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Dipeptidyl-α,β-epoxyesters as potent irreversible inhibitors of the cysteine proteases cruzain and rhodesain

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Abstract—The dipeptidyl epoxyesters **3** and **4** are potent, irreversible inhibitors of cruzain and rhodesain.

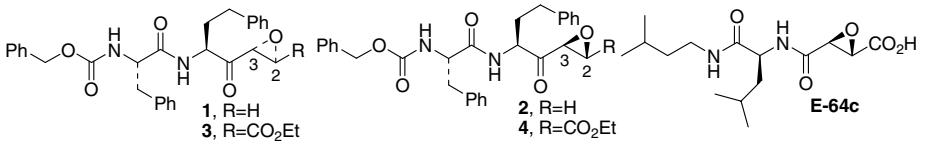
Cysteine proteases are an important class of enzymes involved in the hydrolysis of peptides and proteins.[1,2] The papain family of cysteine proteases includes cathepsins, calpains, and the parasitic cysteine proteases cruzain and rhodesain which are essential for the development and survival of the protozoan *Trypanosoma cruzi* and *Trypanosoma brucei*, respectively.[3] *T. cruzi* causes Chagas' disease in humans in South and Central America,[4,5] whereas *T. brucei* causes sleeping sickness in humans in large areas of central and southern Africa.[6,7] Consequently, inhibition of cysteine proteases has emerged as an important strategy for the treatment of these diseases.

In 1998 dipeptidyl α,β-epoxy ketones **1** and **2** were reported as cysteine protease inhibitors.[8] For improved potency and selectivity, we envisioned a new class of inhibitors, represented by structures **3** and **4**, which incorporated the epoxyester unit.

We report herein the design and synthesis of a new class of cysteine protease inhibitors, represented by structures **3** and **4**, that combine the reactivity of the E-64c[9,10] epoxide electrophile with the Cbz-Phe-HPhe sequence already known as a selective moiety for effective cruzain inhibition.[11–13]

Although the stereochemistry of the epoxide in E-64c is 2(*S*),3(*S*) and precedents[8] of epoxy inhibitors pointed to 2(*S*) as the best selection, we carried out a synthesis of both isomeric epoxides **3** and **4**.

The preparation of these inhibitors involved an enantioselective *anti* aldol[14] reaction using thiazolidinethione **5** (prepared by acylation of thiazolidinethione derived from L-phenylalanine), ethyl 4-oxo butenoate (prepared from furfural),[15] and $MgBr_2$ as a catalyst (Scheme 1). The crude mixture of protected aldols was then treated with 1 M aq HCl and separated by chromatography, affording a 4:1 mixture of free aldols **6** and **7**. Then protection and removal of the chiral auxiliary in **6** with H_2O_2 and LiOH gave carboxylic acid **8** which was submitted to Curtius reaction[16] using DPPA and Et_3N affording isocyanate **9**. Compound **9** was then directly



Keywords: Cysteine proteases inhibitors; Epoxides; Epoxyesters; Dipeptides.
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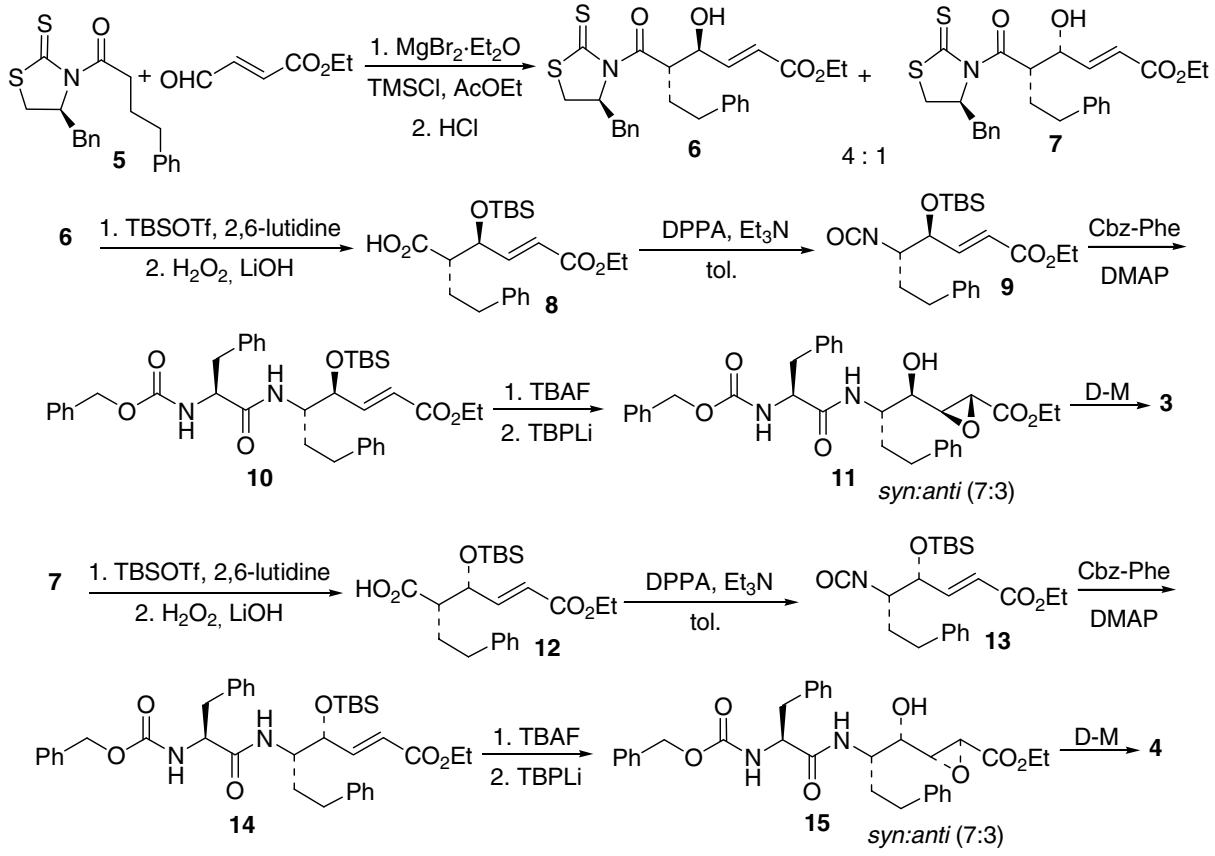

Scheme 1. Synthetic scheme for the preparation of dipeptidyl epoxyesters **3** and **4**.

coupled[17] with Cbz-Phe and 4-DMAP furnishing dipeptidyl enoate **10**. Then compound **10** was deprotected and epoxidized with TBPLi giving a 7:3 mixture of *syn*/*anti* epoxyalcohols which were separated by chromatography. The selectivity of the epoxidation reaction is in agreement with our previous results related to the nucleophilic epoxidation of γ-hydroxy α,β-unsaturated esters.[18] Finally Dess–Martin oxidation[19] of **11** *syn* afforded ketone **3**. The isomeric dipeptidyl epoxyester **4** was prepared by an analogous sequence starting from aldol **7**. In this case, the epoxidation of the dipeptidyl allylic alcohol gave a 7:3 mixture, with the *syn* isomer **15** predominating.

The stereochemical assignments of **6** and **7** were verified by NMR experiments of oxazolidinones **16** and **17**, respectively (Fig. 1). The derivatization of the aldols into the cyclic compounds was accomplished by thiazolidinethione removal and Curtius degradation sequence. The stereochemistries of **16** and **17** were assigned on the basis of coupling constant analysis ($J_{4,5}$ = 5.5 Hz for **16** and $J_{4,5}$ = 7.5 Hz for **17**) and by NOE experiments: **16** gave NOE between H-4 and homobenzylic protons, whilst **17** gave NOE between H-4 and H-5.

The stereochemistries of the epoxides were assigned through NMR experiments applied to lactones **18** and **19** (Fig. 2). Epoxides **11** and **15** were treated with sodium thiophenolate and the resulting diols were further cyclized in acidic media. In both cases the resulting lactones **18** and **19** gave NOE between H-2 and H-4 denoting the relative *syn* stereochemistry of the preceding epoxyalcohols.

The dipeptidyl epoxyesters **3** and **4** and their preceding epoxyalcohols **11** and **15** were screened against cruzain, rhodesain, and *T. brucei* cathepsin B[20] (Table 1). Alcohols **9** and **13** did not inhibit cysteine proteases whilst

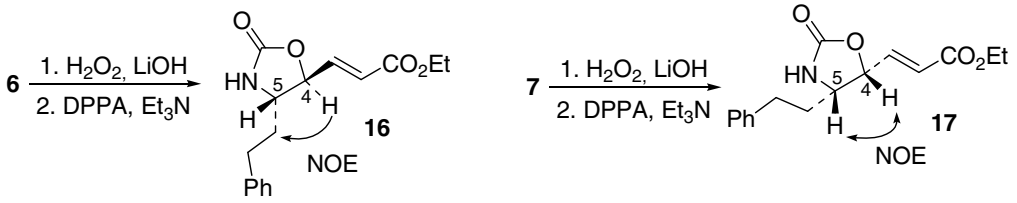

Figure 1. Stereochemical assignments of **6** and **7** determined using the oxazolidinones derivatives **16** and **17**.

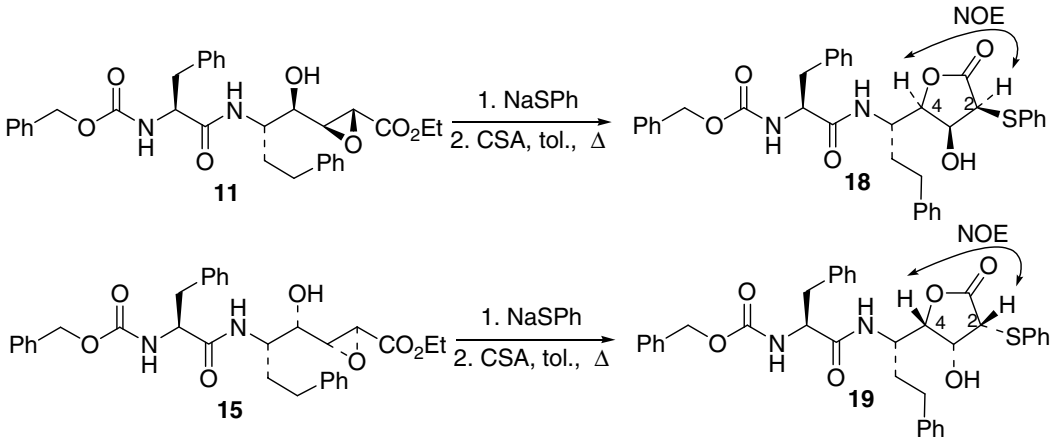

Figure 2. Stereochemical assignments of epoxides **11** and **15** determined from lactone derivatives **18** and **19**.

Table 1. Inhibitory effect and determination of IC_{50} values for novel dipeptidyl epoxyester inhibitors and previous inhibitors

	Inhibition				IC_{50} (nM)		
Compound	Tbb in vitro (%)	Cruzain (%)	Rhodesain (%)	Tbb CathB (%)	Cruzain	Rhodesain	Tbb CathB
11	−37	5	−4	6	n.d.	n.d.	n.d.
15	17	1	3	3	n.d.	n.d.	n.d.
3	42	93	98	51	20	3.5	≫1000
4	−2	55	80	47	50	30	400
1					>1000		
2					10		
K11777	99	100	72		5	5	2000

Tbb, *Trypanosoma brucei brucei*.

3 and **4** inhibited cruzain and rhodesain. IC_{50} determinations[21] indicated that **3** and **4** are potent inhibitors of cruzain and rhodesain, with **3** being more potent than **4**, especially against rhodesain. Curiously, compound **3**, having stereochemistry opposite to that of E-64c, was the most active inhibitor. Neither **3** nor **4** inhibited cathepsin B. In addition, dipeptidyl epoxyester **3** was much more potent than its analog **1**, while **4** was slightly less potent than the corresponding epoxyketone **2**. Compound **3** was also the most active against *Trypanosoma brucei brucei* in vitro.[22]

Kinetic analyses were performed on the most interesting compounds,[23] which confirmed that they are time-dependent inhibitors of cysteine proteases (Table 2). The second order rate constant for inactivation of cruzain by **3** is 3-fold greater than that of **4** and 4-fold greater in the case of rhodesain. Compound **3** displayed a second order rate constant higher than that for E-64c, and both dipeptidyl epoxyesters **3** and **4** showed second order rate constants that were lower than the values for epoxyketones **1** and **2**.

Table 2. Second order rate constants, k_{inact}/K_i or k_{ass} ($s^{-1}\,M^{-1}$), of inhibitors **1**, **2**, **3**, **4** and E-64c against cruzain, rhodesain, and *T. brucei* cathepsin B[21]

Compound	Versus cruzain k_{inact}/K_i	Versus rhodesain k_{inact}/K_i	Versus Tbb CathB k_{ass}
3	82,900	92,090	120
4	25,200	23,500	84.5
1	128,200	—	—
2	330,000	—	—
E-64c	70,600	—	—

Further studies on the development of the dipeptidyl epoxyesters series as cysteine protease inhibitors are ongoing and the results will be reported.

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21. IC_{50} determination. The inhibition of the cysteine proteases cruzain, rhodesain, and Tb CatB was evaluated by quantitation of the fluorescence emission resulting from proteolytic cleavage of the synthetic substrate Z-Phe-Arg-AMC (Bachem). Inhibitors were used at concentrations ranging from 10 nM to 10,000 nM (500–10,000 nM for Tb CatB), with the minimum inhibitor concentration used for calculations, at least 10-fold greater than enzyme; serial dilutions were prepared in DMSO.[20] These assays were performed in a 96-well plate, scanning fluorescence reader (Flex Station, Molecular Devices) at ex 355 and em 460 nm at 25 °C, with delivery of 100 μL of substrate by multichannel pipettes to an equal volume of enzyme inhibitor pre-incubated at 25 °C for 5 min. For each assay set, studies using enzyme with no additions, enzyme with DMSO vehicle, and enzyme in the presence of the previously known, highly effective irreversible inhibitor (*N*-methyl piperazine-Phe- HPhe-(CH=CHSO$_2$ Ph)) 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.) served as controls. Inhibitors, which had IC_{50} values of less than 1 μM, were further analyzed. Inhibition data for all enzymes were determined similarly: rec cruzain at 4 nM with 5 μM Z FR AMC (K_m = 1 μM) in buffer A (100 mM Na acetate pH 5.5 with 5 mM DTT and 0.001% Triton X-100 (Sigma)); rec rhodesain at 4 nM enzyme and 5 μM Z-Phe-Arg-AMC (K_m = 1 μM) in buffer A; and rec Tb CatB at 40 nM enzyme and 5 μM Z-Phe-Arg-AMC (K_m = 60 μM) in buffer A. A direct read of the semi log plot of reaction rate versus inhibitor concentration was used to determine IC_{50}.
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23. Kinetic assays of irreversible inhibitors. Kinetic analyses of the irreversible cysteine protease inhibitors were performed as follows.[20] (Tian, W.-X.; Tsou, C.-L. *Biochemistry* **1982**, *21*, 1028.) Enzyme in 100 μL of assay buffer was added to inhibitor dilutions in 100 μL of 10 μM Z-Phe-Arg-AMC in buffer A (Ref. 20) using the FLEX Station in FLEX mode. Enzyme was added by the instrument while fluorescent emission was being followed, allowing the first seconds of inhibition to be recorded. Progress curves were recorded over 5 min at 25 °C (less than 5% of substrate consumed) over a 10-fold range of dilutions of inhibitor, starting at 10 μM, and 5 μM (10, 5, 1, 0.5, 0.1, 0.05, 0.01, and 0 μM). Inhibitor dilutions which gave simple exponential progress curves over a wide range of k_{obs} were used to determine kinetic parameters (excluding those values where inhibitor concentration was not 10-fold greater than enzyme). The value of k_{obs}, the rate constant for loss of enzyme activity, was determined from an equation for pseudo first order dynamics using Prism3.16 (GraphPad). When k_{obs} varied linearly with inhibitor concentration, k_{ass} was determined by linear regression analysis.[20] If the variation was hyperbolic, indicating saturation inhibition kinetics, k_{inact} and K_i were determined from an equation describing a two-step irreversible inhibitor mechanism ($k_{obs} = k_{inact}$ [I]o/([I]o + K_i * (1 + [S]o/K_m))) and nonlinear regression analysis using Prism.[2] The value of k_{obs}/[I] is given at a single concentration of inhibitor only when the k_{obs} was significantly different from the k_{obs} for solvent, at the highest concentration tested.