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Design, synthesis, and biochemical evaluation of novel cruzain inhibitors with potential application in the treatment of Chagas' disease

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Abstract—A series of compounds bearing tetrahydronaphthalene, benzophenone, propiophenone, and related rigid molecular skeletons functionalized with thiosemicarbazone or unsaturated carbonyl moieties were prepared by chemical synthesis and evaluated for their ability to inhibit the enzyme cruzain. As potential treatment agents for Chagas' disease, three compounds from the group demonstrate potent inhibition of cruzain with IC_{50} values of 17, 24, and 80 nM, respectively.

American trypanosomiasis or Chagas' disease is a parasitic disease endemic to Latin America where it is a major cause of heart disease with 18–20 million people infected and over a hundred million at risk. It is caused by infection with the flagellate protozoan *Trypanosoma cruzi*, which is transmitted to humans by triatomine vectors (kissing bugs) or through contact with infected blood.[1] At the present time, the two clinically accepted drugs, nifurtimox and benznidazole, are associated with toxicity and a poor therapeutic index for the chronic form of the disease. The urgent need for an effective therapy against Chagas' disease has stimulated the search for a suitable drug target in the parasite. The primary cysteine protease, cruzain or cruzipain, is essential for infection of host cells, replication, and metabolism throughout the life cycle of the *T. cruzi* parasite.[2] Several compounds that block the action of cruzain have been used to cure this infection in cell and mouse models.[3,4] Therefore, cruzain has emerged as a validated target for drug development.

Du and coworkers[5] first introduced the thiosemicarbazone functionality into compounds designed to inhibit cruzain. In particular, the 3′-bromopropiophenone thiosemicarbazone derivative **5** is an effective cruzain inhibitor that demonstrates trypanocidal activity at concentrations that exhibit no toxicity for mammalian cells.[5] Further support for the importance of this functional group applied to the design of cysteine protease inhibitors was recently shown by Greenbaum and coworkers as well as Fujii and coworkers who synthesized small libraries of thiosemicarbazone cruzain inhibitors of which several show antiparasitic activity in cell cultures.[6,7] Therefore, we thought that the extension of the thiosemicarbazone functionality into other carbon skeletons might generate improved cysteine protease inhibitors. Cysteine proteases are known to bind to their protein substrates through extended beta sheet structures.[8] Accordingly our design paradigm is to choose rigid molecular skeletons suitable to mimic the extended conformations of substrates. These rigid or semi-rigid molecular scaffolds may be readily elaborated by functional group manipulation to fine-tune substrate binding. Structure–activity relationship (SAR) studies are aided by an examination of available X-ray crystallographic reports of cruzain–inhibitor complexes which reveal a well-defined and relatively rigid active site.[9–12] The structures of cruzain with noncovalent hydroxymethyl ketone inhibitors show that they are in extended conformations at the enzyme's active site.[12]

A variety of tetrahydronaphthalene, benzophenone, and propiophenone derivatives were prepared by chemical synthesis as shown in Schemes 1–4.[13–18] In addition,

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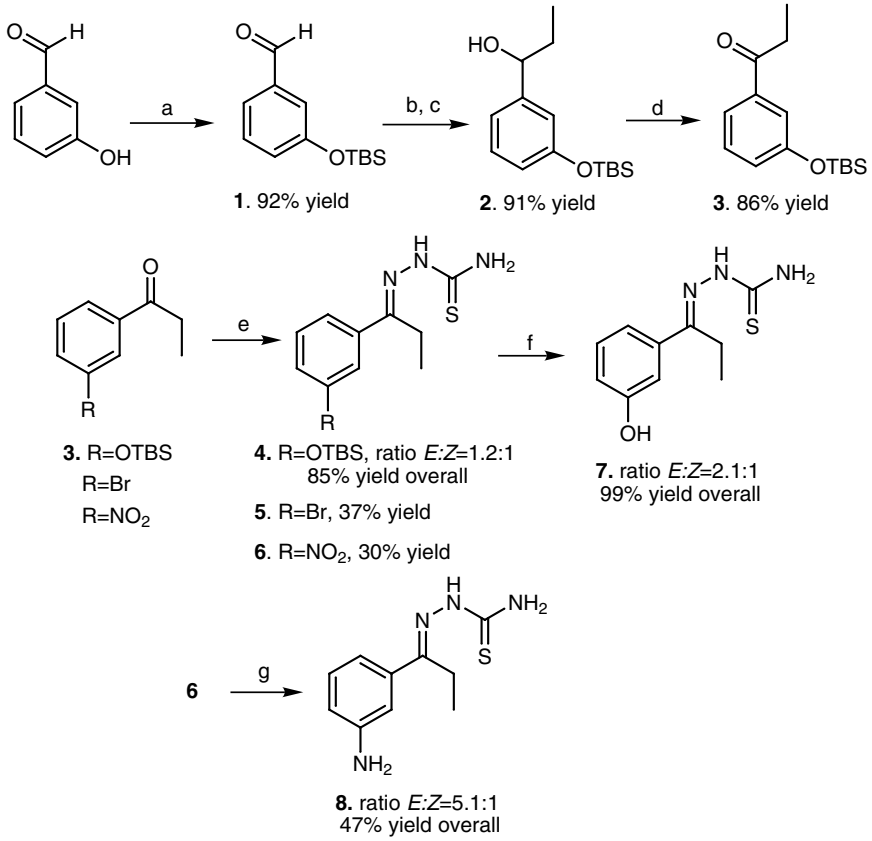
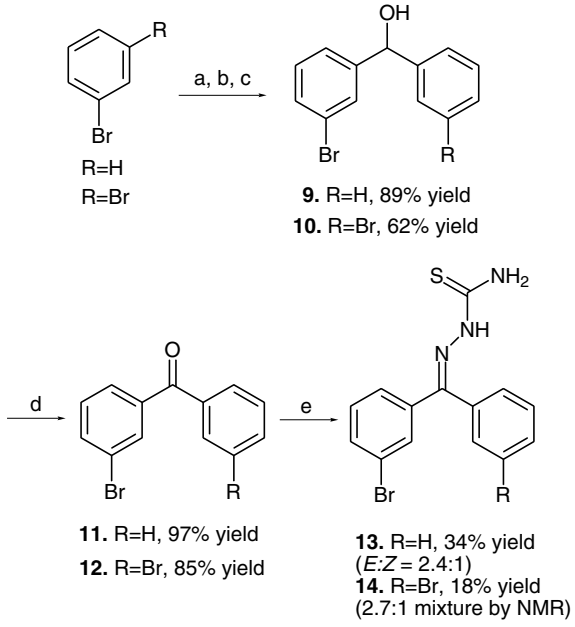


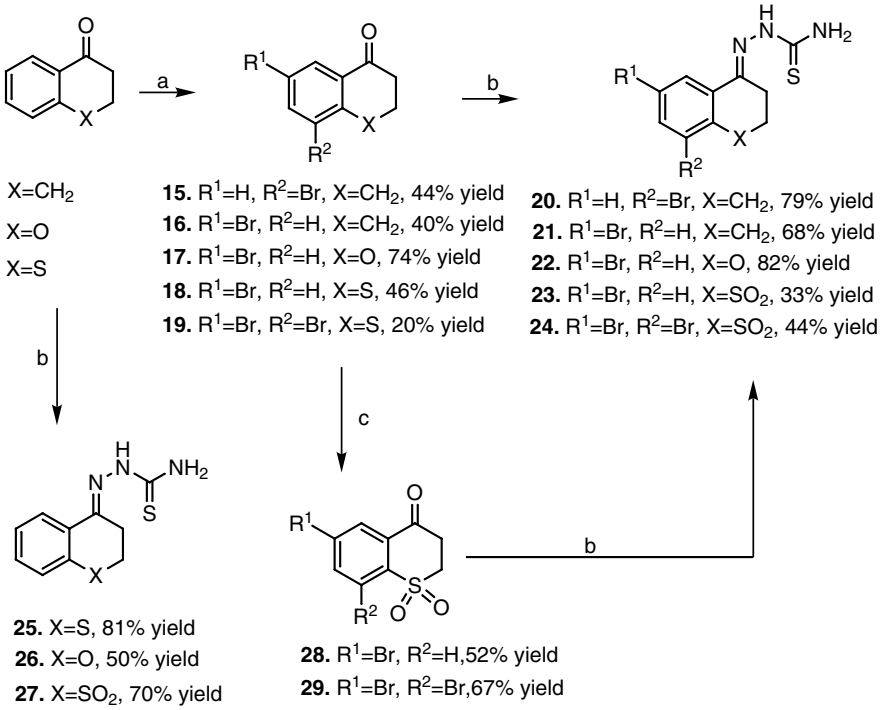
Scheme 1. Synthesis of some representative propiophenone thiosemicarbazones. Reagents and conditions: (a) TBSC1, NEt_3, DMAP, 2.5 h, rt; (b) Mg, EtBr, Et_2O, rt; (c) H_2O, HCl; (d) PCC, CH_2Cl_2, rt; (e) $NH_2NHCSNH_2$, MeOH, 1% AcOH, reflux; (f) TBAF, CH_2Cl_2, 0 °C, 1.5 h; (g) Zn, $CaCl_2$, EtOH–H_2O, 1.5 h.



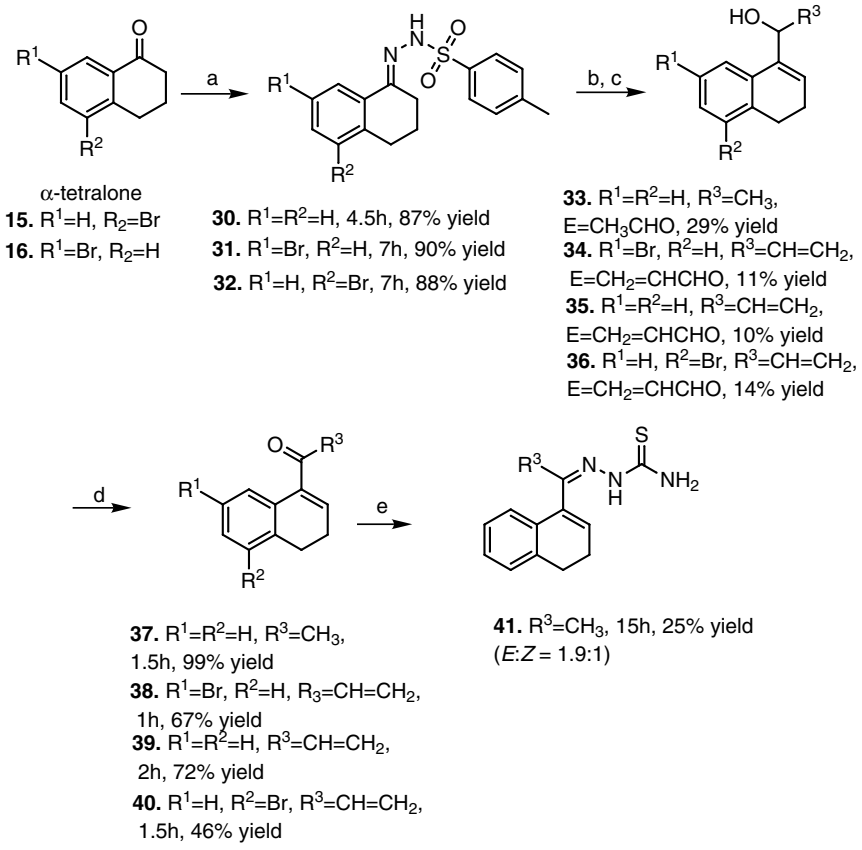
Scheme 2. Synthesis of benzophenone thiosemicarbazones. Reagents and conditions: (a) Mg, Et_2O, 2.5 h, 40 °C; (b) *m*-BrC_6H_5CHO, 2 h, rt; (c) H_2O, HCl; (d) PCC, CH_2Cl_2, 4.5 h, rt; (e) $NH_2NHCSNH_2$, MeOH, 1% AcOH, reflux.

several compounds were prepared which have an α,β-unsaturated carbonyl as an alternative functional group (Scheme 4).[13,14,19] The synthesis of two novel propiophenone thiosemicarbazones **7** and **8** which were prepared following the procedure used by Du and coworkers[5] is shown in Scheme 1. The 3′-bromopropiophenone thiosemicarbazone derivative **5** was prepared as a reference standard.[5] Although thiosemicarbazone **6** has been previously reported,[20] its activity against cruzain had not been determined prior to this study. The synthesis of two new benzophenone thiosemicarbazones is presented in Scheme 2. The synthetic strategy included a Grignard reaction to form secondary alcohols **9** and **10**, followed by a PCC oxidation to afford ketones **11** and **12** which subsequently were reacted with thiosemicarbazide to form the final products **13** and **14**.

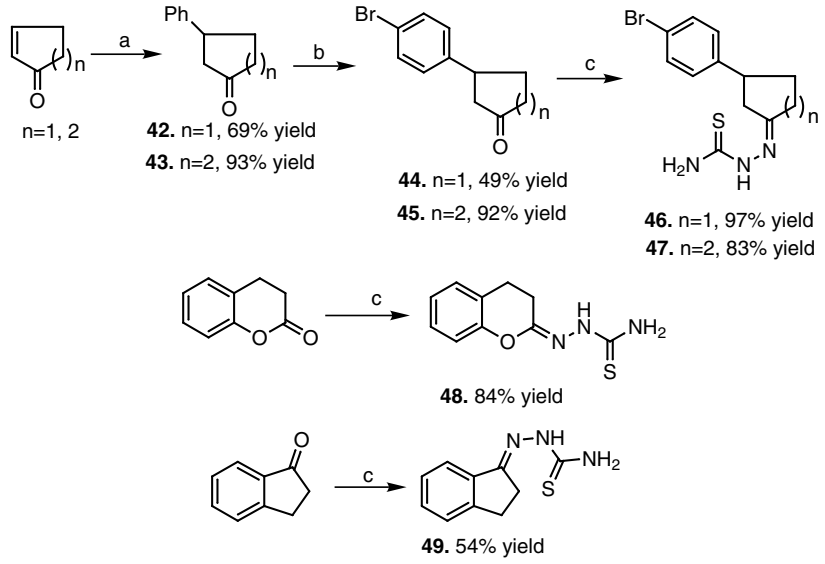
In Scheme 3, the synthesis of α-tetralone thiosemicarbazones **20** and **21** is shown as well as the synthesis of derivatives containing a heteroatom such as oxygen, and sulfur (compounds **22** and **26**, and **25**, respectively) and a sulfone group (compounds **23**, **24**, and **27**). The incorporation (at the correct location) of an α,β-unsaturated carbonyl group into the tetrahydronaphthalene skeleton should allow the inhibitor to react with the nucleophilic thiolate of the enzyme active site Cys25 through a 1,4-Michael addition[19] and hence suppress the catalytic activity of the enzyme. Accordingly, a group of compounds was synthesized (Scheme 4) which contain an exocyclic conjugated carbonyl group (**37**–**40**). In addition, **37** was converted to its thiosemicarbazone **41**.



Scheme 3. Synthesis of α-tetralone thiosemicarbazones. Reagents and conditions: (a) $AlCl_3$, Br_2; (b) $NH_2NHCSNH_2$, MeOH, TsOH, reflux; (c) H_2O_2, AcOH, 1 h, 100 °C.



Scheme 4. Synthesis of *exo*-cyclic unsaturated carbonyl thiosemicarbazones. Reagents and conditions: (a) NH_2NHTs, MeOH, TsOH; (b) BuLi, TMEDA, 1 h; (c) electrophile (E); (d) Dess–Martin reagent, CH_2Cl_2; (e) $NH_2NHCSNH_2$, MeOH, TsOH, reflux.



Scheme 5. Synthesis of other thiosemicarbazones. Reagents and conditions: (a) PheLi, CuCN, THF; (b) $AlCl_3$, Br_2; (c) $NH_2NHCSNH_2$, MeOH, TsOH, reflux.

Finally, the synthesis of other thiosemicarbazones containing different skeletons is shown in Scheme 5. Compounds were assayed (Table 1) for their ability to inhibit purified, recombinant cruzain lacking the C-terminal domain.[21]

A number of the compounds have IC_{50} values less than 1.5 μM including thiosemicarbazone derivatives **6**, **13**, **14**, **20**–**24** and two dihydronaphthalenes with exocyclic unsaturated carbonyls (**37** and **38**). SAR results of the five most active compounds, all with IC_{50} values lower than that of the reference compound **5**, are shown in Figure 1. The benzophenone thiosemicarbazones, Ia and Ib (**13** and **14**), are potent inhibitors of cruzain (IC_{50} values of 80 and 24 nM, respectively). During the course of this investigation, another report of an active benzophenone thiosemicarbazone inhibitor of cruzain appeared emphasizing the efficacy of this series.[7] The thiosemicarbazone derivative of 4-chromanone, IIb (**22**), and of the sulfone, IIc (**23**), are also active with IC_{50} values of 110 and 210 nM, respectively. It is especially noteworthy that the α-tetralone thiosemicarbazone analog IIa (**21**) has an IC_{50} value of 17 nM, which places this compound among the most active known thiosemicarbazone-based inhibitors of cruzain. The SAR gained from these compounds, along with the compounds that are essentially inactive, significantly expands the current data in regard to molecular recognition of small molecule inhibitors of cruzain. In particular, the current work is significant as it expands the SAR knowledge base to include fused-ring analogs such as IIa–c (**21**–**23**) and further functionalized benzophenone congeners Ia,b (**13** and **14** respectively) as potent cruzain inhibitors.

Table 1. Inhibition of cruzain by thiosemicarbazone derivatives and other novel cyclic compounds

Compound	Cruzain inhibition IC_{50} (nM)
5[a]	240(100)[b], (60)[c], (310)[d]
6	860
7	>33,000
8	>3640
13	80
14	24
20	1200
21	17
22	110
23	210
24	820
25	>2000
26	>17,000
27	>20,000
37	1360
38	800
41	>20,000
46	>5000
47	>7000
48	>16,000
49	>17,000

[a] Compound **5** has been previously synthesized and reported in the literature. It is included here for the purpose of comparison (see ref. 5).
[b] Ref. 5.
[c] Ref. 6.
[d] Ref. 7.

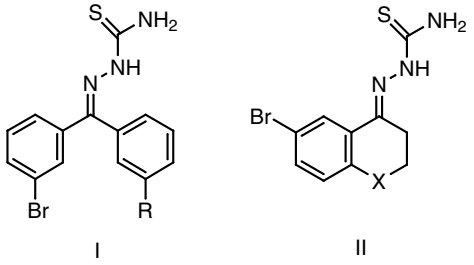


Figure 1. SAR results for the five most active inhibitors of cruzain from this study. IIa, X = CH_2 (**21**) > Ib, R = Br (**14**) > Ia, R = H (**13**) > IIb, X = O (**22**) > IIc, X = SO_2 (**23**).

Acknowledgments

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15. All compounds are well characterized by detailed proton, carbon, and DEPT 135 NMR spectra and X-ray crystallography has also been carried out for three compounds although the results of these studies are not reported herein. X-ray structures confirm the stereochemistry as *E* for compounds **6**, **13**, and **21** and thiosemicarbazone analogs (such as **5**, **6**, **20**–**26**, and **46**–**49**) are indicated as single isomers by NMR studies. X-ray crystallographic data have been deposited with the Cambridge Crystallographic Data Center for compound **21** and assigned the deposition number CCDC 606033. It is important to note that some of the thiosemicarbazone derivatives (such as **4**, **7**, **8**, **13**, **14**, and **41**) appear as mixtures in the NMR. The major isomer in all cases is presumed to be the *E* isomer although this is not confirmed except for **6**, **13**, and **21**. Furthermore, the issue of thiosemicarbazone derivatives appearing as two compounds is reported in the literature and at this point it is not possible to say for certain whether we are actually seeing *E* and *Z* double bond isomers of these compounds in solution by NMR or if instead we are observing tautomeric forms of the thiosemicarbazone functionality,[16,17] or simply rotamers.[18] Since compound **14** cannot exist as double bond isomers due to symmetry, it is highly probable that the mixture observed by NMR is due to tautomeric forms or rotamers. Despite continued efforts to refine this question, including low and high temperature NMR, the issue remains for further study.
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21. Cruzain (0.1 nM) in 100 mM sodium acetate buffer (pH 5.50) containing 5 mM DTT was preincubated for 5 min at 25 °C with inhibitor. The reaction was initiated[5–7] by the addition of 20 μL of substrate, benzyloxycarbonyl-L-phenylalanyl-L-arginyl-7-amido-4-methylcoumarin (Z-Phe-Arg-AMC, Sigma, K_m = 1.1 μM) to the enzyme–inhibitor mixture at 25 °C to give 300 μL with a final concentration of 10 μM substrate and nine to twenty concentrations of inhibitor ranging from 0.001 to 50,000 nM. The increase in fluorescence (excitation at 355 nM and emission at 460 nM) upon release of 7-amino-4-methylcoumarin was recorded for 3 min with a spectrofluorimeter (Jobin Yvon-SPEX Instruments, fluoroMAX-2). Inhibitor stock solutions were prepared in DMSO, and serial dilutions were made in DMSO to give a final concentration of 0.7% DMSO in the assay. Controls were performed using enzyme alone, substrate alone, and enzyme with DMSO. IC_{50} values were determined by non-linear regression analysis using $v_{o(inh)} = V_{max}/\{1 + 10^{(X - \log IC_{50})Hillslope}\}$ and Prism software (GraphPad).