Synthesis and Structure−Activity Relationship Study of Potent Trypanocidal Thio Semicarbazone Inhibitors of the Trypanosomal Cysteine Protease Cruzain

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American trypanosomiasis, or Chagas' disease, is the leading cause of heart disease in Latin America. Currently there is an urgent need to develop antitrypanosomal therapy due to the toxicity of existing agents and emerging drug resistance. A novel series of potent thio semicarbazone small-molecule inhibitors of the *Trypanosoma cruzi* cysteine protease cruzain have been identified. Some of these inhibitors have been shown to be trypanocidal. We initially discovered that 3′-bromopropiophenone thio semicarbazone (**1i**) inhibited cruzain and could cure mammalian cell cultures infected with *T. cruzi*. 3′-Bromopropiophenone thio semicarbazone showed no toxicity for mammalian cells at concentrations that were trypanocidal. Following this lead, more than 100 compounds were designed and synthesized. A specific structure−activity relationship (SAR) was established, and many potent analogues with IC_{50} values in the low nanomolar range were identified. Eight additional analogues were trypanocidal in a cell culture assay, and this indicates that aryl thio semicarbazone is a productive scaffold for killing the parasites. Kinetic studies show that these are time-dependent inhibitors. Molecular modeling studies of the enzyme−inhibitor complex have led to a proposed mechanism of interaction as well as insight into the SAR of the thio semicarbazone series. The nonpeptide nature of this series, small size, and extremely low cost of production suggest this is a promising direction for the development of new antitrypanosome chemotherapy.

Introduction

Trypanosomiasis, malaria, and leishmaniasis are major parasitic diseases in developing countries.[1] American trypanosomiasis, or Chagas' disease, is the leading cause of heart disease in Latin America.[2] At least 16−18 million people are infected with *Trypanosoma cruzi*, resulting in more than 50,000 deaths each year.[3] Benznidazole, the only commercial drug still available for treatment during the chronic stage of Chagas' disease, is associated with significant toxicity.[4] Chagas' disease is transmitted to humans by blood-sucking triatomine vectors with an infectious trypomastigote form of the protozoan parasite *T. cruzi*.[5] The trypomastigote enters the host bloodstream and ultimately invades a cardiac muscle cell, where it transforms into the intracellular amastigote. Amastigotes replicate within cells, transform back to trypomastigotes, and rupture the cell, releasing the infectious form back into the bloodstream and other cells, amplifying the infection.

Cruzain (aka cruzipain) is the major cysteine protease of *T. cruzi*. The protease is expressed in all life cycle stages of the parasite, but delivered to different cellular compartments in each stage. In the epimastigote stage, which occurs in the insect vector, the protease is in a lysosomal compartment where it functions to degrade proteins endocystosed from the insect gut. In the infectious trypomastigote stage, the protease appears at the flagellar pocket, the site of endocytosis and secretion. In the amastigote stage, within the mammalian host cell, the protease is both in the lysosomal compartment and on the surface of the parasite where it may function in nutrition, remodeling of the mammalian cell, or evasion of host defense mechanisms. Addition of a cruzain inhibitor such as Z-Phe-Ala-FMK (benzyloxycarbonyl-L-phenylalanyl L-alanine fluoromethyl ketone) to cultures of mammalian cells exposed to trypomastigotes or to mammalian cells already infected with *T. cruzi* amastigotes blocks replication and differentiation of the parasite,[5] thus arresting the parasite life cycle. Therefore, cruzain is essential for replication of the intracellular parasite. Treatment of *T. cruzi*-infected mice with a vinyl sulfone-derivatized pseudopeptide inhibitor of cruzain, *N*-methyl piperazine-Phe-homoPhe-vinyl sulfone phenyl, has resulted in a cure in a mouse model study.[6] Thus, cruzain is an appealing target for new antitrypanosomal chemotherapy.[7]

Several irreversible peptide-based inhibitor series including halomethyl ketones, diazomethanes, epoxysuccinyl derivatives, and vinyl sulfone derivatives targeting cysteine proteases have been developed.[8] A disadvantage of the chloromethyl ketones is their high reactivity and consequent lack of selectivity. They react with serine proteases and other SH-containing molecules,

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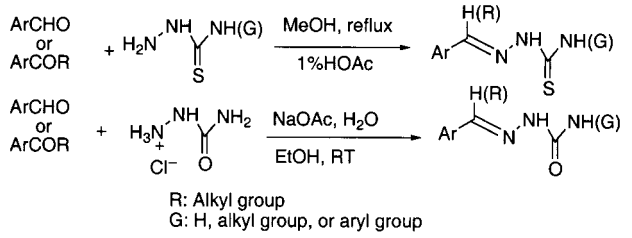
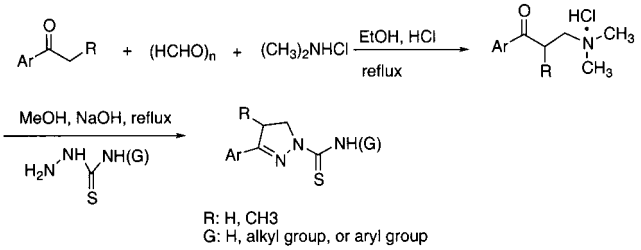
Scheme 1. Synthesis of Thio Semicarbazones and Semicarbazones



Scheme 2. Synthesis of Cyclized Pyrazoline Analogues of Thio Semicarbazones



such as glutathione or nonproteolytic enzymes, and result in toxicity in vivo. To increase selectivity and reduce reactivity and toxicity, less reactive series including monofluoro methyl ketones, epoxy derivatives,[9] and vinyl sulfone derivatives[10] were developed. However, the low oral bioavailability associated with peptidyl inhibitors led us to explore alternative small-molecule inhibitor scaffolds. Initially we developed two series of nonpeptidic noncovalent inhibitors with the aid of the crystal structure of cruzain:[11] the bisaryl acylhydrazide[12] and the bisaryl urea series.[13] In this paper, we report a new series of mechanism-based small-molecule inhibitors discovered during a collaborative screening effect, the thio semicarbazones. Peptidyl semicarbazones inhibit cysteine proteases through the formation of a reversible tetrahedral adduct by attack of the thiolate on the C-5 carbon.[7] This is reminiscent of other reversible covalent inhibitors including 1,3-bis-(acylamino)-2-propanone inhibitors of Cathepsin K. In this case, the X-ray structure of the enzyme−inhibitor complex showed the formation of a thiohemiketal between the ketone carbonyl and the active site Cys25.[14]

We found that selected thio semicarbazone compounds exhibit potent activity against cruzain as well as trypanocidal activity against parasites in cell culture. The nonpeptidic nature of these compounds, coupled with their low cost of synthesis, makes this class of reversible covalent inhibitors very promising candidates for the development of new antitrypanosomal chemotherapy.

Methods

Synthesis of Thio Semicarbazones and Semicarbazones (Scheme 1). Refluxing aldehyde or ketone with a thio semicarbazide generates thio semicarbazones. For aldehydes, the reaction is usually complete in less than 3 h and no acetic acid is required. For ketones, the reaction is usually run overnight with 1% acetic acid. Yields are generally greater than 90% except with a few specific ketones such as the 2-substituted aryl ketones. Synthesis of semicarbazones is done at room temperature. A sodium acetate solution of semicarbazide hydrochloride salt is added to the ethanol solution of aldehyde or ketone.[15] The product usually precipitates out with good yield.

Synthesis of the Cyclized Pyrazoline Analogues (Scheme 2). The Mannich reaction of various ketones with formaldehyde and dimethylamine hydrochloride generates the Mannich base precursor.[16] The reaction is sensitive to both the amount of hydrochloric acid and the amount of solvent present. The reaction works best when a minimum amount of ethanol and 2 μL of acid/mmol ketone is applied. The methyl aryl ketones gave high yields above 80%, while the yields for other alkyl aryl ketones in the Mannich reaction were lower in the range of 30−60%. However, the product mixture contained only unreacted starting material and the Mannich product. The unreacted starting material was recovered for repetitive use. Condensation and cyclization of the precursor with the thio semicarbazide generates the cyclized pyrazoline analogues with yields between 20% and 50% without optimization. The cyclization also works in the substituted thio semicarbazide but with a lower yield.

IC_{50} Determinations. Inhibitors were screened for effectiveness against the *T. cruzi* cathepsin L-like protease (cruzain) using purified recombinant protein. Cruzain (1 nM) was incubated with 20−50000 nM inhibitor in 100 mM sodium acetate−5 mM DTT buffer (pH 5.50, buffer A) for 5 min at room temperature. A 200 μL portion of Z-Phe-Arg-AMC (Bachem, K_m = 1 μM) was added to the enzyme−inhibitor reaction to give a 20 μM substrate concentration. The increase in fluorescence (excitation at 355 nM and emission at 460 nM) was followed with an automated microtiter plate spectrofluorimeter (Molecular Devices, spectraMAX Gemini). Inhibitor stock solutions were prepared at 20 mM in DMSO, and serial dilutions were made in DMSO (0.7% DMSO in assay). Controls were performed using enzyme alone and enzyme with DMSO. IC_{50} values were determined graphically using inhibitor concentrations in the linear portion of a plot of inhibition versus log [I] (seven concentrations were tested, and at least two were in the linear range).

The time dependence of inhibition was determined by incubating cruzain with inhibitors, DMSO, or enzyme alone at room temperature for time points between 90 s and 24 h. The enzymatic activity was determined as above.

Drug Screening in Cell Culture. Mammalian cells are routinely cultured in RPMI-1640 medium supplemented with 5−10% heat-inactivated fetal calf serum (FCS) at 37 °C in 5% CO_2. The Y strain of *T. cruzi* is maintained by serial passage in bovine embryo skeletal muscle (BESM) cells. Infectious trypomastigotes are collected from culture supernatants. For drug assays, J774 macrophages were irradiated (5000 rad) and plated onto six-well tissue culture plates 24 h prior to infection with ~10^6 trypomastigotes/well. Parasites were removed 2 h postinfection, and the medium was supplemented with the appropriate cysteine protease inhibitor (10 μM). Inhibitor stocks (10 mM) in DMSO were stored at 4 °C. J774 monolayers treated with a blank containing DMSO were used as a negative control, and monolayers treated with a known trypanocidal inhibitor, 10 μM *N*-methyl piperazine-Phe-homoPhe-vinyl sulfone phenyl (*N*-Pip-F-hF-VSPh) acted as a positive control.[6] RPMI medium with or without inhibitor was replaced every 48 h. Cultures were maintained for up to 30 days and monitored daily by contrast phase microscopy. *T. cruzi* completed the intracellular cycle in 5−6 days in the untreated controls but was unable to survive in macrophages treated with *N*-Pip-F-hF-VSPh. The comparative effectiveness of each inhibitor was estimated from plots of the duration of the intracellular cycle of *T. cruzi* (days) in treated vs untreated control wells.

Molecular Modeling. DOCK 4.0.1 was used to position putative inhibitors in the enzyme active site and score the quality of the interactions of inhibitors with cruzain.[17] The procedure for constructing the molecular surface and the energy grid of the active site of cruzain can be found in ref 13. The *C* log *P* values were calculated for each compound using the software CLogP 4.61 (Daylight Chemical Information Systems, Santa Fe, NM) on the basis of the work of Hansch.[18]

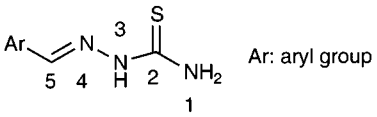


Figure 1. Thio semicarbazone scaffold.

Results

Initial Discovery of the Trypanocidal Activity of Thio Semicarbazones. As part of a collaborative effort with Parke-Davis, a library of Parke-Davis compounds were screened. Forty-five compounds were identified as active against cruzain. We observed a thio semicarbazone scaffold common to several of the inhibitors (Figure 1). The scaffold has advantageous properties: low molecular weight, reasonable *C* log *P*, good hydrogen bond donating and accepting capabilities, and easy, economical synthetic routes. The closely related semicarbazone scaffold has been evaluated for clinical use as an antihypertensive,[19] anticonvulsant,[20] and antiallodynic agent.[21] For example, the semicarbazone compound 4-[4-fluorophenoxy]benzaldehyde semicarbazone has entered clinical trials for the treatment of neuropathic pain.[22] Recently, 5-nitrofurfural *N*-butyl semicarbazone[23] has been shown to have antitrypanosomal activities targeting trypanothione reductase[24] through a nitro anion radical mechanism. To further explore the activity of the thio semicarbazone scaffold, an initial group of 14 compounds were synthesized. In our previous work on noncovalent inhibitors of curzain including bisaryl acylhydrazides and ureas, we found that the most active compounds have a six-membered phenyl ring−five-membered heteroaromatic ring combination (6−5) in the aryl position. This feature was incorporated into some of the compounds. Some of the other alternatives in the aryl position were also tried including fused aromatic rings or single aromatic rings. Four of the 6−5 compounds (**1b**, **1c**, **1e**, **1f**) have IC_{50} values below 300 nM. Two of the alternative compounds (**1i**, **1n**) were active at 100 and 560 nM against the enzyme, respectively (Chart 1). Three (**1i**, **1e**, **1f**) of the most effective compounds were tested against intact trypanosomes in cell culture. Typically, infected host cells die within 5 days without treatment. In contrast, infected host cells treated with **1e** (5 μM) survived 10 days, but the therapeutic index associated with this compound was low with toxicity to host cells observed at 10 μM. Infected host cells treated with **1f** survived 14 days, but this compound crystallized in the cell culture medium. In other words, **1e** and **1f** were trypanostatic, which means the intracellular cycle of the parasite is completed at a slower growth rate and the cells survive longer. Compound **1i** exhibited significant trypanocidal activity without toxicity to host cells or solubility problems. Infected cells treated with **1i** at 10 μM were cured of trypanosomal infection. To confirm trypanocidal activity, infected cells were treated with **1i** for three weeks and then **1i** was removed from the cell culture. No parasites were observed in either the culture supernatants or host cells. Cells were healthy and parasite-free until the experiment was terminated at 6.5 weeks postinfection. Encouraged by this result, we explored the SAR (structure−activity relationship) of **1i** in an effort to find more active, trypanocidal analogues.

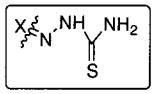
SAR Studies. Aryl Group Activity (Chart 2). A series of **1i** analogues with various substituents on the aryl moiety were synthesized. Compound **2d** did not have any substitution on the aryl ring. Its activity dropped at least 2 orders of magnitude compared to that of **1i** (samples with IC_{50} values greater than 10 μM were not tested further). C-2 substitution with bromine, chlorine, or trifluoromethyl groups[25] resulted in poor activity. Among the C-3 variants, the trifluoromethyl substitution (**2b**) resulted in a better inhibitor with an IC_{50} of 50 nM compared to **1i**. The chlorine substitution was well tolerated (compound **2a**, 220 nM), but was not as potent as **1i**. Fluorine or methoxy substitution at C-3 resulted in a loss of activity of more than 2 orders of magnitude. C-4 substitution with bromine, chlorine, and methoxy groups all resulted in a decrease of activity of more than 2 orders of magnitude. Several disubstituted analogues were synthesized. 3,4-Dichloro (**2i**) or 3,5-bis-(trifluoromethyl) (**2h**) substitution resulted in potent inhibitors with IC_{50} values of 20 nM, about a 5-fold increase in activity over **1i**. The 3,5-dichloro substitution was also tolerated. Dichlorine substituents at C-2 (2,3-Cl_2 or 2,4-Cl_2) resulted in poor activity, consistent with the aforementioned observation that C-2 is a suboptimal site for substitution.

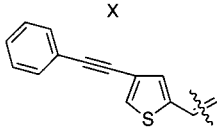
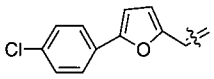
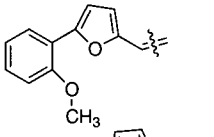
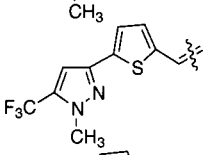
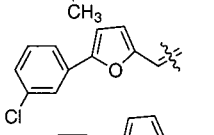
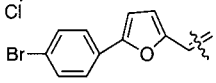
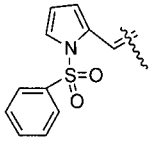
Varying Ethyl Groups at C-5 (Chart 3). The IC_{50} of the compounds decreased from 1300 nM for a hydrogen (**3a**) to 200 nM for a methyl (**3b**) to 100 nM for an ethyl group (**1i)** at C-5. The same trend was observed for other substituents such as trifluoromethyl, bis(trifluoromethyl), and dichloro groups. Compared to those of unsubstituted compounds and methyl-substituted compounds, the IC_{50} of ethyl-substituted compounds improved more than 10-fold and 2−3-fold, respectively. Changing the phenyl ring to bromo- or chloro-substituted thiophenes resulted in a significant loss of activity of ∼20-fold.

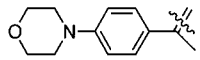
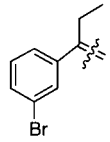
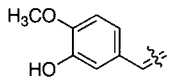
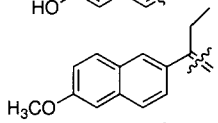
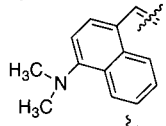
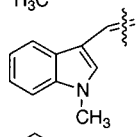
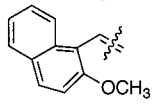
Cyclized Pyrazoline Analogues (Chart 4). A set of analogues with the C-5 ethyl group attached to the C-3 nitrogen to form a pyrazoline ring was synthesized to investigate the influence of the restricted flexibility and the impact of substitution on the C-3 nitrogen. Compound **4a** resulted in a 2-fold decrease in activity as compared to **1i**. However, addition of a methyl group on the pyrazoline ring (**4b**) restored the activity. Compound **4b** had an IC_{50} of 80 nM, slightly better than that of the parent compound **1i**. Similar trends were observed in the chlorine-substituted (**4c**, **4d**) and trifluoromethyl-substituted (**4g**, **4h**) analogues. For dichloro substitutions, an additional methyl group in the ring (**4f**) reduced the activity. Limits in solubility may contribute to the decreased activity of this compound. Compound **4f** has the poorest solubility in methanol compared to the others. Overall, there are five pyrazoline analogues (**4b**, **4e**, **4f**, **4g**, and **4h**) that have IC_{50} values better than that of **1i**.

N1 NH_2 Substitutions (Chart 5). A variety of noncyclized and cyclized pyrazoline thio semicarbazones substituted on the N1 amino group were synthesized. These included alkyl groups (e.g., methyl, dimethyl, ethyl, hexyl), aryl groups (e.g., phenyl, chlorophenyl, trifluoromethylphenyl), methylfuran, and ethylmorpholine. Most of the compounds had an IC_{50} greater than

Chart 1. Structures and Inhibition of the Cruzain Activity (IC_{50}) of the First 14 Thio Semicarbazones Synthesized[a]

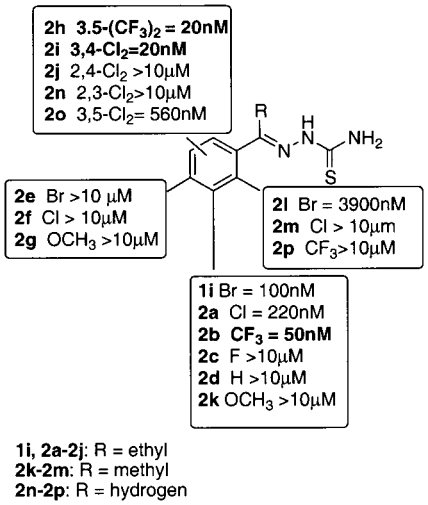


ID	X	IC50(nm)
1a		>10,000
1b		300
1c		280
1d		7000
1e		140
1f		280
1g		2800

ID	X	IC50(nm)
1h		>10,000
1i		100
1j		>10,000
1k		>10,000
1l		>10,000
1m		2800
1n		560

[a] X represents the aryl portion of the thio semicarbazone.

Chart 2. Variation of the Aryl Substituents and Position of the Substituents Produces a Significant Difference in Cruzain Inhibitions[a]



[a] Compounds in bold have better IC_{50} values than **1i**.

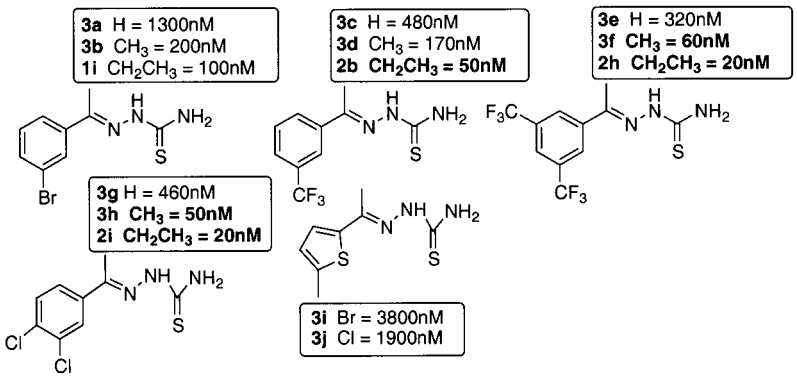
10 μM. A few of them (**5c**, **5d**, **5e**, and **5f**) had moderate activity between 1 and 2 μM.

C=S Double Bond (Chart 6). Semicarbazone analogues of the most active thio semicarbazones were synthesized. All of these compounds had IC_{50} values greater than 10 μM. We take this as evidence that the sulfur in the C=S double bond is critical for activity.

C=N4 Double Bond (Scheme 3). Reduction of **1i** by sodium borohydride generated the saturated C−N4 bond variant of **1i** (**7a**). Compound **7a** exhibited poor activity compared to **1i**, indicating the importance of the double bond.

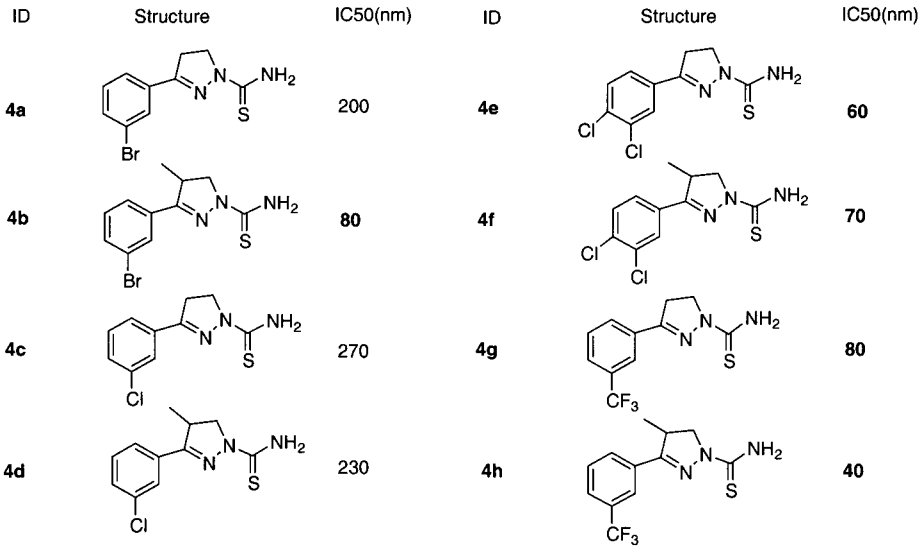
Cell Culture Assays of Some Representative Compounds. In addition to their effect on enzymatic activity, we attempted to understand whether thio semithiocarbazones other than compound **1i** were able to enter cells and exert a trypanocidal effect against this intracellular parasite. Chart 7 contains the trypanocidal properties of some of the representative compounds as judged by the survival of infected host cells. The toxicity of compounds to mammalian host cells was also evaluated in the assay. Of the five 3-bromo-substituted compounds, three (**1i**, **3b**, **4b**) exhibited a trypanocidal effect. No parasites were observed in supernatants or host cells after the inhibitors were removed at day 22. The 3-chloro-substituted compound **2a** was also trypanocidal, but the two disubstituted compounds **2i** and **2h** were not, though one of them was trypanostatic for 20 days. Three more compounds from the initial group of thio semicarbazones were tested but were not effective. Toxicity (**1c**, **1n**) or solubility (**1b**) appears to be a problem for some of the compounds tested in this latter group. Two moderate inhibitors with substitution on the N1 amino group (**5c**, **5d**) had no effect on the parasites. Following this, a second group of inhibitors that have good IC_{50} values was evaluated in the cell culture assay (Chart 8). Among these 10 compounds, two (**4c**, **4g**) were

Chart 3. Compounds Having a C-5 Ethyl Group Inhibit Cruzain Better Than Compounds with a C-5 Methyl Group or a C-5 Hydrogen[a]



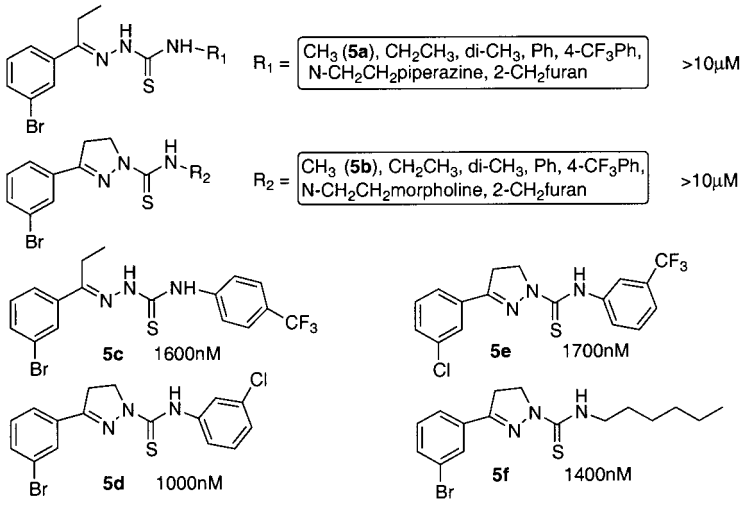
[a] Compounds in bold have better IC_{50} values than **1i**.

Chart 4. Cyclized Pyrazoline Analogues Are Generally Potent Inhibitors of Cruzain[a]



[a] Compounds in bold have better IC_{50} values than **1i**.

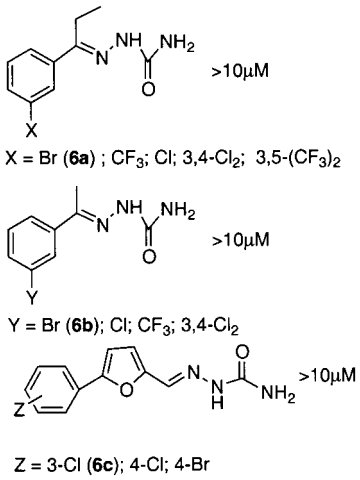
Chart 5. Substitution on the N1 Amino Group Results in Inhibitors with Moderate to Poor IC_{50} Values against Cruzain



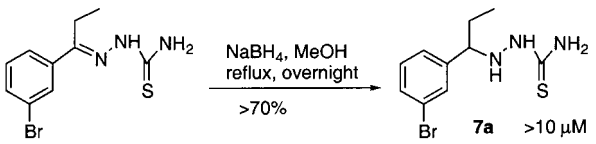
inactive. Three were trypanostatic (**3f**, **3d**, **4f**). The remaining five compounds (**2b**, **3h**, **4d**, **4e**, **4h**) were trypanocidal and cure the infected cells. Altogether nine compounds have been identified to be trypanocidal through the cell culture assay, indicating that the thio semicarbazone is a productive scaffold for potential novel antitrypanosomal therapy.

Mechanism of Inhibition. The most active thio semicarbazone compounds including the pyrazoline analogues are time-dependent inhibitors. This indicates

Chart 6. Semicarbazone Analogues of the Most Potent Thio Semicarbazones Are Poor Inhibitors of Cruzain



Scheme 3. Reduction of the C═N4 Bond Decreases the IC_{50} Value by More Than 2 Orders of Magnitude



that inhibition by the thio semicarbazone series is mechanism-based. A variety of compounds including some of the ones with moderate to poor activity were chosen for a more detailed study of time-dependent inhibition (Figure 2). A known irreversible inhibitor, K002 (morpholino urea-Phe-homoPhe-vinyl sulfone benzene; Axys Pharmaceuticals Inc., South San Francisco, CA), and a known reversible covalent inhibitor, leupeptin, were used as controls for the thio semicarbazones. Both controls were rapidly time-dependent. Inhibitors with a free N1 amino group such as **1i**, **4b**, **3a**, **3b**, and **4a** are time-dependent. Even some of the weak inhibitors such as **5e** and **7a** showed time dependency. The time dependency of **7a** indicates that the C═N4 double bond is not the site for covalent bond formation. Thus, the only logical site for covalent interaction with cruzain in **7a** is the C═S double bond.

Both dialysis and centrifugal filtration of cruzain inhibitor mixtures were done to determine the reversibility of the thio semicarbazones. The same controls (K002 and leupeptin) were used. However, because cruzain activity is not stable after either of these processes, no clear-cut conclusions could be drawn.

To further understand the mechanism of interaction, computational tools were used to dock compound **1i** into the active site of cruzain. While we do not expect quantitative agreement between calculation and the experimental SAR data, these models do provide insight into the activity of these cruzain inhibitors. The calculated best orientation of **1i** with cruzain is shown in Figure 3. The bromophenyl portion of **1i** is oriented toward the deep S2 pocket. Such an interaction is not ideal as judged by the modeling effort. We anticipate that the ethyl group fits into the shallow S1 pocket, while the rest of the thio semicarbazone scaffold is positioned close to Cys25 and His159. The distance between one of the His159 protons and the sulfur in the thio semicarbazone is calculated to be 3.14 Å. In this docked orientation, the distance between the Cys25 thiolate and the carbon (C2) attached to sulfur is 3.78 Å and the distance between the Cys25 thiolate and the carbon in C═N4 is 4.07 Å. The separation between N4 and the proton of His159 (4.31 Å) is not compatible with a direct interaction. Therefore, this orientation of **1i** suggests that the covalent attack of the Cys25 on **1i** is directed toward the C2═S bond, consistent with the time dependency of **7a**. The attack on the C2═S bond would be assisted by the transfer of the His159 proton to the thio semicarbazone sulfur (Figure 4).

If the ethyl group fits into the S1 pocket, this would explain the preference for an ethyl group over a methyl group or a hydrogen in the S1pocket. The unsubstituted pyrazoline ring analogues have a methyl group equivalent on C-4. This might be one of the reasons that the pyrazoline analogue **4a** has 2-fold lower activity when compared to the parent ethyl compound **1i**. Upon addition of a methyl group to the C4 position on the pyrazoline ring, this analogue resembles the original ethyl substituent and its activity is restored.

When the semicarbazone analogue **6a** was docked into the active site, none of the top 20 orientations were similar to that of **1i**. Most orient the semicarbazone portion toward S2 site and the aryl group toward the S′ site. In other words, the modeling suggests that the electronic and volumetric difference between a sulfur and an oxygen atom may make a substantial difference. This is consistent with the substantial activity difference between compounds **6a** and **1i**.

Further optimization efforts based on our modeling to improve interactions with the S2 and S1 pocket are ongoing. Variation under study includes replacing the C-5 alkyl groups predicted to lie in the S1 pocket with longer alkyl chains, aryl groups, alkyl groups containing heteroatoms, or functional groups and replacing aryl groups predicted to lie in the S2 with alkyl or other groups. We anticipate that this will lead to more potent inhibitors. We are also attempting to obtain cocrystals of one or more of the potent thio semicarbazones with cruzain to facilitate our structure-based design efforts and to improve our understanding of the mechanism of action of this class of inhibitors.

Discussion

In this study, over 100 thio semicarbazones have been synthesized and tested as inhibitors of the parasite cysteine protease cruzain. By comparison to the existing antitrypanosomal nitro semicarbazones, we have eliminated nitro groups in these compounds thought to generate nitro anion radicals that are critical to inhibition of trypanothione reductase. We have evaluated a related but different scaffold: the thio semicarbazones (C═S instead of C═O). These thio semicarbazones are active against the cysteine protease cruzain instead of trypanthione reductase. Unlike the SAR of the N1-substituted nitro semicarbazones, substitution at the N1 amino group on the thio semicarbazone scaffold is generally unproductive. Only a few of these compounds have shown moderate inhibitory activity.

The thio semicarbazone series exhibited a specific and consistent structure−activity relationship. Reduction of the C═N4 bond or a change of the C═S bond into a C═

Chart 7. Cell Culture Activity of Representative Thio Semicarbazones

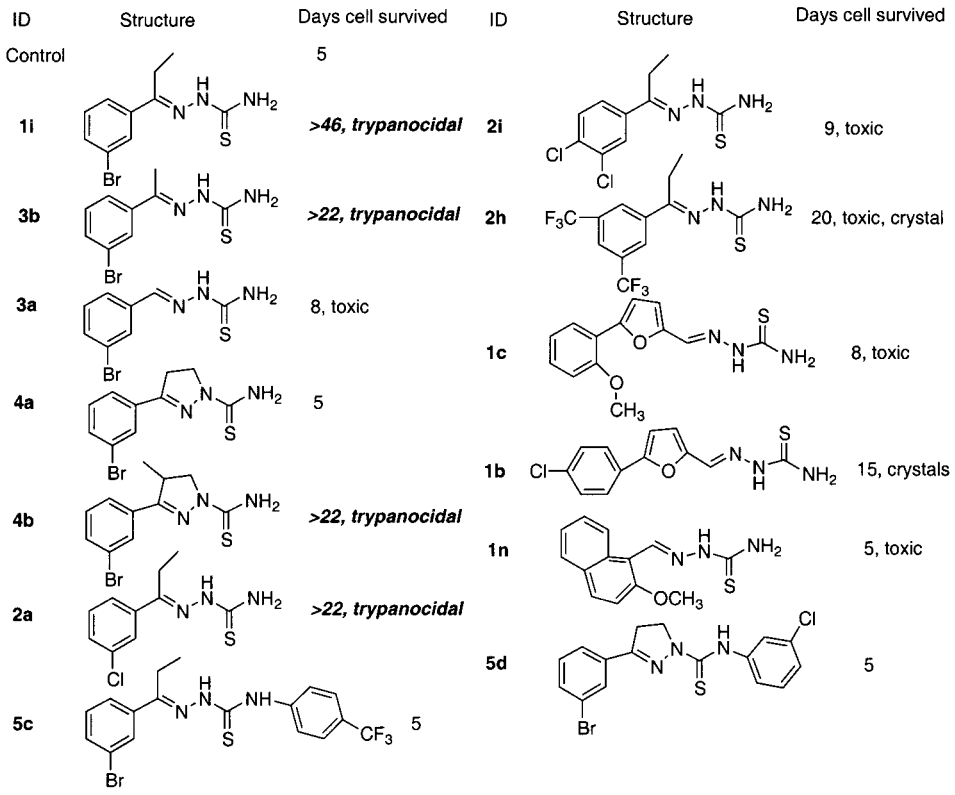
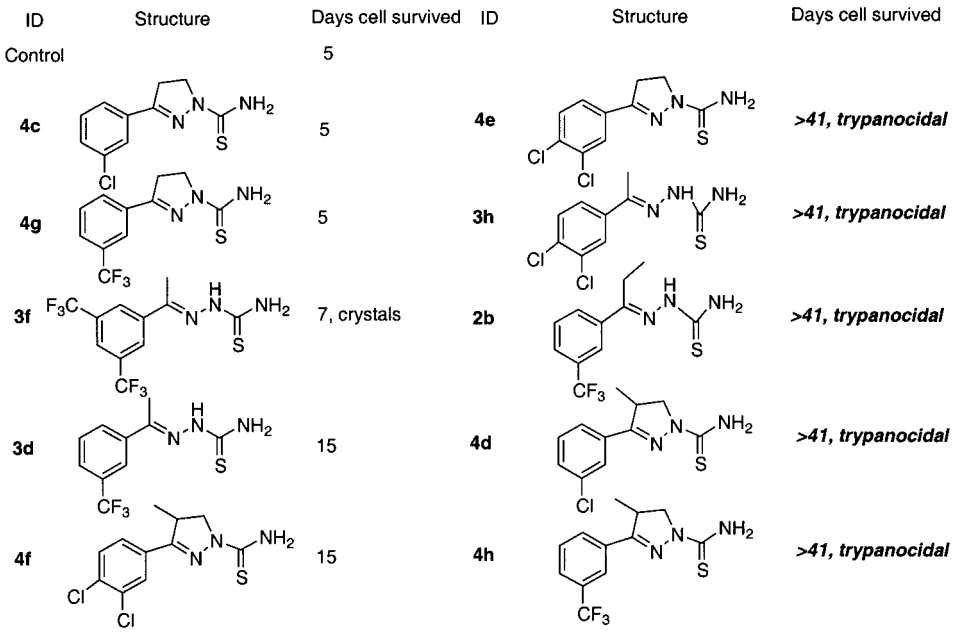


Chart 8. Cell Culture Activity of Some Other Potent Thio Semicarbazones



O bond results in poor activity. For compounds with single phenyl rings, substitution at specific positions can lead to enhanced activity. The most potent compounds have at least one substituent group at C-3. Among the C-3 substituents, trifluoromethyl, bromine, and chlorine result in potent inhibitors while compounds with other groups have poor acitivity. Disubstitution with 3,4-dichlorine or 3,5-bis(trifluoromethyl) results in potent inhibitors of cruzain. By contrast, C-2 or C-4 substitution is not useful. Five compounds (**2b, 2h, 2i, 3f, 3h)** have better IC_{50} values than **1i.** Compounds with the six phenyl−five heteroaromatic group also are effective inhibitors of cruzain. Ethyl substitution at C-5 is favorable over a broad range of compounds with different aryl substituents. Connection of the C-5 ethyl group and the N-3 into cyclized pyrazoline analogues resulted in compounds with potent activities. Some of these compounds have improved IC_{50} values when compared with the noncyclized analogues. These pyrazolines may have improved selectivity for cruzain and improved pharmacokinetic properties.

The cell culture activity involves complicated factors. Compounds that are potent cruzain inhibitors in vitro are not necessarily active in cell culture. Antiparasitic

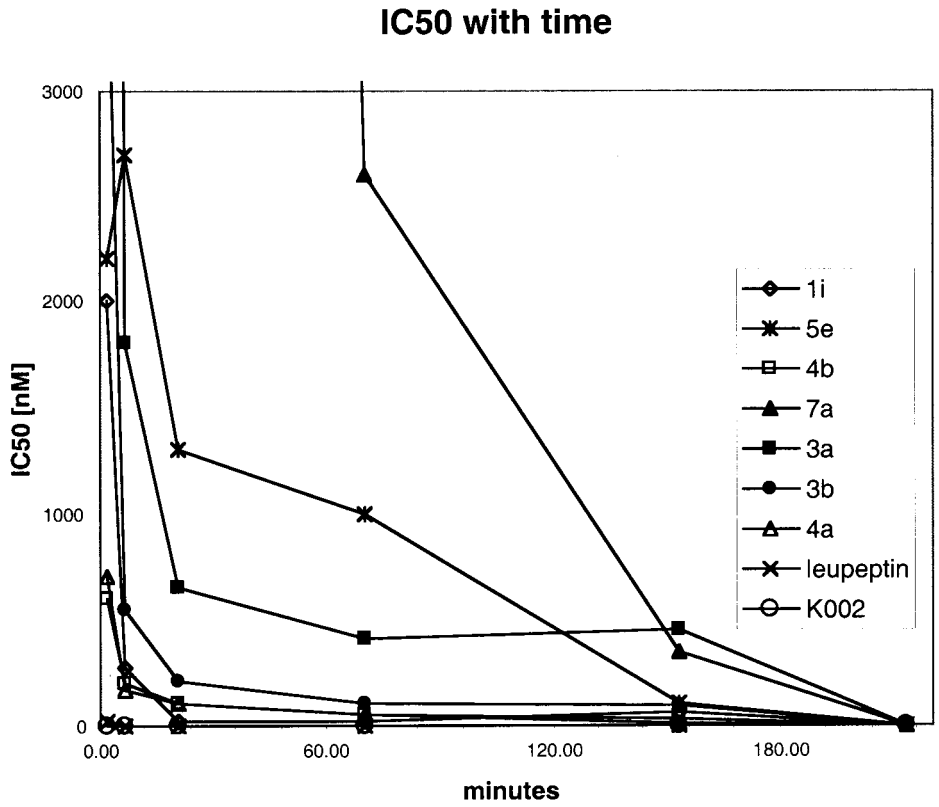


Figure 2. IC_{50} versus time. The data of representative thio semicarbazones are shown.

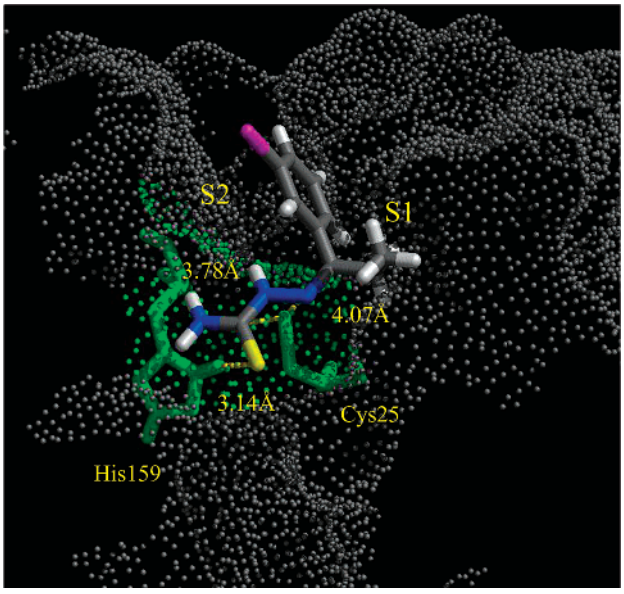


Figure 3. Best calculated orientation of compound **1i** in the active site of cruzain. The surface of the active site of cruzain is represented by gray dots. His159 and Cys25 are colored in green. Compound **1i** is colored according to atom types: bromine, magenta; carbon, gray; hydrogen, white; nitrogen, blue; sulfur, yellow.

inhibitors must be able to cross the macrophage's cell membrane and cross the parasite's cytoplasm in sufficient quantity to significantly inhibit cruzain without killing the host cell. So far, nine compounds in the thio semicarbazone series (three single-phenyl-ring-substituted compounds (**1i**, **2a**, **2b**), two of the methyl-substituted thio semicarbazones (**3b**, **3h**), and four pyrazoline analogues (**4b**, **4d**, **4e**, **4h**) are trypanocidal. The trypanocidal activity of this series represents a

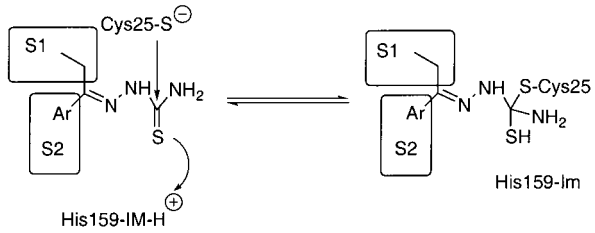


Figure 4. Proposed mechanism of reversible covalent interaction between thio semicarbazones and cruzain based on experimental and modeling results.

significant advance. For example, our best acylhydrazide- and urea-based cruzain inhibitors are only trypanostatic for 18 and 22 days, respectively. Success in animal and human clinical trials will depend in part upon the absorption, distribution, metabolism, and excretion of these thio semicarbazones. To advance the experimental studies, we have looked for computational surrogates to guide our medicinal chemistry effort.

Lipinski described desired ranges for certain properties thought to be important for pharmacokinetics and drug development. They are C log $P < 5$, number of hydrogen bond donors ≤ 5, number of hydrogen bond acceptors ≤ 10, and molecular weight < 500.[26] A compound that fulfills at least three out of the four criteria adheres to Lipinski's rule. Table 1 lists such properties of the nine trypanocidal compounds. All of our most potent antiparasitic agents are fully compatible with Lipinski's rule.

Conclusion

We have identified a novel series of small-molecule compounds that potently inhibit trypanosomal infection and serve as promising agents for antitrypanosomal

Table 1. The Trypanocidal Compounds (for Structures, See Charts 7 and 8) Have Physical Properties Compatible with Reasonable Pharmacokinetics and Drug Availablity

ID	mol wt	*C* log *P*	no. of H bond donors	no. of H bond acceptors	no. of criteria met
rule	<500	<5	<5	<10	at least 3
1i	286	3.79	3	3	all
2a	242	3.64	3	3	all
2b	275	3.81	3	3	all
3b	272	3.26	3	3	all
3g	262	3.71	3	3	all
4b	298	4.33	2	3	all
4d	254	4.18	2	3	all
4e	274	4.25	2	3	all
4h	287	4.35	2	3	all

therapy. These inhibitors have specific SARs which should allow for the development of additional antitrypanosomal analogues and potential inhibitors of other cysteine proteases. Their advantages include

(i) minimal cellular toxicity, (ii) physical properties known to be compatible with desirable pharmacokinetics (low molecular weight, favorable *C* log *P*, favorable hydrogen bond donating and accepting capabilities), (iii) potency and efficacy, with IC_{50} values at the low nanomolar level against cruzain, (iv) evidence of efficacy against parasite-infected macrophages, (v) simple synthetic access and thus low production costs, and (vi) nonpeptidic compounds improving the likelihood of reasonable bioavailability. Further optimization and pharmacokinetics characterization of this series are ongoing.

Experimental Section

Melting points were determined in open capillaries with a Büchi melting point apparatus, B-540 (Switzerland). Infrared spectra were recorded in an Impact 400 spectrophotometer for a representative set of compounds. ^{1}H NMR spectra were obtained with a Varian Inova-400 NMR spectrometer. Unless otherwise noted, all spectra were recorded with DMSO as solvent. Splitting patterns are designated as follows: s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants (*J*) are given in hertz. Mass spectral analyses were performed at the Mass Spectrometry facility, University of California, San Francisco. Elemental analyses were carried out by Atlantic Microlab, Inc., Norcross, GA. Unless otherwise stated, yields for the reactions were higher than 90%.

General Procedure for the Preparation of Thio Semicarbazones. Aldehyde or ketone (0.5 mmol) and thio semicarbazide (0.5 mmol) were added to a dry flask. The mixture was dissolved in 10 mL of anhydrous MeOH. For ketone, 1% acetic acid (0.1 mL) was also added to the reaction. The reaction was heated to reflux under nitrogen. TLC was used to monitor whether the reaction was complete. The reaction time ranged from 3 h for aldehydes to overnight for ketones. The solvent was removed in vacuo, and the resulting solid was rinsed or recrystallized.

Data for 4-(Phenylethynyl)thiophene-2-carboxyaldehyde Thio Semicarbazone (1a): mp 197.6−199.6 °C; ^{1}H NMR δ 7.42 (m, 3H), 7.52 (m, 2H), 7.60 (s, 1H), 7.66 (br s, 1H), 7.95 (s, 1H), 8.19 (br s, 1H), 8.25 (s, 1H), 11.52 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{14}H_{11}N_3S_2$ 285.0394, found 285.0403.

Data for 5-(4-Chlorophenyl)-2-furancarboxaldehyde Thio Semicarbazone (1b): mp 199.2−200.8 °C; ^{1}H NMR δ 7.06 (d, 1H, J = 3.2), 7.16 (d, 1H, J = 3.6), 7.49 (d, 2H, J = 8.4), 7.77 (br s, 1H), 7.85 (d, 2H, J = 8.0), 7.96 (s, 1H), 8.28 (br s, 1H), 11.50 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{12}H_{10}ClN_3OS$ 279.0233, found 279.0237.

Data for 5-(2-Methoxyphenyl)-2-furancarboxaldehyde Thio Semicarbazone (1c): mp 209.5−211.1 °C; IR (neat, cm^{-1}) 3389, 3234, 3138, 3051, 2983, 2930, 2839, 1603, 1545, 1487, 1343, 1299, 1241, 1101, 1019, 923, 845, 797, 759; ^{1}H NMR δ 3.92 (s, 3H), 7.04 (m, 3H), 7.13(d, 1H, J = 8.8), 7.33 (t, 1H, J = 7.8), 7.76 (br s, 1H), 7.94 (d, 1H, J = 7.6), 7.97 (s, 1H), 8.24 (br s, 1H), 11.46 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{13}H_{13}N_3O_2S$ 275.0728, found 275.0720.

Data for 2-(1-Methyl-3-trifluoromethyl)pyrazol-5-yl)-thiophene-5-carboxaldehyde Thio Semicarbazone (1d): mp 222.4 °C dec; ^{1}H NMR δ 4.05 (s, 3H), 7.06 (s, 1H), 7.52 (m, 2H), 7.62 (br s, 1H), 8.23 (s, 1H), 8.28 (br s, 1H), 11.57 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{11}H_{10}F_3N_5S_2$ 333.0330, found 333.0337.

Data for 5-(3-Chlorophenyl)-2-furancarboxaldehyde Thio Semicarbazone (1e): mp 198.8−199.8 °C; ^{1}H NMR δ 7.07 (d, 1H, J = 3.6), 7.24 (d, 1H, J = 3.6), 7.38 (d, 1H, J = 8.0), 7.46 (m, 1H), 7.79 (d, 1H, J = 8.0), 7.83 (br s, 1H), 7.89 (d, 1H, J = 1.6), 7.96 (s, 1H), 8.28 (br s, 1H), 11.51 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{12}H_{10}ClN_3OS$ 279.0233, found 279.0231.

Data for 5-(4-Bromophenyl)-2-furancarboxaldehyde Thio Semicarbazone (1f): ^{1}H NMR (isomer ratio 3:1) (major isomer) δ 7.14 (dd, 1H, J = 1,2, 3,6), 7.29 (d, 1H, J = 1.2), 7.30 (s, 1H), 7.72 (m, 2H), 7.77 (m, 2H), 8.14 (br s, 1H), 8.61 (br s, 1H), 10.53 (s, 1H); (minor isomer) δ 7.06 (d, 1H, J = 2.4), 7.17 (m, 1H), 7.62 (d, 2H, J = 7.6), 7.77 (m, 3H), 7.96 (s, 1H), 8.28 (br s, 1H), 11.50 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{12}H_{10}BrN_3OS$ 322.9728, found 322.9723.

Data for 1-(Phenylsulfonyl)-2-pyrrolecarboxaldehyde Thio Semicarbazone (1g): mp dec upon heating; ^{1}H NMR δ 6.44 (t, 1H, J = 3,4), 7.10 (d, 1H, J = 2.0), 7.55 (s, 1H), 7.68 (t, 2H, J = 7.8), 7.77 (t, 1H, J = 7.4), 7.81 (br s, 1H), 7.96 (d, 2H, J = 8.0), 8.17 (br s, 1H), 8.49 (s, 1H), 11.49 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{12}H_{12}N_4O_2S_2$ 308.0402, found 308.0407.

Data for 4′-Morpholinoacetophenone Thio Semicarbazone (1h): mp 231.2−232.8 °C; ^{1}H NMR δ 2.23 (s, 3H), 3.16 (t, 4H, J = 4.8), 3.73 (t, 4H, J = 4.8), 6.90 (d, 2H, J = 8.8), 7.79 (d, 2H, J = 8.8), 7.80 (s, 1H), 8.14 (br s, 1H), 10.04 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{13}H_{18}N_4OS$ 278.1201, found 278.1210.

Data for 3′-Bromopropiophenone Thio Semicarbazone (1i): mp 144.3−144.1 °C; IR (neat, cm^{-1}) 3413, 3238, 3144, 3059, 2974, 2920, 1584, 1509, 1470, 1420, 1280, 1115, 1051, 857, 787; ^{1}H NMR δ 0.99 (t, 3H, J = 7.4), 2.85 (q, 2H, J = 7.6), 7.33 (t, 1H, J = 8.0), 7.55 (d, 1H, J = 8.0), 7.85 (d, 1H, J = 7.6), 8.04 (br s, 1H), 8.13 (s, 1H), 8.26 (br s, 1H), 10.30 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{10}H_{12}BrN_3S$ 284.9935, found 284.9936. Anal. ($C_{10}H_{12}BrN_3S$) C, H, Br, N, S.

Data for 3-Hydroxy-4-methoxybenzaldehyde Thio Semicarbazone (1j): mp 175.3−177.5 °C; ^{1}H NMR δ 3.79 (s, 3H), 6.92 (d, 1H, J = 8.4), 7.10 (dd, 1H, J = 1.6, 8.4), 7.24 (d, 1H, J = 2.0), 7.78 (br s, 1H), 7.90 (s, 1H), 8.06 (br s, 1H), 9.03 (s, 1H), 11.24 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_9H_{11}N_3O_2S$ 225.0572, found 225.0566.

Data for 6′-Methoxy-2′-propiononaphthone Thio Semicarbazone (1k): mp 170.5−171.2 °C; ^{1}H NMR δ 1.07 (t, 3H, J = 7.4), 2.96 (q, 2H, J = 7.4), 3,87 (s, 3H), 7.17 (dd, 1H, J = 2.4, 8.8), 7.33 (d, 1H, J = 2.4), 7.77 (d, 1H, J = 8.8), 7.89 (d, 1H, J = 8.8), 7.98 (br s, 1H), 8.19 (d, 1H, J = 8.8), 8.26 (s, 1H), 8.30 (br s, 1H), 10.32 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{15}H_{17}N_3OS$ 287.1092, found 287.1090.

Data for 4-Dimethylamino-1-naphthaldehyde Thio Semicarbazone (1l): mp 170.5−171.2 °C; ^{1}H NMR δ 2.87 (s, 6H), 7.10 (d, 1H, J = 8.0), 7.55 (m, 1H), 7.61 (m, 1H), 7.83 (br s, 1H), 8.03 (d, 1H, J = 8.0), 8.18 (m, 2H), 8.42 (d, 1H, J = 8.4), 8.78 (s, 1H), 11.32 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{14}H_{16}N_4S$ 272.1096, found 272.1094.

Data for 1-Methylindole-3-carboxaldehyde Thio Semicarbazone (1m): mp 200.3−201.2 °C; ^{1}H NMR δ 3.80 (s, 3H), 7.15 (t, 1H, J = 7.4), 7.25 (t, 1H, J = 7.6), 7.40 (br s, 1H), 7.47 (d, 1H, J = 8.0), 7.79 (s, 1H), 8.00 (br s, 1H), 8.22 (d, 1H, J = 7.6), 8.26 (s, 1H), 11.13 (s, 1H); HRMS (EI) *m*/*z* (M^+) calcd for $C_{11}H_{12}N_4S$ 232.0783, found 232.0786.

Data for 2-Methoxy-1-naphthaldehyde Thio Semicarbazone (1n): mp 156.2−157.6 °C; ^{1}H NMR δ 3.96 (s, 3H), 7.40 (t, 1H, J = 7.4), 7.48 (d, 1H, J = 9.2), 7.50 (br s, 1H), 7.56 (t, 1H, J = 7.8), 7.88 (d, 1H, J = 8.0), 8.02 (d, 1H, J = 9.2), 8.20 (br s, 1H), 8.80 (s, 1H), 8.92 (d, 1H, J = 8.8), 11.48 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{13}H_{13}N_3OS$ 259.0779, found 259.0772.

Data for 3′-Chloropropiophenone Thio Semicarbazone (2a): mp 136.6−138.3 °C; IR (neat, cm^{-1}) 3408, 3203, 3155, 2970, 2941, 1598, 1511, 1472, 1418, 1301, 1233, 1112, 1053, 1004, 859, 791; ^{1}H NMR δ 0.98 (t, 3H, J = 7.4), 2.86 (q, 2H, J = 7.6), 7.41 (m, 2H), 7.82 (m, 1H), 8.03 (s, 1H), 8.08 (br s, 1H), 8.31 (br s, 1H), 10.36 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{12}ClN_3S$ 241.0440, found 241.0449.

Data for 3′-Trifluoromethylpropiophenone Thio Semicarbazone (2b): mp 156.0−157.9 °C; IR (neat, cm^{-1}) 3427, 3272, 3155, 3053, 2975, 2941, 1618, 1525, 1481, 1345, 1306, 1189, 1146, 1078, 864, 810, 703; ^{1}H NMR δ 1.00 (t, 3H, J = 7.4), 2.92 (q, 2H, J = 7.4), 7.61 (t, 1H, J = 7.8), 7.72 (d, 1H, J = 7.6), 8.11 (br s, 1H), 8.20 (s, 2H), 8.35 (br s, 1H), 10.42 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{11}H_{12}F_3N_3S$ 275.0704, found 275.0709. Anal. ($C_{11}H_{12}F_3N_3S$) C, H, F, N, S.

Data for 3′-Fluoropropiophenone Thio Semicarbazone (2c): mp 138.8−139.5 °C; ^{1}H NMR δ 0.99 (t, 3H, J = 7.6), 2.86 (q, 2H, J = 7.6), 7.20 (dt, 1H, J = 2.4, 8.6), 7.43 (m, 1H), 7.69 (d, 1H, J = 8.0), 7.87 (d, 1H, J = 11.2), 8.07 (br s, 1H), 8.31 (br s, 1H), 10.36 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{12}FN_3S$ 225.0736, found 225.0738.

Data for Propiophenone Thio Semicarbazone (2d): mp 116.8−117.9; ^{1}H NMR δ 1.01 (t, 3H, J = 7.6), 2.86 (q, 2H, J = 7.6), 7.38 (m, 3H), 7.89 (m, 3H), 8.22 (br s, 1H), 10.27 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{13}N_3S$ 207.0830, found 207.0826.

Data for 4′-Bromopropiophenone Thio Semicarbazone (2e): mp 171.3−173.0 °C; ^{1}H NMR δ 0.98 (t, 3H, J = 7.4), 2.84 (q, 2H, J = 7.4), 7.55 (d, 2H, J = 8.4), 7.88 (d, 2H, 8.4), 7.97 (br s, 1H), 8.31 (br s, 1H), 10.38 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{12}BrN_3S$ 284.9935, found 284.9938.

Data for 4′-Chloropropiophenone Thio Semicarbazone (2f): yield 60%; mp 175.1−176.8 °C; ^{1}H NMR δ 0.99 (t, 3H, J = 7.6), 2.84 (q, 2H, J = 7.6), 7.41 (d, 2H, J = 8.8), 7.95 (d, 2H, J = 8.4), 7.96 (br s, 1H), 8.30 (br s, 1H), 10.37 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{12}ClN_3S$ 241.0440, found 241.0447.

Data for 4′-Methoxypropiophenone Thio Semicarbazone (2g): mp 117.8−119.8 °C; ^{1}H NMR δ 0.99 (t, 3H, J = 7.6), 2.83 (q, 2H, J = 7.6), 3.78 (s, 3H), 6.92 (d, 2H, J = 9.2), 7.85 (br s, 1H), 7.86 (d, 2H, J = 8.8), 8.19 (br s, 1H), 10.21 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{11}H_{15}N_3OS$ 237.0936, found 237.0933.

Data for 3′,5′-bis(trifluoromethyl)propiophenone Thio Semicarbazone (2h): mp 188.0−190.2 °C; IR (neat, cm^{-1}) 3245, 3153, 2982, 2936, 1596, 1508, 1466, 1392, 1281, 1171, 1126, 893, 866; ^{1}H NMR δ 0.99 (t, 3H, J = 7.6), 2.98 (q, 2H, t, 3H, J = 7.6), 8.07 (s, 1H), 8.33 (br s, 1H), 8.43 (br s, 1H), 8.49 (s, 2H), 10.51 (s, 1H); HRMS (EI) m/z (M + H^+) calcd for $C_{12}H_{12}F_6N_3S$ 344.0656, found 344.0671.

Data for 3′,4′-Dichloropropiophenone Thio Semicarbazone (2i): mp 185.6−186.4 °C; IR (neat, cm^{-1}) 3420, 3249, 3150, 3041, 2980, 2942, 1603, 1513, 1475, 1390, 1300, 1101, 1063, 888, 860, 793; ^{1}H NMR δ 0.98 (t, 3H, J = 7.6), 2.86 (q, 2H, J = 7.6), 7.61 (d, 1H, J = 8.8), 7.85 (dd, 1H, J = 2.0, 8.6), 8.12 (br s, 1H), 8.22 (d, 1H, J = 2.0) 8.30 (br s, 1H), 10.35 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{11}Cl_2N_3S$ 275.0051, found 275.0053.

Data for 2′,4′-Dichloropropiophenone Thio Semicarbazone (2j): mp 171.8−173.0 °C; ^{1}H NMR δ 1.04 (t, 3H, J = 7.6), 2.49 (q, 2H, J = 7.6), 7.33 (d, 1H, J = 8.4), 7.53 (dd, 1H, J = 2.0, 8.4), 7.75 (d, 1H, J = 2.0), 7.77 (br s, 1H), 8.26 (br s, 1H), 9.42 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{11}Cl_2N_3S$ 275.0051, found 275.0049.

Data for 3′-Methoxyacetophenone Thio Semicarbazone (2k): mp 195.1−196.7 °C; ^{1}H NMR δ 2.28 (d, 3H, J = 3.2), 3.79 (d, 3H, J = 2.8), 6.95 (d, 1H, J = 8.0), 7.29 (dt, 1H, J = 2.8, 8.0), 7.44 (m, 2H), 7.88 (br s, 1H), 8.21 (br s, 1H), 10.12 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{13}N_3OS$ 223.0779, found 223.0777.

Data for 2′-Bromoacetophenone Thio Semicarbazone (2l): yield 40%; mp 144.5−146.2 °C; ^{1}H NMR δ 2.27 (s, 3H), 7.31 (m, 1H), 7.42 (m, 2H), 7.60 (br s, 1H), 7.65 (d, 1H, J = 8.0), 8.24 (br s, 1H), 10.34 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_9H_{10}BrN_3S$ 270.9779, found 270.9775.

Data for 2′-Chloroacetophenone Thio Semicarbazone (2m): yield 45%; mp 155.8−156.7 °C; ^{1}H NMR δ 2.28 (s, 3H), 7.38 (m, 2H), 7.49 (m, 2H), 7.62 (br s, 1H), 8.24 (br s, 1H), 10.34 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_9H_{10}ClN_3S$ 227.0284, found 227.0289.

Data for 2,3-Dichlorobenzaldehyde Thio Semicarbazone (2n): mp 232.3−233.6 °C; ^{1}H NMR δ 7.37 (t, 1H, J = 8.0), 7.65 (m, 1H), 8.17 (br s, 1H), 8.29 (d, 1H, J = 8.0), 8.35 (br s, 1H), 8.48 (s, 1H), 11.67 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_8H_7Cl_2N_3S$ 246.9738, found 246.9730.

Data for 3,5-Dichlorobenzaldehyde Thio Semicarbazone (2o): mp 234.1−235.5 °C; ^{1}H NMR δ 7.57 (t, 1H, J = 1.6), 7.93 (d, 2H, J = 1.6), 7.96 (s, 1H), 8.31 (br s, 1H), 11.59 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_8H_7Cl_2N_3S$ 246.9738, found 246.9737.

Data for 2-Trifluoromethylbenzaldehyde Thio Semicarbazone (2p): mp 248.3−249.4 °C; ^{1}H NMR δ 7.58 (t, 1H, J = 7.6), 7.68 (t, 1H, J = 7.6), 7.75 (d, 1H, J = 8.0), 8.15 (br s, 1H), 8.35 (br s, 1H), 8.43 (s, 1H), 8.50 (d, 1H, J = 8.0), 11.67 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_9H_8F_3N_3S$ 247.0391, found 247.0391.

Data for 3-Bromobenzaldehyde Thio Semicarbazone (3a): mp 200.6−210.9 °C; IR (neat, cm^{-1}) 3388, 3233, 3149, 3024, 2984, 2805, 1604, 1534, 1467, 1355, 1310, 1220, 1101, 941, 897, 832, 792; ^{1}H NMR δ 7.34 (t, 1H, J = 7.6), 7.55 (d, 1H, J = 7.2), 7.68 (d, 1H, J = 7.6), 7.99 (s, 1H), 8.18 (s, 2H), 8.24 (br s, 1H), 11.49 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_8H_8BrN_3S$ 256.9622, found 256.9618.

Data for 3′-Bromoacetophenone Thio Semicarbazone (3b): mp 174.2−174.9 °C; IR (neat, cm^{-1}) 3428, 3263, 3144, 3059, 1604, 1524, 1310, 1116, 871, 802, 732; ^{1}H NMR δ 2.27 (s, 3H), 7.33 (t, 1H, J = 8.0), 7.55 (dd, 1H, J = 1.0, 8.0), 7.88 (d, 1H, J = 8.0), 8.10 (br s, 1H), 8.18 (s, 1H), 8.31 (br s, 1H), 10.22 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_9H_{10}BrN_3S$ 270.9779, found 270.9778.

Data for 3-Trifluoromethylbenzaldehyde Thio Semicarbazone (3c): mp 220.7−221.7 °C; ^{1}H NMR δ 7.62 (t, 1H, J = 7.6), 7.71 (d, 1H, J = 8.0), 8.02 (d, 1H, J = 7.6), 8.10 (s, 1H), 8.25 (br s, 1H), 8.26 (s, 1H), 8.28 (br s, 1H), 11.55 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_9H_8F_3N_3S$ 247.0391, found 247.0395.

Data for 3-Trifluoromethylacetophenone Thio Semicarbazone (3d): mp 197.9−201.2 °C; ^{1}H NMR δ 2.33 (s, 3H), 7.60 (t, 1H, J = 7.6), 7.72 (d, 1H, J = 7.6), 8.13 (br s, 1H), 8.22 (m, 2H), 8.34 (br s, 1H), 10.29 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{10}F_3N_3S$ 261.0548, found 261.0545.

Data for 3,5-Bis(trifluoromethyl)benzaldehyde Thio Semicarbazone (3e): mp 229.8−230.3 °C; ^{1}H NMR δ 8.04 (s, 1H), 8.16 (s, 1H), 8.38 (br s, 1H), 8.46 (br s, 1H), 8.54 (s, 2H), 11.71 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_7F_6N_3S$ 315.0265, found 315.0269.

Data for 3′,5′-Bis(trifluoromethyl)acetophenone Thio Semicarbazone (3f): mp 238 °C dec; ^{1}H NMR δ 2.38 (s, 3H), 8.06 (s, 1H), 8.36 (br s, 1H), 8.42 (br s, 1H), 8.53 (s, 2H), 10.37 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{11}H_9F_6N_3S$ 329.0421, found 321.0429.

Data for 3,4-Dichlorobenzaldehyde Thio Semicarbazone (3g): ^{1}H NMR δ 7.64 (d, 1H, J = 8.4), 7.71 (dd, 1H, J = 1.8, 8.4), 7.98 (s, 1H), 8.24 (s, 1H), 8.27 (br s, 1H), 11.55 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_8H_7Cl_2N_3S$ 246.9738, found 246.9732.

Data for 3′,4′-Dichloroacetophenone Thio Semicarbazone (3h): mp 196.0−197.9 °C; ^{1}H NMR δ 2.27 (s, 3H), 7.61 (d, 1H, J = 8.8), 7.88 (dd, 1H, J = 0.8, 8.4), 8.17 (br s, 1H), 8.27 (s, 1H), 8.34 (br s, 1H), 10.27 (s, 1H); HRMS (EI) m/z (M^+)

calcd for $C_9H_9Cl_2N_3S$ 260.9894, found 260.9891. Anal. ($C_9H_9Cl_2N_3S$) C, H, Cl, N, S.

Data for 2-Acetyl-5-bromothiophene Thio Semicarbazone (3i): mp 205.1−207.3 °C; ^{1}H NMR δ 2.27 (s, 3H), 7.19 (m, 1H), 7.32 (d, 1H, J = 3.6), 7.49 (br s, 1H), 8.31 (br s, 1H), 10.37 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_7H_8BrN_3S_2$ 276.9343, found 276.9349.

Data for 2-Acetyl-5-chlorothiophene Thio Semicarbazone (3j): mp 237.1−238.2 °C; ^{1}H NMR δ 2.27 (s, 3H), 7.09 (d, 1H, J = 4.0), 7.36 (d, 1H, J = 4.0), 7.48 (br s, 1H), 8.31 (br s, 1H), 10.38 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_7H_8ClN_3S_2$ 232.9848, found 232.9845.

General Procedure for Synthesizing Cyclized Pyrazoline Analogues of Thio Semicarbazones. Mannich Reaction. A 20 μL portion of concentrated hydrochloric acid was added to a mixture of ketone (10 mmol), paraformaldehyde (13 mmol, 390 mg), and dimethylamine hydrochloride (13 mmol, 1.059 g) in 5 mL of ethanol. The reaction was refluxed overnight under nitrogen. In some cases, precipitates formed and the product was obtained by filtration in ethanol. If no precipitate was formed, the solvent was removed. A few drops of HCl was added, and the mixture was worked up with dichloromethane and water. The dichloromethane layer was discarded. The aqueous layer was adjusted to basic and extracted with dichloromethane (3×). The dichloromethane layer was combined and dried. The product was obtained by removal of dichloromethane.

Cyclization. Thio semicarbazide or substituted thio semicarbazide (0.5 mmol) was dissolved in MeOH (5 mL) upon refluxing under nitrogen. Sodium hydroxide (50%) (0.18 mL) was added to the reaction. A warm methanol (5 mL) solution of the previous Mannich reaction product (0.5 mmol) was then added dropwise to the reaction mixture. After the reaction was refluxed for 1 h, methanol was removed at reduced pressure. The residue was dissolved in dichloromethane and washed with water. Evaporation of the solvent and purification through chromatography gave the cyclized analogues.

Data for 3-(3-Bromophenyl)-2-pyrazoline-1-thiocarboxamide (4a): Mannich yield 84%, cyclization yield 24%; mp 172.9−174.5 °C, ^{1}H NMR δ 3.28 (t, 2H, J = 10.0), 4.14 (t, 2H, J = 10.0), 7.41 (t, 1H, J = 8.0), 7.64 (d, 1H, J = 8.4), 7.75 (d, 1H, J = 8.0), 7.89 (br s, 1H), 8.00 (br s, 1H), 8.13 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{10}BrN_3S$ 282.9779, found 282.9780.

Data for 3-(3-Bromophenyl)-4-methyl-2-pyrazoline-1-thiocarboxamide (4b): Mannich yield 81%, cyclization yield 62%; mp 107.4−109.9 °C; IR (neat, cm^{-1}) 3430, 3240, 3139, 3064, 2954, 2924, 2859, 1589, 1497, 1466, 1366, 1086, 1006, 901, 790; ^{1}H NMR δ 1.17 (d, 3H, J = 7.2), 3.80 (m, 1H), 3.92 (dd, 1H, J = 4.2, 11.6), 4.19 (t, 1H, J = 11.6), 7.41 (t, 1H, J = 8.0), 7.63 (m, 1H), 7.78 (d, 1H, 8.0), 7.94 (br s, 1H), 8.07 (br s, 1H), 8.16 (m, 1H); HRMS (EI) m/z (M^+) calcd for $C_{11}H_{12}BrN_3S$ 296.9935, found 296.9926.

Data for 3-(3-Chlorophenyl)-2-pyrazoline-1-thiocarboxamide (4c): Mannich yield 87%, cyclization yield 25%; mp 154.3−157.0 °C; ^{1}H NMR δ 3.28 (t, 2H, J = 9.2), 4.14 (t, 2H, J = 10.0), 7.49 (m, 2H), 7.72 (d, 1H, J = 7.2), 7.89 (br s, 1H), 7.99 (s, 2H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{10}ClN_3S$ 239.0284, found 239.0284.

Data for 3-(3-Chlorophenyl)-4-methyl-2-pyrazoline-1-thiocarboxamide (4d): Mannich yield 44%, cyclization yield 47%; mp 131.3 °C dec; IR (neat, cm^{-1}) 3432, 3267, 3147, 3062, 2967, 2927, 2881, 1584, 1499, 1464, 1364, 1118, 1008, 908, 803, 743; ^{1}H NMR δ 1.17 (d, 3H, J = 7.2), 3.80 (m, 1H), 3.94 (dd, 1H, J = 4.4, 11.6), 4.19 (t, 1H, J = 11.2), 7.50 (m, 2H), 7.75 (d, 1H, J = 6.8), 7.93 (br s, 1H), 8.03 (s, 1H), 8.07 (br s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{11}H_{12}ClN_3S$ 253.0440, found 253.0447.

Data for 3-(3,4-Dichlorophenyl)-2-pyrazoline-1-thiocarboxamide (4e): Mannich yield 50%, cyclization yield 20%; mp 199.3−201.4 °C; IR (neat, cm^{-1}) 3427, 3233, 3140, 2921, 2843, 1598, 1501, 1467, 1379, 1102, 888, 815; ^{1}H NMR δ 3.28 (t, 3H, J = 10.0), 4.15 (t, 2H, J = 10.0), 7.74 (m, 2H), 7.94 (br s, 1H), 8.04 (br s, 1H), 8.16 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_9Cl_2N_3S$ 272.9894, found 272.9896.

Data for 3-(3,4-Dichlorophenyl)-4-methyl-2-pyrazoline-1-thiocarboxamide (4f): Mannich yield 28%, cyclization yield 52%; mp 171.2−172.7 °C; IR (neat, cm^{-1}) 3434, 3264, 3145. 3051, 2961, 2927, 2868, 1581, 1457, 1368, 1129, 1027, 907, 809; ^{1}H NMR δ 1.17 (d, 3H, J = 7.2), 3.80 (m, 1H), 3.95 (dd, 1H, J = 4.0, 11.6), 4.19 (t, 1H, J = 11.6), 7.72 (d, 1H, J = 8.0), 7.79 (d, 1H, J = 8.4), 7.99 (br s, 1H), 8.10 (br s, 1H), 8.21 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{11}H_{11}Cl_2N_3S$ 287.0051, found 287.0052.

Data for 3-(3-Trifluoromethylphenyl)-2-pyrazoline-1-thiocarboxamide (4g): Mannich yield 61%, cyclization yield 30%; mp 159.2−161.1 °C; ^{1}H NMR δ 3.34 (t, 2H, J = 10.4), 4.17 (t, 2H, J = 10.0), 7.70 (t, 1H, J = 7.6), 7.81 (d, 1H, J = 8.0), 8.03 (m, 3H), 8.26 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{11}H_{10}F_3N_3S$ 273.0548, found 273.0542.

Data for 3-(3-Trifluoromethylphenyl)-4-methyl-2-pyrazoline-1-thiocarboxamide (4h): Mannich yield 40%, cyclization yield 35%; mp 120.8−121.9 °C; IR (neat, cm^{-1}) 3443, 3264, 3145, 3068, 2966, 2932, 2872, 1589, 1487, 1351, 1129, 907, 805; ^{1}H NMR δ 1.19 (d, 3H, J = 6.8), 3.88 (m, 1H), 3.97 (dd, 1H, J = 4.4, 11.2), 4.22 (t, 1H, J = 11.2), 7.69 (t, 1H, J = 7.6), 7.80 (d, 1H, J = 7.6), 8.02 (br s, 1H), 8.08 (d, 1H, J = 8.4), 8.11 (br s, 1H), 8.30 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{12}H_{12}F_3N_3S$ 287.0704, found 287.0701. Anal. ($C_{12}H_{12}F_3N_3S$) C, H, F, N, S.

For N1-amino-substituted compounds, only the data of the representative compounds are given.

Data for 3′-Bromopropiophenone *N*-Methyl Thio Semicarbazone (5a): yield 61%, with no acetic acid added during reaction; mp 156.6−158.0 °C; IR (neat, cm^{-1}) 3367, 3297, 3197, 3057, 2967, 2932, 1544, 1494, 1469, 1234, 1118, 1048, 793; ^{1}H NMR δ 0.98 (t, 3H, J = 7.6); 2.87 (q, 2H, J = 7.2), 3.03 (s, 3H), 7.36 (t, 1H, J = 8.0), 7.57 (d, 1H, J = 8.0), 7.87 (d, 1H, J = 7.6), 8.11 (s, 1H), 8.53 (br s, 1H), 10.39 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{11}H_{14}BrN_3S$ 299.0092, found 299.0091.

Data for 3-(3-Bromophenyl)-2-pyrazoline-1-(*N*-methyl)thiocarboxamide (5b): cyclization yield 10%; ^{1}H NMR δ 2.98 (d, 3H, J = 4.4), 3.26 (t, 2H, J = 9.6), 4.15 (t, 2H, J = 10.0), 7.43 (t, 1H, J = 8.0), 7.64 (d, 1H, J = 8.4), 7.74 (d, 1H, J = 7.6), 8.13 (s, 1H), 8.48 (d, 1H, J = 4.4); HRMS (EI) m/z (M^+) calcd for $C_{11}H_{12}BrN_3S$ 296.9935, found 296.9939.

Data for 3′-Bromopropiophenone *N*-(4-Trifluoromethylphenyl) Thio Semicarbazone (5c): mp 153.7−155.7 °C; IR (neat, cm^{-1}) 3302, 3210, 2975, 2935, 1592, 1536, 1463, 1325, 1281, 1115, 840; ^{1}H NMR δ 1.04 (t, 3H, J = 7.2), 2.95 (q, 2H, J = 7.2), 7.38 (t, 1H, J = 8.0), 7.61 (d, 1H, J = 8.0), 7.72 (d, 2H, J = 8.8), 7.86 (m, 2H), 7.94 (d, 1H, J = 8.0), 8.17 (s, 1H), 10.30 (s, 1H), 10.98 (s, 1H); HRMS (EI) m/z (M − H) calcd for $C_{17}H_{15}Br^{81}F_3N_3S$ 430.0023, found 430.0009.

Data for 3-(3-Bromophenyl)-2-pyrazoline-1-(*N*-3-chlorophenyl)thiocarboxamide (5d): yield 35%; mp 197.5−198.8 °C; IR (neat, cm^{-1}) 3337, 3081, 2970, 1606, 1559, 1491, 1444, 1363, 1133, 878; ^{1}HNMR δ 3.36 (t, 2H, J = 10.0), 4.25 (t, 2H, J = 10.0), 7.19 (dd, 1H, J = 1.2, 8.0), 7.38 (t, 1H, J = 8.0), 7.45 (t, 1H, J = 8.0), 7.58 (d, 1H, 8.0), 7.68 (dd, 1H, J = 1.2, 8.0), 7.73 (t, 1H, J = 1.6), 7.86 (d, 1H, J = 8.0), 8.26 (t, 1H, J = 1.6), 10.19 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{16}H_{13}Br^{81}ClN_3S$ 394.9682, found 394.9694.

Data for 3-(3-Chlorophenyl)-2-pyrazoline-1-(*N*-3-trifluoromethylphenyl)thiocarboxamide (5e): cyclization yield 6%; mp 151.8−152.9 °C; ^{1}H NMR δ 3.38 (t, 2H, J = 9.6), 4.27 (t, 2H, 9.6), 7.56 (m, 4H), 7.83 (d, 1H, J = 7.2), 7.95 (t, 1H, J = 7.2), 8.00 (d, 1H, J = 7.6), 8.14 (s, 1H), 10.31 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{17}H_{13}ClF_3N_3S$ 383.0471, found 383.0474.

Data for 3-(3-Bromophenyl)-2-pyrazoline-1-(*N*-hexyl)-thiocarboxamide (5f): cyclization yield 18%; mp 117.0−118.2 °C;^{1}H NMR δ 0.86 (m, 3H), 1.27 (m, 6H), 1.56 (m, 2H), 3.25 (t, 2H, J = 10.4), 3.51 (q, 2H, J = 6.8), 4.15 (t, 2H, J = 10.4), 7.42 (t, 1H, J = 7.6), 7.64 (d, 1H, J = 7.6), 7.75 (d, 1H, J = 7.6), 8.12 (d, 1H, J = 1.2), 8.49 (t, 1H, J = 5.6); HRMS (EI) m/z (M^+) calcd for $C_{16}H_{22}BrN_3S$ 367.0718, found 367.0724.

General Procedure for the Preparation of Semicarbazones. Semicarbazide hydrochloride (1 mmol) and sodium acetate (1 mmol) were dissolved in 1 mL of distilled water.

The solution was slowly added to an ethanol (5 mL) solution of aldehyde or ketone (1 mmol) and the mixture stirred for 2 h. The resulting precipitate was filtered, washed with water and methanol, and dried. Only the data of the representative compounds are given.

Data for 3′-Bromopropiophenone Semicarbazone (6a): mp 182.4−184.2 °C; IR (neat, cm^{-1}) 3473, 3302, 3209, 3068, 2974, 1713, 1593, 1491, 1419, 1142, 1065, 831, 801; ^1H NMR δ 0.97 (t, 3H, $J = 7.6$), 2.71 (q, 2H, $J = 7.6$), 6.54 (br s, 2H), 7.31 (t, 1H, $J = 8.0$), 7.51 (m, 1H), 7.79 (d, 1H, $J = 8.0$), 8.03 (s, 1H), 9.53 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{10}H_{12}BrN_3O$ 269.0164, found 269.0158.

Data for 3′-Bromoacetophenone Semicarbazone (6b): mp 229.3−231.3 °C; ^1H NMR δ 2.15 (s, 3H), 6.55 (br s, 2H), 7.31 (t, 1H, $J = 7.6$), 7.51 (d, 1H, $J = 7.6$), 7.80 (d, 1H, $J = 8.4$), 8.04 (s, 1H), 9.37 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_9H_{10}BrN_3O$ 255.0007, found 255.0000.

Data for 5-(3-Chlorophenyl)-2-furancarboxaldehyde Semicarbazone (6c): mp 192.1−194.8 °C; ^1H NMR δ 6.44 (s, 2H), 6.92 (d, 1H, $J = 3.6$), 7.20 (d, 1H, $J = 3.6$), 7.36 (dd, 1H, $J = 1.2$, 8.0), 7.45 (t, 1H, $J = 8.0$), 7.75 (d, 1H, $J = 6.8$), 7.85 (s, 1H), 10.33 (s, 1H); HRMS (EI) m/z (M^+) calcd for $C_{12}H_{10}ClN_3O_2$ 263.0462, found 263.0467.

Reduction of Thio Semicarbazone 1i to 1-(3′-bromophenyl)propylaminothiourea (7a): Thio semicarbazone (100 mg) was dissolved in anhydrous methanol. Sodium borohydride (excess) was added in small portions every 30 min. The reaction was heated to reflux under nitrogen. The reaction was stopped when most of the starting material was reacted according to TLC. Then the reaction was worked up with saturated ammonium chloride solution and ethyl acetate. After concentration, chromatography gave pure **7a** in 70% yield: mp 110.4−112.4 °C; IR (neat, cm^{-1}) 3403, 3224, 3144, 3064, 2965, 2930, 2870, 1589, 1470, 1270, 1066, 872, 787; ^1H NMR δ 0.64 (t, 3H, $J = 7.2$), 1.43 (m, 1H), 1.68 (m, 1H), 3.74 (m, 1H), 7.25 (t, 1H, $J = 7.6$), 7.34 (d, 1H, $J = 7.6$), 7.38 (br s, 1H), 7.43 (d, 1H, $J = 8.0$), 7.57 (br s, 1H), 7.61 (s, 1H), 8.51 (s, 1H); HRMS (EI) m/z ($M + H^+$) calcd for $C_{10}H_{14}BrN_3S$ 288.0170, found 288.0159.

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