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Potency and selectivity of P2/P3-modified inhibitors of cysteine proteases from trypanosomes

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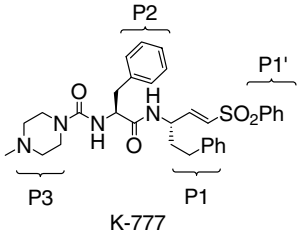
Abstract—A systematic study of P2 and P3 substitution in a series of vinyl sulfone cysteine protease inhibitors is described. The introduction of a methyl substituent in the P2 phenylalanine aryl ring had a favorable effect on protease inhibition and conferred modest selectivity for rhodesain over cruzain. Rhodesain selectivity could be enhanced further by combining these P2 modifications with certain P3 amide substituents.

Chagas' disease is a trypanosomiasis caused by the protozoan parasite *Trypanosoma cruzi*. Millions are affected in Latin America where the disease is associated with significant morbidity and mortality and has become the leading cause of cardiomyopathy. Current therapies for Chagas' disease include the nitroheterocyclics nifurtimox and benznidizole. The use of these drugs is restricted however, due to significant toxicity (neuropathies, gastrointestinal symptoms, myelosuppresion) and concerns about the potential mutagenicity of these agents. Furthermore, although the existing therapies can be effective in treating acute disease, they are not effective against the chronic infection that ultimately results in cardiomyopathy.

In the hope of identifying safer and more selective antitrypanosomals, significant interest has been directed at parasitic proteases as potential drug targets. Along these lines, inhibitors of cruzain, the major cysteine protease in *T. cruzi*, have been shown to kill parasites within infected macrophages and to cure a *T. cruzi* infection in rodents.[1,2] Among the cruzain inhibitors currently under investigation is the vinyl sulfone K-777 shown below. This substrate-like inhibitor bears hydrophobic P1 and P2 residues, a more hydrophilic *N*-methylpiperazine moiety at P3, and a vinyl sulfone 'warhead' that reacts irreversibly with the thiol function of the active site cysteine in cruzain.[3] The homophenylalanine-derived moiety at P1 confers stability towards endopeptidases, leading to superior stability in vivo as compared to analogs bearing a natural amino acid side chain at P1.[1] The vinyl sulfone warhead is modestly electrophilic, reacting only with active site cysteines, and only when properly oriented in the protease active site.[3] Hence, cysteine protease inhibitors containing a vinyl sulfone warhead can exhibit good selectivity and a favorable in vivo safety profile despite the irreversible nature of inhibition. Indeed, K-777 has proven well tolerated in preclinical animal toxicology and efficacy studies aimed at enabling the clinical evaluation of this compound as a treatment for Chagas' disease.[4] In an effort to further optimize this class of inhibitors and possibly identify analogs effective against other trypanosome proteases, we synthesized and evaluated a series of analogs closely related to K-777.



K-777

Keywords: Cysteine protease inhibitors; Trypanosomes; Vinyl sulfones; Cysteine proteases; Antiparasitics; Antitrypanosomals.
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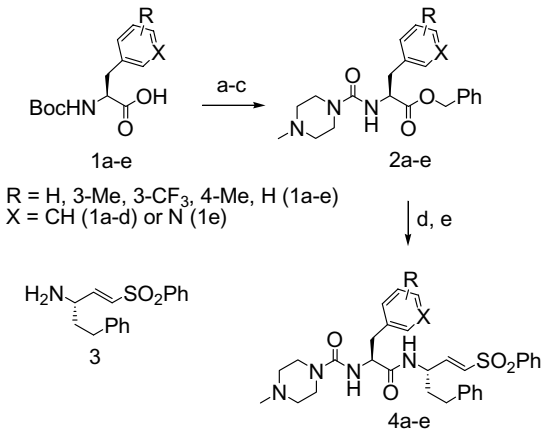
Vinyl sulfones such as K-777 were originally investigated in the context of human cathepsin inhibition.[3] An early study[5] of cathepsin specificity using vinyl sulfones showed that the size (surface area) of the P2 side chain was positively correlated with potency, and that cathepsin S was able to tolerate greater P2 diversity than cathepsins K or L. In this study, cathepsin L was most effectively inhibited by vinyl sulfones bearing Leu or Phe residues at P2.

Cruzain is considered to be most similar to cathepsin L, and is distinguished by an S2 pocket that permits the binding of basic (Arg) as well as hydrophobic (Phe) side chains. This dual-specificity is conferred by a glutamate residue (Glu205) at the base of the S2 pocket that is capable of interacting with Arg-containing substrates, but swings away from the pocket upon binding more lipophilic P2 side chains.[6] Notably, this glutamate residue is absent in the parasite proteases rhodesain and cathepsin-B-like protease[7] (TbCatB), potential drug targets in the causative agent of Sleeping Sickness, *Trypanosoma brucei*. These observations, together with the inspection of published cruzain crystal structures,[8,9] suggested that the introduction of small aliphatic substituents at the meta or para position of the P2 Phe moiety in K-777 might confer improved potency while impacting selectivity for these three parasitic proteases.

In addition to modifications at P2, we sought to remove or attenuate the basicity of the *N*-methylpiperazine substituent at P3 in K-777. Although the piperazine ring is quite common in drug structures, the presence of this ring system in a series of cathepsin K inhibitors has been associated with lysosomotropism—the accumulation of drug in acidic (e.g., lysosomal) compartments.[10] This can result in an effective loss of enzyme selectivity in cellular contexts, even for compounds that exhibit exquisite selectivity in biochemical assays. On the other hand, for an antiparasitic indication the accumulation of drug within acidic compartments of *parasites* may in fact be a favorable property. To better understand these issues, we examined analogs of K-777 bearing P3 groups of attenuated basicity.

The synthesis of substituted phenylalanine analogs was accomplished using a slight modification of the procedure reported previously for the preparation of K-777 and derivatives.[11,12] Starting from commercially available Boc-phenylalanines (**1a–e**), we prepared the corresponding benzyl esters and then removed the amine protecting group using standard methods. Next, the urea function was introduced via reaction of an intermediate isocyanate with *N*-methylpiperazine, affording analogs **2a–e** (Scheme 1). Hydrogenolysis of the benzyl esters **2a–e** and EDC-mediated coupling of the resulting acids with the vinyl sulfone amine **3**[11] afforded the desired P2-modified analogs **4a–e** with no detectable epimerization.

Although installation of the vinyl sulfone prior to urea formation could in principle save two steps, a facile formation of hydantoin product upon isocyanate formation precluded this approach. In the case of analogs

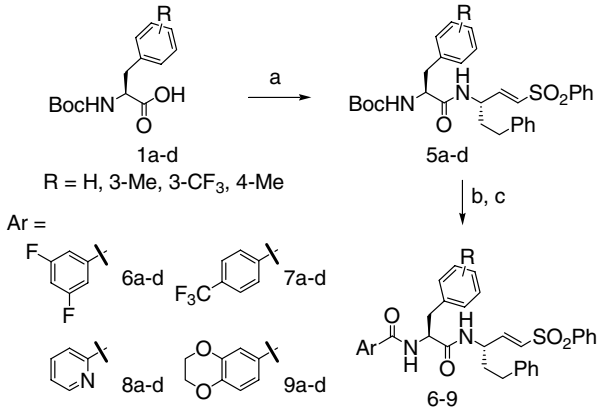


Scheme 1. Reagents: (a) BnOH, DIC, DMAP, DMF; (b) HCl, dioxane; (c) triphosgene, DIEA, CH_2Cl_2, then *N*-methylpiperazine; (d) H_2, Pd/C, EtOH; (e) **3**(HCl), EDC, HOBt, DIEA, DMF.

with an amide connection at P3, hydantoin formation is precluded and hence a shorter three-step sequence afforded the sixteen P2/P3 modified analogs **6a–9d** (Scheme 2).

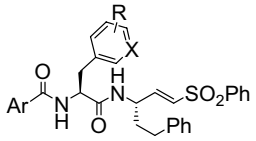
For each of the new analogs, second-order rate constants of enzyme deactivation were determined[13,14] against three important parasitic proteases: cruzain from *T. cruzi*, and both rhodesain and cathepsin-B-like protease (TbCatB) from *T. brucei*. The data are presented in Table 1 as k_{inact}/K_i or k_{ass} values (M^{-1} s^{-1}).[13] The individual K_i (nM) values and rate constants for enzyme inactivation k_{inact} (s^{-1}) are provided when the kinetic data allowed for the calculation of these values.[13] The separate contributions of K_i and k_{inact} to activity are discussed in those cases where such an analysis is informative.

The effects of P2 modification alone are revealed by considering the data for analogs **4a–e** in Table 1. K-777 (**4a**) itself exhibits little selectivity for cruzain over rhodesain and is much less effective (ca. 500-fold) against the cathepsin-B-like protease (TbCatB) in *T. brucei*. The introduction of a methyl substituent at the meta or para



Scheme 2. Reagents: (a) **3**(HCl), EDC, HOBt, DIEA, CH_2Cl_2; (b) HCl, dioxane; (c) ArCOOH, EDC, HOBt, DIEA, DMF.

Table 1. Inhibition kinetics of P2/P3 modified vinyl sulfones against three trypanosome proteases



Compound	R	X	Ar	Cruzain			Rhodesain			TbCatB
				k_{inact}/K_i (M^{-1} s^{-1})	K_i (nM)	k_{inact} (s^{-1})	k_{inact}/K_i (M^{-1} s^{-1})	K_i (nM)	k_{inact} (s^{-1})	k_{inact}/K_i (M^{-1} s^{-1})
K-777 (**4a**)	H	CH	*N*-MePip	510,000	220	0.11	432,000	270	0.11	1000[a]
4b	3-Me	CH	*N*-MePip	257,000	3300	0.86	517,000	170	0.088	19,000
4c	3-CF_3	CH	*N*-MePip	53,000	170	0.009	81,000[a]	—	—	400[a]
4d	4-Me	CH	*N*-MePip	440,000[a]	—	—	852,000	120	0.11	10,000
4e	H	N	*N*-MePip	45,000	3600	0.16	55,000[a]	—	—	800[a]
6a	H	CH	3,5-DiFPh	31,000	980	0.31	317,000	66	0.021	<10[b]
7a		CH	4-CF_3Ph	100,000	97	0.0097	104,000	87	0.0090	<5[a]
8a		CH	2-Pyridyl	120,000	250	0.030	150,000	910	0.14	70[a]
9a		CH	DHBD	70,000	80	0.0056	223,000	85	0.019	540[a]
6b	3-Me	CH	3,5-DiFPh	105,000	50	0.0052	456,000	50	0.023	16,000
7b		CH	4-CF_3Ph	38,000	92	0.0035	116,000	43	0.0050	50[b]
8b		CH	2-Pyridyl	36,000	280	0.010	173,000	230	0.040	100[a]
9b		CH	DHBD	14,000	71	0.0010	252,000	37	0.0093	650[a]
6c	3-CF_3	CH	3,5-DiFPh	2400[b]	—	—	52,000	33	0.0017	<10[b]
7c		CH	4-CF_3Ph	55[b]	—	—	900[b]	—	—	<10[b]
8c		CH	2-Pyridyl	3100	150	0.00047	6000	280	0.0017	500[a]
9c		CH	DHBD	3300[b]	—	—	2350[b]	—	—	35[b]
6d	4-Me	CH	3,5-DiFPh	34,000	350	0.012	366,000	30	0.011	<10[b]
7d		CH	4-CF_3Ph	109,000	45	0.0049	420,000	33	0.014	<10[b]
8d		CH	2-Pyridyl	16,000	1700	0.027	327,000	880	0.290	60[a]
9d		CH	DHBD	311,000	19	0.0060	1,570,000	20	0.031	600[a]

TbCatB, cathepsin-B-like protease in *T. brucei*; *N*-MePip, *N*-methyl piperazine; DHBD, 2,3-dihydro-1,4-benzodioxin-6-yl.
[a] Value is k_{ass} (M^{-1} s^{-1}).
[b] Value is k_{obs}/[I] (M^{-1} s^{-1}) at a single inhibitor concentration.

position (**4b** and **4d**) confers a subtle effect on enzyme selectivity. Thus, the substituted analogs are marginally *less* potent than K-777 against cruzain while exhibiting slightly improved potency against rhodesain and a 10- to 20-fold improvement against TbCatB. The improved rhodesain activity of **4b** and **4d** derives from an approximately twofold improvement in K_i, presumably a result of the additional binding affinity contributed by the additional methyl substituent. That no comparable gains in potency are realized versus cruzain possibly reflects an unfavorable interaction between the introduced methyl substituent and the side chain of Glu205. In support of this notion, we note that the 3-methyl analog **4b** exhibits a 20-fold weaker K_i for cruzain than for rhodesain while K-777 shows similar K_i values for the two enzymes. Perhaps surprisingly then, the isosteric 3-trifluoromethyl analog **4c** exhibits a cruzain K_i much closer to that of K777 than **4b**. In the case of **4c**, the rate constant of enzyme inactivation k_{inact} is notably affected.

The P2 effects noted above are to some extent recapitulated in the case of analogs bearing non-piperazine P3 substituents (Table 1). Hence, compared to the parent P2 Phe analogs **6a–9a**, the corresponding 4-methyl analogs **6d–9d** exhibit improved rhodesain activity while showing only comparable activity against cruzain. In the case of P2/P3 modified analogs **6–9**, substitution at the 4 position is clearly favored over 3-substitution, with 3-methyl analogs **6b–9b** no more potent than the desmethyl analogs **6a–9a**. As was the case with **4c**, the introduction of an electron-withdrawing trifluoromethyl substituent results in significant losses in potency (analogs **6c–9c**). Overall, the SAR at P2 suggests good tolerance for small alkyl substituents and augurs that substitution with larger groups may be a fruitful strategy for engineering improved activity against rhodesain and TbCatB.

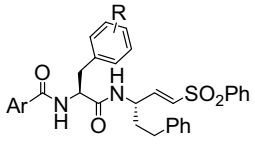
To explore SAR at P3, we replaced the *N*-methylpiperazine ring of analogs **4a–d** with one of four aromatic or heteroaromatic ring systems (Scheme 2). As measured by the k_{inact}/K_i ratio, these new analogs (**6–9**) display activities generally inferior to the parent piperazine analogs, but with an enhanced selectivity profile that favors rhodesain over cruzain—in some cases by a significant margin (e.g., **8d**). Looking solely at k_{inact}/K_i, it is difficult to discern a consistent SAR trend with respect to P3 substitution. Examined separately however, the k_{inact} and K_i values reveal a more interesting story. Thus, compared to the parent piperazine analogs **4**, the non-basic benzoyl P3 congeners **6**, **7**, and **9** exhibit superior

binding affinities (K_i as low as 19 nM) but significantly reduced rate constants of inactivation (k_{inact}). Apparently, while a lipophilic P3 substituent can confer stronger binding through enhanced interactions with S3, the vinyl sulfone moiety may, as a result, be nudged into a less favorable orientation for efficient reaction. Interestingly, analogs **8a–d** bearing a more hydrophilic P3 group (pyridyl) display K_i and k_{inact} values that are more comparable to the parent piperazine analogs.

In some cases the combination of P2 and P3 modification confers a noteworthy synergistic effect. For example, the combination of a 3-Me Phe at P2 with either 3,5-difluorophenylamide or benzodioxane moieties at P3 produces analogs (**6b** and **9b**) with noteworthy selectivity for rhodesain over cruzain (up to eighteenfold). The combination of a benzodioxane P3 substituent with 4-Me Phe at P2 affords **9d**, a rhodesain-selective analog that is fourfold more potent than K-777. Finally, the combination of a pyridyl P3 group with a 4-Me Phe P2 substituent affords **8d**, the most highly rhodesain-selective (twentyfold) analog described in this study. Interestingly, in each of these cases (**6b**, **9b**, **9d**, **8d**) rhodesain selectivity derives not from increased binding affinity (K_i) relative to cruzain but from faster rate constants of inactivation (k_{inact}).

Next, we examined selected analogs for their ability to prolong survival of *T. cruzi*-infected J774 macrophages using an established protocol[1] (Table 2). This assay provides information about antiparasitic efficacy and also provides a qualitative measure of compound toxicity to the macrophage itself. The experiment was carried out over 42 days and a number of the new P2/P3-modified analogs were found to exert an antitrypanosomal effect (Table 2). The 3-methyl and 3-trifluoromethyl analogs **4b** and **4c** were as effective as K-777, although the latter showed some toxicity. A number of P3 benzoyl amide analogs were also examined in this assay and showed varying degrees of efficacy but were generally less effective than the P3 piperazine analogs. Of the non-piperazine analogs examined, only the P3 pyridyl analog **8a** was as effective as K-777. While it is tempting to attribute this effect to the presence of a basic nitrogen in **8a**, the 2-pyridyl ring in **8a** is unlikely to be significantly protonated, even at lysosomal pH. Instead, the activity of **8a** may derive from improved permeability (or active transport) into the parasite and/or sequestration to high concentration within particular compartments. Certainly, the superior efficacy of **8a** as compared to **6a–c** and **7a** and **7b** cannot be explained solely on the basis of relative enzymatic activities.

Table 2. Efficacy of vinyl sulfones in a *T. cruzi*-infected J774 macrophage assay (10 μM compound)



Compound	R	Ar	J774 macrophage survival (days)	Macrophage toxicity
No drug	—	—	5	—
K-777 (**4a**)	H	*N*-MePip	42	No
4b	3-Me	*N*-MePip	42	No
4c	3-CF_3	*N*-MePip	42	Yes
6a	H	3,5-DiFPh	12	Yes
6b	3-Me	3,5-DiFPh	12	Yes
6c	3-CF_3	3,5-DiFPh	23	No
7a	H	4-CF_3Ph	8	No
7b	3-Me	4-CF_3Ph	8	Yes
8a	H	2-Pyridyl	42	No

In summary, we have conducted a systematic exploration of P2 and P3 substitution in a series of vinyl sulfone-based cysteine protease inhibitors. Our results suggest that the introduction of small substituents on the phenyl ring of the P2 side chain represents a viable strategy for generating more rhodesain-selective inhibitors. We continue to explore P2/P3 modification in this context and plan a more extensive biological evaluation of these analogs, particularly with respect to activity against *T. brucei* parasites.

Acknowledgments

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13. Kinetic analyses were performed as follows: Cruzain (2 nM), rhodesain (2 nM), or TbCatB (40 nM) in 100 μL assay buffer was added to inhibitor dilutions in 100 μL of 10 μM *Z*-Phe-Arg-AMC in buffer A. Progress curves were determined for 150 s (cruzain) or 300 s (rhodesain and TbCatB) at room temperature for inhibitor concentrations ranging from 10 to 0.01 μM (10–0.1 μM for TbCatB). Inhibitor dilutions which gave simple exponential progress curves over a wide range of k_{obs} were used to determine kinetic parameters. The value of k_{obs}, the rate constant for loss of enzymatic activity, was determined from an equation for pseudo first-order dynamics using Prism 3.16 (GraphPad). When k_{obs} varied linearly with inhibitor concentration, k_{ass} was determined by linear regression analysis. If the variation was hyperbolic, indicating saturation inhibition kinetics, k_{inact} and K_i were determined from an equation describing two-step irreversible inhibitor mechanism $[k_{obs} = k_{inact}[I]o/([I]o + K_i^*(1+ [S]o/K_m))]$ and non-linear regression analysis using Prism.
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