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DESIGN AND SYNTHESIS OF DIPEPTIDYL α',β'-EPOXY KETONES, POTENT IRREVERSIBLE INHIBITORS OF THE CYSTEINE PROTEASE CRUZAIN

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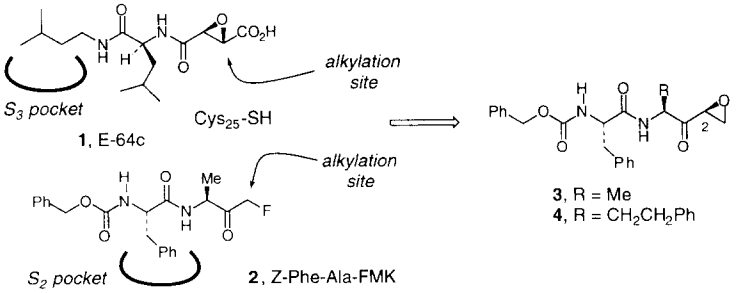
Abstract: The dipeptidyl epoxy ketone **4** is a potent, irreversible inhibitor of cruzain, a cysteine protease isolated from *Trypanosoma cruzi*, with an apparent second order inhibition rate constant of 330,000 $sec^{-1}M^{-1}$.

Cysteine proteases are an important class of enzymes involved in the hydrolytic processing of peptides and proteins.[1,2] They are ubiquitous in nature and play vital roles in numerous pathological processes, including viral replication, bacterial infection, arthritis, osteoporosis, Alzheimer's disease, cancer cell invasion, and apoptosis.[1–3] Cysteine proteases are also essential to the life cycles of many pathogenic protozoa.[4,5] One such parasite is *Trypanosoma cruzi*, the etiologic agent of Chagas' disease. Cruzain,[6,7] the major cysteine protease of *T. cruzi*, has been identified as a potential therapeutic target for treatment of Chagas' disease.[7–9]

We report herein the design and synthesis of a new class of cysteine protease inhibitors,[2,10,11] represented by structures **3** and **4**, that incorporate α,β-epoxy ketone units as the electrophilic warhead targeting the active site cysteine residue (Cys-25 in cruzain). These inhibitors were designed by combining features of the known cysteine protease inhibitors E-64c (**1**)[12,13] and the dipeptidyl fluoromethyl ketone, Cbz-Phe-Ala-FMK (**2**). Examination of X-ray crystal structures of the papain•E-64 or E-64c complexes,[14–16] the cruzain•Cbz-Phe-Ala-FMK complex[7,17] and several papain•chloromethyl ketone complexes[18] reveals that the peptide backbone of E-64c orients in a direction opposite to that of the chloro- or fluoromethyl ketone inhibitors. As a consequence, the L-leucyl side chain of **1** does not optimally occupy the enzyme S_2 binding pocket. We reasoned that epoxy ketones **3** and **4** would be more potent than E-64c as inhibitors of cruzain, since the peptide backbones of **3** and **4** would be expected to interact more productively with the S_1, S_2 and S_3 binding sites (as is also the case with the dipeptidyl halomethyl and α-acyloxymethyl ketone inhibitors).[19] While our work was in progress, Spaltenstein and coworkers reported the first example of a peptidyl α,β-epoxy ketone protease inhibitor, specifically a tripeptidyl epoxy ketone inhibitor targeting the proteasome.[20,21] Other studies on the development of peptidyl epoxides as cysteine or aspartyl protease inhibitors have been reported, although thus far the best inhibitors in these series have displayed very weak activity.[22–24]



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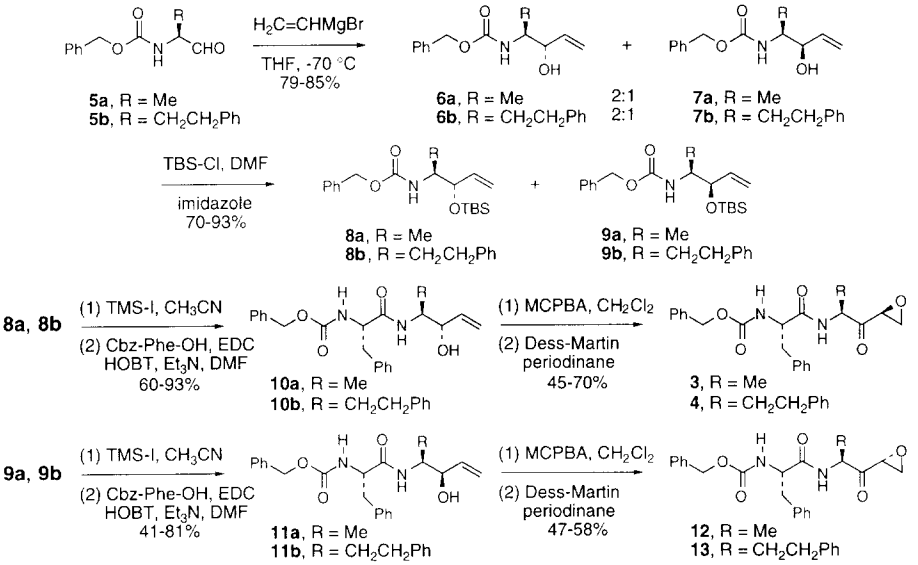
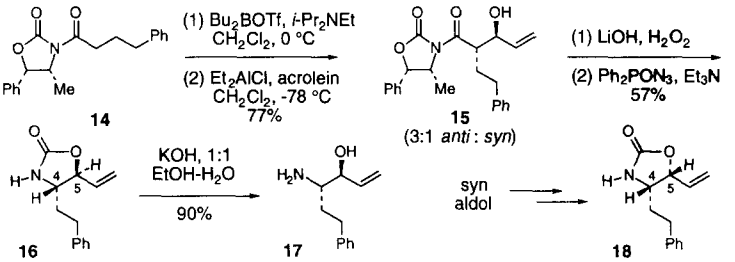
Although we anticipated at the outset that inhibitors **3** and **4** with 2(*S*) stereochemistry would be more potent than their 2(*R*) diastereomers (**12** and **13**), we elected to develop a synthesis that would provide access to both isomers. Thus, treatment of Cbz protected L-alaninal (**5a**)[25] and L-homophenylalaninal (**5b**)[25] with H_2C=CHMgBr in THF at -70 °C provided 2 : 1 mixtures of allylic alcohols **6** and **7**,[26] which were separated chromatographically following protection of the alcohols as the *tert*-butyldimethylsilyl (TBS) ethers **8** and **9**. Treatment of the *threo* β-hydroxy carbamate derivatives **8a** and **8b** with trimethylsilyl iodide (TMS-I) removed both the Cbz and TBS protecting groups, giving the corresponding amino alcohols in good yield. These intermediates were then acylated with Cbz-Phe-OH under standard peptide coupling conditions (EDC, HOBT, Et_3N, DMF),[27] thereby providing the dipeptidyl allylic alcohols **10a** and **10b** in 60-93% yield for the two steps. Epoxidation of **10a** and **10b** with *m*-chloroperoxybenzoic acid in CH_2Cl_2 followed by oxidation of the epoxy alcohol intermediates with the Dess-Martin periodinane[28] then provided the targeted dipeptidyl 2(*S*)-epoxy ketones **3** and **4**. The epimeric 2(*R*)-epoxy ketones **12** and **13** were prepared by an analogous sequence starting from the *erythro* amino alcohol derivatives **9a** and **9b**. It should be noted that while only one epoxide stereoisomer, the *erythro* isomer, was detected in the epoxidations of **10b** and **11b** in the homophenylalanine series, 3-4:1 mixtures of two epoxides were obtained in the epoxidations of **8a** and **9a** in the alanine series. It is surprising that these reactions proceed with this stereoselectivity, since peracid epoxidation of simpler 1-vinyl carbinols generally give 60:40 mixtures favoring the *threo* isomer.[29] The stereochemistry of the epoxide units in the epoxy alcohol precursors to **3**, **4**, **12** and **13** was assigned according to the method of Spaltenstein.[20]



A second route to **4** has also been explored, involving an enantioselective *anti* aldol reaction. Enolization of **14** (prepared by acylation of the (1*S*,2*R*)-norephedrine derived oxazolidinone with $PhCH_2CH_2CH_2COCl$)[30] with Bu_2BOTf and *i*-Pr_2NEt in CH_2Cl_2 at -78°C followed by addition of the enol borane intermediate into a -78 °C solution of the complex generated from acrolein and Et_2AlCl in CH_2Cl_2 provided **15** in 77% yield, as a 3 : 1 mixture of the indicated *anti* aldol and its 2,3-*syn* isomer.[31] Hydrolysis of **15** with H_2O_2 and LiOH[32] followed by Curtius degradation (diphenylphosphoryl azide, Et_3N, C_6H_6, reflux)[33]

of the resulting β-hydroxy carboxylic acid gave the trans oxazolidinone **16** in 57% overall yield. Finally, hydrolysis of **16** provided *threo* amino alcohol **17**, which was identical in all respects to the same compound prepared by deprotection of **8b**. The stereochemistry of **16**, and hence also **17**, was verified by comparative NMR studies with the *syn* oxazolidinone **18**, prepared by hydrolysis and Curtius degradation of the 2,3-*syn* aldol diastereomer. Whereas it was not possible to make definitive assignments on the basis of coupling constant analysis ($J_{4,5}$ = 6.5 Hz for **16**, vs. $J_{4,5}$ = 7.5 Hz for **18**), the cis stereochemistry of **18** was easily assigned on the basis of NOE experiments (irradiation of H(4) led to an enhancement of H(5)); in contrast. NOE's were not observed between H(4) and H(5) of **16**.



Enzyme kinetic assays indicate that **3** and especially **4** are potent, irreversible inhibitors of cruzain.[34] The second order rate constant for inhibition of cruzain by **4** (k_{inact}/K_i = 330,000 $M^{-1}sec^{-1}$) is four–fold greater than that of E-64c (70,600 M^{-1} sec^{-1}).[35] In addition, IC_{50} determinations[36] indicate that both **3** and **4** with 2(*S*) epoxy ketone stereochemistry are substantially more active than their 2(*R*) epimers **12** and **13**. Interestingly, it is the 2(*R*)-epoxy ketone that is most active inhibitor of the proteosome in Spaltenstein's work.[20] Further studies on the development of these compounds as cysteine protease inhibitors will be reported in due course.

Compound	k_{inact}/K_i ($M^{-1}sec^{-1}$)	IC_{50} (μM)
3	128,200	0.08
4	330,000	0.01
E-64c	70,600	--
12	n.d.	1
13	n.d.	>1

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35. Kinetic assays of irreversible inhibitors were determined using purified recombinant cruzain lacking the C terminal domain.[6] Cruzain (4 nM) in 100 μL of assay buffer was added to inhibitor dilutions selected by progress curve anaylsis, in 100 μL of 5 μM Z-Phe-Arg-AMC (Bachem; K_m = 1 μM). Progress curves were followed for 5 min at room temperature (less than 5% of substrate used). The value of k_{obs}, the pseudo-first order rate constant for loss of enzyme activity, was determined from an equation for pseudo first order dynamics using UltraFit (Biosoft). K_i and k_{inact} were determined from the *x* intercept and the slope, respectively, of double reciprocal plot of k_{obs} vs inhibitor concentration when $r^2 \geq 0.95$.[33]
36. Inhibitor IC_{50}'s were determined as follows. Purified recombinant cruzain lacking the C terminal domain[6] (4 nM) was incubated with 1 nM to 10,000 nM inhibitor in 100 mM NaAc (pH 5.5) and 10 mM DTT for 5 min at room temperature. Z-Phe-Arg-AMC (Bachem, K_m = 1 μM) was added to a final concentration of 20 μM in a final volume of 200 μL. The increase in fluorescence (excitation at 355 nm and emission at 460 nm) was followed with an automated microtiter plate spectrofluorometer (Labsytems Fluoroskan II), and the initial velocities determined. Inhibitor stocks were prepared at 20 mM in DMSO, and serial dilutions were made in DMSO.[33] Controls of enzyme alone, enzyme with DMSO and enzyme with a previously known irreversible inhibitor [Mu-Phe-HPhe-CH=CH_2–SO_2Ph ("Mu" = morpholine urea), Arris Pharmaceuticals Inc., South San Francisco, CA (Palmer, J. T.; Rasnick, D.; Klaus, J. L.; Brömme, D. *J. Med Chem.* **1995**, *38*, 3193, and Brömme, D.; Klaus, J. L.; Okamoto, K.; Rasnick, D.; Palmer, J. T. *Biochem. J.* **1996**, *315*, 85)] were run with each assay set.
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