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Design and Synthesis of Potent Substrate-based Inhibitors of the *Trypanosoma cruzi* Dihydroorotate Dehydrogenase

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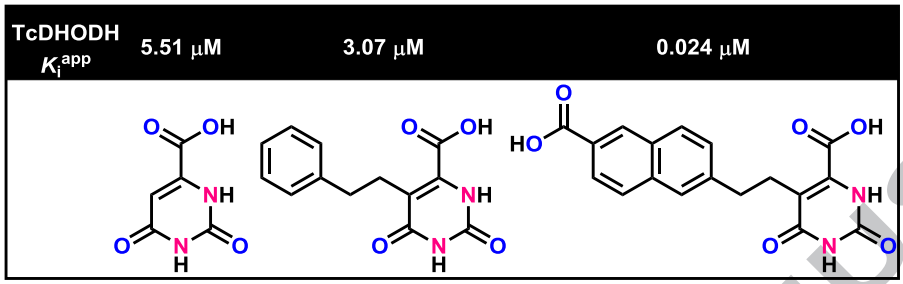
Abstract

Chagas disease, caused by the parasitic protozoan *Trypanosoma cruzi*, is the leading cause of heart disease in Latin America. *T. cruzi* dihydroorotate dehydrogenase (DHODH), which catalyzes the production of orotate, was demonstrated to be essential for *T. cruzi* survival, and thus has been

considered as a potential drug target to combat Chagas disease. Here we report the design and synthesis of 75 compounds based on the orotate structure. A comprehensive structure-activity relationship (SAR) study revealed two 5-substituted orotate analogues (**5u** and **5v**) that exhibit K_i^{app} values of several ten nanomolar level and a selectivity of more than 30,000-fold over human DHODH. The information presented here will be invaluable in the search for next-generation drug leads for Chagas disease.



Main Text

Introduction

Chagas disease (American trypanosomiasis), caused by the parasitic protozoan *Trypanosoma cruzi*, is the leading cause of heart disease in Latin America.[1,2] Today, at least 12 million people are infected with the parasite, resulting in more than 50,000 deaths each year. Chemotherapy for Chagas disease using the currently available drugs, nifurtimox and benznidazole, is unsatisfactory due to significant toxic side effects.[3] This toxicity, together with emerging drug resistance, has created an urgent need for an effective therapy against Chagas disease.

Dihydroorotate dehydrogenase (DHODH) catalyzes the oxidation of (*S*)-dihydroorotate to orotate in the *de novo* biosynthesis of pyrimidine (Figure 1), which is an important component in the synthesis of DNA, RNA, glycoprotein, and membrane lipids.[4] Oxidization of dihydroorotate, the first half reaction, is coupled with the reduction of flavin mononucleotide (FMN) cofactor in both *T. cruzi* DHODH (TcDHODH) and human DHODH (HsDHODH). In the second half reaction, the reduced FMN enzymatically converts ubiquinone to ubiquinol in human, whereas fumarate is used as the electron acceptor to generate succinate in *Trypanosoma cruzi*.[5] Recently, Nara et al. demonstrated that

TcDHODH-knockout *T. cruzi* could not survive even in the presence of substrates for enzymes of the pyrimidine salvage pathway.[6,7] Therefore, development of an inhibitor of TcDHODH would lead to the generation of promising chemotherapeutic agents to combat infections with this pathogen.[8,9,10,11] Here we report the design and synthesis of 75 compounds based on the orotate structure. Using the results of a comprehensive structure-activity relationship (SAR) study, we found a number of substrate-based compounds with potent inhibitory activity against TcDHODH as well as high selectivity for TcDHODH from HsDHODH. These features make this class of inhibitors a novel starting point for the development of new drug leads for Chagas disease.

Results and Discussion

A comparison of the X-ray crystal structures of TcDHODH[12] and HsDHODH[13,14] in complexes with orotate provided the basis for the rational design of selective substrate-based inhibitors (Figure 2).[15,16] The bound orotate in TcDHODH stacks parallel to FMN and forms multiple hydrogen bonds with the backbone of M69, G70 and L71 and the side chain of K43, N67, N127, N194 and S195. While the hydrogen bond network is well-conserved within the active sites of the two enzymes, M69, L71, C130 and S195 in TcDHODH differ from the corresponding residues (Y147, F149, S215 and T285) in HsDHODH. Importantly, replacement of F149 of HsDHODH with the L71 of TcDHODH enlarges the space around position 5 of orotate. Therefore, we exploited this vacant space to design selective TcDHODH inhibitors. Specifically, substructures which vary in shape, bulkiness, rigidity and polarity were planned to be synthetically attached at the 5-position of orotate, then the products were to be screened to identify TcDHODH selective inhibitors.

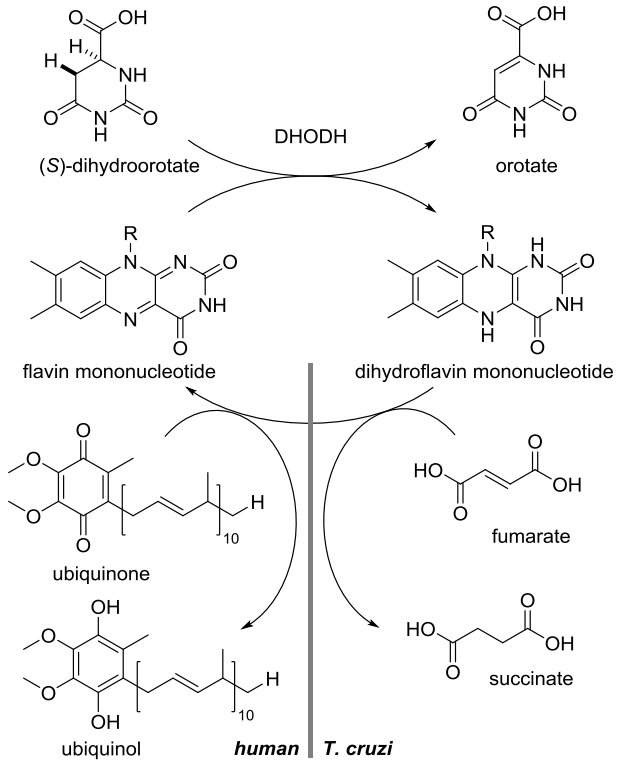


Figure 1. Reactions of human DHODH (HsDHODH) and *T. cruzi* DHODH (TcDHODH). Oxidation of dihydroorotate to orotate is coupled with the reduction of a flavin mononucleotide (FMN) cofactor in the first half-reaction of both DHODHs. In the second half-reaction, HsDHODH and TcDHODH use ubiquinone and fumarate, respectively, as the electron acceptor. R = $CH_2CH(OH)CH(OH)CH(OH)CH_2OP(OH)_2$

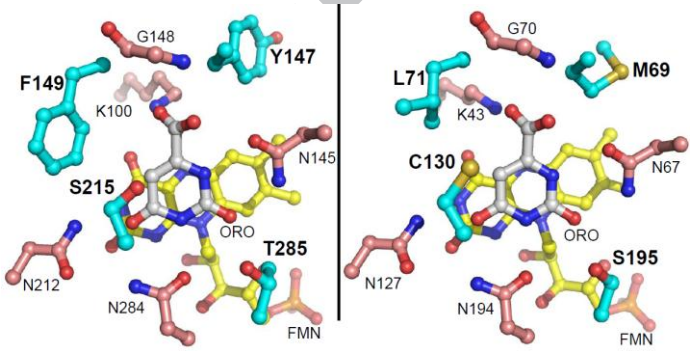
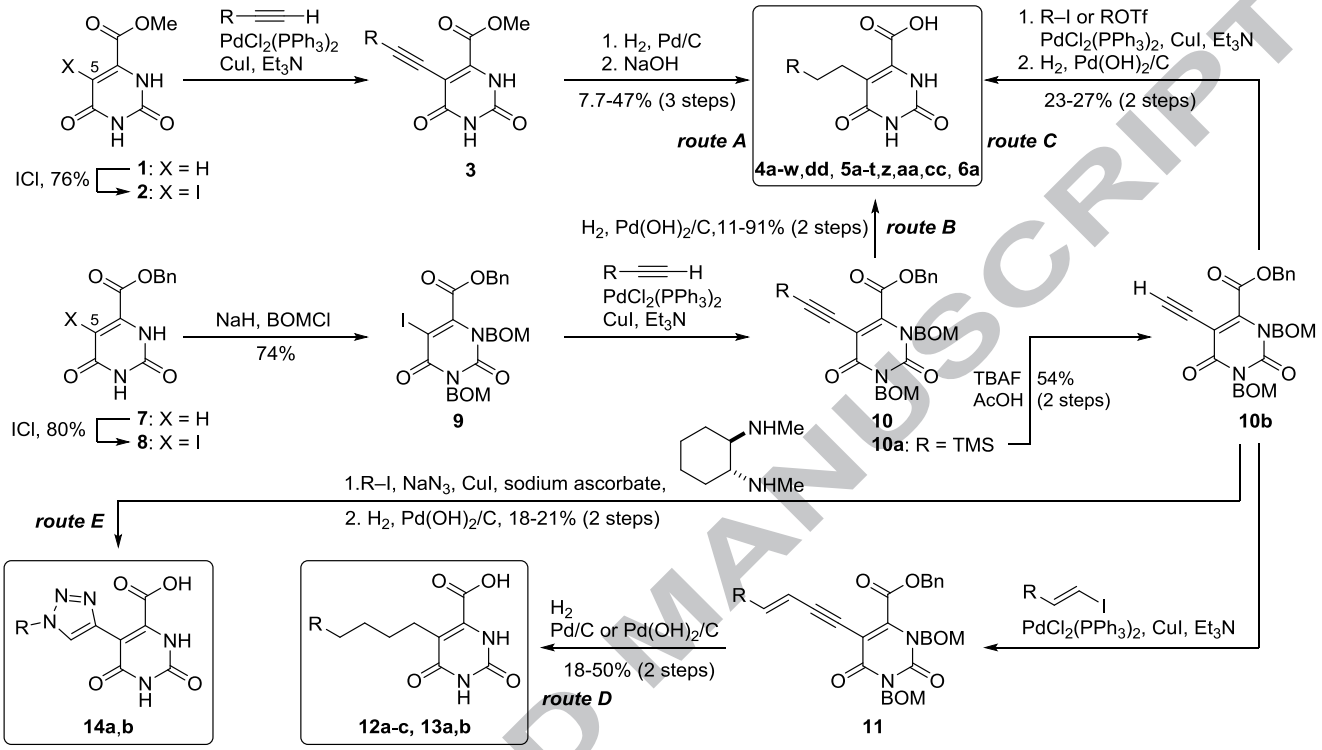
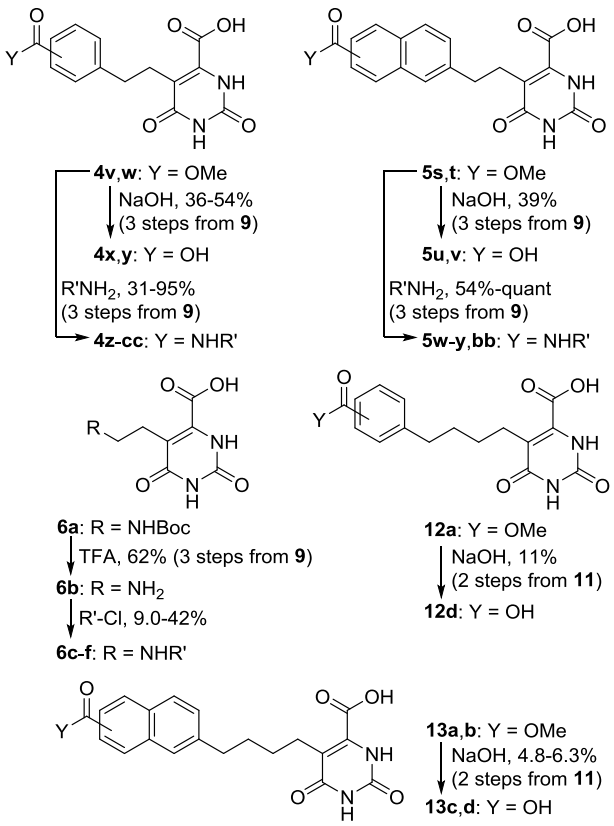


Figure 2. Comparison of the active site in human DHODH (left, PDB: 1D3H)[10] and *T. cruzi* DHODH (right, 2E6A).[9] DHODHs complexed with orotate (ORO). ORO, flavin mononucleotide (FMN) and amino acid residues interacting with ORO are shown in ball-and-stick representation. Oxygen, nitrogen, sulfur and phosphate atoms are colored in red, blue, dark yellow and orange, respectively. Residues

with salmon and cyan carbon atoms represent conserved and non-conserved residues, respectively, between the two DHODHs. Non-conserved residues are labeled in bold to emphasize their structural differences. Carbon atoms from ORO and FMN are colored in white and yellow for both DHODHs.



Scheme 1. Synthesis of the various 5-substituted orotates. Chemical yield of each compound is described in Table S1 or S2.



Scheme 2. Further functional group transformations to the various 5-substituted orotates. Chemical yield of each compound is described in Table S1 or S2.

To efficiently explore the chemical space and to access the optimized inhibitors, the 5-substituted orotate structures were diversified by both terminal aromatic and linker moieties (Schemes 1 and 2). Namely, a variety of phenyl and naphthyl structures were attached to C5 of the orotate ring through two-carbon (**4** and **5**), four-carbon (**12** and **13**), triazole (**14**) or ethanamine (**6**) spacer. Sonogashira coupling was selected as the key reaction for extending the carbon chain from C5.

Phenyl **4** and naphthyl derivatives **5** with the two-carbon spacer were first synthesized through route A (Scheme 1). Methyl orotate **1** was converted into the corresponding iodide **2**, followed by Sonogashira coupling with the acetylene moiety in the presence of catalytic $PdCl_2(PPh_3)_2$, CuI and Et_3N to provide **3**.[17,18] After chemoselective hydrogenation of the alkyne moiety of **3**, the methyl ester was hydrolyzed under basic conditions to generate **4** or **5**. Despite its conciseness, route A was often irreproducible, and gave products in variable yields. The insoluble aggregates, which are presumably

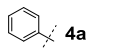
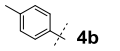
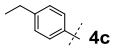
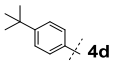
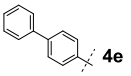
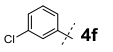
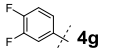
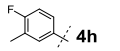
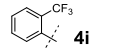
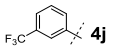
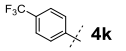
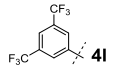
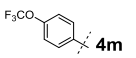
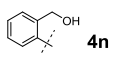
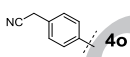
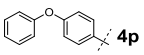
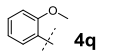
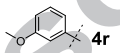
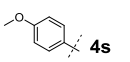
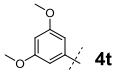
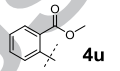
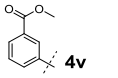
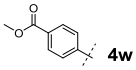
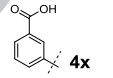
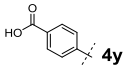
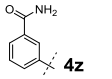
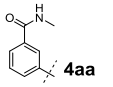
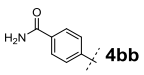
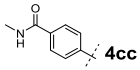
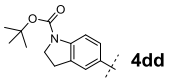
formed due to intermolecular hydrogen bonds between NH and C=O of the orotate core, decreased the efficiency of the transformation and purification of **3**, **4** or **5**. Accordingly, route A was abandoned.

In route B, the fully protected **9** was used as the common intermediate (Scheme 1). Iodination of benzyl orotate **7** resulted in the formation of vinyl iodide **8**, which was treated with BOMCl and NaH to produce bis-BOM protected **9**.[19] Sonogashira reaction of **9** with the various alkyne reagents proceeded smoothly, leading to **10**. Finally, hydrogenation of the alkyne and hydrogenolysis of the Bn and BOM groups of **10** were simultaneously achieved under a hydrogen atmosphere in the presence of $Pd(OH)_2/C$ to afford the requisite **4**-**6**. Route B simplified the handling and purification procedures because of the minimized hydrogen bond donors of the intermediates, and only required the same number of steps as route A.

Routes C, D and E stemmed from alkyne **10b**, which was prepared from **10a** using TBAF and AcOH (Scheme 1). Coupling of **10b** with aryl iodide or triflate and subsequent hydrogenation gave rise to **5** (route C). Alternatively, the use of vinyl iodide with **10b** in the Sonogashira reaction, followed by treatment of **11** with H_2 in the presence of Pd/C or $Pd(OH)_2/C$, provided analogue **12** or **13** with the four-carbon spacer (route D). In route E, triazole analogues **14** were synthesized from alkyne **10b** by coupling of aryl iodide with in situ generated aryl azides.[20,21]

Further transformations produced another set of analogues (Scheme 2). Basic hydrolysis of the methyl ester of **4v**,**w**, **5s**,**t**, **12a** and **13a**,**b** resulted in formation of the corresponding carboxylic acid **4x**,**y**, **5u**,**v**, **12d** and **13c**,**d**, respectively. Ester/amide exchange from **4v**,**w**/**5s**,**t** was realized using the various amines, leading to **4z**,**aa-cc**/**5w-y**,**bb**. Alternatively, deprotection of **6a** gave the primary amine **6b**, which was treated with the electrophilic reagents to give **6c-f**. Overall, the Sonogashira coupling strategies successfully produced the divergent 5-substituted orotates (**4**, **5**, **6**, **12**, **13** and **14**) in an efficient and reliable fashion.

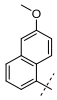
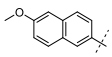
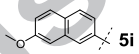
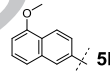
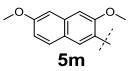
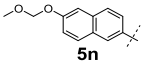
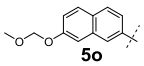
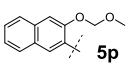
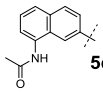
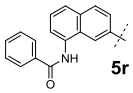
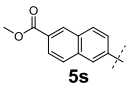
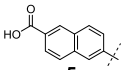
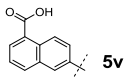
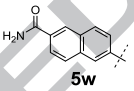
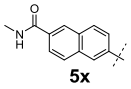
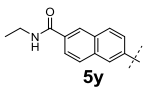
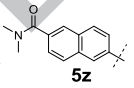
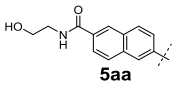
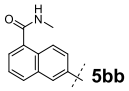
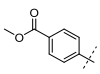
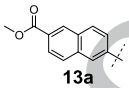
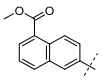
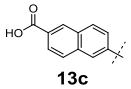
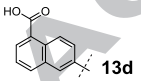
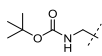
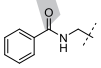
Table 1. Activity of phenylethyl-substituted orotates against TcDHODH and HsDHODH (μM). S.I. = selectivity index. green: K_i^{app} (T_CDHODH) = <0.3 μM.

compound (R)	K_i^{app} (Tc) / K_i^{app} (Hs)	S.I.	compound (R)	K_i^{app} (Tc) / K_i^{app} (Hs)	S.I.	compound (R)	K_i^{app} (Tc) / K_i^{app} (Hs)	S.I.
 **4a**	3.07 / >2500	>814	 **4b**	0.427 / 2.88	6.75	 **4c**	1.19 / 248	208
 **4d**	1.10 / 8.40	7.64	 **4e**	0.788 / 0.485	0.615	 **4f**	1.61 / 1285	798
 **4g**	1.97 / >2500	>1269	 **4h**	1.02 / 147	144	 **4i**	0.739 / >2500	>3338
 **4j**	0.616 / 142	231	 **4k**	1.07 / 360	336	 **4l**	0.886 / 5.70	6.43
 **4m**	0.497 / 41.2	82.9	 **4n**	3.09 / >2500	>809	 **4o**	0.749 / >2500	>3338
 **4p**	1.11 / 8.30	7.48	 **4q**	0.714 / >2500	>3501	 **4r**	0.581 / 484	833
 **4s**	2.66 / 1255	472	 **4t**	0.297 / 530	1785	 **4u**	7.78 / >500	>64.3
 **4v**	0.169 / 382	2260	 **4w**	2.11 / 1395	661	 **4x**	0.200 / 1200	6000
 **4y**	0.130 / >125	>961	 **4z**	0.818 / >500	>611	 **4aa**	2.34 / 1035	442
 **4bb**	1.61 / >500	>311	 **4cc**	0.896 / >500	>558	 **4dd**	0.180 / 195	1080

The inhibitory activities of the synthesized 5-substituted orotates were evaluated against TcDHODH and HsDHODH (Tables 1 and 2). The obtained IC_{50} numbers were converted to K_i^{app} (apparent inhibition constant), then the selectivity indexes were calculated from the K_i^{app} values of both DHODHs.[12,22] Table 1 shows the activities of the 5-phenethyl-orotate derivatives (**4a-z**,**aa-dd**). Although the inhibition constants of the original orotate (5.51 μM) and 5-phenethyl-orotate **4a** (3.07 μM) were comparable, **4a** exhibited negligible inhibition against HsDHODH (>2,500 μM). The high selectivity index of **4a** validated our inhibitor design, based on the subtle spatial differences of the enzyme pockets. Attachment of hydrophobic (**4b-e**), halogen (**4f-l**) and polar substituents (**4m-z**,**aa-dd**) to the benzene ring of **4a** generally enhanced the inhibitory activities, while retaining the selectivity indexes. Among the 30 phenethyl derivatives, the bis(methoxy) (**4t**), methoxycarbonyl (**4v)** and carboxy (**4x**,**y**) benzene analogues and the indoline structure (**4dd**) were found to display K_i^{app} values

less than 0.3 μM (indicated in green), suggesting that the functional groups, capable of forming hydrogen bonds, were beneficial for increased potency toward TcDHODH.

Table 2. Activity of 5-substituted orotate analogues against TcDHODH and HsDHODH (μM). S.I. = selectivity index. red: K_i^{app} (T_cDHODH) = <0.1 μM, green: K_i^{app} (T_cDHODH) = <0.3 μM.

compound (R)	K_i^{app} (Tc) / K_i^{app} (Hs)	S.I.	compound (R)	K_i^{app} (Tc) / K_i^{app} (Hs)	S.I.	compound (R)	K_i^{app} (Tc) / K_i^{app} (Hs)	S.I.
 5a	0.763 / 217	284	 5b	0.222 / 23.1	104	 5c	0.138 / 2.07	15
 5d	0.247 / >625	>2530	 5e	0.372 / 1300	3495	 5f	0.128 / 34.1	266
 5g	0.106 / 10.2	96.2	 5h	0.142 / 23.1	163	 5i	0.291 / 6.65	22.8
 5j	0.223 / 19.9	89.2	 5k	0.046 / 11.3	246	 5l	0.110 / 10.3	93.6
 5m	0.206 / 23.9	116	 5n	0.882 / 139	158	 5o	0.305 / 15.8	51.8
 5p	0.044 / 22.2	505	 5q	0.847 / 1980	2338	 5r	3.41 / 262	76.8
 5s	0.175 / 38.8	222	 5t	0.317 / 22.2	70	 5u	0.024 / 893	37210
 5v	0.033 / >2500	>75760	 5w	0.272 / >2500	>9191	 5x	0.788 / 1111	1410
 5y	0.259 / 236	911	 5z	0.311 / 637	2048	 5aa	0.154 / >500	>3247
 5bb	0.561 / 149	266	 5cc	1.76 / 20.2	11.5	 12a	1.40 / 102	72.9
 12b	2.14 / 73.5	34.3	 12c	0.763 / 217	284	 12d	2.21 / 319	144
 13a	1.07 / 26.1	24.4	 13b	0.481 / 12.0	24.9	 13c	0.377 / 986	2615
 13d	0.197 / 76	386	 6a	2.96 / >313	>106	 6b	2.04 / >313	>153
 6c	7.44 / >313	>42.1	6d	5.57 / >625	>112	6e	6.75 / >625	>92.6
6f	2.61 / >313	>120	14a	>4.92 / >250	n.d.	14b	2.50 / 420	168

As shown in Table 2, more than half of the 29 naphthylethyl analogues (**5a-z**,**aa-cc**) showed an inhibitory activity below 0.3 μM. In comparison to 5-phenethyl-orotate **4a** (3.3 μM), 1-naphthyl (**5a**), 2-naphthyl (**5b**), 9-phenanthrenyl (**5c**), 3-quinolyl (**5d**) and 4-quinolyl (**5e**) derivatives all possessed decreased K_i^{app} values (0.138-0.763 μM). The extended π-systems of these analogues would contribute to favorable hydrophobic and/or CH-π interactions[23,24] with the hydrophobic portion of the amino acid residues of TcDHODH. Further substitution of the 1-naphtyl (**5f**,**g**) or 2-naphthyl ring (**5h-z**,**aa-cc**) with the polar functional groups variably influenced potency toward TcDHODH. Most importantly, 8-methoxy-2-naphthyl (**5k**), 3-methoxymethoxy-2-naphtyl (**5p**), 6-carboxy-2-naphtyl (**5u**) and 5-carboxy-2-naphtyl (**5v**) substituted analogues exhibited K_i^{app} values smaller than 0.05 μM (indicated in red), and the selectivity indexes of **5u** and **5v** were found to be larger than 30,000. These inhibitors would attain the high inhibitory activities by forming the hydrogen bonds between their polar functionalities and the proximally positioned residues of TcDHODH. The greater potency of **5u** and **5v** compared to esters **5s**,**t** and amides **5w-z**,**aa-cc** would originate from the intrinsically stronger hydrogen bonds of carboxylic acids than esters and amides.

Replacement of the two carbon spacer with the four carbon spacer had a negative effect on the potency of the inhibitors (**12a-d**, **13a-d**, Table 2), even when the molecules contained the naphthalene rings and polar functional groups. For instance, the carboxylic acid analogous **13c** and **13d** were approximately 16- and 6-times less potent than **5u** and **5v** with the shorter spacer, respectively.

The optimal nature of the two carbon spacer was further corroborated by evaluating the activities of **6a-f**, containing the ethanamine spacer, and **14a**,**b** with the triazole spacer (Table 2). None of these compounds displayed K_i^{app} values of the one micromolar level, indicating the importance of the appropriate length and flexibility of the spacer that connects the orotate and aromatic moieties.

Conclusions

Substrate-based inhibitors of TcDHODH were rationally designed based on the structural differences between the enzyme pockets of TcDHODH and HsDHODH. Efficient and robust routes using

Sonogashira coupling as the key reaction were developed to generate a series of 5-substituted orotate analogues, varying in shape, bulkiness, rigidity and polarity. A comprehensive SAR study of the 75 synthesized analogues clarified the importance of the naphthyl ring and the two carbon spacer at C5 for potent inhibitory activity toward TcDHODH. Significantly, the two orotate analogues **5u** and **5v** were found to exhibit K_i^{app} values of 0.024 μM and 0.033 μM, respectively, and 37210-fold and >75760-fold selectivities, respectively, over HsDHODH. The information obtained in this study will be invaluable in the search for next-generation drug leads for Chagas disease.[25]

Supporting Information.

Experimental procedure, spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

Acknowledgement

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25. Activities of the synthesized 5-substituted orotate analogues towards *T. Cruzi* proliferation will be evaluated, and reported in due course.