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POTENT SELECTIVE THIENOXAZINONE INHIBITORS OF HERPES PROTEASES

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Abstract: Thieno[3,2-d]oxazinones are potent, selective, mechanism-based inhibitors of the herpes proteases with good aqueous stability. Specificity between the HSV and CMV proteases varies across the series: compounds **14b** and **14c** are submicromolar HSV protease inhibitors with modest CMV protease inhibition, **14g** is a selective CMV protease inhibitor, and **32** inhibits both enzymes with an IC_{50} of about 1 μM.
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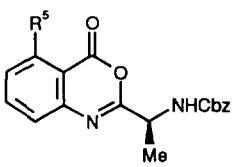
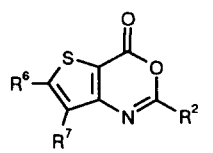
The recent discovery that a protease is encoded by the UL26 gene of herpes simplex type 1 (HSV-1)[1] and by the homologous UL80 gene of cytomegalovirus (CMV),[2] has afforded a potential new target for therapy of herpesvirus infections. This protease plays an essential role in virus capsid maturation, cleaving a scaffold protein which is encoded in-frame with the C-terminal part of the gene.[3] The full-length protease also undergoes self-cleavage at two 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. The protease shows a varying degree of sequence homology across the herpesvirus family and a highly conserved P_4-P_1' cleavage motif in which proteolysis occurs between alanine and serine residues. The herpes proteases do not show homology with any known proteases and the recent determination of the crystal structure of CMV protease indicates that they belong to an entirely new family of serine proteases with a novel catalytic Ser-His-His catalytic triad.[4]

We recently reported the first potent mechanism-based inhibitors of the herpes proteases.[5,6] These inhibitors included the known serine protease inhibitor class of 2-substituted benzoxazinones such as **1**,[5] and spiro-oxazolones and imidazolones which represented novel serine protease inhibitor classes.[6]



1a R_5 = Me
1b R_5 = H



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It was noted that for a series of 5-substituted benzoxazinones with a 2-substituent derived from Cbz-alanine, the HSV-1 protease inhibitory potency was inversely dependent on the size of the 5-substituent. Thus in the general structure **1** compounds with R^5 as chloro or ethyl were not inhibitory, whereas compound **1a** with R^5 as methyl had low potency and **1b** with R^5 as hydrogen was moderately potent. In order to reduce the

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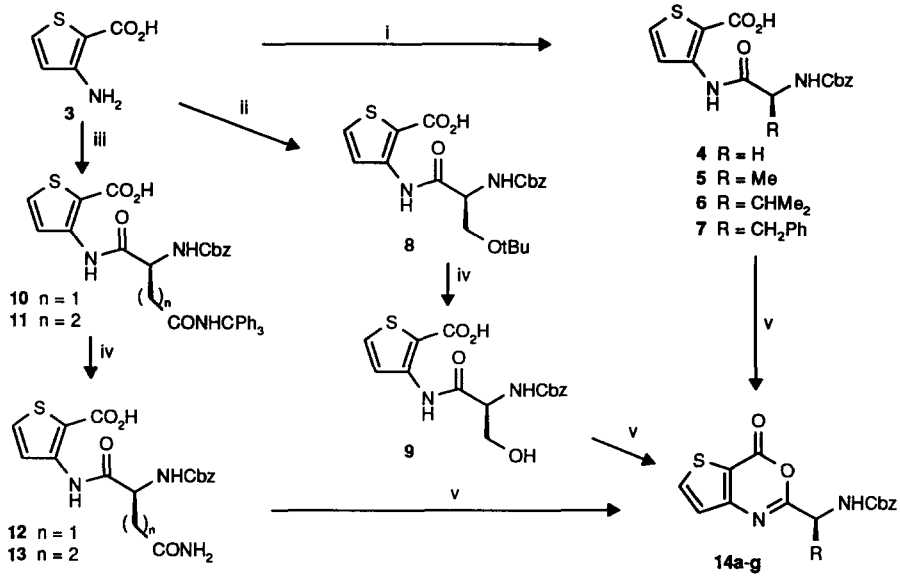
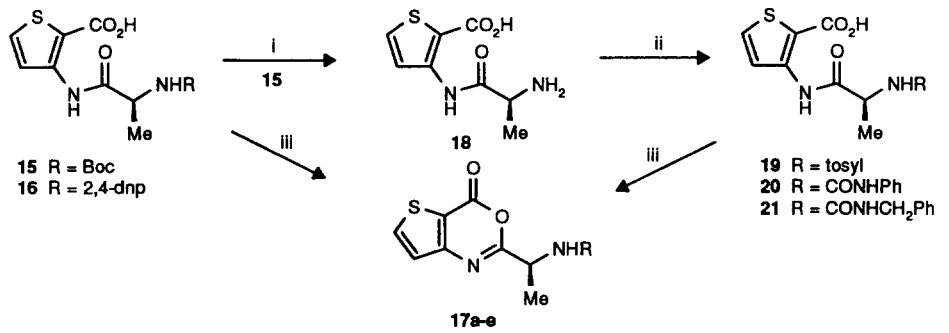
steric bulk at this position still further, we decided to prepare a series of derivatives of the 4*H*-thieno[3,2-d][1,3]oxazin-4-one ring system **2**.

Synthesis of 2-substituted thienoxazinones with different branching groups was achieved by acylation of 3-aminothiophene-2-carboxylic acid **3** with a variety of amino acid derivatives using the mixed anhydride from *iso*-butyl chloroformate (Scheme 1 and Table 1). Non-functionalised amino acid derivatives **4-7** were then cyclised with water soluble carbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC). Attempted one-step acylation/cyclisation using DEC resulted in decarboxylation of the thiophene ring. In the case of serine the O-*t*-butyl protecting group was used and this was removed at the intermediate stage **8** with trifluoroacetic acid (TFA). For the asparagine and glutamine side chains, trityl protection was used and this was again removed with TFA at the intermediate stage (**10** and **11**).



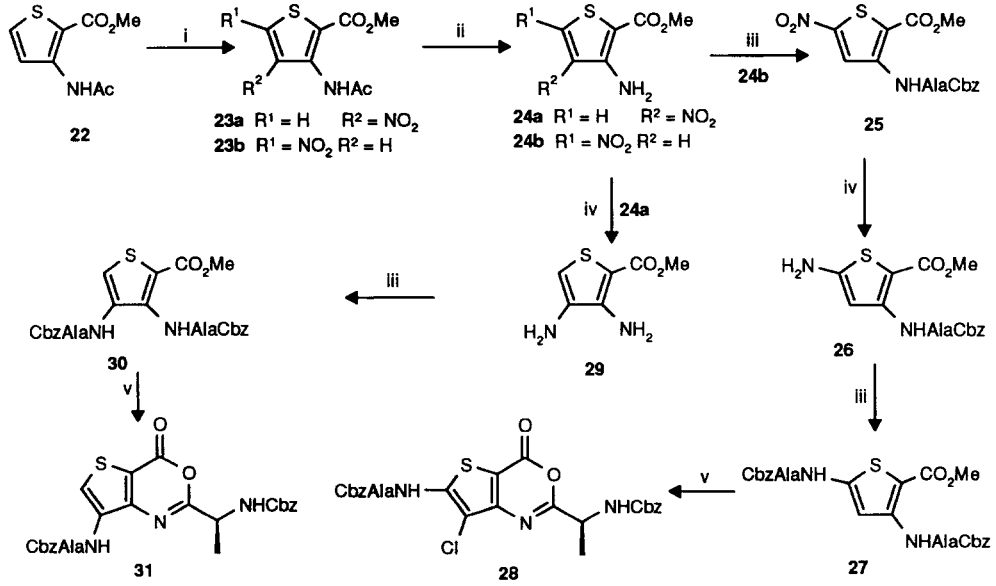
Scheme 1. i. iBuOCOCl/N-methylmorpholine(NMM)/DMF/Cbz-Gly, Cbz-Ala, Cbz-Val or Cbz-Phe; ii. iBuOCOCl/NMM/DMF/Cbz-Ser(tBu); iii. iBuOCOCl/NMM/DMF/Cbz-Asn(trityl) or Cbz-Gln(trityl); iv TFA/CH_2Cl_2; v. DEC/DMF.

Analogues of **14b** in which the Cbz group was replaced by Boc or 2,4-dinitrophenyl were prepared by acylation of the aminothiophene **3** with the appropriately protected alanine affording the intermediates **15** and **16** which were cyclised by DEC treatment to afford **17a** and **17b** (Scheme 2 and Table 1). Deprotection of **15** yielded the amine **18** as the hydrochloride in 55% yield. Reaction of **18** with tosyl chloride or the appropriate isocyanate afforded **19-21** which were cyclised in the usual way to **17c-e**. In the case of tosyl chloride some spontaneous cyclisation occurred in the tosylation reaction.



Scheme 2 i. 6M HCl/dioxane/anisole; ii TsCl/iPr$_2$EtN/DMF or RNCO/pyridine/60°C; iii. DEC/DMF.

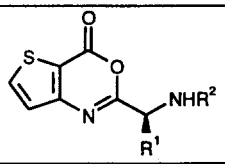
The 6-phenyl and 7-methyl derivatives of **14b** were prepared in the usual way from the relevant substituted thiophene amino acid. From our studies of benzoxazinone inhibitors,[5] aminoacyl substituents in the thiophene ring were of interest and the synthesis of these compounds commenced with nitration of the 3-amidothiophene ester **22**. When the nitration of **22** was carried out between -30 and -25°C,[7] the 5-nitro isomer **23b** was obtained exclusively in an isolated yield of 38%. However, nitration of **22** under conditions similar to those described by Elliott *et al.*,[8] afforded a mixture of 4- and 5-nitrothiophenes **23a** and **23b** in a ratio 1:1.4 which was separated by chromatography on silica gel. Since literature reports regarding the assignment of the site of nitration of thiophene **22** are inconsistent,[7-9] we decided to determine unequivocally the regiochemistry of isomers **23a** and **23b**. Initial analysis of the NMR chemical shifts and 2D heteronuclear correlations were not conclusive and definitive evidence for assignment of the regioisomers was acquired using secondary isotope shifts observed after partial NH deuteration.[10] The acetyl groups in **23a** and **23b** were removed to give the



Scheme 3. i. H_2SO_4/HNO_3; ii. HCl/MeOH; iii. Cbz-Ala/iBuOCOCl/NMM; iv. Fe/AcOH; v. $PCl_5/CCl_4/CHCl_3$.

aminothiophenes **24a** and **24b**.[8] Acylation of the amino function in **24b** with the mixed anhydride of Cbz-Ala provided compound **25** which was reduced to the 5-aminothiophene **26** and this in turn was acylated to afford diamide **27**. The isomeric diamide **30** was prepared similarly from **24a** but in this case reduction to **29** preceded diacylation. Cyclisation of **27** and **30** to the oxazinones **28** and **31** was effected with PCl_5/CCl_4[11] and proceeded with concomitant chlorination of the thiophene ring in the case of **27**.

The thiophene oxazinones were evaluated in hplc assays of the peptidolytic activity of the HSV-1, HSV-2 and CMV proteases.[12] The Cbz-alanine derivative **14b** was found to be a submicromolar inhibitor of HSV-1 protease (Table 1) with a 100-fold improvement on potency over the benzoxazinone analogue **1b**. The serine derived compound **14c** was also a potent HSV-1 protease inhibitor. Both compounds inhibited HSV-2 protease at similar levels to HSV-1 protease, as might be expected from the high sequence homology of the two proteins. The nature of the R^1 group (Table 1) had a profound effect on inhibitory potency. For optimal HSV-1 protease inhibition, small R^1 groups are preferred and potency is reduced with larger groups to the point where the phenylalanine **14g** is completely ineffective. In contrast, compounds with small R^1 groups were much less effective inhibitors of CMV protease and in this case compound **14g** was optimal.



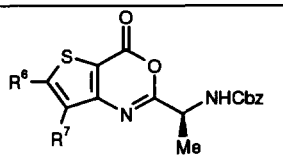
No.	R^1	R^2	IC_{50} (μM) or inhibition at 10 μM HSV-1	HSV-2	CMV
14a	H	Cbz	2.7	ND	45
14b	Me	Cbz	0.48	0.42	9.4
14c	CH_2OH	Cbz	0.65	0.33	20
14d	CH_2CONH_2	Cbz	10	ND	18
14e	$(CH_2)_2CONH_2$	Cbz	6.3	ND	ND
14f	$CHMe_2$	Cbz	31	ND	26
14g	CH_2Ph	Cbz	>300	ND	3.5
17a	Me	Boc	ND	32%	15%
17b	Me	2,4-dinitrophenyl	ND	2.9	3.3
17c	Me	tosyl	ND	13%	30%
17d	Me	CONHPh	ND	>100	>100
17e	Me	$CONHCH_2Ph$	ND	1%	20%

Table 1. Inhibition of herpes proteases by 2-substituted thieno[3,2-d]oxazinones.

Replacement of the Cbz group with Boc, sulphonamide or urea substituents (**17a**, c-e) resulted in a significant decrease in HSV-2 protease potency but the dinitrophenyl derivative **17b** afforded increased CMV inhibition, a similar IC_{50} being obtained for both HSV-2 and CMV enzymes.

Our previous studies had shown that a Cbz-alanine amide substituent on the 7-position of the aryl ring of benzoxazinones increased HSV-1 protease inhibition.[5] In thiophene oxazinones the Cbz-Ala seems to be

preferred at the 6-position (**32**, Table 2). This substituent notably enhanced CMV protease potency so that **32** had a similar effect on the HSV-2 and CMV enzymes. In contrast, substitution at this position with a phenyl ring significantly reduced CMV potency (compound **30**).



No.	R^6	R^7	IC_{50} (μM) or inhibition at 10 μM HSV-1	HSV-2	CMV
30	Ph	H	3.3	ND	>300
31	H	Me	3.1	ND	12
32	Cbz-Ala-NH	Cl	ND	1.6	1.3
33	H	Cbz-Ala-NH	ND	50%	43%

Table 2. Inhibition of herpes proteases by ring substituted thieno[3,2-d]thienoxazinones.

The SAR of these thieno[3,2-d]oxazinones for HSV and CMV proteases clearly do not run in parallel. Compound **30** has about 100-fold selectivity for HSV-1 over CMV protease whereas compound **14g** has similar selectivity in the opposite direction. Despite their similar cleavage pattern, there is significant sequence divergence between the HSV and CMV enzymes (30% identity). However, compounds such as **32** do have micromolar potency for both HSV and CMV proteases, suggesting pan-herpetic inhibition may be achievable.

In order to check that protease inhibition was occurring by formation of a covalent adduct as anticipated, compound **14b** was incubated with HSV-2 protease and adduct formation was monitored by electrospray mass spectroscopy. At both 2 min and 60 min time-points a monoadduct with mass increment of 330 was observed, consistent with the thienoxazinone acting as a mechanism-based inhibitor of HSV-2 protease by formation of an acyl-enzyme complex.

The selectivity of **14b** with respect to representatives of the major serine protease families, elastase and subtilisin was examined. Virtually no inhibition of subtilisin was observed up to an inhibitor concentration of 1 mM. Compound **14b** was also very selective with respect to elastase, with an IC_{50} of 120 μM. Although the absolute value of the selectivity ratio depends on the assay conditions, it is interesting that decreasing the steric bulk in the region peri to the oxazinone carbonyl ring from compound **1b** through to **14b** results in a 10^5-fold increase in the relative selectivity ratio (Table 3). Analysis of the aqueous stability of **14b** as described previously[5] also indicated that it had enhanced stability relative to the benzoxazinones **1a** and **1b** (Table 3).

Compound	IC_{50} (μM) HSV-1 pr.	elastase	elast./HSV-1 pr.	$t_{1/2}$ (h)
1a	120	0.22	0.002	33
1b	50	45	1.1	14
14b	0.48	120	250	>100

Table 3. Comparison of **14b** with 5-substituted benzoxazinone analogues **1a** and **1b**.

Replacement of the benzene ring of 2-carba benzoxazinones with a thiophene ring thus resulted in thieno[2,3-d]oxazinone inhibitors such as **14b**, **14c** and **32** which are potent and selective mechanism based inhibitors of the herpes proteases with enhanced stability relative to benzoxazinones.

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10. The magnitude of the $^{1}J_{CH}$ heteronuclear coupling constants for the protonated carbons of compounds **23a** and **23b** (194.2 Hz and 186.2 Hz respectively in $CDCl_3$) suggested that **23a** possessed a C5 protonated carbon and **23b** possessed a C4 protonated carbon although the difference in $^{1}J_{CH}$ values for the two compounds was smaller than expected from literature data for thiophenes.[9b] Secondary isotope shifts, $^{n}\Delta\delta$ where $\Delta\delta = \delta C(D)-\delta C(H)$ and n = the number of intervening bonds, were determined in d_6-acetone. For **23b**, large $^{2}\Delta\delta$ effects were observed for C3 (-134 ppb) and 3-NHCO (-97 ppb) and smaller $^{3}\Delta\delta$ effects for C2 (-38 ppb) and the protonated carbon (-52 ppb). On this basis the protonated carbon for **23b** was assigned as C4 with the position of nitro substitution therefore at C5. Based on this conclusion, together with corroborating $^{1}J_{CH}$ values, thiophene **23a** must possess a C5 protonated carbon with nitro-substitution at C4. The observed $^{n}\Delta\delta$ for **23a** were consistent with this assignment, where $^{n}\Delta\delta$ of C3, 3-NHCO, C2 and 3-NHCOMe were of a similar magnitude for **23a** as for **23b** but no $^{n}\Delta\delta$ was observed to either the protonated or nitrated carbon of **23a**. The lack of the expected $^{n}\Delta\delta$ for C4 of **23a** could be explained by the extensive quadrupolar broadening observed for the nitrated carbon of both **23a** and **23b** arising from the adjacent nitrogen. The $^{n}\Delta\delta$ values are consistent with literature data for indoles and pyrroles[13] and the conclusions corroborate those drawn from $^{1}J_{CH}$ values above and in reference 8.
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