

A number of these benzoxazinones were analysed for their stability in aqueous solution by monitoring the long-wavelength uv absorption of the parent oxazinone ring system. The half-life at pH 7.5 (phosphate buffer-acetonitrile, 80:20) and 25°C of compounds with $IC_{50} \leq 10$ μM is shown in Table 3. The inhibitors had a wide range of half-lives and there was no correlation of potency with reactivity. Compound **15** is of particular interest as a micromolar inhibitor with high aqueous stability.

No.	IC_{50} (μM)	$t_{1/2}$ (h)
26	1.5	1.0
29	2.5	20
15	5	171
24	7	4
28	10	6.9

Table 3. Half-life in aqueous solution of selected benzoxazinone HSV-1 protease inhibitors

In summary, benzoxazinones have been identified as mechanism based inhibitors of HSV-1 protease with micromolar potency. Formation of a 1:1 adduct with the protease has been shown by MALDI-TOF mass spectroscopy and this is consistent with the formation of an acyl-enzyme adduct. That these compounds have a specific molecular interaction with the HSV-1 protease is also suggested by demonstrable SAR trends at the 2-, 5- and 7- positions of the benzoxazinone system, including some stereoselectivity, and by the fact that the inhibition is not correlated with inhibitor reactivity.

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7. A solution of inhibitor was added to HSV-1 protease (protease domain of the UL26 protein, residues 1 to 247, with the addition of an amino terminal MDLPRHHHHHHS tag; 0.5 - 1 μM) diluted in buffer (50 mM sodium phosphate, 100 mM sodium chloride; pH 7.8) to give a final inhibitor concentration of 0.01 to 300 μM in a total volume of 39 μl. Following a 30 min preincubation, of a solution of the substrate Ac-HTYLQASEKFKMWG (1 μl) was added to give a final substrate concentration of 250 μM. The sample was incubated at 37°C for 1 h and then quenched by addition of 10% trifluoroacetic acid in water (10 μl). The sample was analysed by HPLC to quantitate the amounts of the substrate peptide and of the N-terminal and C-terminal cleavage fragments and the percentage cleavage was calculated.
8. Isocyanate parallel synthesis method: To a solution of anthranilic acid (0.41 g, 3 mmol) in dry pyridine (4 ml) at RT was added isocyanate (6.3 mmol) and the solution was heated at 70°C for 1 h. The solution was poured into water (50 ml) and the precipitate was filtered, washed with water and hexane and dried.
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