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Potent inhibitor scaffold against *Trypanosoma cruzi trans*-sialidase

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ABSTRACT

The protozoan *Trypanosoma cruzi*, the causative agent of Chagas' disease, can infect the heart, causing cardiac arrest frequently followed by death. To treat this disease, a potential molecular drug target is *T. cruzi trans*-sialidase (TcTS). However, inhibitors found to date are not strong enough to serve as a lead scaffold; most inhibitors reported thus far are derivatives of the substrate sialic acid or a transition state analogue known as 2,3-dehydro-3-deoxy-*N*-acetylneuraminic acid (DANA) with an IC_{50} value of more than hundreds of micromolar. Since natural products are highly stereodiversified and often provide highly specific biological activity, we screened a natural product library for inhibitors of TcTS and identified promising flavonoid and anthraquinone derivatives. A structure–activity relationship (SAR) analysis of the flavonoids revealed that apigenin had the minimal and sufficient structure for inhibition. Intriguingly, the compound has been reported to possess trypanocidal activity. An SAR analysis of anthraquinones showed that 6-chloro-9,10-dihydro-4,5,7-trihydroxy-9,10-dioxo-2-anthracenecarboxylic acid had the strongest inhibitory activity ever found against TcTS. Moreover, its inhibitory activity appeared to be specific to TcTS. These compounds may serve as potent lead chemotherapeutic scaffolds against Chagas' disease.

1. Introduction

The protozoan *Trypanosoma cruzi* is the causative agent of Chagas' disease and its infection of the heart leads to cardiac arrest frequently followed by death.[1] Chagas' disease is widely distributed in Central and South America, with 18–20 million people infected and 40 million people at risk according to the WHO report of 2002.[2] It was estimated that there are up to 200,000 new cases and 21,000 deaths every year.[2,3] Only two approved drugs, nifurtimox and benznidazole, are capable of curing at least 50% of infections.[4,5] However, these drugs are only efficient during the acute phase of Chagas' disease.[6] Moreover, these drugs have severe side effects such as anorexia, vomiting, peripheral polyneuropathy and allergic dermopathy, which can lead to treatment discontinuation.[3] In treatment trials for acute infections, patients responded to the two drugs with varied sensitivities, probably because of the diversity of *T. cruzi* strains. Another problem is that in the region where Chagas' disease is prevalent, there is an insufficient supply of injection syringes, which could lead to repeated use by more than one person, thus leading to increased incidence of other infectious diseases such as HIV. Considering this situation, effective oral chemotherapy is what is urgently needed for the millions who are already infected.

In recent years, some trials have been conducted for the development of chemotherapeutic drugs against Chagas' disease. One potential molecular drug target is *T. cruzi trans*-sialidase (TcTS).[7] TcTS is a membrane-anchored protein which, unlike eukaryotic sialyl transferases, is capable of transferring sialic acid from sialyl-glycoconjugates, not from CMP–sialic acid.[8–10] When *T. cruzi* invades mammalian cells, TcTS transfers sialic acid from host sialyl-glycoconjugates to mucin-like acceptor molecules expressed on its cell surface. Sialylated mucin-like molecules have been implicated in adhesion and invasion of *T. cruzi* to the host cells as well as in its resistance to the host immune system.[11,12] In a previous preclinical study, an increased survival rate of *T. cruzi*-infected mice was reported with anti-TcTS antibody treatment.[13] These evidences make TcTS one of the most appealing therapeutic targets.

Strong inhibitors that can be considered as lead scaffolds have not been found to date; most inhibitors reported thus far are derivatives of the substrate sialic acid[7] or a transition state analogue known as DANA with IC_{50} values of more than hundreds of

Abbreviations: SAR, structure–activity relationship; DANA, 2,3-dehydro-3-deoxy-*N*-acetylneuraminic acid; TcTS, *Trypanosoma cruzi trans*-sialidase; SAPA, shed acute-phase antigen; MuNANA, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; Neu2, neuramidase 2.

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micromolar.[7,10,14] Thus, it might be difficult to find a novel TcTS inhibitor scaffold from sialic acid mimetics. It is well accepted that natural products are highly stereodiversified and often provide highly specific biological activity.[15–17] As highlighted in a recent survey, the majority of new drugs introduced between 1981 and 2002 were either natural products or their derivatives.[18] From these viewpoints, highly potent TcTS inhibitors that could serve as a lead scaffold might be found from natural products libraries.

In search of highly potent TcTS inhibitors that do not have the sialic acid scaffold, we screened a library of 2283 purified natural organic products. The screening yielded 16 promising compounds. Considering structural availability for further investigations, we selected two compounds for further characterization, compound **1**, myricetin, a flavone and compound **19**, 6-chloro-9,10-dihydro-4,5,7-trihydroxy-9,10-dioxo-2-anthracenecarboxylic acid. Structure–activity relationship (SAR) analyses of derivatives of compound **1**, revealed that compound **6**, apigenin, has the minimal and sufficient structure for inhibition. Compound **19** had the highest inhibitory effect among inhibitors reported thus far and appears highly specific for TcTS in that it did not inhibit human neuramidase 2.

2. Results

2.1. Expression and purification of TcTS

The recombinant TcTS was produced in *Escherichia coli*. Highly homogeneous TcTS, 21 mg, was purified from 1 L of culture by cation exchange chromatography, nickel affinity chromatography, and gel filtration chromatography (Fig. 1). The TcTS kinetics followed Michaelis behavior with K_m and k_{cat} values of 78 μM and 2.9 s^{-1}, respectively.

2.2. Screening for inhibitors of TcTS

TcTS preferentially transfers sialic acid from sialyl-glycoconjugates to the terminal β-galactosyl residues in acceptor molecules. However, TcTS can also act as sialidase by releasing free sialic acid from sialyl-glycoconjugates. The same active site of TcTS has been shown to engage in both mechanisms,[19] therefore we employed hydrolysis assay for monitoring TcTS activity. The screening was conducted by the discontinuous fluorometric method using 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MuNANA) as the substrate. The fluorescence of the released product 4-methylumbelliferone (Mu) was monitored. Hit compounds were selected via four screening steps (Fig. 2). In the primary screening step, a natural product library consisting of 2283 compounds was tested for inhibitory activity of TcTS at the single concentration of 1 μM (Fig. 3). The hit threshold was set at 40% inhibition by considering experimental data variation. In the second step, 103 compounds selected in the primary screening were subjected to IC_{50} evaluation, which showed that 50 of the 103 compounds had IC_{50} of less than tens of micromolar. In the third step, in order to eliminate promiscuous inhibitors, IC_{50} evaluation was repeated in the same buffer used in the primary screening except that 0.1% (v/v) Triton X-100 was included instead of 0.01%.[20,21] In this step, IC_{50} values were determined for 16 out of the 50 compounds, ranging between 10 nM and 17 μM. They include tannins, one flavonoid derivative, and two quinone derivatives. It is well accepted that orally active

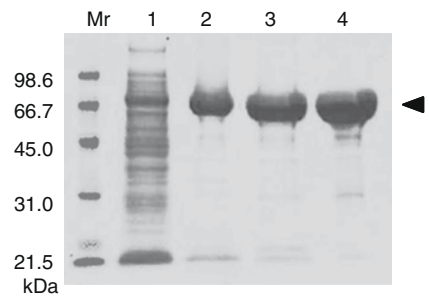


Figure 1. Purification analysis of TcTS. SDS–PAGE gel stained with Quick-CBB. Lane 1, crude extract; lane 2, eluate from Ni affinity chromatography; lane 3, eluate from cation exchange chromatography; lane 4, eluate from gel filtration chromatography. The arrow head indicates the position of recombinant TcTS. Highly homogeneous TcTS, 21 mg, was purified from 1 L of culture.

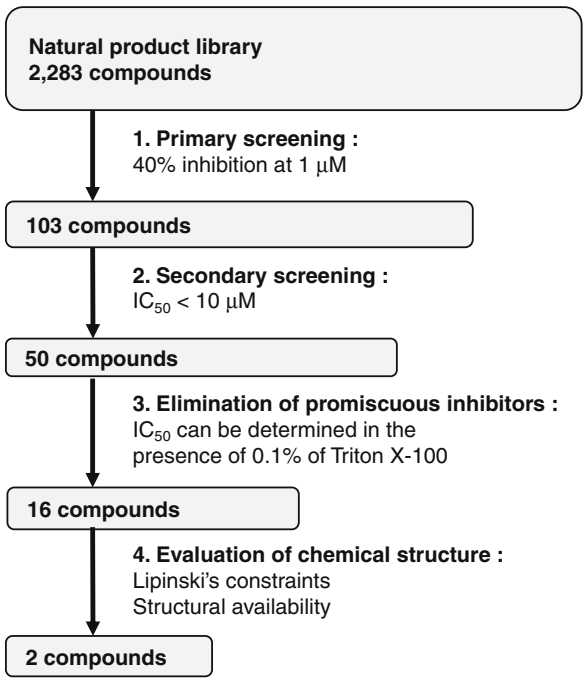


Figure 2. Scheme of screening of TcTS inhibitors. Hit compounds were selected via four screening steps. In the primary screening, the natural product library consisting of 2283 compounds was tested for inhibitory activity of TcTS at the single concentration of 1 μM. The hit threshold was set at 40% inhibition (shown with dotted line) and 103 compounds were selected. In the second step, IC_{50} evaluation showed that 50 compounds had IC_{50} of less than tens of micromolar. In the third step, in order to eliminate promiscuous inhibitors, IC_{50} evaluation was repeated in the presence of 0.1% (v/v) Triton X-100. In this step, IC_{50} values of 16 compounds were determined out of 50. In the final step, considering Lipinski's constraints and structural availability, two compounds were selected for further analyses.

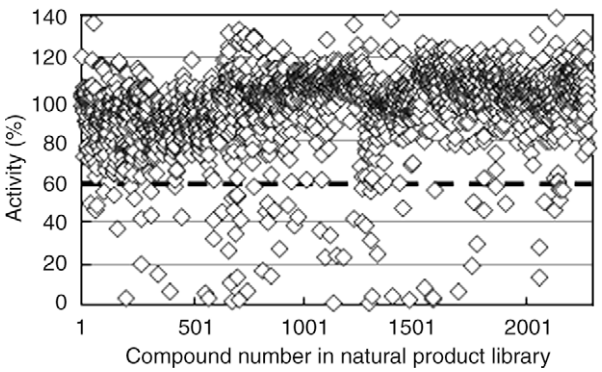


Figure 3. Primary screening of natural product library at 1 μM. Vertical and horizontal axes represent TcTS activity and compound number in natural compound library, respectively. Compounds suppressing more than 40% activity (shown with dotted line) were subjected to the secondary analysis.

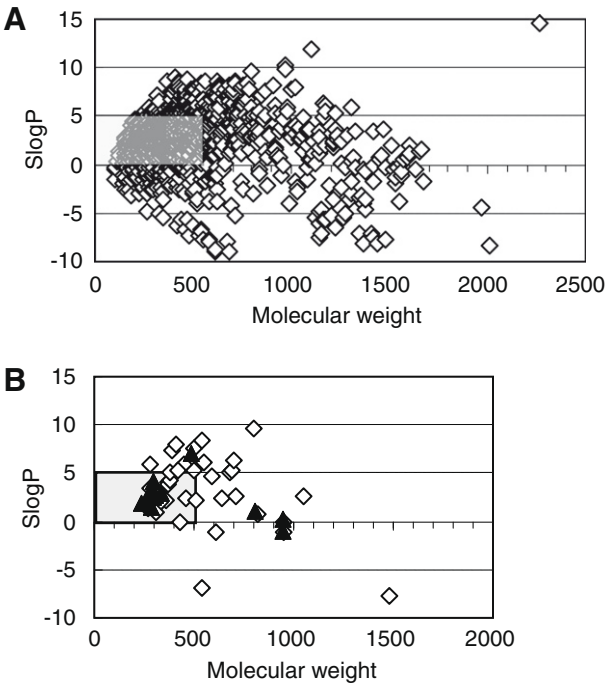


Figure 4. Profile of *S*log *P* and molecular weight. Vertical and horizontal axes represent *S*log *P* and molecular weight, respectively. The area that fulfills two of four of Lipinski's constraints is shown with a gray background. (A) The profile of natural product library. (B) The profile of compounds selected in the second and the third steps. Open diamond and closed triangle represent the profile of compounds screened under assay conditions including 0.01% and 0.1% Triton X-100, respectively. Seven compounds that fulfill two of Lipinski's constraints were identified in the forth step.

drugs obey Lipinski's rule of 5.[22] Therefore, seven compounds that fulfill two Lipinski's constraints, $0 < S\log P < 5$, molecular weight <500, were selected in the fourth step (Fig. 4). Five of the seven compounds were too stereodiverse to proceed to SAR analyses. Considering structural availability for further investigations, we selected two compounds, myricetin (**1**, IC_{50} = 17 μM), and 6-chloro-9,10-dihydro-4,5,7-trihydroxy-9,10-dioxo-2-anthracenecarboxylic acid (**19**, IC_{50} = 0.58 μM).

Compound **1** is a flavonoid consisting of three rings, A, B, and C, and compound **19** is an anthraquinone. Both compounds have several hydroxyl groups (Fig. 5). As described later, in order to elucidate which hydroxyl groups are critical for TcTS inhibitory activity, several structurally related compounds with altered numbers and positions of functional groups were further analyzed (Table 1).

As Shoichet and co-workers have reported, a wide range of molecules, even those including approved drugs, can form colloidal aggregates through self-association and cause inhibition of protein activity in the concentration above several μM.[21,23] In another report, they claim that at 30 μM, up to 19% of drug-like molecules can form aggregates.[24] Therefore, we tested whether each compound forms colloidal aggregates under the assay buffer conditions by measuring dynamic light scattering (DLS). As shown in Figure 6, the DLS analyses showed that about half of the compounds, **3**, **6**, **7**, **8**, **9**, **13**, **14**, **15**, **16**, **18**, **19**, **20**, and **22** formed colloidal aggregates in the assay buffer containing 0.01% Triton X-100, whereas almost all compounds showed less degree of aggregation in the presence of 0.1% Triton X-100. It was suspected that the inhibition observed in the presence of 0.01% Triton X-100 might be due to compound aggregation. As in the case reported by Shoichet,[20,21] our investigation also showed that an assay condition containing 0.1% Triton X-100 was effective for decreasing compound aggregation. Based on this observation, we employed this condition to avoid selecting the false positive inhibitors for further analyses.

2.3. SAR analyses

2.3.1. Myricetin derivatives

Myricetin belongs to flavonoids, which are classified based on the functional group on the C-ring, and those flavonoids tested in this study can be classified into five categories, flavone, flavonol, isoflavone, flavanone and flavan-3-ol (Fig. 5). As shown in Table 1

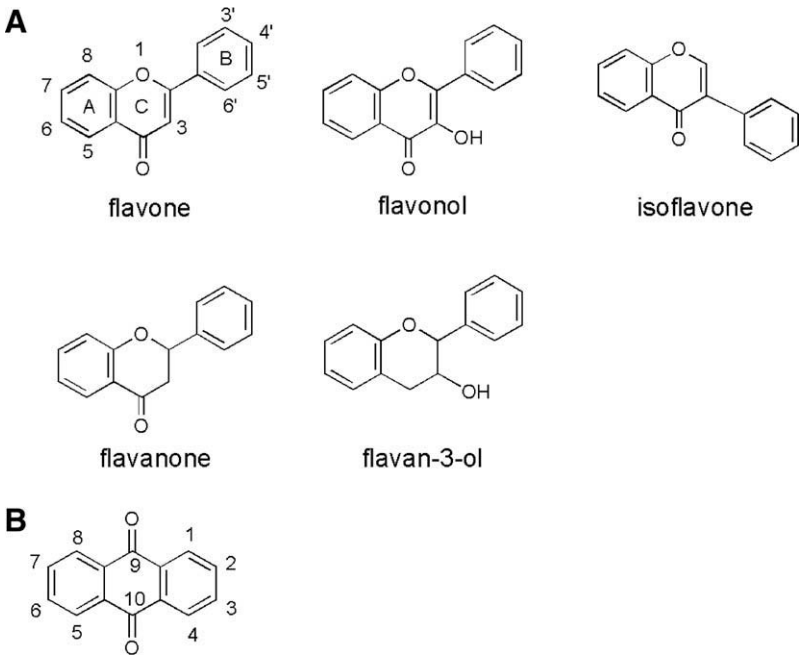


Figure 5. Structure of flavonoids and anthraquinone. (A) Structures of flavonoids. Flavonoid consists of three rings, A, B, and C. Flavonoids tested in this study are classified into five groups: flavone, flavonol, isoflavone, flavanone, and flavan-3-ol. (B) Structure of anthraquinone.

(see also Supplementary data 1A), the IC_{50} values of the compounds ranging between 17 μM and >1000 μM provided some foundation for the structure–activity relationship. The 4′-, 5- and 7-hydroxyl groups were important for inhibitory activity and we could deduce that compound **6** should represent the minimal structure required for inhibitory activity of compound **1** (see compounds **1**–**9**). The position of the hydroxyl group on the B-ring was also important because compound **16** showed less inhibitory activity than **6**. Although flavone **6** and isoflavone **17** bear the same substituents, isoflavone **17** with an altered B-ring position decreased inhibitory activity compared with flavone **6**. Flavan-3-ol **10** that lacks the carbonyl group compared with flavonol **2** decreased inhibitory activity and, flavanone **18** that lacks sp^2 carbon compared with flavone **6** and **14** showed decreased inhibitory activity (Fig. 7). In search of stronger inhibitors, we also conducted SAR analyses around compound **6** by methylation of hydroxyl groups and alteration of the position of hydroxyl groups. Unfortunately, there was no inhibitor as strong as compound **6** (**6** > **11**, **12**, **13**, **14**, **15**, **16**). We concluded that compound **6** has the minimal and sufficient structure for inhibition among the tested flavonoids.

2.3.2. Compound 19 derivatives

Compound **19**, an anthraquinone, possessed the highest inhibitory effect among the inhibitors reported thus far. In order to optimize the scaffold, we conducted SAR analyses. As shown in Table 1 (see also Supplementary data 1A), they provided some foundation for the structure–activity relationship in a similar manner to the flavonoid derivatives. The IC_{50} values of this series of compounds range between 0.58 μM and >1000 μM. SAR analyses revealed that elimination of the functional groups from compound **19** led to loss of inhibitory activity. The best inhibitor in the series of compounds was compound **19** (IC_{50} = 0.58 μM).

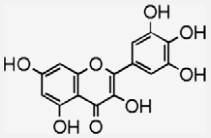
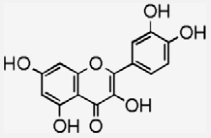
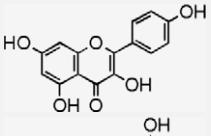
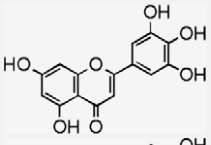
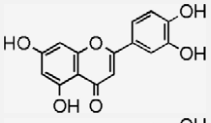
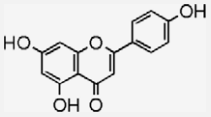
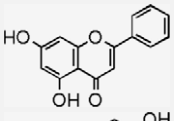
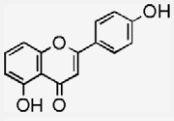
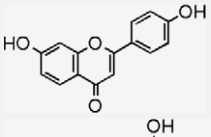
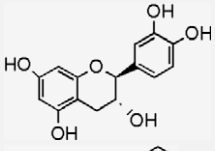
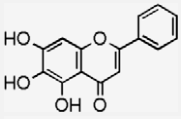
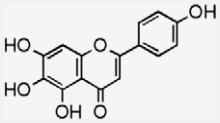
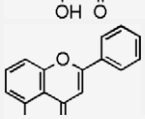
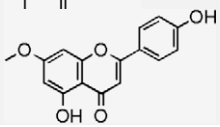
2.4. TcTS specificity

We determined the inhibitory activity of these compounds against human neuramidase 2 (Neu2)[25] to evaluate their specificities. As shown in Table 1 (see also Supplementary data 1B), the flavonoids tend to weakly inhibit Neu2 with IC_{50} values around hundreds of micromolar. The ratios of IC_{50} values against TcTS and Neu2 ($IC_{50(Neu2)}/IC_{50(TcTS)}$) range from 0.2 to 8.8. As in the case of inhibitory activity, compound **6** represents the minimal structure among the derivatives that maintain the similar specificity of compound **1** to TcTS. In addition, most anthraquinones also showed moderate specificity toward TcTS as well as flavonoids, however, compound **19** showed the high specificity toward TcTS, with IC_{50} value of 0.58 μM and IC_{50} value more than 100 μM against Neu2, respectively, attaining the specificity ($IC_{50(Neu2)}/IC_{50(TcTS)}$) >170. This value represents the highest specificity toward TcTS among the inhibitors reported thus far.

2.5. Inhibitory mechanism of 6 and 19

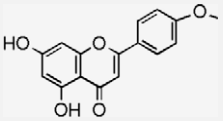
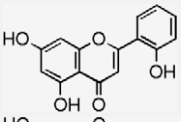
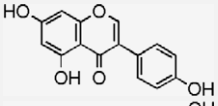
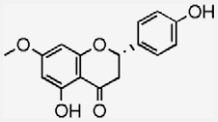
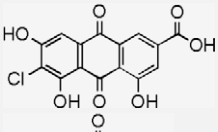
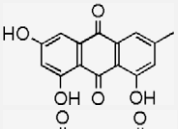
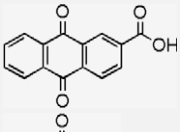
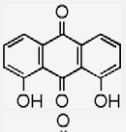
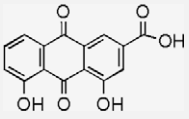
After determining the minimal structures required for inhibition of the two series, the modes of inhibition of compound **6** and **19** were examined. The enzyme kinetics showed that these inhibitory effects were reversible and not due to mechanism-based inhibition (data not shown). We further characterized the inhibitory mechanism of these compounds by enzyme kinetics. Lineweaver–Burk plots revealed that all the regression lines converged on the *X*-axis, strongly suggesting that both compounds **6** and **19** behaved in a noncompetitive manner. The K_i values of compound **6** and **19** were estimated to be 60 μM and 0.89 μM, respectively (Fig. 8).

Table 1
Inhibitory activities against TcTS and neuraminidase 2

Compound number	Structure	IC_{50} (μm)		
		TcTS	Neu2	Specificity
Flavonoids				
1		17	150	8.8
2		58	160	2.8
3		110	230	2.1
4		39	130	3.3
5		22	130	5.9
6		78	570	7.3
7		340	890	2.6
8		>1000	>1000	n.d.[a]
9		>1000	470	<0.5
10		>1000	>1000	n.d.[a]
11		100	130	1.3
12		50	55	1.1
13		>1000	>1000	n.d.[a]
14		140	>1000	7.1

(*continued on next page*)

Table 1 (*continued*)

Compound number	Structure	IC_{50} (μm)		
		TcTS	Neu2	Specificity
15		>1000	>1000	n.d.
16		360	70	0.2
17		230	380	1.7
18		>1000	890	<0.9
Anthraquinones				
19		0.58	>100	>170
20		110	760	6.9
21		430	800	1.9
22		710	>1000	>1.4
23		14	130	9.3

Specificity for TcTS was defined by the ratios between IC_{50} values against TcTS and Neu2 ($IC_{50(Neu2)}/IC_{50(TcTS)}$).

[a] n.d. = not determined.

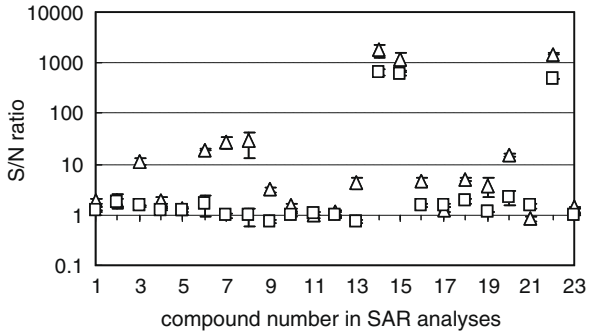


Figure 6. DLS analysis of aggregate formation of compound. Vertical axis represents signal-to-noise (S/N) ratio between the derived counter rate with and without the compound. Signal means the derived counter rate with compound. Noise means the derived counter rate of buffer. Horizontal axis represents the compound number in SAR analyses (see Table 1). Compound that shows more than one in S/N ratio is suspected to form colloidal aggregates. Squares and triangles indicate the S/N ratio evaluated in the presence of 0.1% and 0.01% Triton X-100, respectively.

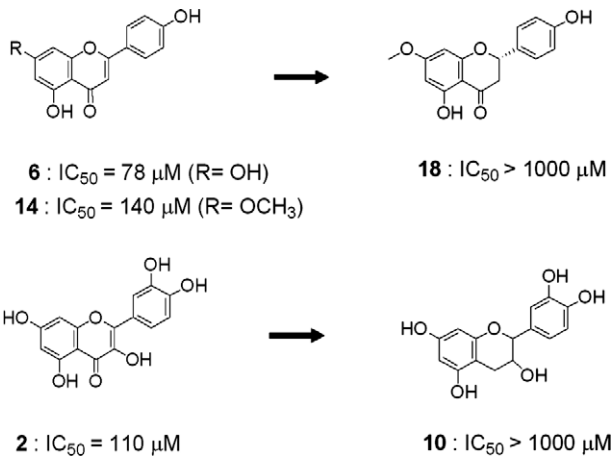


Figure 7. SAR analysis of flavonoids. Compound **10**, which lacks the carbonyl group and unsaturated carbon bond of the C-ring, showed drastically repressed inhibitory activity compared with compound **2**. Compound **18**, which lacks unsaturated carbon bond, showed much lower inhibitory activity than compound **14**. Based on these results, it seems that the C-ring might interact with TcTS via the π electron cloud.

3. Discussion

Flavonoids consist of three rings with functional groups, and we conducted SAR analysis focusing on each ring one by one. With respect to the isolated B-ring, compounds **2** and **3** have fewer hydroxyl groups than compound **1** and showed decreased inhibition in proportion to the number of eliminated hydroxyl groups. Compound **7**, which lacks all hydroxyl groups on the B-ring, showed further decreased inhibition. The hydroxyl groups on the B-ring seem to be the essential moiety for the inhibitory effect of compound **1**. In the case of compound **16** with the 6′-hydroxy group on the B-ring, the inhibition was less than that of compound **6.** Thus, the position as well as the number of the hydroxyl group appears to be important for achieving full inhibitory activity. Moreover, compound **17**, with an altered B-ring position, showed decreased inhibition compared with compound **6**. The position of the B-ring would be one of the important elements for interaction with TcTS. Methylation of the 4′-hydroxy group of compound **6** led to decreased inhibitory activity (compound **15**). These results may imply the possibility that 4′-hydroxy group on the B-ring forms a hydrogen bond with some residues of TcTS as a proton donor.

With respect to the C-ring, compound **4**, which lacks the 3-hydroxy group, did not affect inhibition compared with compound **1**. On the other hand, compound **10**, which lacks the carbonyl group and unsaturated carbon bond of the C-ring compared with compound **2**, showed drastically repressed inhibitory activity. This result suggests that the 3-hydroxyl group does not contribute to the interaction with TcTS but the carbonyl group or unsaturated carbon bond does. In addition, compound **18**, which lacks unsaturated carbon bond, showed much lower inhibitory activity than compound **14**. Based on these findings, we suppose that the C-ring might interact with TcTS via the π electron cloud. On the A-ring, compounds **8** and **9**, which lack the 7- and 5-hydroxyl group, respectively, showed drastically decreased inhibitory activity compared with compound **6**. Methylation of the 7-hydroxy group of compound **6** had no significant effect on inhibitory activity (compound **14**). These observations suggest that both 5-hydroxyl group and 7-hydroxyl group may form hydrogen bonds to TcTS. Our SAR analyses indicated that compound **6** should be the minimal structure required for inhibitory activity among flavonoids.

Schinor et al. reported that compound **6** induced lysis of *T. cruzi* with the IC_{50} value of 0.68 mM.[26] Our findings suggested that such

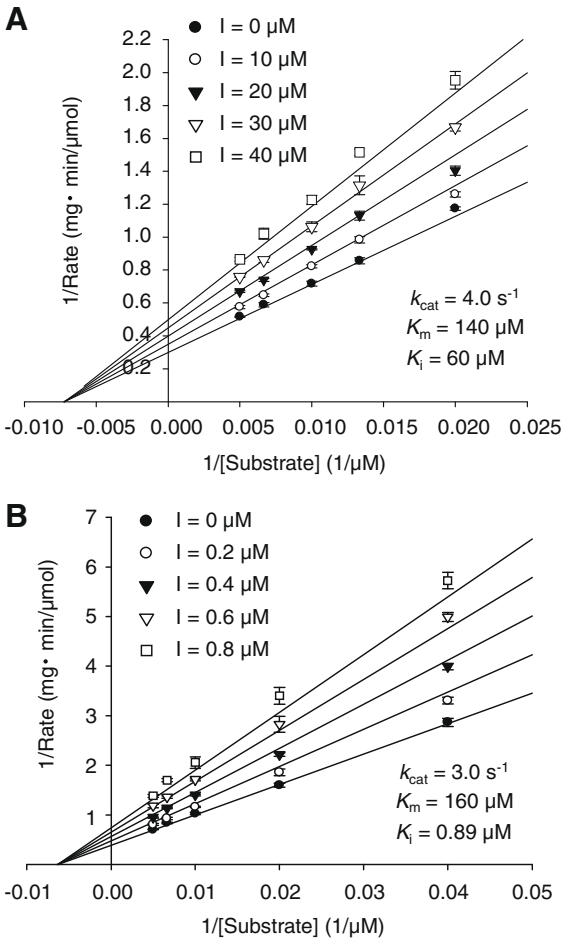


Figure 8. Kinetics evaluation of inhibitory mechanism of action. TcTS was kinetically analyzed as described in Section 5. Data points represent the average of triplicate experiments and bars indicate standard deviation. I and substrate in the figure represent inhibitor and MuNANA, respectively. The Lineweaver–Burk plots illustrate the noncompetitive inhibition mode of compound **6** with the K_i value of 60 μM (A), and the noncompetitive inhibition mode of compound **19** with the K_i value of 0.89 μM (B).

trypanocidal activity of compound **6** is due to its inhibitory action towards TcTS. More detailed studies on the trypanocidal activity of compound **6** should reveal the relationship between TcTS and mechanisms of action.

For anthraquinones, we compared five compounds. Compound **23** showed 24-fold decreased inhibitory activity compared with **19**, suggesting that the 6-chloro and 7-hydroxyl group in **19** interact with TcTS. Compound **22**, which lacks the carboxylic acid found in **23**, showed 50-fold lower inhibition compared with compound **23**. And, compound **21** lacking the 4,5-dihydroxy groups had 30-fold lower inhibitory activity than **23**. These results indicate that 2-carboxylic acid 4,5-dihydroxyl groups play important roles in inhibition of TcTS activity. Taken all together, compound **19** would represent the minimal structure required for inhibitory activity of anthraquinones.

Recently sulfonamide chalcones were reported to possess the highest inhibitory effect with IC_{50} values around one micromolar.[27] Considering that chalcones are precursor of flavonoids, a derivative of flavonoids could have possibility to yield more potent TcTS inhibitor.

By inspecting the TcTS structure (PDB code 1MS0),[28] we noticed that it has a relatively wide and shallow active site for substrate binding. It seems difficult for small molecules such as compounds **6** and **19** to bind firmly in a competitive manner. The fact that many noncompetitive inhibitors were obtained from our screening, supports this notion.

We have also been screening competitive inhibitors from sialic acid derivatives, however, strong inhibitors that can be considered as lead scaffolds have not been found to date (we will publish in future). Our success in finding TcTS inhibitors might be attributed to the fact that natural products cover a wide range of chemical territory compared with combinatorial libraries.[16] Most natural products are highly stereodiversified, which can mislead the SAR interpretations in designing derivatives.[17] In this study, merit of the designed derivatives of compounds **1** and **19** is clear because they do not have any chiral centers and bridgehead atoms. Although it should be noted that myricetin and some related compounds show significant cytotoxicity,[29,30] this feature may be the reasons for our successful SAR analyses and might be quite beneficial for further stage in drug discovery research.

4. Conclusion

In this study, we identified flavonoid and anthraquinone derivatives as potential TcTS inhibitors from a natural product library. Our SAR analyses revealed that compound **6**, known to have trypanocidal activity, involved the minimal and sufficient structure for the inhibition of TcTS among the flavonoids tested. Among the anthraquinones, compound **19** showed the strongest inhibitory activity against TcTS thus far found. Moreover, it did not inhibit Neu2, demonstrating that its inhibition is quite specific to TcTS. These compounds may become a promising novel class of TcTS inhibitors, though we must pay attention to further precise evaluation of the specificity of the inhibitory effect by compound **19** on various known bacterial and viral sialidases as well as TcTS.

5. Experimental

5.1. TcTS used for analyses

The structure of TcTS basically consists of three regions: an N-terminal catalytic domain, a central lectin-like domain and a C-terminal region with 12 amino acid repeats called the shed acute-phase antigen (SAPA repeat), which is linked to the membrane via the GPI anchor. Our final goal is structure-based drug design. Generally, a smaller protein is more feasible and easier to crystallize. We chose TcTS without the C-terminal SAPA repeat, which is not necessary for activity.[31]

The *T. cruzi* genome has several hundreds of TcTS genes.[32] Schenkman reported that the kinetic properties of recombinant TcTS had no major differences from kinetic parameters of the enzyme directly purified from parasites.[33] Therefore, recombinant TcTS could be used as a representative for parasitic TcTS, however, it is uncertain which mutant is suitable for screening inhibitors. Considering the capability of the structural approach, we chose a TcTS mutant registered to the PDB (PDB code 1MS0).[28]

5.2. Construction of TcTS and human Neu2 expression plasmid

Plasmid expressing TcTS was kindly provided by Professor José Osvaldo Previato (Cidade Universitaria, Brazil). Sequence analysis of the open reading frame of TcTS revealed that it had SAPA repeats at the C-terminus and eight mutations in both the catalytic domain (N58F, R200 K) and the lectin-like domain (S495 K, V496G, E520 K, D593G, I597D, H599R) compared with the objective sequence. To prepare TcTS, the PCR-base two-step DNA synthesis (PTDS) method[34,35] was used because site-directed mutagenesis is very laborious and time-consuming for multiple mutations. In the first PCR,

six DNA fragments were amplified by using 12 oligonucleotide primers (see below) which were designed to cover the five regions with objective mutations. The following primer combinations were used in the first PCR: TcTSNhe1 (GGTATGGCTAGCCTGGCACCCG GATCGAGCCG) and TcTSR1 (CAATGAGGGAGTTGTCAAAGGATGTTT CGTAGC), TcTSF1 (GCTACGAAACATCCTTTGACAACTCCCTCATTG) and TcTSR2 (GATCTTGGAAAAAACTTGCTTTTTTTTGTTCGTAACCTGC ACAGGG), TcTSF2 (CCCTGTGCAGGTTACGAACAAAAAAAAGCAAG TTTTTTCCCA AGATC) and TcTR3 (CGATTGACGAGGTTCCGAAAGGC GCGAGTCCTTTGCTGGG), TcTSF3 (CCCAGCAAAGGACTCGCGCCTTT CGGAACCTCGTGAATCG) and TcTSR4 (GCTGCCACTGGTGCCTTTTGT CGTACGAGAGCCCC), TcTSF4 (GGGGCTCTCGTAGGACAAAAGGCAC CAGTGGCAGC) and TcTSR5 (GTAAAGAAGAACATTATTCACCGT CACACGGCTATCGGTTGGCATGCCACTCCTTTTATACCCGCC), TcTSF5 (GGCGGGTATAAAAGGAGTGGCATGCCAACCGATAGCCGTGTGACGG TGAATAATGTTCTTCTTTAC) and TcTSRXbaI (CTATCTAGATTAATCCA TGTGTGCTTCCGTGCCAATCAGG). One percent agarose gel electrophoresis was conducted to ensure the precise extension. Six fragments, in which each of them has complement sequences, extracted from agarose gel were mixed and joined together in the second PCR to synthesize full-length of 1905 bp TcTS. After digesting with *Nhe*I and *Xba*I (Takara bio, Japan), the full-length TcTS gene was cloned into *NheI*/*Xba*I restriction sites of pTrcHisA vector (Invitrogen, USA) to generate pTrcHisA-TcTS and then sequenced with a DNA sequencer (Applied BioSystems, USA). All molecular cloning was performed according to Molecular Cloning third Edition.[36] The open reading frame of the human neuraminidase 2 (NEU2) gene was amplified from the plasmid (Open Biosystems) of the National Institutes of Health mammalian gene collection clone (clone ID: 40004620) by using primers 5HsNEU2-Nde (5′-GCCCATATGGCGTCCCTTCCTGTC-3′) and 3HsNEU2-Bam (5′-CGGGGATCCTCACTGAGGCAGGTA-3′). After digesting with *Nde*I and *Bam*HI, the amplified fragment was cloned into *Nde*I/*Bam*HI restriction sites of the pET-15b vector (Novagen) to generate pET15b-HsNEU2. This plasmid expresses the Neu2 protein tagged with six histidine consecutive residues followed by a thrombin recognition site at the N-terminus.

5.3. Expression and purification of enzyme

Competent *E. coli* JM109 (Promega, UK) cells were transformed with pTrcHisA-TcTS. The transformant was cultured in TB at 37 °C to midlog phase before the addition of isopropyl-α-D-thiogalactopyranoside (IPTG) (Sigma, USA) to the final concentration of 0.2 mM. The induction was maintained at 20 °C for 16–18 h. Cells were harvested by centrifugation at 5000 *g* for 10 min and frozen at −80 °C until they were used. After thawing, the pellet was resuspend in buffer A (50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.6), and cells were lysed using Microfluidizer processor M-110EH (Microfluidics, USA). The supernatant obtained by centrifugation at 45,000 *g* for 60 min was applied to 20 ml SP Sepharose FF resin (GE Healthcare, USA). The protein was eluted with a linear NaCl gradient from 0 M to 0.5 M in buffer A. The active fraction was directly applied to 5 ml His-Trap HP column (GE Healthcare, USA). The protein was eluted with a linear imidazole gradient from 0 M to 0.5 M. The active fraction had been diluted with buffer A and applied to Source 15S PE 4.6/100 (GE Healthcare, USA). The protein was eluted with a linear NaCl gradient from 0 M to 0.5 M in buffer A. The active fraction was concentrated to 1 ml by using Amicon Ultra-15 with ultrafiltration membrane (10 kDa, Millipore, USA) and applied to HiLoad 16/60 Superdex 200 pg (GE Healthcare, USA). The active fraction was pooled and analyzed for purity using SDS–PAGE stained with Quick-CBB (Wako, Japan) and the concentration was determined with BCA protein assay reagent (Thermo Scientific, USA) by using BSA as a standard. One litter of culture yielded 21 mg of highly purified homogeneous TcTS.

Competent *E. coli* BL21(DE3) cells were transformed with, pET15b-HsNEU2 and the transformed cells were grown in 2×YT-ampicillin medium at 37 °C to midlog phase before the addition of IPTG to 0.5 mM. After growth at 25 °C for 12 h, cells were harvested and lysed by sonication. The supernatant obtained after centrifugation at 12,000 *g* for 15 min was subjected to further purification using DEAE FF and His-Trap HP columns (GE Healthcare, USA).

5.4. Compounds and reagents

Unless otherwise stated, all commercially available solvents and reagents were obtained from Wako Pure Chemical Industry Co., Ltd and used without purification. A total of 2283 purified natural organic products were supplied in DMSO from Shionogi & Co., Ltd. Compounds **1**, **4**, **8** and **23** were purchased from Sigma, Specs i.s.o. SPECS and BioSPECS, Alfa Aesar, and BIOMOL, respectively. Compounds **2**, **10**, **17**, **20**, and **21** were obtained from Tokyo Chemical Industry Co., Ltd. Compounds **7** and **22** were from Acros Organics. Compounds **9** and **16** were purchased from Indofine chemical company, Ltd. Compounds **12**, **14**, **15**, and **18** were obtained from Nakalai tesque. Compounds **3**, **5**, **6**, **10**, **11**, and **13** were from Wako Pure Chemical Industry Co., Ltd.

5.5. Enzyme assay

The activity assay was conducted by the discontinuous fluorometric method using 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MuNANA) (Nakalai tesque, Japan) as a substrate. All fluorescence measurements were performed on EnVision plate reader (Perkin Elmer, USA) by using black flat-bottomed 384-well plates (Greiner, Germany). Reaction solutions were dispensed with Cybi®-well 96-channel simultaneous pipettor (Cybio, Germany). Hydrolysis assay for screening of inhibitors was conducted in 50 mM Tris–HCl pH 7.5, 0.01% or 0.1% Triton X-100, 50 μM MuNANA, 10% DMSO, and 8 ng/ml TcTS at 25 °C. We adopted a low concentration of MuNANA so as not to miss weak inhibitors (K_m = 78 μM). After the incubation of 30 min, the reaction was quenched by adding 50 μl of 0.2 M Na_2CO_3 solution (pH 10). The fluorescence of the released product 4-methylumbelliferone (Mu) was monitored with excitation and emission filters of 355 nm and 460 nm, respectively. Initial velocity constants were assessed by the amount of products Mu and measured in two or three replicates. The raw inhibition values are supposed to include two values derived from enzyme inhibition and fluorescence interference which is seen in several compounds at a high concentration, then we corrected for fluorescence interference by using the tested compounds with the method reported previously.[37] Evaluation of this assay system constantly showed a Z' value of over 0.5. It was considered reliable and was used for screening inhibitors.[38] The same method was used to examine Neu2 activity. The velocity versus MuNANA concentration profile obeyed Michaelis–Menten kinetics with K_m = 0.38 mM in Neu2 (data not shown). The hydrolysis assay for screening inhibitor was conducted in 50 mM Tris–HCl pH 7.5, 0.1% Triton X-100, 10% DMSO and 0.2 mM MuNANA at 25 °C. The IC_{50} value was calculated by fitting experimental data to the equation in GraphPad Software (GraphPad Software Inc., USA); $(Y = \text{bottom} + (\text{top}-\text{bottom})/(1 + 10^{((\text{Log}IC_{50}-X)\times \text{Hillslope})})$ where X is the logarithm of concentration of each compound, and Y is the percent activity. Y starts at the bottom and goes to the top with a sigmoidal shape. The experiments for K_i determination were performed in 25–200 μM MuNANA concentrations and several concentrations of inhibitor. Unbiased assignments of the best-fit inhibitory mode of each compound were conducted using the SigmaPlot Software (Systat Inc., USA) and the K_i value for noncompetitive inhibition was derived from the Lineweaver–Burk (double reciprocal) plot.

5.6. Dynamic light scattering assay

Inhibitors were delivered as concentrated DMSO stocks and diluted to 200 μM with filtered enzyme assay buffer: 50 mM Tris–HCl, pH 7.5, 0.01% or 0.1% Triton X-100. Measurements were done using a Zeta sizer nano (Malvern, UK) with 4 mW laser at 663 nm. The detector angle was 173°. The derived counter rate (kilocounts per second) was represented by an average of three independent measurements for 10 s at 25 °C and used as the index of compound aggregation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.12.062.

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