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Synthesis, Biological Evaluation, and Structure−Activity Relationships of Potent Noncovalent and Nonpeptidic Cruzain Inhibitors as Anti-*Trypanosoma cruzi* Agents

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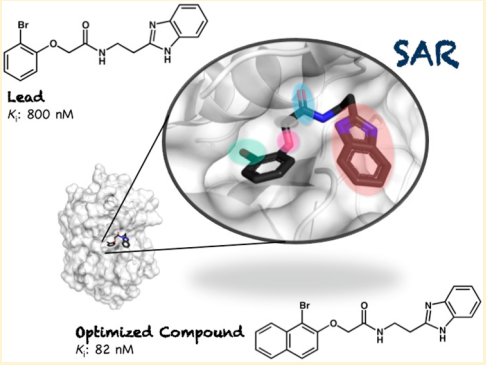
Supporting Information

ABSTRACT: The development of cruzain inhibitors has been driven by the urgent need to develop novel and more effective drugs for the treatment of Chagas' disease. Herein, we report the lead optimization of a class of noncovalent cruzain inhibitors, starting from an inhibitor previously cocrystallized with the enzyme (K_i = 0.8 μM). With the goal of achieving a better understanding of the structure−activity relationships, we have synthesized and evaluated a series of over 40 analogues, leading to the development of a very promising competitive inhibitor (**8r**, IC_{50} = 200 nM, K_i = 82 nM). Investigation of the in vitro trypanocidal activity and preliminary cytotoxicity revealed the potential of the most potent cruzain inhibitors in guiding further medicinal chemistry efforts to develop drug candidates for Chagas' disease.



INTRODUCTION

Over a century after its discovery, treating Chagas' disease still presents a major challenge. Despite its high impact in Latin America, where approximately 10 million people are infected and thousands die annually, nifurtimox and benznidazole are the only two drugs available.[1−4] These drugs exhibit a number of drawbacks, including serious side effects and low efficacy, especially in the treatment of the chronic stage of the disease.[5] Therefore, there is an urgent need for the development of safe and effective new medicines to meet the therapeutic needs of the affected individuals. Cruzain (EC 3.4.22.51), the major cysteine protease from *Trypanosoma cruzi*, is essential for parasite nutrition, metacyclogenesis, immune evasion, and host cell invasion.[6−8] This enzyme, expressed during the parasite life cycle, is among the most important biological targets for Chagas' disease.[5,9,10] Moreover, cruzain has been validated through the development of several classes of inhibitors with potent trypanocidal activity.

Most cruzain inhibitors were developed on the basis of knowledge of its substrate specificity[11] and are irreversible peptidic inhibitors that bind covalently to the enzyme. For instance, the vinyl sulfone K777 (compound **1**), a proof-of-concept compound, has shown efficacy against parasite cell cultures[12] and mouse[13] and dog[14] animal models. These results encouraged the search for new classes of inhibitors through the application of a variety of techniques, such as identification of quantitative structure−activity relationships (QSARs),[15,16] chemical modification of hits identified in substrate library screening,[17] and optimization of compounds discovered by high-throughput screening (HTS)[18] and virtual screening (VS).[19,20] In addition to the traditional irreversible peptidic compounds, the application of these drug design approaches allowed the identification of potent nonpeptidic inhibitors (e.g., compounds **2**−**5**),[17,18,21−23] including several compounds that bind noncovalently to the enzyme (e.g., reversible inhibitors **3**−**5**)[20] (Figure 1). Several crystal structures of complexes of cruzain with inhibitors have been determined and are available,[18,20,21,24−26] providing a solid basis for the application of structure-based drug design (SBDD) methods.

On the basis of the structural information available, the potency of the compound in the low micromolar range (K_i = 2 μM) and its leadlike properties,[27] we have selected a previously described competitive inhibitor (compound **5**) of the target enzyme (PDB ID 3KKU)[20] as a starting point for a lead optimization program. A number of regions of the lead

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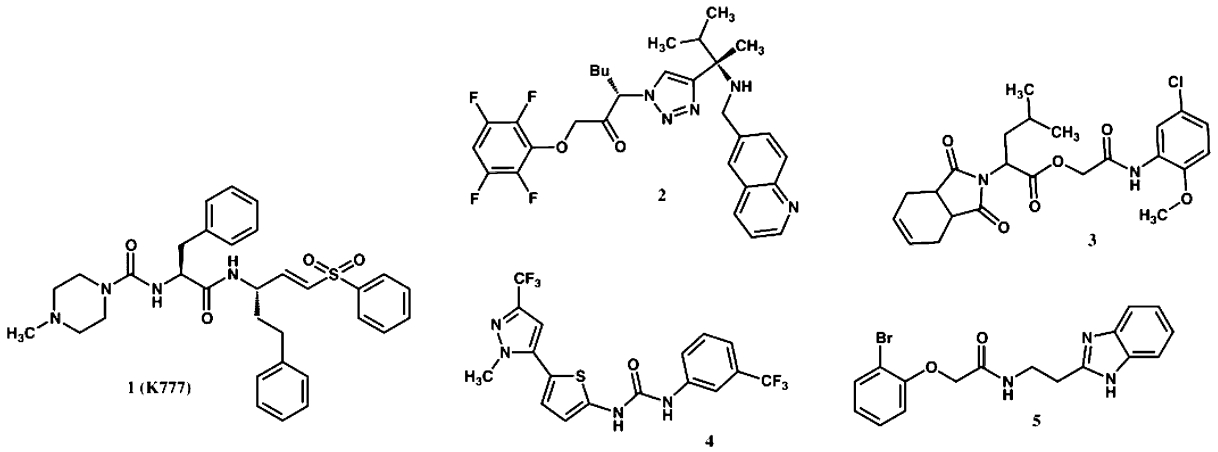
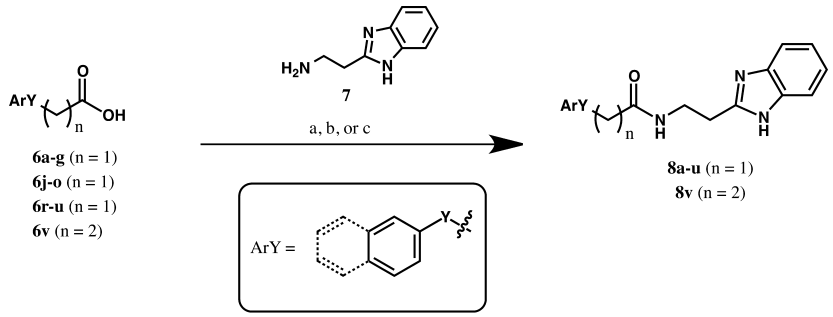


Figure 1. Structures of compound K777 (**1**) and other *T. cruzi* cruzain inhibitors **2**–**4**, including the lead compound **5** employed in this study.

Scheme 1. Synthesis of Benzimidazoles 8[a]



8a, ArY = phenoxy
8b, ArY = *o*-fluorophenoxy
8c, ArY = *o*-chlorophenoxy
8d, ArY = *o*-bromophenoxy
8e, ArY = *o*-iodophenoxy
8f, ArY = *o*-cyanophenoxy
8g, ArY = *o*-nitrophenoxy
8h, ArY = *o*-aminophenoxy
8i, ArY = *o*-acetamidophenoxy
8j, ArY = *o*-methylphenoxy
8k, ArY = *m*-bromophenoxy
8l, ArY = *p*-bromophenoxy
8m, ArY = α-naphthoxy
8n, ArY = β-naphthoxy
8o, ArY = α-carboxyaldehyde-β-naphthoxy
8p, ArY = α-hydroxymethylene-β-naphthoxy
8q, ArY = α-hydroxy-β-naphthoxy
8r, ArY = α-bromo-β-naphthoxy
8s, ArY = *o*-bromobenzyl
8t, ArY = thiophenoxy
8u, ArY = H (acetamide of 2)
8v, ArY = phenoxy (n = 2)

[a]Reagents and conditions: (a) PivCl, Et_3N, DCM, 0 °C, 1 h, then **7**, Et_3N, THF, −78 °C, 2 h; (b) oxalyl chloride, DMF (cat.), DCM, rt, 2 h, then *N*-hydroxysuccinimide, Et_3N, DCM, 0 °C to rt, 2 h, then **7**, DCM, Et_3N, 15 min, rt; (c, for **8u** only) Ac_2O, Et_3N, DCM, 0 °C to rt, overnight; (d) H_2, 5% Pd/C, HCl, EtOH, 1 atm, rt, 3 h; (e) Ac_2O, Py, DCM, 0 °C, 2 h; (f) $NaBH_4$, EtOH, rt, 2 h; (g) MCPBA, EtOH, rt, overnight. Compound **8d** is the same as the lead compound **5** shown in Figure 1.

molecule were explored, leading to an understanding of crucial SAR requirements of this series of analogs and to the development of new inhibitors with anti-*T. cruzi* activity.

RESULTS

Chemistry. α-Phenoxyacetic acids of type **6** were prepared from corresponding substituted phenols and α-haloacetic acid following the described procedures (Scheme 1).[28,29] Subsequent activation of acids of type **6**, followed by coupling with 2-(2-aminoethyl)benzimidazole (**7**), led to formation of the target-substituted benzimidazoles **8**. Initially, the activation of acids of type **6** was performed using pivaloyl chloride (PivCl), but the reaction of **7** with the mixed anhydrides thus generated was not completely regioselective. Consequently, the coformation of 2–3 mol % of the undesired pivalamide of **7** was observed, even at −78 °C. Because this byproduct could not be eliminated by fractionated precipitation, we sought an alternative coupling method. The more reactive acyl chlorides derived from acids of type **6** tended to concomitantly react to a certain extent with the less reactive secondary NH-group of the benzimidazole moiety of **7**. Nevertheless, the reactivity of the acyl chlorides was drastically attenuated through their conversion into corresponding *N*-hydroxysuccinimide esters. These active esters selectively reacted with the terminal NH_2-group of **7**, leading to the production of the desired benzimidazoles **8** in yields higher than 90% and with excellent purity.

To probe the relevance of the benzimidazole moiety for the cruzain inhibitory activity of compounds of type **8**, some related amides of type **10** and compound **12** were prepared from selected acids of type **6** (Scheme 2). The amides of 2-(piperidin-1-yl)ethanamine (**9B**) or the biogenic amines tryptamine (**9C**) and histamine (**9D**) were prepared analogously to compounds of type **8**. In contrast to the results

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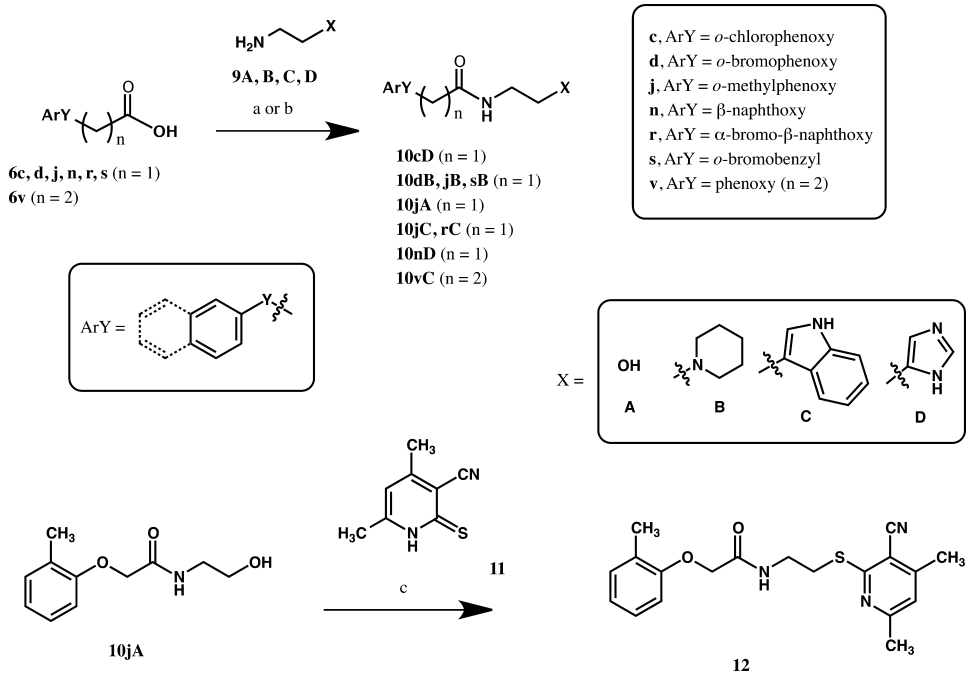
Scheme 2. Synthesis of Compounds 10 and 12[a]

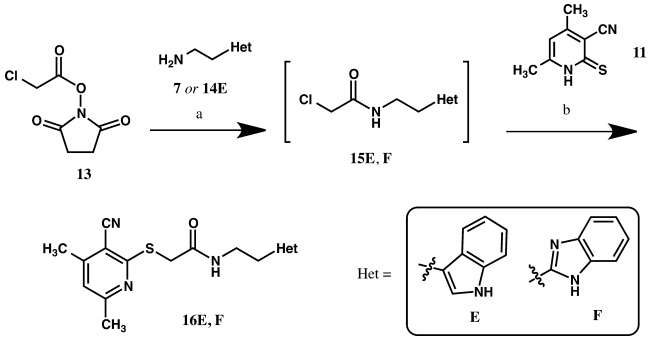


[a]Reagents and conditions: (a) PivCl, Et_3N, DCM, 0 °C, 1 h, then **9A–C**, Et_3N, THF, −78 °C, 2 h; (b) oxalyl chloride, DMF (cat.), DCM, rt, 2 h, then *N*-hydroxysuccinimide, Et_3N, DCM, 0 °C to rt, 2 h, then **9D**, DCM, Et_3N, 0 °C, 15 min, rt, 2 h; (c) mesyl chloride, Et_3N, DCM, 0 °C, 1 h, then Et_3N, DCM, rt, 40 h.

obtained with the amine **7**, the reaction of amines **9A–C** with pivaloyl-mixed anhydrides of acids of type **6** showed satisfactory regioselectivity, and the coformation of undesired pivalamides was limited to trace amounts only. Then, histamine derivatives **10cD** and **10nD** were obtained through intermediacy of the corresponding *N*-hydroxysuccinimide esters. Compound **12** was obtained from **10jA** following a two-step one-pot strategy. Accordingly, the hydroxyl group attached to the ethylene linker was first mesylated and then replaced with the nucleophilic thiopyridone **11**.[30]

The *o*-bromophenoxy moiety of the template compound (**8d**) was also substituted with the thiopyridone **11**,[30] giving rise to analogues **16E** and **16F** (Scheme 3). For the synthesis of these compounds, however, a new synthesis strategy had to be developed because the α-substituted acetic acid that resulted from the *S*-alkylation of **11** with α-bromoacetic acid (correspondent of **6**, structure not shown) could not be converted into the respective acyl chloride (the strategy exploited in Scheme 1). To circumvent this limitation, the active ester **13** was prepared through the straightforward acylation of *N*-hydroxysuccinimide with α-chloroacetyl chloride. Next, amines **7** and **14E** were both acylated with **13** to give the chlorinated intermediates **15E** and **15F**, which were then submitted to reaction in situ with **11**, leading to the target compounds **16E** and **16F**.

Scheme 3. Synthesis of Compounds 16E and 16F[a]



[a]Reagents and conditions: (a) Et_3N, DCM, 0 °C, 1 h; (b) Et_3N, DCM, 0 °C to rt, overnight.

The terminal aromatic amide function of bisamides of type **18** was assumed to replace the benzimidazole moiety of the template compound (**8d**) to achieve structural simplification. Two different strategies were followed to synthesize the key intermediate acids **17** (Scheme 4). According to the first strategy, the precursor acid **6j** was activated with PivCl, and the resulting mixed anhydride was treated with β-alanine ethyl ester. However, this reaction showed poor regioselectivity, and a considerable amount of the undesired pivalamide of β-alanine ethyl ester was coformed. Nevertheless, after saponification of this mixture of products, the undesired pivaloyl derivative was rinsed off with diethyl ether, leaving the pure acid **17j** behind. In the second, more effective strategy, acids **6g**, **6n**, and **6r** were first converted into the respective acyl chlorides and then esterified with *N*-hydroxysuccinimide. The active esters thus obtained were then converted to the corresponding acids **17g**, **17n**, and **17r** upon reaction with free β-alanine in a binary system composed of ethyl acetate and water under conditions of phase transfer catalysis. Afterward, acids **17g**, **17j**, and **17n** were activated with PivCl, and the resulting mixed anhydrides were allowed to react with the matching aromatic amine ("**G–L**") to give the respective bisamides **18**. The reaction of (less nucleophilic) aromatic amines showed satisfactory regioselectivity, and coformation of the undesired pivalamides was minimized.

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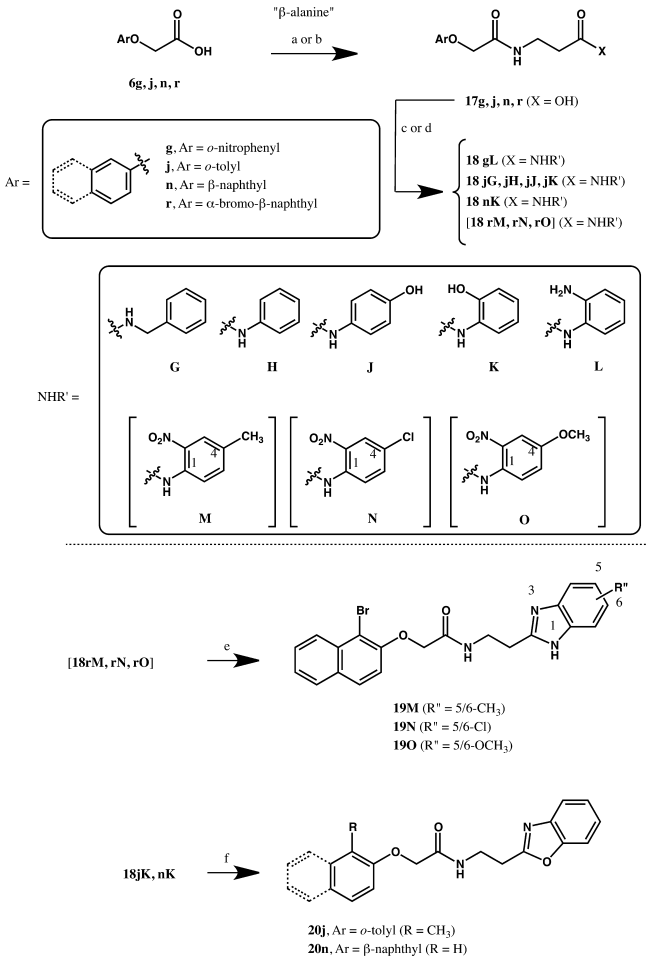
Scheme 4. Synthesis of Compounds 18−20[a]



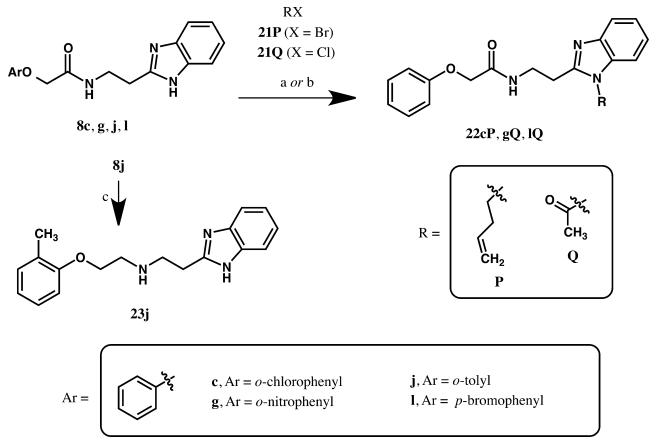
[a]Reagents and conditions: (a) for **17j**, PivCl, Et_3N, THF, 0 °C, 1 h, then *β*-alanine ethyl ester hydrochloride, Et_3N, THF, −78 °C, 2 h, then 2 M NaOH (aq), EtOH, rt, 1 h; (b) for **17g**, **17n**, and **17r**, oxalyl chloride, DMF (cat.), DCM, rt, 2 h, then *N*-hydroxysuccinimide, Et_3N, DCM, 0 °C, 2 h, then *β*-alanine, Na_2CO_3, $(n\text{-}Bu)_4NBr$ (cat.), ethyl acetate, H_2O, rt 0.5 h; (c) PivCl, Et_3N, DCM, 0 °C to rt, 2 h, then matching amine (**G**−**L**), Et_3N, THF, −78 °C, 2 h; (d) for **18rM**, **18rN**, and **18rO**, oxalyl chloride, DMF (cat.), DCM, rt, 2 h, then matching amine ("**M**−**O**"), Py, 0 °C, 0.5 h; (e) Fe powder, acetic acid, *n*-butanol, reflux, 2.5 h; (f) propionic acid, reflux, 4.5 h.

Conversely, the mixed anhydride method proved ineffective for preparation of bisamides **18rM**−**18rO**. Nonetheless, the low-nucleophilic nitroanilines "**M**−**O**" were satisfactorily acylated with the acyl chlorides generated from the acid **17r**. The bisamides **18rM**−**18rO** were not isolated but rather were directly converted into the corresponding *C*-substituted benzimidazo-1-yl derivatives **19M**−**O**. This transformation comprised the reduction of the nitro group with metallic iron in boiling acetic acid[31] and the concomitant cyclization of intermediate amines to the target compounds.[32] Following this one-pot strategy, *C*-substituted benzimidazoles **19M**−**19O** were isolated in good yield and excellent purity. However, it should be noted that the hydrogen atom of the NH-group may move between both nitrogen atoms within the benzimidazole moiety. As a result, compounds **19M**−**19O** present two tautomeric forms in which the 5- and 6-positions are scrambled.

The bisamides **18jJ** and **18nK**, both bearing an *o*-hydroxy group, are amenable to cyclize to the respective benzoxazoles **20j** and **20n** in refluxing propionic acid.[32]

Next, the effect of the *N*-substitution of the benzimidazole moiety on inhibitory activity was probed. Thus, the NH group of the benzimidazole system of a set of three selected compounds (**8c**, **8g**, and **8l**) was modified through *N*-alkylation or *N*-acylation, giving rise to compounds of type **22** (Scheme 5). Moreover, the general role of the amide-carbonyl of

Scheme 5. Synthesis of Compounds 22 and 23[a]



[a]Reagents and conditions: (a) for **22cP**, K_2CO_3, acetonitrile, 60 °C, 24 h; (b) for **22gQ** and **22lQ**, Py, 4-DMAP (cat.), acetyl chloride, DCM, 0 °C to rt, 4 h; (c) $LiAlH_4$, THF, rt, 3 days.

compounds of type **8** in the binding and inhibition of cruzain was also evaluated. For this purpose, compound **8j** was reduced to the corresponding amine **23j**.

Biological Activity and Molecular Modeling. The biochemical evaluation of the effects of this series of compounds against the enzyme cruzain allowed a comprehensive description of the SAR for this scaffold. Four rounds of synthesis allowed us to exploit the impact of changes in the phenyl ring, the modifications in the linker between the aromatic rings, and the replacement or modification of the benzimidazole moiety. Such understanding was aided by molecular modeling studies, which were conducted in parallel with the enzymatic assays.

We began our investigation using *α*-phenoxyacetic acid derivatives (Figure 2, Table 1). Modifications of the aromatic ring that occupies the S2 pocket of cruzain were tolerated. Replacement of the bromine atom by other halogens (**8b**, **8c**, and **8e**) or by a methyl (**8j**) resulted in a modest decrease in potency against the enzyme (2−6-fold) relative to the lead compound **8d**, which suggests that bromine has an optimal size for occupation of the S2 pocket. Additional modifications with the electron-withdrawing groups NO_2 (**8g**) and CN (**8f**) and with the electron-donor NH_2 (**8h**) in the ortho-position resulted in, respectively, 15-, 80-, and >100-fold decrease in potency. The analogue containing the acetylated amine (**8i**) substituent was even less potent. A shift of the Br to the meta-position (**8k**) resulted in an equally potent compound, whereas the modification to the para-position (**8l**) caused a 4-fold decrease in potency. Therefore, it is possible to change the position of the bromine atom while conserving activity against cruzain. However, its removal strongly affects the potency

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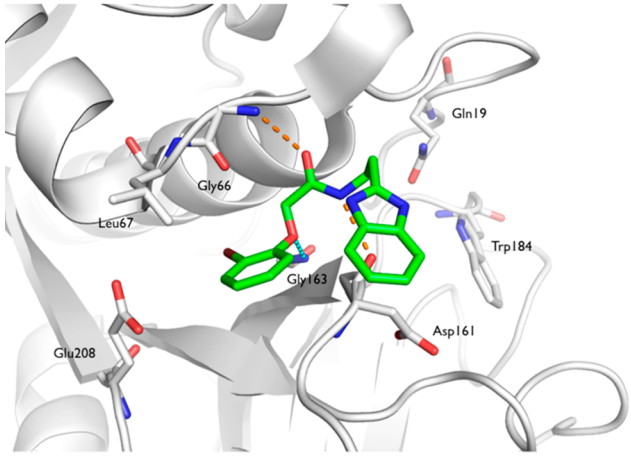


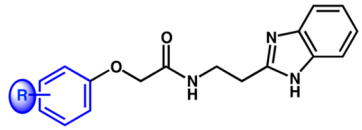
Figure 2. Crystallographic binding mode of the lead compound **5** (PDB ID 3KKU).[20] Hydrogen bonds are displayed as dashed orange lines, and the dipole−dipole interaction is displayed as a cyan dashed line. The residues involved in these interactions are highlighted. The figure was prepared using PyMOL.[33]

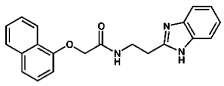
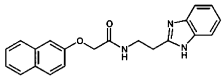
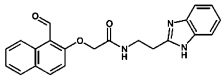
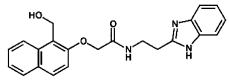
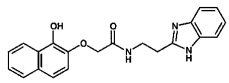
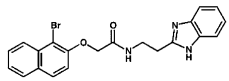
because the IC_{50} value of the unsubstituted analog (**8a**) was 14-fold lower than that of the lead compound (**8d**).

The α-bromo-β-naphthoxyacetic analog (**8r**), the most potent compound of this series, showed a 4-fold higher potency than the lead compound. Again, the importance of the bromine was demonstrated because the unsubstituted β-naphthoxy analog **8n** was 15-fold less potent than the brominated version (**8r**). Conversely, α-naphthoxy (**8m**) and β-naphthoxy (**8n**) analogues exhibited approximately the same potency. Further modifications of the naphthoxy moiety, such as the replacement of bromine with a carboxyaldehyde group (**8o**), a hydroxymethylene group (**8p**), or a hydroxyl group (**8q**), were tolerable, although the cruzain inhibitory activity decreased by at least 1 order of magnitude.

Subsequent investigations concentrated on evaluation of the impact of modifications in the linker between the phenyl (or naphthalene) ring and the benzimidazole moiety (Table 2). The importance of the ether functionality was demonstrated by comparing compound **5** with the ether derivative **8s**, in which the oxygen atom was replaced by a methylene group, and also with compound **8t**, in which the oxygen was replaced by a sulfur atom. These modifications resulted in 100- and 50-fold decreases in potency, respectively. Analysis of the crystallographic binding mode of the lead compound **5** revealed that the linker oxygen atom forms a dipole−dipole interaction with the nitrogen atom of the main chain of Gly163 (Figure 2). The docking results of the synthesized analogues showed that the sulfur atom of the thioether analogue (**8t**) could still interact with this residue, even though the interaction would be weaker because it involves a weaker hydrogen bond acceptor. Naturally, when the ether functionality is substituted by a methylene group (**8s**), such an interaction would not be possible. Elongation of the spacer between the ether and the amide functionalities by adding one methylene group led to compound **8v**, which exhibits no inhibitory activity. On the basis of the crystallographic binding mode of the lead compound **8d**, modifications at the amide functionality would have a significant impact on cruzain inhibitory activity. Indeed, the amine (**23j**) obtained through reduction of the parent amide (**8j**) showed no inhibitory activity against the enzyme.

Table 1. Structures and Activity Profiles of Compounds 8a−8q against *T. cruzi* Cruzain



Compd	R			% Cruzain inhibition (100 μM)[a]	IC_{50} (μM)[b]
	ortho	*meta*	*para*		
8a	H	-	-	88	10.9 ± 1
8b	F	-	-	92	5.3 ± 1.1
8c	Cl	-	-	92	3 ± 0.6
8d (lead)	Br	-	-	89	0.8 ± 0.1
8e	I	-	-	96	1.6 ± 0.2
8f	CN	-	-	81	65.1 ± 1.2
8g	NO_2	-	-	93	13.2 ± 1.2
8h	NH_2	-	-	43	ND
8i	$NHCOCH_3$	-	-	25	ND
8j	CH_3	-	-	88	4.1 ± 1.1
8k	-	Br	-	96	0.8 ± 0.5
8l	-	-	Br	96	2.7 ± 0.4
8m				84	5.2 ± 0.7
8n				90	3 ± 0.5
8o				80	13.5 ± 0.6
8p				80	12.7 ± 0.8
8q				51	8.2 ± 0.9
8r				92	0.21 ± 0.04

[a]The percent inhibition values of the compounds against *T. cruzi* cruzain are the average of at least three measurements, and the errors are within 10%. [b]The IC_{50} values of the compounds against *T. cruzi* cruzain were independently determined by obtaining rate measurements in triplicate for at least six inhibitor concentrations. The values represent the means of at least three individual experiments. ND = not determined.

This drastic change in potency may be explained by the loss of the hydrogen bond between the carbonyl oxygen and the nitrogen atom of the main chain of Gly66, an interaction commonly observed in cruzain-small molecule complex structures.[21] Another possible effect of removing this functional group is the higher flexibility of the compound, which could result in a considerable loss of entropy upon formation of the complex.

Considering that in the inhibitor−enzyme complex, the benzimidazole moiety is highly exposed to the solvent, we exploited alternatives to replace this bicyclic heterocycle (Table

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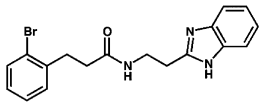
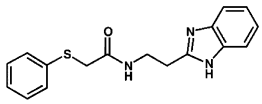
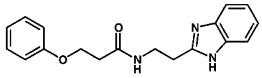
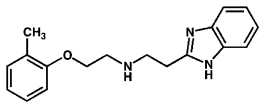
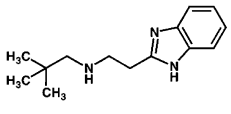
Table 2. Structures and Activity Profiles of Derivatives 8s, 8t, 8v, and 23j against *T. cruzi* Cruzain

Compd	Structure	% Cruzain inhibition (100 μM)[a]	IC_{50} (μM)[b]
8s		51	77.5 ± 1.4
8t		66	38.4 ± 1.2
8v		25	ND
23j	 (~90 mol%)  coproduct (~10 mol%)	8	ND

[a]The percent inhibition values of the compounds against *T. cruzi* cruzain are the average of at least three measurements, and the errors are within 10%. [b]The IC_{50} values of the compounds against *T. cruzi* cruzain were independently determined by obtaining rate measurements in triplicate for at least six inhibitor concentrations. The values represent the means of at least three individual experiments. ND = not determined.

3). Several modifications were performed, including replacement by a piperidin-1-yl ring (**10dB**, **10jB**, and **10sB**), 1*H*-indol-3-yl residue (**10jC** and **10rC**), 1*H*-imidazol-5-yl residue (**10cD** and **10nD**), a tetrasubstituted pyridine (**12**), and some simple acyclic bisamides (**18jG**, **18jH**, **18jJ**, **18jK**, **18gL**, and **18nJ**). In all cases, the activity against cruzain was lost, indicating that the benzimidazole system is essential for cruzain inhibition. To further evaluate the role of the benzimidazole system, benzoxazo-2-yl derivatives **20j** and **20n** were synthesized. It is worth noting that even this minor structural change (NH/oxygen exchange) caused a complete loss of inhibitory activity. The difference between the activity of benzimidazo-2-yl and benzoxazo-2-yl derivatives cannot be easily explained on the basis of the analysis of the crystal structure of the cruzain−inhibitor complex.

To investigate the tolerance of substituents in the benzimidazole ring, analogues carrying either *N*-alkylated (**22cP**) or *N*-acetylated (**22gQ** and **22lQ**) benzimidazole moieties were synthesized. These modifications gave rise to active compounds, although their potency was approximately 1 order of magnitude lower than that of the parent NH-free analogues (Table 4). Hence, the NH group of the benzimidazole moiety, which may act as a hydrogen donor, is not essential for inhibitory activity, even though the entire benzimidazole moiety seems to be crucial.

We next explored the influence of steric and/or electronic effects by synthesizing analogues carrying electron-donating or -withdrawing groups on the benzimidazole moiety (**19N**−**19O**). These three analogues showed a similar potency against cruzain when compared with **8r**, regardless of the electron-withdrawing (**19N**) or electron-donating (**19M** and **19O**) nature of the substituent.

The results of the SAR analysis are summarized in Figure 3. On the basis of these results, the design of additional analogues with potentially higher cruzain potency and affinity is currently underway. Along these lines, substituents should be linked to the benzimidazole moiety in a manner that allows them to occupy either the S1′ or the S3 pocket of the enzyme.

Given the competitive mode of inhibition of lead compound **8d**, we expected that the optimized compounds in this series would also be competitive inhibitors. Through the evaluation of the cruzain inhibition at several substrate and inhibitor concentrations, we confirmed that **8r**, one of the most potent compounds in the series, was a competitive inhibitor (K_i = 82 nM), as shown in the Lineweaver−Burk plot (Figure 4).

In Vitro Evaluation of Compound Activity against *T. cruzi*. Because some compounds in this series showed potent inhibitory activity against cruzain, their in vitro activity against *T. cruzi* was also evaluated. A set of 17 compounds was assayed using the standard drug benznidazole (**BZ**) as a positive control. Several compounds showed similar or higher trypanocidal activity than the standard drug. Among them, lead compound **8d** presented an IC_{50} value of 1.6 μM and was thus 3-fold more potent than **BZ**.

Although the phenyl ring of the inhibitor, which binds to the S2 pocket of the enzyme, is amenable to modifications that do not significantly affect the inhibitory activity against cruzain, several such modifications were highly detrimental to the trypanocidal activity. For instance, analogues containing halogens other than bromine in the ortho-position (**8b**, **8c**, and **8e**), those carrying the bromine in the meta-position (**8k**), or those containing the α-naphthyl ring (**8l**) showed no activity against the parasite. In contrast, replacing the bromine atom with a cyano (**8f**), nitro (**8g**), or a methyl group (**8j**) or shifting it to the para-position resulted in compounds with IC_{50} < 10 μM against *T. cruzi*. Among the synthesized analogues, the most potent inhibitor **8r** showed a 4-fold higher potency than lead compound **8d** against cruzain. However, **8r** was 10-fold less active in vitro against the parasite than compound **8d**. The *N*-acetylated compounds **22lQ** and **22gQ** also showed similar potencies in this assay. Surprisingly, compound **20j**, which showed only approximately 30% enzyme inhibition at 100 μM, was quite potent against *T. cruzi*, with an IC_{50} value of 2.9 μM.

Pre-Toxicity Assessment. The potency of this compound series against the enzyme and the parasite encouraged us to investigate its toxicity profile. Therefore, all compounds evaluated in vitro against *T. cruzi* were also submitted to acute toxicity assays. Each compound was evaluated in one female BALB/c mouse and administered by the intraperitoneal route (ip). During a 6-h time period, four doses were injected, resulting in a cumulative dose of 150 mg/kg (Table 6). The animals were then observed for 48 h. Mice treated with the compounds **8j**, **8t**, **8b**, **8f**, **8l**, **8k**, and **8e** presented some acute toxicity signs after the last dose (Table 7); however, after 24 h, these signs were no longer observed. The preliminary toxicity assays indicated that all compounds seem to be safe because no toxicity signs were observed. The only exception was compound **8e**, in which the mouse treated was dead after 48 h, suggesting that this compound may be toxic.

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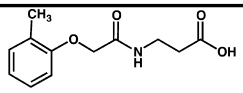
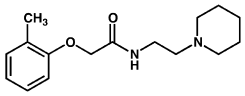
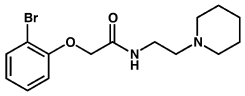
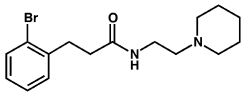
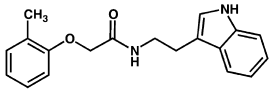
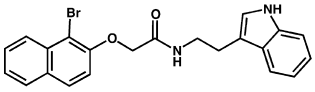
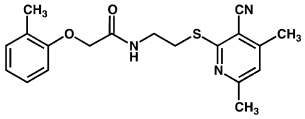
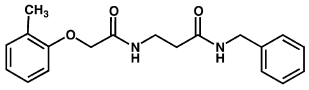
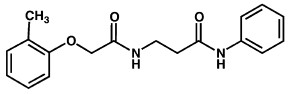
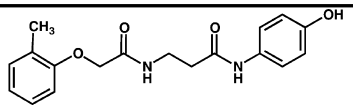
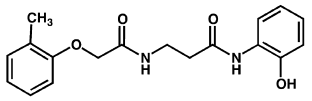
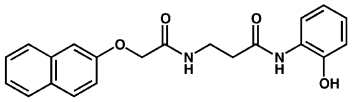
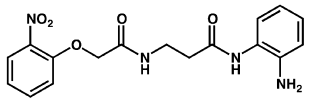
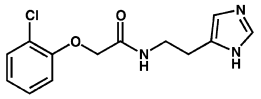
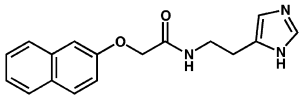
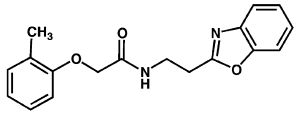
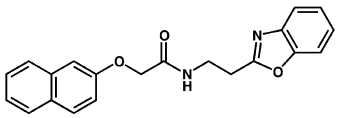
Table 3. Structures and *T. cruzi* Cruzain Inhibitory Activity Profiles of Derivatives with a Replaced Benzimidazole Moiety

Compd	Structure	% Cruzain inhibition (100 μM)[a]	IC_{50} (μM)[b]
17j		8	ND
10jB		3	ND
10dB		1	ND
10sB		7	ND
10jC		5	ND
10rC		2	ND
12		13	ND
18jG		8	ND
18jH		4	ND
18jJ		5	ND
18jK		2	ND
18nJ		14	ND
18gL		4	ND
10cD		14	ND
10nD		7	ND
20j		33	ND
20n		22	ND

[a]The percent inhibition values of the compounds against *T. cruzi* cruzain are the average of at least three measurements, and the errors are within 10%. [b]The IC_{50} values of the compounds against *T. cruzi* cruzain were independently determined by obtaining rate measurements in triplicate for at least six inhibitor concentrations. The values represent the means of at least three individual experiments. ND = not determined.

The assay performed in this study involved only one mouse per compound to minimize the number of animals tested. Therefore, the acute toxicity findings reported here do not have statistical value. However, they still serve as an important pilot study to encourage further investigation of this series of compounds, which could serve as an interesting platform for the generation of new antichagasic agents.

DISCUSSION

Through the combination of drug design, organic synthesis, molecular modeling, enzymatic and parasitic assays, and toxicity assessments, this paper contributes to the field of Chagas' disease research by validating a new class of cruzain inhibitors as promising leads for drug development.

The synthesis of over 40 analogues of the original lead **8r** allowed a comprehensive study of the SARs in this series of compounds. We were able to characterize and delineate the functional groups essential for improved potency and selectivity against cruzain. The SAR studies revealed several useful structural insights, which are consistent with the known experimental binding mode[20] and molecular modeling results, thus providing a detailed interpretation of the binding mode and mechanism of inhibition within this series of inhibitors. Accordingly, we have shown that the new analogues are competitive inhibitors, following the mechanism of the original lead compound.[20] Therefore, we provide important data to guide further SBDD efforts, which we are already undertaking or planning for subsequent rounds of investigation.

Another important result obtained here was the demonstration of biological activity for a class of competitive, reversible inhibitors of cruzain. Usually, compounds that are active against *T. cruzi* are irreversible inhibitors, as demonstrated in several studies.[34−36] Herein, we describe 12 compounds active against the *T. cruzi* parasite, eight of which had IC_{50} values lower than 10 μM. These inhibitors are candidates for preclinical studies in animal models of the disease.

Considering the high failure rates of compounds in clinical trials because of toxicity, it is important to consider this property. Herein, we show that only one of the compounds evaluated in mouse models was potentially toxic, and therefore, this series seems to have a promising safety profile. Because the studies conducted here are only pilot studies, further assays will be important to evaluate this property in more detail.

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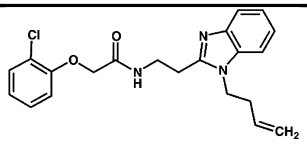
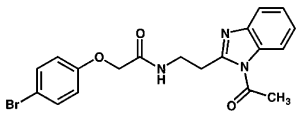
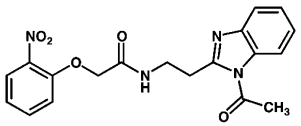
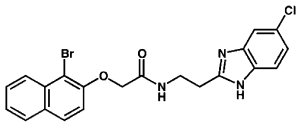
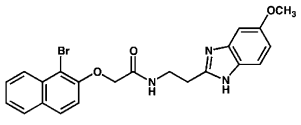
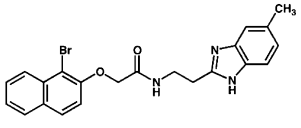
Table 4. Structures and Cruzain Inhibitory Profiles of Compounds Carrying *C*- or *N*-Substituted Benzimidazo-2-yl Residues

Compd	Structure	% Cruzain inhibition (100 μM)[a]	IC_{50} (μM)[b]
22cP		89	23.9 ± 1.2
22lQ		84	9.9 ± 0.9
22gQ		47	ND
19N		90	0.6 ± 0.2
19O		93	0.54 ± 0.2
19M		97	0.62 ± 0.32

[a]The percent inhibition values of the compounds against *T. cruzi* cruzain are the average of at least three measurements, and the errors are within 10%. [b]The IC_{50} values of the compounds against *T. cruzi* cruzain were independently determined by obtaining rate measurements in triplicate for at least six inhibitor concentrations. The values represent the means of at least three individual experiments. ND = not determined.

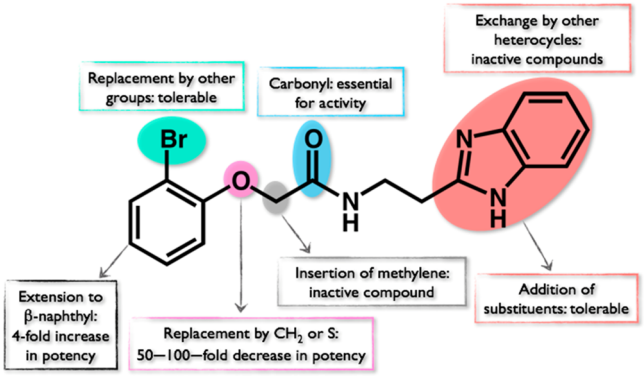


Figure 3. Summary of SAR investigations for optimization of the lead compound **5**.

Taking into account all experiments performed in this study, compounds **8d** (the original lead) and **8r** are particularly promising. **8d** had IC_{50} values of 0.8 μM against *T. cruzi* cruzain and 1.6 μM against the parasite, whereas **8r** was more potent against the enzyme (IC_{50} = 0.2 μM, K_i = 80 nM), and although less potent against *T. cruzi*, it was still active (IC_{50} = 16.2 μM).

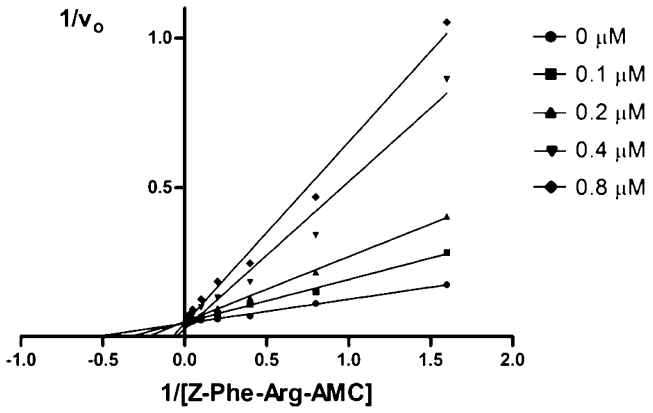


Figure 4. Lineweaver–Burk plot for the highly potent inhibitor **8r**.

Table 5. In Vitro Activity Profiles of the Most Potent Cruzain Inhibitors against *T. cruzi*

compd	IC_{50} (μM)[a]
8j	2.8 ± 0.7
8s	34.6 ± 2.5
8t	>100
8b	>100
20j	2.9 ± 0.7
8f	6.5 ± 2.1
8k	>100
22lQ	5.3 ± 2.1
8m	>100
8d	1.6 ± 0.6
8r	16.2 ± 3.5
8c	>100
22cP	11.1 ± 3.2
8g	7.9 ± 2.13
8l	6.7 ± 2.4
8e	>100
8n	46.1 ± 6.2
BZ	4.8 ± 0.5

[a]The IC_{50} values of the compounds against the *T. cruzi* Tulahuen strain in the in vitro assay represent the means of at least three individual experiments. BZ = benznidazole (*N*-benzyl-2-(2-nitro-1*H*-imidazol-1-yl)acetamide).

Table 6. Cumulative Doses of Compounds Evaluated against Mice

time point (h)	dose injected (mg/kg)	cumulative dose (mg/kg)
0	20	20
2	30	50
4	50	100
6	50	150

The analogue **8r** was also highly efficient (LE = 0.4 kcal mol^{-1}/non-H atom), as an LE = 0.30 mol^{-1}/non-H atom is necessary to reach low nanomolar affinities with druglike compounds.[37] Additionally, the physicochemical properties of both compounds are within the Lipinski rule of 5,[38] and the mouse assay for acute toxicity indicates that they are safe.

CONCLUSION

In summary, a new series of benzimidazole derivatives that can act as trypanocidal cruzain inhibitors was discovered through structural optimization and SAR analysis, and the series shows

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Table 7. Acute Toxicity of Selected Compounds[a]

time (h)	8j	8d	8s	8r	8t	8c	8b	22cP	20j	8g	8f	8l	8k	8e	22lQ	8n	8m
0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	C	+	+	+	+	+	+	+	+	+	C	T	C	C	+	+	+
24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
48	+	+	+	+	+	+	+	+	+	+	+	+	+	D	+	+	+

[a]In vivo assays with BALB/c mice. Key: +, without acute toxicity signs; C, chill; T, thirst; D, death.

promising inhibitory potency and in vitro activity against the parasite. These cruzain inhibitors interact noncovalently with the enzyme, revealing an interesting profile for further lead optimization and new chemical entity (NCE) discovery. Several compounds with IC_{50} values in the nanomolar or low micromolar range against the enzyme, and in several cases with activity against *T. cruzi* at low micromolar concentrations, were described. The most potent inhibitor, **8r**, showed excellent inhibitory activity (IC_{50} = 200 nM, K_i = 82 nM) and considerable in vitro potency against *T. cruzi*. Furthermore, the most promising inhibitors showed a good safety profile in pre-toxicity assays in mice. Together with the SAR description and the structural information available for lead compound **8d**, these results should foster further development of this series as leads for the treatment of Chagas' disease. Structural studies to characterize new *T. cruzi* cruzain−inhibitor complexes and efforts to improve the pharmacodynamic and pharmacokinetic properties are under way.

EXPERIMENTAL SECTION

Chemistry: Materials and Methods. Dichloromethane (DCM), triethylamine (Et_3N), and pyridine (Py) were distilled from CaH_2. Tetrahydrofuran (THF) and diethyl ether were distilled from sodium/benzophenone. Flash column chromatography was conducted using silica gel (230−400 mesh). Analytical thin-layer chromatography was performed on silica gel 60 and GF (5−40-μm thickness) plates, and the visualization was accomplished using UV light and phosphomolybdic acid followed by heating. ^{1}H and proton-decoupled ^{13}C NMR spectra were recorded in DMSO-d_6, $CDCl_3$, or CD_3OD at 250 MHz (^{1}H) and 60 MHz (^{13}C), 400 MHz (^{1}H) and 100 MHz (^{13}C), 500 MHz (^{1}H) and 125 MHz (^{13}C), or 600 MHz (^{1}H) and 150 MHz (^{13}C). The chemical shifts (δ) are reported in ppm using tetramethylsilane (TMS) at 0.00 ppm or the solvent signal as an internal standard ($CDCl_3$ at 7.26 ppm, CD_3OD at 3.30 ppm, and DMSO-d_6 at 2.49 ppm for ^{1}H NMR spectra and $CDCl_3$ at 77.0 ppm, CD_3OD at 49.0 ppm, and DMSO-d_6 at for ^{13}C NMR spectra). Data are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, bs = broad singlet, dd = doublet of doublets, ddd = doublet of doublet of doublets, m = multiplet; coupling constant(s) in Hz; integration. High-resolution mass spectrometry (HRMS) was performed using the electrospray ionization (ESI) technique. The parent ions ($[M + H]^+$ or $[M + Na]^+$) are cited. The purity (≥95%) of all compounds evaluated in this work was determined by ^{1}H NMR.

Note that the NH-free benzimidazo-2-yl system of compounds of type **8** presents two possible tautomeric forms (i.e., the hydrogen atom may shift between both nitrogen atoms). As a result, some carbon atoms of this moiety usually give broad, low-intensity ^{13}C NMR signal or no ^{13}C NMR signal at all when spectra are recorded in DMSO-d_6. Most likely, the rate of tautomer interconversion is influenced by the water content of DMSO-d_6. Upon derivatization of the free NH-group of the benzimidazo-2-yl moiety (e.g., **22cP**, **22lQ**, and **22gQ**), the whole set of ^{13}C NMR signals becomes apparent. In contrast, when the ^{13}C NMR spectrum is recorded in a protic solvent (CD_3OD, e.g., compound **8v**) the NH-free benzimidazo-2-yl system becomes "symmetric" and is reduced to only four detectable ^{13}C NMR signals.

Synthesis Chemistry. General Procedures for Preparation of Compounds of Type 8. *Method A.* The matching 2-phenoxyacetic acid (**6**, 15 mmol) was dissolved in absolute DCM (50 mL) containing dry Et_3N (22.5 mmol) at 0 °C under an Ar atmosphere. Neat PivCl (15 mmol) was added dropwise within 15 min, and a white precipitate formed and was separated. The slurry was stirred for 20 min, the ice bath was removed, and then stirring was continued for 1 h. The mixture containing the crude mixed anhydride was stored at 0 °C. In a separate reaction flask, commercial 2-(2-aminoethyl)benzimidazole dihydrochloride (**7**, 0.20 g, 0.85 mmol) was suspended in absolute THF under an Ar atmosphere. Dry Et_3N (3.06 mmol) was added, and the mixture stirred until complete dissolution of 2-dihydrochloride occurred (eventually, gentle warming was required). The THF solution was cooled down to −78 °C, and the cold DCM solution containing the crude mixed anhydride (3 mL) was added portionwise within 15 min. Stirring was continued for 2 h at −78 °C. The reaction mixture was allowed to warm up to room temperature, and the solvent blend was evaporated under reduced pressure. The evaporation residue was portioned between ethyl acetate and water. The organic layer was washed with water (three times), diluted $NaHCO_3$ solution (two times), and brine; dried over Na_2SO_4; and concentrated to dryness in vacuum. The crude solid product was suspended in diethyl ether, and the resulting slurry was stirred for 1 h at room temperature. The precipitate was filtered off, rinsed with diethyl ether, collected, and dried in vacuum.

Method B. The matching 2-phenoxyacetic acid (1.5 mmol) was suspended in absolute DCM (15 mL) at room temperature. A catalytic amount of *N*,*N*-dimethylformamide (DMF, 0.10 mL) was added, and then neat oxalyl chloride (0.63 g, 5 mmol) was combined portionwise within 10 min. Stirring was continued until gas evolution ceased. The clear solution that resulted was cooled down to 0 °C and combined with solid *N*-hydroxysuccinimide (0.69 g, 6 mmol), and then neat dry Et_3N (1.52 g, 15 mmol) was added portionwise within 10 min. After 15 min, the mixture was removed from the ice bath, and stirring was continued for 2 h. The solvent was evaporated in vacuum, and the solid evaporation residue was partitioned between ethyl acetate and ice-cold water. The organic layer was washed with cold diluted $NaHCO_3$ solution and brine, dried over Na_2SO_4, and concentrated in vacuum. The crude *N*-hydroxysuccinimide ester thus obtained was suspended in diethyl ether (3 mL), and the mixture was stirred at room temperature (15 min). Hexane (3 mL) was added, and the mixture was kept in a freezer overnight. The solid was filtered off, rinsed with cold diethyl ether, collected, dried in vacuum, and stored. In a separate reaction flask, commercial 2-(2-aminoethyl)-benzimidazole dihydrochloride (**7**, 0.25 g, 1.07 mmol) was suspended in ordinary DCM (30 mL). Neat, ordinary Et_3N (0.36 g, 3.52 mmol) was added, and the matching solid *N*-hydroxysuccinimide ester was added to the clear solution that resulted. The mixture was stirred at room temperature for 15 min. The solvent was evaporated in vacuum, and the evaporation residue was portioned between ethyl acetate and water. The organic layer was washed with diluted NaOH solution and brine, dried over Na_2SO_4, and concentrated to dryness in vacuum. The crude solid product was suspended in diethyl ether, and the resulting slurry was stirred for 1 h at room temperature. The precipitate was filtered off, rinsed with diethyl ether, collected, and dried in vacuum. Unless otherwise stated, no further purification was required.

General Procedures for Preparation of Compounds of Type 10. *Method A.* The matching 2-phenoxyacetic acid (**6**, 15 mmol) was

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dissolved in absolute DCM (50 mL) containing dry Et_3N (22.5 mmol) at 0 °C under an Ar atmosphere. Neat PivCl (15 mmol) was added dropwise over 15 min, whereupon a white precipitate separated. The slurry was stirred for 20 min, the ice bath was removed, and stirring was continued for 1 h. The mixture containing the crude mixed anhydride was stored at 0 °C. In a separate reaction flask, the matching amine of type **9** (2 mmol) was dissolved in absolute THF (10 mL). Neat, dry Et_3N (0.20 g, 2 mmol) was added. The mixture was cooled down to −78 °C, and then the cold DCM solution containing the crude mixed anhydride (12 mL, ca. 3.6 mmol) was added portionwise within 10 min. The mixture was stirred for 2 h. The reaction mixture was concentrated in vacuum, and the evaporation residue was portioned between ethyl acetate and water. The organic layer was washed with water (once), saturated $NaHCO_3$ solution (twice), and brine; dried over Na_2SO_4; and concentrated to dryness in vacuum to give the crude products.

Method B. The matching 2-phenoxyacetic acid (**6**, 5 mmol) was suspended in absolute DCM (15 mL) at room temperature. A catalytic amount of DMF (0.10 mL) was added, and then neat oxalyl chloride (0.63 g, 5 mmol) was combined portionwise within 10 min. Stirring continued until gas evolution ceased. The clear solution that resulted was cooled down to 0 °C, solid *N*-hydroxysuccinimide (0.69 g, 6 mmol) was combined, and then neat dry Et_3N (1.52 g, 15 mmol) was added portionwise within 10 min. After 15 min, the mixture was removed from the ice bath, and stirring was continued for 2 h. The solvent was evaporated in vacuum, and the solid evaporation residue was partitioned between ethyl acetate and ice-cold water. The organic layer was washed with cold diluted $NaHCO_3$ solution and brine, dried over Na_2SO_4, and concentrated in vacuum. The crude *N*-hydroxysuccinimide ester thus obtained was suspended in diethyl ether (3 mL), and the mixture was stirred at room temperature (15 min). Hexane (3 mL) was added, and the mixture was kept in a freezer overnight. The solid was filtered off, rinsed with cold diethyl ether, collected, dried in vacuum, and stored. In a separate reaction flask, commercial 2-(1*H*-imidazol-4-yl)ethanamine dihydrochloride (**9D**, 0.195 g, 1.06 mmol) was suspended in ordinary ethyl acetate (40 mL). Neat, ordinary Et_3N (0.21 g, 2.12 mmol) was added, and the mixture was stirred for 30 min at room temperature. The matching solid *N*-hydroxysuccinimide ester was added, and the mixture was stirred at room temperature for 15 min. The reaction solution was washed with water and then extracted with 2 M HCl solution. The organic phase was discarded. The aqueous phase was alkalinized using 2 M NaOH solution and extracted with ethyl acetate (three times). The combined organic phases were washed with brine, dried over Na_2SO_4, and concentrated to dryness in vacuum. The products were obtained in pure form, and no purification was required.

General Procedures for Preparation of Compounds 16E and 16F. *2,5-Dioxopyrrolidin-1-yl 2-chloroacetate (**13**).* *N*-Hydroxysuccinimide (1.15 g, 10 mmol) was dissolved in a mixture of absolute DCM (20 mL) and dry Et_3N (1.39 mL, 10 mmol) at 0 °C. Neat 2-chloroacetyl chloride was combined portionwise within 5 min, whereupon a white precipitate separated. The mixture was stirred at 0 °C for 1 h. The ice bath was removed, and stirring was continued for 2 h. The reaction mixture was diluted with DCM; washed with ice-cold water (once), diluted $NaHCO_3$ solution (twice), and brine; dried over Na_2SO_4; and concentrated in vacuum. The crude product showed excellent purity, and no purification was performed: 1.20 g (63%), pale solid; ^{1}H NMR (250 MHz, $CDCl_3$) δ 4.35 (s, 2H), 2.84 (s, 4H); ^{13}C NMR (60 MHz, $CDCl_3$) δ 168.42, 163.19, 37.79, 25.50.

Typical Procedures for Preparation of Compounds of Type 17. *Preparation of N-Hydroxysuccinimide Esters (2,5-Dioxopyrrolidin-1-yl 2-(2-Nitrophenoxy)acetate).* 2-(*o*-Nitrophenoxy)acetic acid (1 g, 15 mmol) was suspended in absolute DCM (30 mL) at room temperature. A catalytic amount of DMF (0.20 mL) was added, and then neat oxalyl chloride (2.28 g, 18 mmol) was combined portionwise within 10 min. Stirring was continued until gas evolution ceased. The clear solution that resulted was cooled to 0 °C, solid *N*-hydroxysuccinimide (2.10 g, 18 mmol) was combined, and then neat dry Et_3N (2.27 g, 22.5 mmol) was added portionwise within 10 min; stirring was continued for 1 h at 0 °C. The reaction mixture was diluted with ordinary DCM (50 mL), acidified with a few drops of acetic acid, washed with ice-cold water (once) and then washed with ice-cold diluted Na_2CO_3 solution (three times) and brine, dried over Na_2SO_4, and concentrated to dryness in vacuum. The crude *N*-hydroxysuccinimide ester thus obtained was suspended in diethyl ether (10 mL), and DCM (1−2 mL) was added. The slurry was vigorously stirred at room temperature for 1 h and then kept in a freezer overnight. The solid material was filtered off, rinsed with diethyl ether, and dried in vacuum: 3.97 g (90% yield), pale solid; ^{1}H NMR (250 MHz, DMSO-d_6) δ 7.90 (m, 1H), 7.67 (m, 1H), 7.37 (m, 1H), 7.20 (m, 1H), 5.24 (s, 2H), 2.82 (s, 4H); ^{13}C NMR (60 MHz, DMSO-d_6) δ 169.85, 164.79, 149.49, 139.90, 134.23, 125.10, 122.13, 115.31, 63.86, 25.48.

General Procedures for Preparation of Bisamides 18. The matching substituted propanoic acid of type **17** (1.0 equiv) was dissolved in absolute DCM (50 mL) containing dry Et_3N (1.0 equiv) at 0 °C under an Ar atmosphere. Neat PivCl (1.0 equiv) was added dropwise within 15 min, whereupon a white precipitate separated. The slurry was stirred for 20 min, and then the ice bath was removed, and stirring was continued for 1 h. The reaction mixture containing the mixed anhydride was kept at 0 °C. In a separate reaction flask, the matching aromatic amines (1.2 equiv of **G**, **H**, or **L** and 2.4 equiv of **J** or **K**) were dissolved in absolute THF under an Ar atmosphere. Dry Et_3N (1.0 equiv) was added, and the solution was cooled to −78 °C. The cold DCM solution containing the preformed mixed anhydride was carefully transferred to the reaction mixture by means of a syringe, and stirring at −78 °C was continued for 2 h. The reaction mixture was concentrated under reduced pressure. The evaporation residue was portioned between ethyl acetate and water. The organic layer was washed with water (three times), diluted $NaHCO_3$ solution (two times), and brine; dried over Na_2SO_4; and concentrated to dryness in vacuum to produce the crude products.

Typical Procedure for Preparation of Compounds 19M−19O. *2-(1-Bromonaphthalen-2-yloxy)-N-(2-(5-chloro-1H-benzo[d]-imidazol-2-yl)ethyl)acetamide (**19N**).* The substituted propionic acid **17r** (1. 0 g, 2.85 mmol) was suspended in absolute DCM (30 mL) at room temperature. A catalytic amount of DMF (0.10 mL) was added, and then neat oxalyl chloride (0.54 g, 4.27 mmol) was combined portionwise within 10 min. Stirring was continued until gas evolution ceased (ca. 1 h). The clear solution that resulted was cooled to 0 °C for addition of solid 4-chloro-2-nitroaniline (**N**, 0.49 g, 2.85 mmol). Neat, dry Py (0.69 mL, 8.55 mmol) was then combined portionwise within 10 min. Stirring was continued at 0 °C for 30 min, acetic acid (2 mL) was added in one shot, and the DCM was evaporated in vacuum. The wet evaporation residue was suspended in *n*-butanol (20 mL). Iron powder (0.48 g, 8.85 mmol) was added, and the slurry was heated to reflux temperature for 3 h. The oil bath was removed, and after some time, ethyl acetate (150 mL) was carefully added to the still hot (ca. 80−90 °C) reaction mixture under vigorous stirring. The resulting dark brown slurry was allowed to cool to room temperature, and the solid material was filtered out (suction through a paper filter). The limpid filtrate was washed with water (three times), diluted aq sodium carbonate solution (three times), and brine. The organic phase was vigorously stirred in the presence of anhydrous Na_2SO_4 for a few minutes, and then silica gel (ca. 1 g) was added, and stirring continued for a few minutes. The solid material was filtered off, and the solution was concentrated in vacuum. Finally, the bulk part of residual *n*-butanol was coevaporated with ethanol. The wet crude product was suspended in a few milliliters of ethanol, and the slurry was kept in a freezer overnight. The precipitate was filtered off, rinsed with cold ethanol, and dried in vacuum: 735 mg (57% overall yield, based on acid **17r**), pale, amorphous solid, excellent purity according to ^{1}H NMR; ^{1}H NMR (250 MHz, DMSO-d_6) δ 12.10 (bs, 1H), 8.20 (t, 1H, $J \sim 5$ Hz), 8.05 (d, 1H, $J = 8.5$ Hz), 7.87 (m, 2H), 7.69−7.37 (m, 4H), 7.36−7.26 (m, 1H), 7.20−7.07 (m, 1H), 4.72 (s, 2H), 3.68 (q, 2H, $J \sim 6$ Hz), 3.04 (t, 2H, $J = 6.5$ Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.29, 154.60, 154.15, 152.26, 144.22, 142.07, 135.06, 133.11, 132.10, 129.63, 129.19, 128.27, 128.16, 126.14, 126.00, 125.34, 125.26, 124.66, 121.65, 121.21, 119.38, 117.66, 115.30, 112.15, 110.71, 107.69, 68.30,

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36.51, 28.59; HRMS (ES+) m/z ([M + H]$^+$) calcd for $C_{21}H_{18}BrClN_3O_2$ 458.0271, found 458.0247.

Typical Procedure for Preparation of Compounds 20j and 20n. *N-(2-(Benzo[d]oxazol-2-yl)ethyl)-2-(o-tolyloxy)acetamide (20j).* The bisamide **18jK** (0.25 g, 0.76 mmol) was suspended in propionic acid (3.0 mL), and the slurry was refluxed for 6 h. The propionic acid was evaporated in vacuum (70 °C, 45 mbar). The wet evaporation residue was dissolved in diethyl ether, and the ethereal solution was washed with diluted $NaHCO_3$ solution (three times) and brine. The ethereal solution was vigorously stirred in the presence of anhydrous Na_2SO_4 for a few minutes, and then silica gel (ca. 1 g) was added, and stirring continued for a few minutes. The solid material was filtered off, and the solution was concentrated in vacuum. The liquid crude product was dissolved in a minimal amount of DCM and precipitated with hexane. The solvent blend was evaporated to dryness in vacuum, and the tacky, solid evaporation residue was suspended and stirred in a blend of diethyl ether and hexane (1:1 v/v). The slurry was kept in a freezer overnight, and the solid material was filtered off, rinsed with fresh cold solvent blend, and dried in vacuum: 150 mg (64% yield), pale solid; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.77−7.56 (m, 2H), 7.52−7.40 (m, 1H), 7.37−7.26 (m, 2H), 7.16−7.01 (m, 2H), 6.90−6.78 (m, 1H), 6.71 (d, 1H, J = 8.0 Hz), 4.48, (s, 2H), 3.93 (q, 2H, J = 6.0 Hz), 3.16 (t, 2H, J = 6.0 Hz), 2.24 (s, 3H); ^{13}C NMR (60 MHz, $CDCl_3$) δ 168.40, 164.88, 155.16, 150.73, 140.83, 130.89, 126.96, 126.41, 124.84, 124.31, 121.53, 119.52, 110.91, 110.42, 67.03, 35.04, 28.60, 16.35; HRMS (ES+) m/z ([M + H]$^+$) calcd for $C_{18}H_{19}N_2O_3$ 311.1396, found 311.1370.

Procedure for Preparation of Compounds of Type 22. *N-(2-(1-(But-3-enyl)-1H-benzo[d]imidazol-2-yl)ethyl)-2-(2-chlorophenoxy)acetamide (22cP).* Compound **8c** (0.10 g, 0.30 mmol) and potassium carbonate (0.42 g, 3.0 mmol) were suspended in acetonitrile (10 mL). The mixture was warmed to 60 °C, and 4-bromobut-1-ene (**21P**, 0.04 g, 0.30 mmol) was added. The reaction progress was monitored by TLC ($CHCl_3$/methanol 20:3) every 2 h, and extra **21P** (a huge excess of the alkylating agent was required) was added until **8c** was completely consumed. The acetonitrile was evaporated under reduced pressure. The evaporation residue was portioned between diethyl ether and water. The organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated to dryness in vacuum. The tacky crude product was rinsed (three times) with a blend of diethyl ether and hexane (1:1 v/v): 110 mg (95% yield), colorless, crystalline solid; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.04 (t, 1H, $J \sim$ 5.5 Hz), 7.76−7.66 (m, 1H), 7.35−7.19 (m, 4H), 7.13 (dt, 1H, J = 1.6, 7.9 Hz), 6.91−6.76 (m, 2H), 5.85−5.64 (m, 1H), 5.10−4.96 (m, 2H), 4.51 (s, 2H), 4.15 (t, 2H, J = 7.2 Hz), 4.02 (q, 2H, J = 6.1 Hz), 3.08 (t, 2H, J = 6.1 Hz), 2.52 (q, 2H, J = 7.2 Hz); ^{13}C NMR (60 MHz, $CDCl_3$) δ 167.67, 152.69, 152.12, 142.24, 134.74, 133.39, 130.31, 127.69, 122.99, 122.44, 122.34, 122.03, 119.34, 118.46, 113.28, 109.29, 67.73, 43.07, 35.75, 33.78, 27.25; HRMS (ES+) m/z ([M + H]$^+$) calcd for $C_{21}H_{23}N_3O_2Cl$ 384.1479, found 384.1416.

Procruzain Expression and Purification. Procruzain truncated at the C-terminal was expressed and purified using a modified version of a previously published protocol.[39] Overnight cultures of *Escherichia coli* (SG13009) were diluted 10-fold into fresh LB medium containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL) and cultured for 1 h at 37 °C. Cruzain expression was then induced with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside), and the cultures were grown with agitation at 30 °C for 4 h. The cells were harvested by centrifugation and resuspended in 100 mL of lysis buffer (100 mM sodium phosphate, 10 mM Tris-HCl, 8 M urea ultrapure, pH 8) per liter of culture. After 1 h of incubation at 4 °C, the solution was centrifuged, and the supernatant was collected. The soluble fraction was purified in a Ni−NTA column (Qiagen) by employing a wash buffer (100 mM sodium phosphate, 10 mM Tris-HCl, 8 M urea ultrapure, pH 6.3) and eluted with the same buffer plus 200 mM imidazole. The fractions obtained were analyzed in an SDS−PAGE gel to determine which fractions would be pooled. The purified protein at 1−2 mg/mL was incubated in 10 mM DTT at 37 °C for 45 min and immediately diluted 100-fold in ice-cold renaturation buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8, 250 mM L-arginine, 20% glycerol), in which it was stored at 4 °C for 20 h. Refolded cruzain was then concentrated to 0.5 mg/mL for activation.

Procruzain Activation and Purification of Mature Cruzain. The enzyme was activated following a previously published protocol.[18] Briefly, a 0.5 mg/mL solution of procruzain (in 100 mM sodium acetate, pH 5.5, 10 mM EDTA, 5 mM DTT, and 1 M NaCl, pH adjusted to 5.3) was activated at 37 °C. During activation, cruzain activity was measured at 30 min intervals on the basis of the assay described below. When the enzyme activity was observed to be stable over time, and the activity stopped increasing (usually after approximately 3 h), the solution was transferred to an ice bath. After activation, the mature enzyme was diluted 20-fold in binding buffer (20 mM sodium phosphate, pH 7.2, 150 mM NaCl) to reduce the DTT concentration and allow binding to a thiopropyl Sepharose 6B gel (Pharmacia), as previously reported.[3] After overnight incubation at 4 °C, the gel incubated with cruzain was transferred to a column, and cruzain was eluted with binding buffer plus 20 mM DTT. Fractions containing cruzain, as confirmed by an SDS−PAGE gel, were pooled. The buffer was exchanged with 0.1 M sodium acetate, pH 5.5, and the solution was stored at −80 °C.

Cruzain Inhibition Assays. Cruzain activity was measured by monitoring the cleavage of the fluorogenic substrate Z-Phe-Arg-aminomethylcoumarin (Z-FR-AMC). All cruzain assays were performed in 0.1 M sodium acetate, pH 5.5, and in the presence of 5 mM dithiothreitol (DTT) and 0.01% Triton X-100.[4] The final concentration of cruzain was 1.5 nM, and the substrate concentration was 5.0 μM (K_m = 1.6 μM), except for K_i determination, for which several substrate concentrations were employed. The assays were monitored for 5 min, and the activity was calculated on the basis of initial rates compared with a DMSO control. All assays were performed in triplicate. Values of IC_{50} were independently determined by taking rate measurements for at least six inhibitor concentrations, each one evaluated in triplicate, and the reported error refers to the fitting error of the curve. To determine the mechanism of cruzain inhibition for compound **8r**, eight Z-FR-AMC substrate concentrations and four inhibitor concentrations (one corresponding to the IC_{50} value and the others equivalent to half, twice, and 4-fold IC_{50}) were used in the assays, each one in triplicate. Kinetic parameters were determined from the collected data employing the SigmaPlot enzyme kinetics module. The values represent the means of at least three individual experiments.

Molecular Docking. Mol2 files of the docked compounds were prepared from the corresponding SMILES by upload in the ZINC Web site.[40] The molecules were then docked with the program GOLD 3.1[41,42] (Cambridge Crystallographic Data Centre, Cambridge, UK) against the cruzain structure (PDB ID 3KKU).[20] Receptor preparation consisted of removing all crystallographic waters and adding hydrogens with Sybyl. The active site residues Cys25 and His162 were treated as an ionic pair, with the Cys25 negatively charged and His162 protonated. Compounds were docked by applying default GOLD parameters, except that the efficiency was changed to 200% and scaffold constraints were added to favor superposition to the crystallographic atomic coordinates of a substructure of the lead compound. The generated poses were evaluated using the Goldscore scoring function.

Parasites and Culture Procedures. Assays were performed using *T. cruzi* strain Tulahuen parasites genetically engineered to express the *E. coli* β-galactosidase gene, *lacZ*,[43] which catalyzes a colorimetric reaction with chlorophenol red β-D-galactopyranoside (CPRG, Sigma Chemical Co., St. Louis, MO) as the substrate. The Tulahuen strain of *T. cruzi* was kindly provided by Frederick S. Buckner (University of Washington, Seattle, WA). Trypomastigotes were grown on monolayers of human fibroblasts, and epimastigotes were grown in liver infusion tryptone (LIT) with 10% fetal calf serum, penicillin, and streptomycin. Cultures assayed for β-galactosidase activity were grown in RPMI 1640 medium without phenol red plus 10% fetal calf serum, penicillin, and streptomycin.

Assay against the Intracellular Amastigote form of *T. cruzi* Tulahuen *lacZ*. Drug screening against β-galactosidase-expressing strains of *T. cruzi* was performed as described previously.[43] The assays

were performed in 96-well tissue culture plates (Becton Dickinson). Human fibroblasts were seeded at 2×10^3 per well in 80 μL volumes (RPMI 1640 without phenol red) and incubated overnight. The next day, β-galactosidase-expressing trypomastigotes (Tulahuen strain) were added at 1.0×10^4 per well in 20 μL volumes (RPMI 1640 without phenol red). After 24 h, the synthesized compounds were added in serial dilutions in 50 μL volumes covering a range from 300 to 0.4 μM. Each dilution was tested in triplicate. Previously, the stock solutions were prepared in DMSO and diluted in RPMI 1640 without phenol red. After 72 h of incubation, the plates were inspected under an inverted microscope to ensure growth of the controls and sterility. Then, 50 μL of the substrate containing CPRG and Nonidet P-40 (0.1% final concentration) was added to all wells. In wells with β-galactosidase activity, the media changed color from yellow to red, and the color change was measured at 570 nm in an automated microplate reader. Data were transferred into Sigma Plot to determine IC_{50} values. The drug benznidazole (**BZ**; Sigma Chemical Co., St. Louis, MO) was used as a positive standard in this assay, and untreated parasite cultures were used as negative controls.

Pre-Toxicity Assays. For in vivo assays, BALB/c mice (18−20 g) were obtained from the Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo and maintained under standard conditions in the animal house of the CBEM-USP. The procedures performed in this study were approved by the Ethics Committee on Animal Research of The Federal University of São Carlos, which are in agreement with the International Ethics Committee on Animal Research. In addition, the 3R principles (replacement, reduction, refinement) were followed, which propose alternative methods to the use of animals and the refinement of techniques aiming to diminish, whenever possible, the suffering and the number of animals used for scientific study. The synthesized compounds that showed in vitro activity against *T. cruzi* were submitted for acute toxicity tests. This assay was designed following the Guidelines for Testing Chemicals with some modifications. In the assay, 6 mg of each of the compounds (**8j**, **8d**, **8s**, **8t**, **8c**, **8b**, **8g**, **8l**, **8k**, and **8m**) was suspended in DMSO (30%) plus Tween 80 (10%), and compounds **8r**, **22cP**, **20j**, **8f**, **8e**, **22lQ**, and **8n** were suspended in DMSO (20%) plus Tween 80 (10%). The stock solutions were diluted to a final concentration of 5 mg/mL with sterile distilled water. Next, one female BALB/c mouse weighing 18−20 g was used for cumulative application of each test compound via the ip route. Six doses were injected during a period of 6 h, resulting in a final dose of 150 mg/kg. After the administration, the mice were observed for 48 h for signs of acute toxicity or death and then were euthanized.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full description of the synthesis procedures and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

SBDD, structure-based drug design; SAR, structure−activity relationship; QSAR, quantitative structure−activity relationship; HTS, high-throughput screening; VS, virtual screening; PDB, protein data bank; PivCl, pivaloyl chloride; Et_3N, triethylamine; DCM, dichloromethane; THF, tetrahydrofuran; DMF, *N*,*N*-dimethylformamide; Ac_2O, acetic anhydride; Pd/C, palladium on carbon; HCl, hydrochloric acid; EtOH, ethanol; Py, pyridine; $NaBH_4$, sodium borohydride; MCPBA, *m*-chloroperoxybenzoic acid; NaOH, sodium hydroxide; Na_2CO_3, sodium carbonate; K_2CO_3, potassium carbonate; DMAP, 4-dimethylaminopyridine; $LiAlH_4$, lithium aluminum hydride; **BZ**, benznidazole, (*N*-benzyl-2-(2-nitro-1*H*-imidazol-1-yl)acetamide); NCE, new chemical entity; CaH_2, calcium hydride; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; DMSO-d_6, deuterated DMSO; $CDCl_3$, deuterated chloroform; CD_3OD, deuterated methanol; TMS, tetramethylsilane; HRMS, high-resolution mass spectrometry; ESI, electrospray ionization; $NaHCO_3$, sodium bicarbonate; Na_2SO_4, sodium sulfate; TLC, thin-layer chromatography; LB, Luria broth; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Z-FR-AMC, Z-Phe-Arg-aminomethylcoumarin; LIT, liver infusion tryptone; RPMI, Roswell Park Memorial Institute media; CPRG, chlorophenol red-β-D-galactopyranoside; Ni−NTA, nickel−nitrilotriacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis

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