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Article

Biochemical Evaluation of a Series of Synthetic Chalcone and Hydrazide Derivatives as Novel Inhibitors of Cruzain from *Trypanosoma cruzi*

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A doença de Chagas, uma infecção parasitária amplamente distribuída na América Latina, é um problema grave de saúde pública com conseqüências devastadoras em termos de morbidade e mortalidade humana. A enzima cruzaína é a principal cisteíno protease do *Trypanosoma cruzi*, agente etiológico da tripanossomíase Americana ou doença de Chagas, e foi selecionada como alvo atrativo para o desenvolvimento de novos fármacos tripanocidas. No presente trabalho, a síntese e os efeitos inibitórios de uma série de trinta e três chalconas e sete hidrazidas são descritos contra a enzima cruzaína de *T.cruzi*. A maioria dos compostos mostraram inibição promissora *in vitro* (valores de IC_{50} na faixa de 20-60 μM), o que sugere o potencial desses compostos como candidatos a líderes para contínuo desenvolvimento. Doze compostos são inéditos, sendo que quatro destes (**7**, **13**, **16** e **18**) estão entre os inibidores mais potentes da série.

Chagas' disease, a parasitic infection widely distributed throughout Latin America, is a major public health problem with devastating consequences in terms of human morbidity and mortality. The enzyme cruzain is the major cysteine protease from *Trypanosoma cruzi*, the etiologic agent of American trypanosomiasis or Chagas' disease, and has been selected as an attractive target for the development of novel trypanocidal drugs. In the present work, we describe the synthesis and inhibitory effects of a series of thirty-three chalcone and seven hydrazide derivatives against the enzyme cruzain from *T. cruzi*. Most of the compounds showed promising *in vitro* inhibition (IC_{50} values in the range of 20-60 μM), which suggest the potential of these compounds as lead candidates for further development. Twelve compounds have not been reported before, and four of them (**7**, **13**, **16** e **18**) are among the most potent inhibitors of the series.

Keywords: chalcones, *Trypanosoma cruzi*, cruzain, Chagas' disease, inhibition

Introduction

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a major cause of illness, morbidity, long-term disability, and death in Latin America. It is estimated that about 16-18 million people are infected and other 100 million are at risk of infection, with more than 50,000 deaths each year. In spite of the alarming health, economic, and social consequences of this parasitic infection, the limited existing drug therapy (nifurtimox and benznidazole) suffers from a combination of drawbacks including poor efficacy, and serious side effects. Therefore, there is an urgent need for new chemotherapeutic agents with novel mechanisms of action.[1-3]

Enzymes are extremely attractive targets for small molecule drug intervention in human diseases.[1,2] One important drug target is the major cysteine protease from *T. cruzi*, cruzain (EC 3.4.22.51). This enzyme is implicated in several vital processes of the parasite, playing a pivotal role during the infection of host cells, replication, and metabolism. The identification and design of potent and selective cruzain inhibitors is, therefore, of great importance. About one decade ago, in a review of inhibitors of cysteine proteases, Otto and Schirmeister[4] cited a series of chalcone

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derivatives as inhibitors of malarial cysteine proteases. However, the potential of this class of inhibitors has not been fully explored, in spite of its synthetic versatility and potential low toxicity.[5] To the best of our knowledge, only two chalcones were reported as inhibitors of *T. cruzi* cruzain.[6] In that respect, there is a justifiable interest in developing structure-activity relationships (SAR) for this class of compounds. On the other hand, acylhydrazides (a class of compounds structurally related to chalcones) have been successfully investigated against *T. cruzi* cruzain.[7] In addition, both chalcone and acylhydrazide derivatives were active *in vitro* against *T. brucei* and *T. cruzi*.[8,9]

As part of our research program aimed at discovering novel *T. cruzi* cruzain inhibitors, we have synthesized and evaluated a series of chalcones and hydrazides.[10-12]

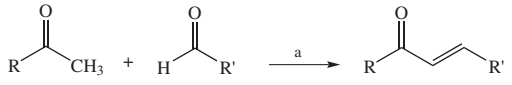
Results and Discussion

Synthesis

The chalcone derivatives (Table 1) were prepared by aldolic condensation of aromatic aldehydes and corresponding acetophenone (Scheme 1), with yields ranging from 18 to 92%.[12] Chalcones **1** and **2** are derived from 3,4-methylenedioxybenzaldehyde; chalcones **3**-**6** are derived from 2-hydroxy-3-bromo-4,6-dimethoxyacetophenone (prepared as previously described, with yield of 95%);[13] chalcones **7**-**20** are derived from 3,4-methylenedioxyacetophenone; chalcones **21**-**27** are derived from 2-naphthylacetophenone; chalcones **28**-**31** are derived from 2,4,5-trimethoxyacetophenone (prepared as previously described, with yield of 81%);[14] and chalcones **32** and **33** are derived from 2,4-dimethoxyacetophenone. The benzylated vanillin (3-methoxy-4-(phenylmethoxy)-benzaldehyde) used to prepare chalcone **33** was obtained according to the procedure of Tsai and Klinman,[15] with yield of 88%.

The hydrazides (**34**-**40**) were prepared by condensation of the obtained 3,4,5-trimethoxybenzohydrazide (**41**) (previously prepared as described, with yield of 80%)[16] and corresponding aromatic aldehydes, with yields ranging from 61 to 72% (Scheme 2).[17]

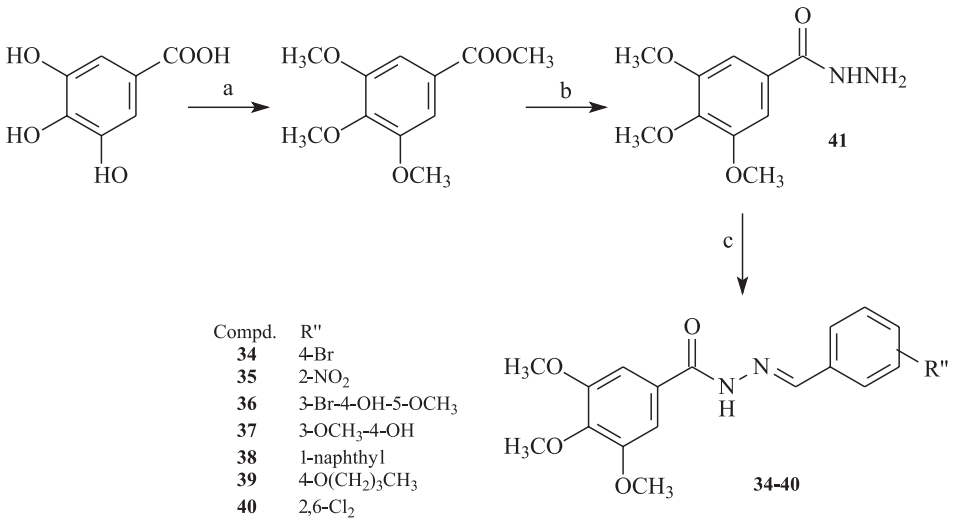
All synthesized compounds, including those that had been previously reported (chalcones **1**, **2**, **4**, **5**, **8-12**, **14**, **17**, **19**, **21**, **23-26**, **28**, **31-33**; and hydrazides **34**, **35**, **38** and **40**)[18,19] were well characterized by ^{1}H NMR, ^{13}C NMR, IR and elemental analysis. Detailed spectral characterization (^{1}H NMR, ^{13}C NMR, IR and elemental analysis) for all novel compounds (**3**, **6**, **7**, **13**, **15**, **16**, **18**, **20**, **22**, **27**, **29**, **30**, **36**, **37** and **39**) is presented in the experimental part. ^{1}H NMR spectra revealed that all the structures are configured E ($J_{H\alpha\text{-}H\beta}$ 15-16 Hz).



1-33

Compd.	R	R'	Compd.	R	R'
1	4-Br-Ph	3,4-methylenedioxy-Ph	**2**	2-OH-Ph	3,4-methylenedioxy-Ph
3	2-OH-3-Br-4,6-OCH_3-Ph	1-naphthyl	**4**	2-OH-3-Br-4,6-OCH_3-Ph	3-NO_2-Ph
5	2-OH-3-Br-4,6-OCH_3-Ph	4-OCH_3-Ph	**6**	2-OH-3-Br-4,6-OCH_3-Ph	4-$O(CH_2)_3CH_3$-Ph
7	3,4-methylenedioxy-Ph	2,6-Cl-Ph	**8**	3,4-methylenedioxy-Ph	4-Br-Ph
9	3,4-methylenedioxy-Ph	4-OCH_3-Ph	**10**	3,4-methylenedioxy-Ph	2,4,5-OCH_3-Ph
11	3,4-methylenedioxy-Ph	3,4,5-OCH_3-Ph	**12**	3,4-methylenedioxy-Ph	4-CH_3-Ph
13	3,4-methylenedioxy-Ph	1-naphthyl	**14**	3,4-methylenedioxy-Ph	Ph
15	3,4-methylenedioxy-Ph	2-Cl-Ph	**16**	3,4-methylenedioxy-Ph	2-CH_3-furan
17	3,4-methylenedioxy-Ph	3-OCH_3-Ph	**18**	3,4-methylenedioxy-Ph	tiophene
19	3,4-methylenedioxy-Ph	3-OCH_3-4-OBn-Ph	**20**	3,4-methylenedioxy-Ph	4-$O(CH_2)_3CH_3$-Ph
21	2-naphthyl	4-OCH_3-Ph	**22**	2-naphthyl	2,6-OCH_3-Ph
23	2-naphthyl	2,4,5-OCH_3-Ph	**24**	2-naphthyl	4-F-Ph
25	2-naphthyl	3,4-methylenedioxy-Ph	**26**	2-naphthyl	2-naphthyl
27	2-naphthyl	2-CH_3-furan	**28**	2,4,5-OCH_3-Ph	2,4,5-OCH_3-Ph
29	2,4,5-OCH_3-Ph	1-naphthyl	**30**	2,4,5-OCH_3-Ph	tiophene
31	2,4,5-OCH_3-Ph	3-OCH_3-Ph	**32**	2,4-OCH_3-Ph	2,4,5-OCH_3-Ph
33	2,4-OCH_3-Ph	3-OCH_3-4-OBn-Ph			

Scheme 1. Synthesis of chalcones **1**-**33**. a: KOH 50% m/v, methanol, rt, 24 h.



Scheme 2. Synthesis of 3,4,5-trimethoxybenzohydrazide and hydrazide derivatives **34**-**40**. a: $(CH_3)_2SO_4$, K_2CO_3, acetone, TBAI, reflux, 12 h. b: H_2NNH_2. H_2O, MeOH, reflux, 5 h. c: respective aldehyde, MeOH, reflux, 2 h.

Biochemical evaluation of the synthetic compounds

As can be seen (Table 1), among the 40 derivatives evaluated against *T. cruzi* cruzain, compounds **1**, **3**-**10**, **12**-**14**, **16**, **18**, **20**, **21**, **25**, **28**-**30**, **33**-**36**, and **38**-**40** have IC_{50} values in the range of 20-100 μM.

The IC_{50} values of compounds **15, 22, 26, 27** (IC_{50s} > 25 μM), **11**, **17, 19, 23, 24,** and **37** (IC_{50s} > 50 μM), as well as of compounds **2**, **31** and **32** (the weakest inhibitors of the series with IC_{50s} > 100 μM), could not be determined accurately due to solubility limitations that appeared to decrease the sensitivity of the assay. The most potent inhibitors of the series, **1**, **5**, **7**, **10**, **13**, **16**, and **18** (IC_{50} values between 20 and 25 μM) represent a new class of lead candidates for further development. Furthermore, with a single exception of compound **5**, all chalcone derivatives possess the methylenedioxyphenyl (1,3-benzodioxole) moiety, which suggests a significant contribution of this group to the observed activity. In addition, it is worth noting that chalcones **7**, **13**, **16** and **18** are newly synthesized compounds and are among the most promising compounds for further SAR studies. On the other hand, the hydrazide derivatives (**34**-**40**) investigated in this work showed only moderate inhibitory potency, with IC_{50} values in the range of 40-60 μM.

Conclusions

A series of chalcones and hydrazides have been prepared and evaluated *in vitro* against cruzain from *T. cruzi*. Most compounds showed promising inhibitory activity against the parasite enzyme, with IC_{50} values in the low micromolar range. Our results demonstrate the potential of these compounds as a new class of small molecule inhibitors of *T. cruzi* cruzain. Future efforts to optimize these compounds will involve the design, synthesis and SAR studies of a variety of more water-soluble derivatives, as well as investigation about the importance of methylenedioxyphenyl moiety side in the structure of chalcones through the determination of the possible mechanism of enzyme inhibition and inhibitor binding mode. Efforts in this direction are in progress, and will be reported in due course.

Experimental

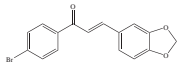
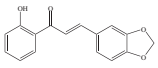
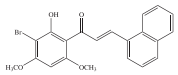
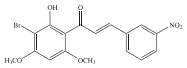
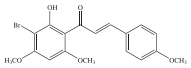
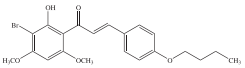
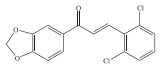
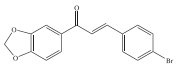
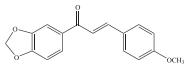
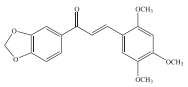
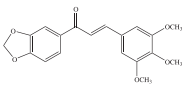
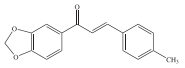
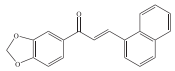
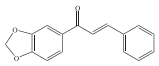
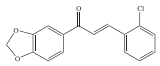
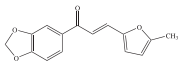
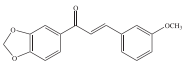
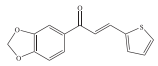
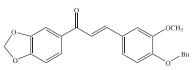
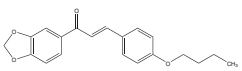
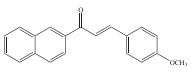
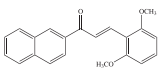
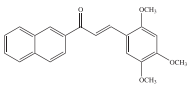
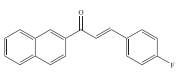
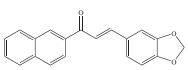
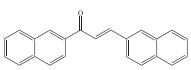
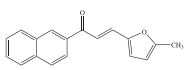
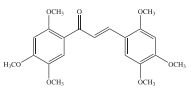
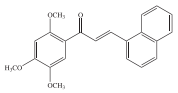
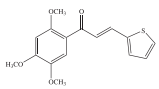
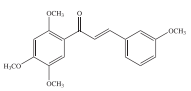
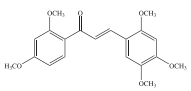
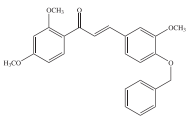
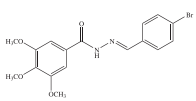
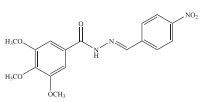
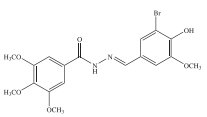
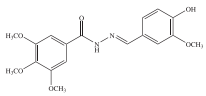
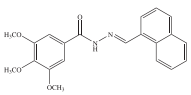
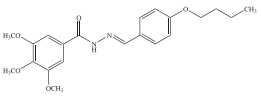
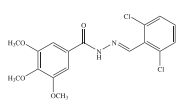
Physicochemical data of the synthesized compounds

The purified compounds were obtained in yields ranging from 18 to 92%. Melting points were determined with a Microquímica MGAPF-301 apparatus and are uncorrected. IR spectra were recorded with an Abb Bomen FTLA 2000 spectrometer on KBr disks. NMR (^1H and ^{13}C NMR) were recorded on a Varian Oxford AS-400 (400 MHz) spectrometer, using tetramethylsilane as an internal standard. Elemental analysis was carried out with a CHNS EA 1110. Percentages of C and H were in agreement with the product formula (within + 0.4% of theoretical values of C).

General procedure for the synthesis and purification of chalcones (***1***-***33***)

The chalcones were synthesized as shown in Scheme 1. All reagents used in this work were analytical grade and were purchased from Merck and Sigma-Aldrich. Exceptions

Table 1. Structures and IC_{50} values of a series of synthetic chalcone and hydrazide derivatives as inhibitors of *T. cruzi* cruzain

Compd.	Structure	IC_{50} [a] (μM)	Compd.	Structure	IC_{50} [a] (μM)	Compd.	Structure	IC_{50} [a] (μM)
1		20	**2**		>100	**3**		37
4		45	**5**		20	**6**		82
7		20	**8**		30	**9**		35
10		25	**11**		>50	**12**		30
13		25	**14**		50	**15**		>25
16		23	**17**		>50	**18**		22
19		>50	**20**		46	**21**		60
22		>25	**23**		>50	**24**		>50
25		50	**26**		>25	**27**		>25
28		100	**29**		48	**30**		100
31		>100	**32**		>100	**33**		37
34		55	**35**		60	**36**		50
37		>50	**38**		40	**39**		47
40		50						

[a] IC_{50} values are representative of three independent experiments performed in triplicate and the standard deviations were within 10% of the mean values reported for each compound.

are 2-hydroxy-3-bromo-4,6-dimethoxyacetophenone, 2,4,5-trimethoxyacetophenone and 3-methoxy-4-(phenylmethoxy)-benzaldehyde, which were prepared as previously described.[13-15] The chalcones (**1**-**33**) were prepared by magnetic stirrer of the acetophenone derivative (1.2 mmol), methanol (20 mL), KOH 50% m/v (5 mL), and the corresponding aldehyde (1.2 mmol), at room temperature for 24 h. Distilled water and hydrochloric acid (10%) were added to the reaction for total precipitation of the products. The compounds were then obtained by vacuum filtration and later recrystallized from dichloromethane and hexane. Chalcones **1**, **2**, **4**, **5**, **8-12**, **14**, **17**, **19**, **21**, **23-26**, **28**, **31**-**33** were previously described in the literature,[18] while compounds **3**, **6**, **7**, **13**, **15**, **16**, **18**, **20**, **22**, **27**, **29** and **30** are novel compounds which have not been reported. The chalcones (**3**-**6**) showed yields in the range of 40-50% due the presence of the bromine atom in the starting material acetophenone, which stabilizes the enolate intermediate of the reaction making it less reactive. This effect was not observed for the chalcones (**7**-**20**), which showed yields in the range of 70-98%.

*(2E)-1-(2'-Hydroxy,3'-bromo,4',6'-dimethoxyphenyl)-3-(1-naphthyl)-2-propen-1-one (**3**)*

Yellow solid; mp 218-219ºC; yield: 42%; ^{1}H NMR (acetone-d_6) δ 4.06 (s, 3H, *o*-OCH_3), 4.14 (s, 3H, *p*-OCH_3), 6.51 (s, 1H, H5'), 7.60 (d, 1H, *J* 7.6 Hz, H10), 7.61 (m, H5), 7.62 (d, J 7.6 Hz, H8), 7.70 (t, 1H, *J* 7.6 Hz, H9), 8.03 (m, 3H, H4, H6), 8.09 (d, 1H, *J* 15.4 Hz, Hα), 8.38 (d, 1H, *J* 8.4 Hz, H3), 8.66 (d, 1H, *J* 15.4 Hz, Hβ); ^{13}C NMR (CDCl3) δ 56.39 (*p*-OCH3), 56.62 (*o*-OCH3), 87.41 (C3'), 92.24 (C5'), 107.20 (C1'), 123.90 (Cα), 125.46 (C10), 125.68 (C3), 126.52 (C9), 127.15 (C5), 128.96 (C4), 129.79 (C8), 130.86 (C6), 132.00 (C2), 133.00 (C7), 133.97 (C1), 140.42 (Cβ), 162.27 (C6'), 162.55 (C2'), 163.46 (C4'), 192.91 (C=O); IR (KBr) ν_{max}/cm^{-1}: 3438 (OH), 1624, 1218 (C=O), 1552 (C=C), 1347, 1040 (C-O), 2952, 1415, 1314, 1250, 1129, 973, 800, 767, 659 (Ar); Anal. calc. for $C_{21}H_{17}BrO4$: C 61.03; H 4.15. Found: C 59.99; H 4.01.

*(2E)-1-(2'-Hydroxy-3'-bromo-4',6'-dimethoxyphenyl)-3-(4-buthoxyphenyl)-2-propen-1- one (**6**)*

Yellow solid; mp 172-173 ºC; yield: 47%; ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, -C**H**$_3$), 1.47-1.60 (m, 2H, -C**H**$_2$CH$_3$), 1.75-1.81 (m, 2H, -C**H**$_2$CH$_2$CH$_3$), 3.98 (s, 3H, OCH_3), 3.99 (s, 3H, OCH_3), 4.01 (t, 2H, -OC**H**$_2$-), 6.06 (s, 1H, H5'), 6.91 (d, 2H, *J* 8.4 Hz, H3, H5), 7.54 (d, 2H, *J* 8.4 Hz, H2, H6), 7.75 (d, 1H, *J* 15.6 Hz, Hα), 7.83 (d, 1H, *J* 15.6 Hz, Hβ), 14.96 (OH); ^{13}C NMR (acetone-d_6) δ 13.42 (**C**H$_3$), 19.18 (-**C**H$_2$CH$_3$), 31.28 (- **C**H$_2$CH$_2$CH$_3$), 56.25 (OCH_3), 67.86 (-O**C**H$_2$-), 88.37 (C5'), 94.61(C3'), 104.43 (C1'), 115.19 (C3, C5), 124.46 (Cα), 128.04 (C1), 130.71 (C2, C6), 143.72 (Cβ), 160.02 (C4), 168.46 (C2'), 168.82 (C6'), 176.12 (C4'), 198.07 (C=O); IR (KBr) ν_{max}/cm^{-1} 3450, 2945, 1615, 1554, 1221, 963; Anal. Calc. for $C_{21}H_{23}BrO_5$: C 57.94; H 5.33. Found: C 57.27; H 5.27.

*(2E)-1-(1,3-Benzodioxol-5-yl)-3-(2,6-dichlorophenyl)-2-propen-1-one (**7**)*

Light yellow solid; mp 118-119 ºC; yield: 90%; ^{1}H NMR ($CDCl_3$) δ 6.08 (s, 2H, -OCH_2O-), 6.90 (d, 1H, *J* 8.0 Hz, H5'), 7.21 (t, 1H, *J* 7.6 Hz, H4), 7.38 (d, 2H, *J* 8.4 Hz, H3, H5), 7.54 (s, 1H, H2'), 7.62 (d, 1H, *J* 16.0 Hz, Hα), 7.64 (d, 1H, *J* 8.0 Hz, H6'), 7.83 (d, 1H, *J* 16.0 Hz, Hβ); ^{13}C NMR ($CDCl_3$) δ 102.16 (-O**C**H$_2$O-), 108.23 (C5'), 108.76 (C2'), 125.40 (C6'), 128.00 (Cα), 129.07 (C3, C5), 129.99 (C1'), 130.49 (C4), 132.75 (C6), 132.98 (C2), 135.40 (C1), 137.50 (Cβ), 148.62 (C3'), 152.23 (C4'), 188.22 (C=O); IR (KBr) ν_{max}/cm^{-1} 1655, 1242 (C=O), 1595 (C=C), 1310, 1033 (C-O), 2905, 1503, 1439, 1107, 979, 927, 786, 743 (Ar); Anal. calc. for $C_{16}H_{10}Cl_2O_3$: C 59.84; H 3.14. Found: C 59.69; H 3.26.

*(2E)-1-(1,3-Benzodioxol-5-yl)-3-(1-naphthyl)-2-propen-1-one (**13**)*

Yellow solid; mp 137-139 ºC; yield: 91%; ^{1}H NMR ($CDCl_3$) δ 6.09 (s, 2H, -OCH_2O-), 6.93 (d, 1H, *J* 8.0 Hz, H5'), 7.53 (d, 1H, *J* 16.0 Hz, Hα), 7.54 (d, 1H, *J* 8.0 Hz, H10), 7.58 (m, 1H, H4), 7.59 (d, 1H, *J* 8.0 Hz, H6'), 7.61 (s, 1H, H2'), 7.61 (m, 1H, H5), 7.72 (dd, 1H, *J* 8.0/1.0 Hz, H9), 7.90 (m, 1H, H8), 7.93 (d, 1H, *J* 8.0 Hz, H6), 8.28 (d, 1H, *J* 8.0 Hz, H3), 8.66 (d, 1H, *J* 16.0 Hz, Hβ); ^{13}C NMR ($CDCl_3$) δ 102.13 (-O**C**H$_2$O-), 108.19 (C2'), 108.73 (C5'), 109.99 (C10), 123.80 (C6'), 124.63 (Cα), 125.05 (C3), 125.23 (C9), 125.66 (C5), 126.52 (C4), 127.17 (C8), 128.96 (C6), 130.90 (C2), 131.99 (C1'), 133.77 (C7), 133.96 (C1), 141.46 (Cβ), 148.60 (C3'), 153.81 (C4'), 188.29 (C=O); IR (KBr) ν_{max}/cm^{-1} 1646, 1253 (C=O), 1584 (C=C), 1343, 1037 (C-O), 2919, 1498, 1444, 1307, 1115, 978, 930, 806, 773, 732, 709 (Ar); Anal. calc. for $C_{20}H_{14}O_3$: C 79.46; H 4.67. Found: C 78.53; H 4.80.

*(2E)-1-(1,3-Benzodioxol-5-yl)-3-(2-chlorophenyl)-2-propen-1-one (**15**)*

Cream solid; mp 112-113 ºC; yield: 87%; ^{1}H NMR ($CDCl_3$) δ 6.08 (s, 2H, -OCH_2O-), 6.90 (d, 1H, *J* 8.0 Hz, H5'), 7.31 (m, 1H, H4), 7.34 (m, 1H, H5), 7.44 (m, 1H, H3), 7.45 (d, 1H, *J* 16.0 Hz, Hα), 7.54 (s, 1H, H2'), 7.65 (dd, 1H, *J* 8.0/1.0 Hz, H6'), 7.74 (m, 1H, H6), 8.16 (d, 1H, *J* 16.0 Hz, Hβ); ^{13}C NMR ($CDCl_3$) δ 101.91 (-O**C**H$_2$O-), 107.94 (C2'), 124.52 (C6'), 124.89 (Cα), 127.04 (C5), 127.75 (C6), 130.30 (C3), 131.03 (C4), 132.72 (C1'),

133.38 (C1), 135.43 (C2), 140.06 (Cβ), 148.36 (C3'), 151.85 (C4'), 188.15 (C=O); IR (KBr) ν_{max}/cm^{-1} 1653, 1241 (C=O), 1598 (C=C), 1319, 1037 (C-O), 2900, 1489, 1435, 1106, 975, 924, 813, 751, 569 (Ar); Anal. calc. for $C_{16}H_{11}ClO_3$: C 67.03; H 3.87. Found: C 67.14; H 4.05.

*(2E)-1-(1,3-Benzodioxol-5-yl)-3-(5-methylfuran-2-yl)-2-propen-1-one (**16**)*

Light orange solid; mp 98-99 ºC; yield: 87%; ^{1}H NMR ($CDCl_3$) δ 2.39 (CH3), 6.06 (s, 2H, -OCH_2O-), 6.13 (d, 1H, *J* 4.0 Hz, H4), 6.61 (d, 1H, *J* 4.0 Hz, H5), 6.89 (d, 1H, *J* 8.0 Hz, H5'), 7.33 (d, 1H, *J* 16.0 Hz, Hα), 7.52 (d, 1H, *J* 16.0 Hz, Hβ), 7.67 (d, 1H, *J* 8.0 Hz, H6'), 7.54 (s, 1H, H2'); ^{13}C NMR ($CDCl_3$) δ 14.25 (CH_3), 102.02 (-O**C**H_2O-), 108.10 (C2'), 108.60 (C5'), 109.52 (C4), 117.46 (C5), 118.21 (C6'), 124.73 (Cα), 130.71 (Cβ, C1), 133.34 (C1'), 148.44 (C3'), 150.64 (C1), 151.73 (C4'), 155.95 (C3), 187.98 (C=O); IR (KBr) ν_{max}/cm^{-1} 1651, 1251 (C=O), 1601 (C=C), 1360, 1036 (C-O), 2908, 1567, 1492, 1438, 1291, 1192, 1107, 1010, 994, 936, 906, 846, 792, 612, 539 (Ar); Anal. calc. For $C_{15}H_{12}O_4$: C 70.31; H 4.72. Found: C 70.09; H 4.79.

*(2E)-1-(1,3-Benzodioxol-5-yl)-3-(thiophen-2-yl)-2-propen-1-one (**18**)*

Beige solid; mp 101-103 ºC; yield: 91%; ^{1}H NMR ($CDCl_3$) δ 6.07 (s, 2H, -OCH_2O-), 6.90 (d, 1H, *J* 4.0 Hz, H5'), 7.09 (d, 1H, *J* 8.0 Hz, H4), 7.29 (d, 1H, *J* 16.0 Hz, Hα), 7.35 (d, 1H, *J* 4.0 Hz, H5), 7.41 (d, 1H, *J* 4.0 Hz, H3), 7.52 s (H2'), 7.63 (d, 1H, *J* 8.0 Hz, H6'), 7.93 (d, 1H, *J* 16.0 Hz, Hβ); ^{13}C NMR ($CDCl_3$) δ 102.09 (-O**C**H_2O-), 108.16 (C2'), 108.59 (C5'), 120.68 ($C6'), 124.81 (Cα), 128.54 (C4), 128.79 (C5), 132.13 (Cβ), 133.12 (C1'), 136.94 (C3), 140.72 (C1), 148.52 (C3'), 151.93 (C4'), 187.87 (C=O); IR (KBr) ν_{max}/cm^{-1} 1644, 1264 (C=O), 1581 (C=C), 1361, 1040 (C-O), 1500, 1443, 1325, 1301, 1202, 1109, 960, 910, 799, 697, 567 (Ar); Anal. calc. for $C_{14}H_{10}O_3S$: C 65.10; H 3.90. Found: C 64.73; H 3.63.

*(2E)-1-(1,3-Benzodioxol-5-yl)-3-(4-buthoxyphenyl)-2-propen-1-one (**20**)*

Cream solid; mp 102-104 ºC; yield: 92%; ^{1}H NMR ($CDCl_3$) δ 0.99 (t, 3H, *J* 8.0 Hz, CH_3), 1.51 (m, -C**H**$_2$- CH_3), 1.79 (m, -C**H**$_2$-CH_2-CH_3), 4.01 (t, 2H, *J* 8.0 Hz, -O-C**H**$_2$-), 6.06 (s, 2H, -OCH2O-), 6.89 (d, 1H, *J* 8.0 Hz, H5'), 6.92 (d, 1H, *J* 8.0 Hz, H3, H5), 7.37 (d, 1H, *J* 16.0 Hz, Hα), 7.53 (s, 1H, H2'), 7.58 (d, 1H, *J* 8.0 Hz, H2, H6), 7.65 (d, 1H, *J* 8.0 Hz, H6'), 7.77 (d, 1H, *J* 16.0 Hz, Hβ); ^{13}C NMR ($CDCl_3$) δ 13.84 (CH_3), 19.22 (-**C**H_2-CH_3), 31.21 (-**C**H_2-CH_2-CH_3), 67.87 (-O-**C**H_2-), 101.81 (-O**C**H_2O-), 107.87 (C2'), 108.44 (C5'), 114.88 (C3, C5), 119.18 (C6'), 124.46 (Cα), 127.46 (C1), 130.14 (C1', C2, C6), 144.25 (Cβ), 148.22 (C3'), 152.11 (C4'), 161.22 (C4), 181.79 (C=O); IR (KBr) ν_{max}/cm^{-1} 1654, 1240 (C=O), 1596 (C=C), 1330, 1030 (C-O), 2953, 2863, 1504, 1438, 1291, 1173, 1110, 973, 924, 807, 571 (Ar); Anal. calc. for $C_{20}H_{20}O_4$: C 74.06; H 6.21. Found: C 74.28; H 6.46.

*(2E)-1-(2-Naphthyl)-3-(2,6-dimethoxyphenyl)-2-propen-1-one (**22**)*

Cream solid; mp 109- 111 ºC; yield: 79%; ^{1}H NMR ($CDCl_3$) δ 3.94 (s, 6H, OCH_3), 6.61 (d, 1H, *J* 8.0 Hz, H3, H5), 7.31 (t, 1H, *J* 8.0 Hz, H4), 7.55 (dd, 1H, *J* 8.0/1.0 Hz, H5'), 7.60 (dd, 1H, *J* 8.0/1.0 Hz, H6'), 7.90 (d, 1H, *J* 8.0 Hz, H4'), 7.93 (d, 1H, *J* 8.0 Hz, H10'), 8.00 (d, 1H, *J* 8.0 Hz, H9'), 8.11 (d, 1H, *J* 8.0 Hz, H7'), 8.13 (d, 1H, *J* 16.0 Hz, Hα), 8.33 (d, 1H, *J* 16.0 Hz, Hβ), 8.53 (s, 1H, H2'); ^{13}C NMR ($CDCl_3$) δ 56.15 (OCH3), 104.00 (C3, C5), 113.19 (C1), 125.08 (Cα), 125.40 (C10'), 126.76 (C5'), 128.01 (C7'), 128.25 (C9'), 128.51 (C6'), 129.76 (C2'), 130.06 (C4), 131.76 (C4'), 132.84 (C8'), 135.52 (C1'), 136.07 (Cβ), 136.53 (C3'), 160.62 (C2, C6), 192.40 (C=O). IR (KBr) ν_{max}/cm^{-1} 1645, 1256 (C=O), 1581 (C=C), 1320, 1011 (C-O), 2934, 2833, 1470, 1439, 1177, 1110, 851, 757, 618, 535, 459 (Ar); Anal. calc. for $C_{21}H_{18}O_3$: C 79.23; H 5.70. Found: C 79.14; H 5.69.

*(2E)-1-(2-Naphthyl)-3-(5-methylfuran-2-yl)-2-propen-1-one (**27**)*

Gold yellow solid; mp 85-87 ºC; yield: 92%; ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 3H, CH_3), 6.15 (d, 1H, *J* 4.0 Hz, H4), 6.66 (d, 1H, *J* 4.0 Hz, H5), 7.54 (d, 1H, *J* 16.0 Hz, Hα), 7.58 (m, 1H, H5'), 7.61 (m, 1H, H6'), 7.62 (d, 1H, *J* 16.0 Hz, Hβ), 7.90 (d, 1H, *J* 8.0 Hz, H4'), 7.94 (d, 1H, *J* 8.0 Hz, H10'), 8.01 (d, 1H, *J* 8.0 Hz, H9'), 8.12 (d, 1H, *J* 8.0 Hz, H7'), 8.57 (s, 1H, H2'); ^{13}C NMR ($CDCl_3$) δ 14.09 (CH_3), 109.43 (C4), 117.52 (C5), 118.36 (C10'), 124.51 (Cα), 126.67 (C5'), 127.80 (C7'), 128.25 (C9'), 128.46 (C6'), 129.56 (C2'), 129.76 (C4'), 130.80 (Cβ), 132.63 (C8'), 135.43 (C1'), 135.75 (C3'), 150.45 (C1), 155.96 (C3), 189.69 (C=O); IR (KBr) ν_{max}/cm^{-1} 1648, 1284 (C=O), 1557 (C=C), 1362, 1012 (C-O), 3056, 1595, 1182, 1114, 819, 780, 710, 614, 535, 473 (Ar); Anal. calc. for $C_{18}H_{14}O_2$: C 82.42; H 5.38. Found: C 81.68; H 5.30.

*(2E)-1-(2',4',5'-Trimethoxyphenyl)-3-(1-naphthyl)-2-propen-1-one (**29**)*

Yellow solid; mp 162-164 ºC; yield: 87%; ^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H, *o*-OCH_3), 3.92 (s, 3H, *m*-OCH_3), 3.98 (s, 3H, *p*-OCH_3), 6.56 (s, 1H, H3'), 7.45 (s, 1H, H6'), 7.51 (t, 1H, *J* 8.0 Hz, H9), 7.53 (m, 1H, H5), 7.59 (d, 1H, H4), 7.72 (d, 1H, *J* 16.0 Hz, Hα), 7.85 (d, 1H, *J* 8.0 Hz,

H8), 7.88 (m, 2H, H6, H10), 8.31 (d, 1H, *J* 8.0 Hz, H3), 8.55 (d, 1H, *J* 16.0 Hz, Hβ); ^{13}C NMR ($CDCl_3$) δ 56.37 (*o*-OCH_3), 56.49 (*m*-OCH_3), 57.01 (*p*-OCH_3), 97.26 (C3'), 113.50 (C6'), 120.67 (C1'), 123.99 (Cα), 125.21 (C10), 125.67 (C3), 125.95 (C9), 126.23 (C5), 128.87 (C4), 130.11 (C8), 130.36 (C6), 132.03 (C2), 133.96 (C7), 138.96 (Cβ), 141.46 (C5'), 143.67 (C1), 153.94 (C2'), 155.18 (C4'), 191.12 (C=O); IR (KBr) ν_{max}/cm^{-1} 3456, 774 (C-S), 1660, 1214 (C=O), 1610 (C=C), 1321, 1027 (C-O), 2834, 1516, 846 (Ar).

*(2E)-1-(2',4',5'-Trimethoxyphenyl)-3-(thiophen-2-yl)-2-propen-1-one (**30**)*

Yellow solid; mp 117-119 ºC; yield: 86%; ^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H, *o*-OCH_3), 3.94 (s, 3H, *m*- OCH_3), 3.96 (s, 3H, *p*-OCH_3), 6.54 (s, 1H, H3'), 7.07 (t, 1H, *J* 4.0 Hz, H4), 7.31 (d, 1H, *J* 4.0 Hz, H5), 7.37 (d, 1H, *J* 4.0 Hz, H3), 7.39 (s, 1H, H6'), 7.47 (d, 1H, *J* 16.0 Hz, Hα), 7.84 (d, 1H, *J* 16.0 Hz, Hβ); ^{13}C NMR ($CDCl_3$) δ 56.37 (*o*-OCH_3), 56.45 (*m*-OCH_3), 57.01 (*p*-OCH_3), 97.33 (C3'), 113.38 (C6'), 120.56 (C1'), 126.51 (Cα), 128.09 (C5), 128.41 (C4), 131.31 (C3), 134.63 (C1', Cβ), 141.38 (C5'), 153.88 (C2'), 155.13 (C4'), 189.21 (C=O); IR (KBr) ν_{max}/cm^{-1}: 3099, 729 (C-S), 1651, 1267 (C=O), 1606 (C=C), 1307, 1023 (C-O), 2997, 2940, 2829, 1582, 1504, 1406, 1206, 1137, 847, 510 (Ar).

*General procedure for the synthesis and purification of 3,4,5-trimethoxybenzohydrazide (**41**)*[16]

3,4,5-trimethoxybenzohydrazide was obtained from a mixture of gallic acid (6 mmol), dimethyl sulphate (28 mmol), K_2CO_3 (26 mmol) and tetrabutylammonium iodide (TBAI) (0.1 g) in acetone (30 mL). The mixture was refluxed for 12 h, after which the precipitate was filtered and washed with acetone. The ester obtained (6 mmol) was treated with a solution of 99% N_2H_4 (4.4 mmol) in methanol (20 mL) and refluxed for 5 h and then kept at room temperature overnight. The solid obtained was filtered and recrystallized from hot methanol to afford the 3,4,5-trimethoxybenzohydrazide (80%).

*General procedure for the synthesis and purification of N´-substituted benzylidene-3,4,5-trimethoxybenzohydrazide (**34-40**)*[17]

The hydrazides were synthesized as shown in Scheme 2. 3,4,5-trimethoxybenzohydrazide (2 mmol) was mixed with the appropriate aldehyde (2 mmol) in methanol (15 mL) and refluxed for 2 h. After cooling, the crude product was collected by filtration, washed and recrystallized from hot ethanol to give white solids. Hydrazides **34**, **35**, **38** and **40** were previously described in the literature,[19] while **36**, **37** and **39** are novel compounds which have not been reported.

*(2E)-N'-(3'-Bromo-4'-hydroxy-5'-methoxybenzilidene)-3,4,5-trimethoxybenzohydrazide (**36**)*

White solid, mp 244-250 ºC; yield: 72%; ^{1}H NMR (DMSO-d_6) δ 3.73 (s, 3H, *m*-OCH_3), 3.87 (s, 6H, *m*-OCH_3), 3.90 (s, 3H, *p*-OCH_3), 7.22 (s, 2H, H2, H6), 7.33 (s, 1H, H2'), 7.43 (s, 1H, H6'), 8.33 (s, 1H, -N=C**H**), 10.03 (br, 1H, OH), 11.68 (s, 1H, -CON**H**); ^{13}C NMR (DMSO-d_6) δ 56.78 (*m*-OCH_3), 56.72 (*p*-OCH_3), 60.81 (*m*- OCH_3), 105.85 (C2, C6), 108.88 (C6'), 110.03 (C3'), 125.05 (C1), 127.30 (C2'), 129.29 (C1'), 142.10 (C4), 146.49 (-N=**C**H), 147.46 (C4'), 149.32 (C5'), 153.37 (C3, C5), 163.16 (-**C**ONH); IR (KBr) ν_{max}/cm^{-1} 3364 (N-H), 1646, 1251 (C=O), 1569 (C=N), 1309, 1027 (C-O), 2985, 1505, 1426, 1296, 1143, 1002 (Ar).

*(2E)-N'-(4'-Hydroxy-5'-methoxybenzilidene)-3,4,5-trimethoxybenzohydrazide (**37**)*

White solid; mp 198ºC; yield: 72%; ^{1}H NMR (DMSO-d_6) δ 3.71 (s, 3H, *m*-OCH_3), 3.82 (s, 6H, *m*-OCH_3), 3.85 (s, 3H, *p*-OCH_3), 6.84 (d, 1H, *J* 8.0 Hz, H3'), 7.08 (d, 1H, *J* 8.0 Hz, H2'), 7.20(s, 2H, H2, H6), 7.30 (s, 1H, H6'), 8.34 (s, 1H, -N=C**H**), 9.55 (br, 1H, OH), 11.52 (s, 1H, -CON**H**); ^{13}C NMR (DMSO-d_6) δ 56.25 (*p*-OCH_3), 56.77 (*m*-OCH_3), 60.81 (*m*-OCH_3), 105.78 (C2, C6), 109.64 (C6'), 116.15 (C3'), 122.83 (C2'), 126.36 (C1'), 129.45 (C1), 140.96 (C4), 148.74 (-N=**C**H), 149.08 (C4'), 149.73 (C5'), 153.37 (C3, C5), 162.99 (-**C**ONH); IR (KBr) ν_{max}/cm^{-1} 3418 (N-H), 1664, 1230 (C=O), 1584 (C=N), 1338, 1030 (C-O), 3265, 2885, 1612, 1495, 1411, 1097 (Ar).

*(2E)-N'-(4'-buthoxybenzilidene)-3,4,5-trimethoxybenzohydrazide (**39**)*

White solid; mp 119ºC; yield: 61%; ^{1}H NMR ($CDCl_3$) δ 0.96(t,CH3), 1.47(m,-C**H**$_2$-CH_3), 1.71(m,-C**H**$_2$-CH_2-CH_3), 4.04 (t, -O-C**H**$_2$-), 3.39 (s, 9H, *m*-OCH_3), 3.45 (s, 3H, *p*-OCH_3), 3.85 (s, 3H, OCH_3), 7.04 (d, 2H, *J* 8.0 Hz, H3', H5'), 7.24 (s, 2H, H2, H6), 7.68 (d, 2H, *J* 8.0 Hz, H2', H6'), 8.41 (s, 1H, -N=C**H**), 11.61 (s, 1H, -CON**H**); ^{13}C NMR ($CDCl_3$) δ 14.38 (CH_3), 19.41 (-**C**H$_2$-CH_3), 31.36 (-**C**H$_2$-CH_2-CH_3), 56.78 (*m*-OCH_3), 60.83 (*p*-OCH_3), 68.02 (-O-**C**H2-), 105.80 (C2, C6), 115.50 (C3', C5'), 127.37 (C1'), 129.35 (C1), 129.40 (C2', C6'), 148.41 (-N=**C**H), 153.37 (C3, C5), 161.03 (C4'), 163.07 (C=O); IR (KBr) ν_{max}/cm^{-1} 3236 (N-H), 1652, 1237 (C=O), 1577 (C=N), 1330, 1027 (C-O), 2961, 2875, 1509, 1413, 1237, 1123, 998, 848 (Ar).

Biochemical evaluation of the synthetic compounds

Materials

Cruzain truncated in the C-terminal extension was obtained from *Escherichia coli* (strain M15 or DH5α containing the expression plasmid) following the previously reported procedure.[20] All reagents for buffer preparation, the standard inhibitor E-64 and the substrate Z-Phe-Arg-AMC were purchased from Sigma–Aldrich. Substrate and inhibitors candidates at 10 mM stock solutions in neat DMSO were stored at −20°C and at −4°C, respectively.

Procedure

The activity of the enzyme was measured and quantified through active-site titration with the irreversible inhibitor E-64, as described previously.[21] For the biochemical evaluation against *T. cruzi* cruzain, the highly purified enzyme (0.64 nM) in 50 mM sodium phosphate, 100 mM sodium chloride, 5 mM EDTA, pH 6.5, containing 5 mM DTT, was incubated with the compounds (chalcones **1**-**33** and hydrazides **34**-**40**, Table 1) for 5 min at room temperature followed by the addition of the fluorogenic substrate *Z*-Phe-Arg-AMC.[22] Fluorescence was monitored on a Wallac 1420-042 PerkinElmer spectrofluorometer and measurements were done using 355 nm as the excitation wavelength and 460 nm as the emission wavelength, as previously described.[10] Cruzain activity was measured as an increase of fluorescence intensity of liberated aminocoumarin when Z-Phe-Arg-AMC was used as the substrate. The percentage of inhibition was calculated according to the following equation:

$$\% \text{ of Inhibition} = 100 \times (1 - V_i / V_0)$$

where V_i and V_0 are the initial velocities (enzyme activities) determined in the presence and in the absence of inhibitor, respectively. Values of IC_{50} were independently determined by making rate measurements for at least six inhibitor concentrations (inhibition range: 20-85%). The IC_{50} values correspond to the concentration of compound required for 50% inhibition of cruzain, and were determined from the collected data by nonlinear regression analysis employing the Sigma-Plot enzyme kinetics module.

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