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Flavonoid Dimers as Novel, Potent Antileishmanial Agents

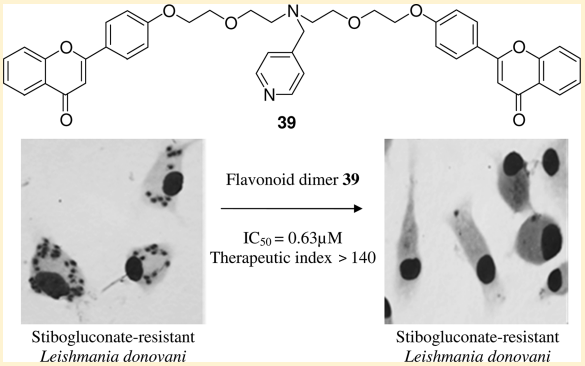
Iris L. K. Wong,[§,†] Kin-Fai Chan,[§,†] Tak Hang Chan,[*,†,‡] and Larry M. C. Chow[*,†]

[†]Department of Applied Biology and Chemical Technology and the State Key Laboratory for Chirosciences, The Hong Kong Polytechnic University, Hung Hom, Hong Kong SAR, China, and State Key Laboratory in Chinese Medicine and Molecular Pharmacology, Shenzhen, China

[‡]Department of Chemistry, McGill University, Montreal, Quebec, H3A 2K6 Canada

Supporting Information

ABSTRACT: The present study found that synthetic flavonoid dimers with either polyethylene glycol linker or amino ethyleneglycol linker have marked leishmanicidal activity. Compound **39** showed very consistent and promising leishmanicidal activity for both extracellular promastigotes (IC_{50} ranging from 0.13 to 0.21 μM) and intracellular amastigotes (IC_{50} = 0.63 μM) irrespective of the drug-sensitivity of parasites. Moreover, compound **39** displayed no toxicity toward macrophage RAW 264.7 cells (IC_{50} > 100 μM) and primary mouse peritoneal elicited macrophages (IC_{50} > 88 μM). Its high value of therapeutic index (>140) was better than other highly potent antileishmanials such as amphotericin B (therapeutic index = 119). Compound **39** is therefore a new, safe, and effective antileishmanial candidate compound which is even effective against drug-refractory parasites.



INTRODUCTION

Leishmaniasis is one of the six major parasitic diseases targeted by the World Health Organization. It is endemic in 88 countries. More than 350 million people worldwide are at risk of infection. Out of the annual 2 million cases, 500 000 of them belong to visceral leishmaniasis (VL). VL is caused by *Leishmania donovani* amastigotes infecting macrophages in a patient's reticuloendothelial organs such as liver and spleen. VL could be fatal if left untreated.[1] Currently, there are no effective vaccines for leishmaniasis. Primary means of chemotherapy include pentavalent antimonials (Sb^V) which is a decade-old drug. Antimonial treatment, however, is less than ideal due to the difficulty in administration (slow intravenous injection), severe side effects, poor patient compliance, and emergence of antimonials-resistant cases. Newer antileishmanials include amphotericin B (with or without liposomal formulations), miltefosine, and paromomycin. All of them, however, have different types of limitations including nephrotoxicity, incomplete efficacy, and expensive or inconvenient treatment protocol. There is an urgent need to speed up the development of a new generation of more effective and safe antileishmanials.

Recent findings suggested that plant-derived products have potent and selective antiparasitic properties.[2] Among these plant-derived products, flavonoids represent a large family of polyphenolic compounds found in vegetables and fruits. As humans consume large amounts of flavonoids every day, it is generally accepted that flavonoids are safe and not toxic. The most common flavonoids are flavone and isoflavone.[3] Naturally occurring flavones have been reported to have potent leishmanicidal activity.[4] We have previously synthesized and characterized a series of flavonoid dimers, as illustrated in Chart 1, to be used in reversing drug resistance in cancer and *Leishmania* by inhibiting membrane transporters.[5−10] In the course of the investigation, it became necessary to assess the *in vitro* leishmanicidal activity of these compounds and draw structure−activity relationships, if possible.

In the present study, we have selected structurally related flavonoid dimers to assess their antileishmanial activity by screening against insect stage parasites (promastigotes) as well as measuring their toxicity toward mouse macrophage cell line. Compounds with promising antipromastigotes activity and low toxicity were then screened against human host stage (amastigotes). At the same time, medicinal chemistry was carried out to further modify structure of promising lead compounds to examine structure−activity relationships. Through these processes, we have discovered novel compounds with potent antileishmanial activity and high therapeutic index which was comparable or superior to other current antileishmanials.

RESULTS

***In Vitro* Antipromastigote Activity of Flavonoid Dimers Reported Previously.** *A. Polyethylene Glycol (PEG) Linked Flavonoid Dimers **1**−**30**.* We investigated if synthetic flavonoid dimers **1**−**30** have any antipromastigotes activity toward *L. donovani* (wild-type LdAG83, sodium stibogluconate (SSG)-resistant Ld39 and pentamidine-resistant LdAG83PentR50)

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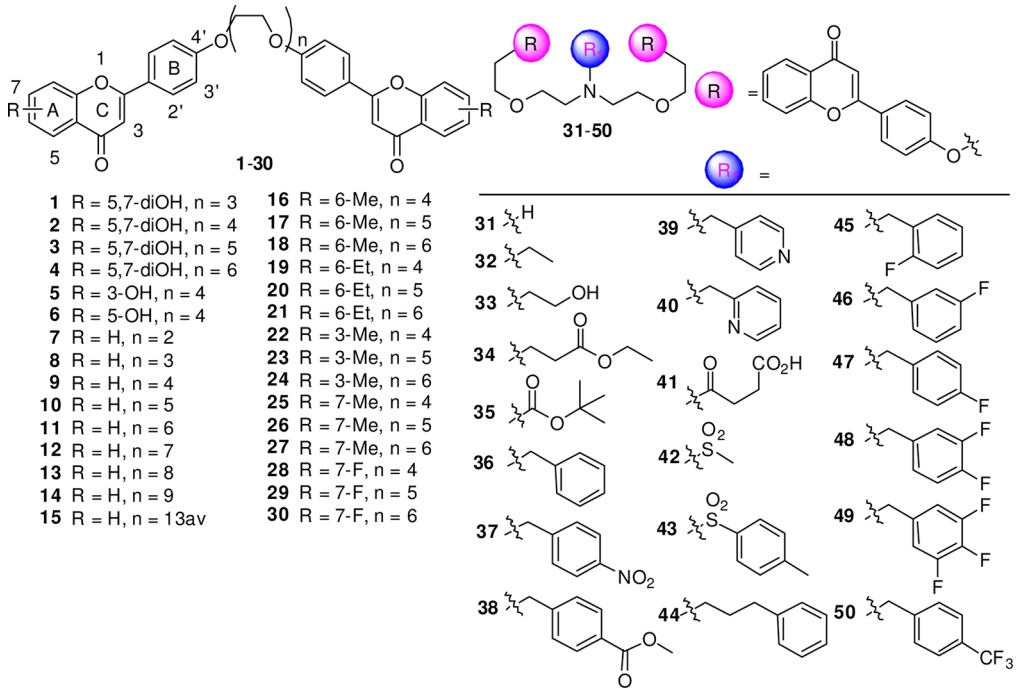
Chart 1. Chemical Structures of Previously Reported Flavonoid Dimers 1−50[a]



[a]PEG 600 with average number of EG unit of 13 was used to synthesize compound **15**.

promastigotes. Cytotoxicity toward mouse macrophage cell line RAW 264.7 was also determined as an indicator for the toxicity of flavonoid dimers. Flavonoid dimers **1−30** contained two flavonoid moieties linked together by a PEG linker. Structural differences of these flavonoid dimers included different substituents in the flavone ring A and C as well as different number of ethylene glycol (EG) units.

As shown in Table 1, all flavonoid dimers in series **A** contain OH groups in the ring A or ring C with linker lengths varied from 3 to 6 EG units. Compounds **1−3** with 3−5 EG units displayed antipromastigotes activity toward both drug sensitive and resistant *L. donovani* at micromolar level. Their IC_{50} ranges from 1.8 to 6.7 μM. No cytotoxicity was observed toward the macrophage RAW 264.7 cells (IC_{50} > 200 μM). Compound **4** with 6 EG units was less effective in killing wild-type and pentamidine-resistant LdAG83 promastigotes. Compound **5** with hydroxyl group at C-3 position of C ring or compound **6** with hydroxyl group at C-5 position of A ring did not have significant antipromastigotes activity.

Flavonoid dimers in series **B** have all hydroxyl groups removed from A ring. As shown in Table 1, flavonoid dimers with shorter linker length (compounds **7−9** with 2−4 EG units) displayed no significant antipromastigotes activity and cytotoxicity toward RAW 264.7 macrophages. On the other hand, dimers with longer linker length (compounds **10−15** with 5−13av EG units) showed antipromastigotes activity toward *Leishmania* promastigotes as well as cytotoxicity toward RAW 264.7 macrophages with IC_{50} smaller than 11 μM. Thus, removal of hydroxyl groups from A ring and/or having linker length longer than 5 EG units seems not favorable.

In flavonoid dimers series **C** of Table 1, hydrophobic substitutions at 3-, 6-, or 7-position, such as methyl group (compounds **16−18**, **22−27**), ethyl group (compounds **19−21**), or fluoro group (compound **28−30**) were introduced into ring A or C. Compounds **20**, **26**, **28**, and **29** showed potent antipromastigotes activity (IC_{50} from 2.3 to 7.9 μM) without cytotoxicity toward macrophage RAW 264.7 (IC_{50} higher than 80 μM). Some structural modifications, particularly with methyl groups at the 3-position of ring C (compounds **22−24**), resulted in cytotoxic effect on both *Leishmania* promastigotes and RAW 264.7 macrophages.

Although some flavonoid dimers listed in Table 1 have significant antipromastigotes activity without cytotoxicity toward macrophage RAW 264.7 cells, these compounds are too hydrophobic and are only sparingly soluble in aqueous medium. Attempts to use some of these compounds in *in vivo* animal experiments proved to be impractical.[5] In order to improve their physicochemical properties as potential drug candidates, we have to introduce some hydrophilic groups into the structure.

B. Amine-Linked Flavonoid Dimers. We have previously reported the synthesis of a new class of flavonoid dimers containing 4 EG units with an amine group in the middle of the linker.[10] The amino group should confer better aqueous solubility and thus better physicochemical properties. Compounds **31−50**, with different substituents on the amine nitrogen, were tested for their antipromastigotes activity (Table 2). Compound **31**, with R = H, was cytotoxic to both promastigotes and macrophage RAW 264.7 cells with IC_{50} value 1.1−3.3 μM, respectively. Comparing compound **31** with **9**, it is clear that the replacement of central oxygen by an amine in the linker changed the activity dramatically. Replacing the hydrogen with an ethyl group (compound **32**), hydroxyethyl group (compound **33**), or ethyl propanoate group (compound **34**) on the amine group did not relieve toxicity toward macrophage RAW 264.7 cells (IC_{50} for RAW 264.7 = 6.4−16.0 μM).

Interestingly, when bulkier R groups were introduced to the amine nitrogen, the flavonoid dimers thus generated (compound **35−44**) were generally nontoxic to RAW 264.7 cells with IC_{50} values ranging from 45.7 μM to greater than 100 μM (Table 2). For example, the *tert*-butyloxycarbonyl (Boc) group (compound

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Table 1. Antipromastigotes Activity of Synthetic Flavonoid Dimers with PEG Linkers[a]

	IC_{50} (μM)			
	promastigotes			macrophages
compd[b]	LdAG83	Ld39	LdAG83 PentR50	RAW 264.7
	Series A			
1	5.6 ± 3.8	2.7 ± 0.3	6.7 ± 2.2	>200.0
2	2.2 ± 0.7	1.9 ± 0.7	3.1 ± 1.4	>200.0
3	2.3 ± 0.7	1.8 ± 0.5	2.6 ± 1.7	>200.0
4	>24.0	1.4	11.8	ND
5	>24.0	>24.0	ND	ND
6	>24.0	>24.0	ND	ND
	Series B			
7	>24.0	>24.0	>24.0	>100.0
8	>24.0	>24.0	>24.0	>95.0
9	>24.0	>24.0	>24.0	>100.0
10	5.3 ± 0.3	7.8 ± 1.5	10.4 ± 1.4	8.9 ± 2.3
11	4.8 ± 0.7	4.3 ± 0.5	4.9 ± 0.7	7.1 ± 1.2
12	6.2 ± 1.3	10.3 ± 0.4	7.3 ± 2.0	4.8 ± 0.3
13	5.0 ± 0.2	4.6 ± 0.4	6.0 ± 2.0	4.4 ± 0.4
14	9.4 ± 0.7	8.6 ± 0.4	10.6	8.1 ± 1.2
15	9.2 ± 0.8	8.2 ± 1.0	7.9 ± 2.8	8.4 ± 2.4
	Series C			
16	>24.0	>24.0	>24.0	>100.0
17	>24.0	4.3 ± 1.3	>24.0	>100.0
18	2.2 ± 0.3	3.6 ± 1.3	>24.0	>100.0
19	>24.0	>24.0	>24.0	>100.0
20	4.7 ± 0.3	4.6 ± 0.4	6.3 ± 2.4	>200.0
21	3.1 ± 0.4	2.5 ± 0.2	3.3 ± 0.8	5.5 ± 0.7
22	6.9 ± 1.4	>19.0	>20.0	8.1 ± 0.6
23	4.8 ± 1.7	4.2 ± 0.9	4.6 ± 2.1	3.1 ± 1.3
24	1.9 ± 0.3	2.8 ± 0.4	2.5 ± 0.3	2.3 ± 0.4
25	>24.0	>24.0	>24.0	88.6 ± 4.5
26	4.4 ± 0.6	7.9 ± 3.3	3.8 ± 0.3	>80.0
27	6.7 ± 1.1	5.3 ± 1.1	4.1 ± 0.7	5.3 ± 2.1
28	2.6 ± 0.5	2.4 ± 0.6	2.3 ± 0.5	>200.0
29	4.1 ± 0.6	4.8 ± 0.6	3.7 ± 0.9	>100.0
30	4.2 ± 0.7	4.2 ± 0.3	4.6 ± 1.4	7.8 ± 1.1

[a]Synthetic flavonoid dimers were divided into three series (**A**–**C**) with various linker lengths containing different EG units (n). Substitutions were made at A-ring of flavone. IC_{50} values for antipromastigotes activity towards LdAG83, Ld39, and LdAG83PentR50 were shown. Toxicity towards macrophage RAW 264.7 cell line was also included. The values were presented as mean ± standard error of mean. N = 1–4 independent experiments. ND = not determined. [b]All compounds were dissolved in DMSO, and the highest % of DMSO used was 1% at which no toxicity to promastigotes and RAW 264.7 was observed.

35) displayed a marked antipromastigotes activity without toxicity toward RAW 264.7 cells. Introduction of a benzyl group (compound **36**) into the amino linker generated a compound with low antipromastigotes activity without any toxic effect to RAW 264.7 cells. However, when the benzyl group contained polar nitro group (compound **37**) or carboxylic ester group (compound **38**) at C-4 position, the antipromastigotes activity was completely lost. In addition, the benzyl group containing a fluorine atom at C-2 (compound **45**), C-3 (compound **46**), C-4 (compound **47**) positions or two fluorine atoms at C-3 and C-4 (compound **48**) positions also resulted in no improved antipromastigotes activity. Nevertheless, a benzyl group with three fluorine atoms (compound **49**) or a trifluoromethyl group at C-4 position (compound **50**) improved antipromastigotes activity slightly when compared to compounds **45**–**48**. Placing the phenyl group further away from the nitrogen (compound **44**) resulted in low antipromastigotes activity similar to that of compound **36**.

Flavonoid dimers **39** and **40** are of particular interest. Introduction of a pyridine ring as part of R resulted in a very strong selective antipromastigotes activity. Compound **39** with nitrogen on position 4 of the pyridine ring has the highest antipromastigotes activity among all flavonoid dimers. No toxicity to the RAW 264.7 cells was observed. Compound **40**, containing nitrogen at position 2 of the pyridine ring, displayed about 20-fold lower antipromastigotes activity than compound **39**, indicating that the position of nitrogen atom on the pyridine ring is critical in antipromastigotes activity. Flavonoid dimer with *N*-mesylate group (compound **42**) also displayed a significant antipromastigotes activity. Interestingly, replacing the *N*-mesylate with *N*-tosylate (compound **43**) resulted in almost complete loss of antipromastigote activity with IC_{50} values greater than 100 μM. Similarly, a succinamide group (compound **41**) gave poor activities.

Design, Synthesis, and Biological Activity of New Flavonoid Dimers Based on Compound 39. *A. Chemistry.* Compound **39** was found to display promising antipromastigotes activity. Using compound **39** as a template, we synthesized a series of flavonoid dimer to study structure–activity relationships. Structural modifications were made by (1) shortening the linker length, (2) changing the attachment position of the two flavones to the amino PEG linker, (3) modifying the two flavones with different substitutions, and (4) further modifying pyridine ring at the amino PEG linker. As shown in Scheme 1, selective alkylation of amino group of compound **51** furnished compound **52**, which was then subjected to Mitsunobu reaction by using 2 equiv of 4′-hydroxyflavone, triphenyl phosphine, and diisopropyl azodicarboxylate (DIAD) in tetrahydrofuran (THF). Compound **53** with shorter linker length was obtained in reasonable yield. Similarly, by selective alkylation of amino group of compound **54** followed by Mitsunobu reaction with different hydroxyflavones, compounds **56**–**61** were furnished in good yield (Scheme 2). With the same chemistry but varying the pyridyl methyl halides used in the selective alkylation of amino group of compound **54**, followed by Mitsunobu reaction with 4′-hydroxyflavones, compounds **68**–**73** were furnished in good yield (Scheme 3).

B. In Vitro Antipromastigote Activity. As illustrated in Table 2, compound **53** (with shorter amino PEG linker than compound **39**) has lost the antipromastigotes activity against SSG-resistant Ld39. This result reiterated the importance of PEG linker length in antipromastigotes activity of flavonoid dimers. Attachment position of the two flavones to the amino PEG linker is also important. Attachment at C-3′ position of B-ring (compound **56**) did not change the antipromastigotes activity significantly compared to compound **39** which has linker attached at C-4′ position. However, attachment at C-2′ position of B-ring (compound **57**) or C-3 position of C-ring (compound **58**) resulted in at least 10-fold reduction in antipromastigotes activity as compared to parent compound **39**.

C3′-methoxy substitution in the B-ring (compound **59**) caused a remarkable cytotoxicity toward both promastigotes and host peritoneal elicited macrophage (PEM) cells. On the contrary, C3-methoxy substitution in the C-ring (compound **60**) or addition of a fluorine atom at C-6 position of A-ring (compound **61**) did not cause any toxic effect toward the PEM cells and retained the promising antipromastigotes activity.

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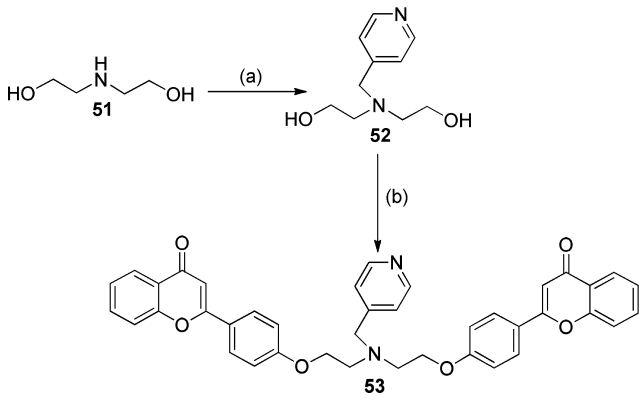
Table 2. Antipromastigotes Activities of Synthetic Flavonoid Dimers with Amino PEG Linkers[a]

	IC_{50} (μM)				
	promastigotes			macrophages	
compd[b]	LdAG83	Ld39	LdAG83PentR50	RAW 264.7	PEM
31	3.3	2.3	1.1	2.1 ± 0.9	ND
32	3.2 ± 0.8	5.5 ± 1.1	ND	8.3 ± 1.0	ND
33	3.2 ± 1.1	7.2 ± 2.2	ND	6.4 ± 1.5	ND
34	3.8 ± 0.8	6.6 ± 0.2	ND	16.0 ± 6.2	ND
35	3.2	1.6	1.1	>100.0	ND
36	8.4 ± 6.1	12.3 ± 6.0	ND	>79.0	ND
37	>100.0	>100.0	ND	70.3 ± 9.7	ND
38	>100.0	>100.0	ND	45.7 ± 19.1	ND
39	0.20 ± 0.03	0.21 ± 0.06	0.13 ± 0.04	>100.0	>88.0
40	5.0 ± 1.5	4.2 ± 1.5	ND	53.0 ± 12.7	>33.0
41	>100.0	>100.0	27.0	65.0	ND
42	0.75 ± 0.25	0.90 ± 0.60	ND	96.0 ± 4.0	ND
43	100.0	>100.0	ND	>100.0	ND
44	8.9	18.4	ND	85.0	ND
45	>100.0	ND	ND	ND	ND
46	>100.0	ND	ND	ND	ND
47	>100.0	ND	ND	ND	ND
48	>100.0	ND	ND	ND	ND
49	33.3	ND	ND	ND	ND
50	14.1	ND	ND	ND	ND
53	1.4 ± 0.3	>50.0	ND	ND	>58.0
56	0.19 ± 0.02	0.44 ± 1.04	ND	ND	>50.0
57	2.3 ± 0.2	4.0 ± 1.1	ND	ND	>11.0
58	2.5 ± 0.3	3.1 ± 0.7	ND	ND	>33.0
59	0.63 ± 0.14	0.69 ± 0.25	ND	ND	2.7 ± 0.3
60	0.98 ± 0.10	0.98 ± 0.17	ND	ND	>50.0
61	0.31 ± 0.03	0.36 ± 0.07	ND	ND	>100.0
68	0.56 ± 0.07	0.98 ± 0.19	ND	ND	33.1 ± 18.9
69	1.8 ± 0.5	>50.0	ND	ND	>100.0
70	1.1 ± 0.1	>50.0	ND	ND	>92.0
71	0.65 ± 0.07	>50.0	ND	ND	>100.0
72	0.44 ± 0.05	0.58 ± 0.11	ND	ND	>33.0
73	20.4 ± 8.8	ND	ND	ND	ND

[a]Flavonoid dimers containing amino EG linker (compounds **31**–**73**) with different substitutions on the linker were synthesized and tested for their cytotoxicity towards promastigotes (LdAG83, Ld39, and LdAG83PentR50) and RAW 264.7 cells. IC_{50} values were presented as mean ± standard error of mean. N = 1–4 independent experiments. ND = not determined. [b]All compounds were dissolved in DMSO, and the highest percentage of DMSO used was 1% at which no toxicity to promastigotes and RAW 264.7 macrophages was observed.

Scheme 1. Synthesis of Compound 53[a]



[a]Reagents and conditions: (a) 4-(chloromethyl)pyridine hydrochloride, K_2CO_3, acetonitrile, reflux, 3 h; (b) 4′-hydroxyflavone, DIAD, PPh_3, THF, reflux, 12 h.

We tried to modify the pyridine ring on the amino linker in order to improve the potency of compound **39** (Table 2). First, we changed the position of the nitrogen atom in the pyridine ring and found that the rank order of antipromastigotes activities was as follows: *para*-position (compound **39**; IC_{50} = 0.20 μM against LdAG83) > *meta*-position (compound **68**; IC_{50} = 0.56 μM) ≫ *ortho*-position (compound **40**; IC_{50} = 5.0 μM). Second, a bromo substitution at *meta*-position (compound **69**) or *ortho*-position (compound **71**) and a cyano group at *meta*-position (compound **70**) of the pyridine ring reduced the antipromastigotes activity, especially in the Ld39 promastigotes. Third, replacement of pyridine ring by pyrimidine ring (compound **72**) exhibited at least 2-fold lower antipromastigotes activity as compared to the parent compound **39**. Finally, compound **73** completely lost the antipromastigotes activity probably due to bulkiness of quinidine ring. Among all the flavonoid dimers, **39** has the strongest antipromastigotes activity (IC_{50} ranging from 0.13 to 0.21 μM against LdAG83, Ld39, and LdAG83PentR50) and very low toxicity toward RAW 264.7 cells (IC_{50} > 100 μM). Importantly,

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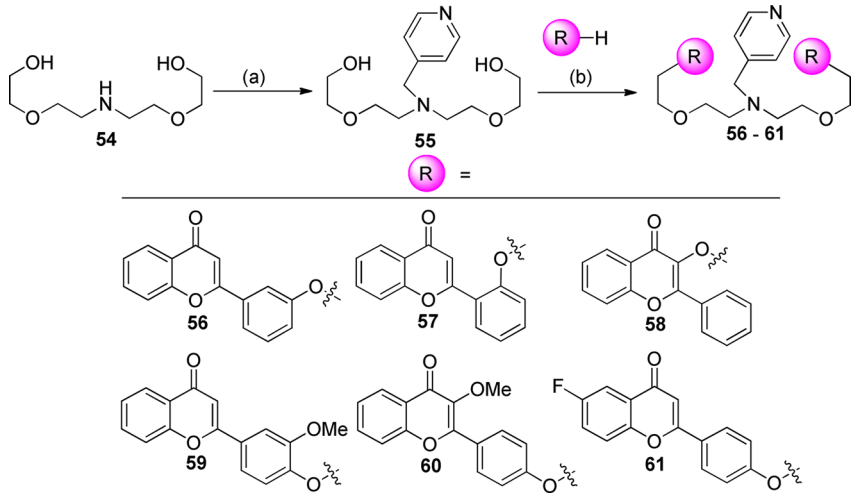
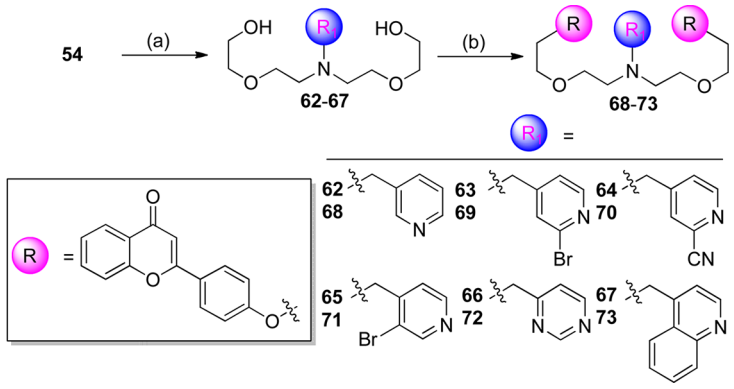
Scheme 2. Synthesis of Compounds 56−61[a]



[a]Reagents and conditions: (a) 4-(chloromethyl)pyridine hydrochloride, K_2CO_3, acetonitrile, reflux, 3 h; (b) corresponding hydroxyflavone, DIAD, PPh_3, THF, reflux, 12 h.

Scheme 3. Synthesis of Compounds 68−73[a]



[a]Reagents and conditions: (a) for compounds **62−67**, RX, K_2CO_3, acetone, reflux, 3 h; (b) for compounds **68−73**, 4′-hydroxyflavone, DIAD, PPh_3, THF, reflux, 12 h.

Table 3. Antipromastigotes Activities and Therapeutic Index of Standard Antileishmanials Compared to Flavonoid Dimer 39[a]

	IC_{50} (μM)					therapeutic index		
	promastigotes			macrophages		promastigotes		
compd	LdAG83	Ld39	LdAG83PentR50	RAW 264.7	PEM	LdAG83	Ld39	LdAG83PentR50
SSG[b]	2215.5 ± 166.2	7089.6 ± 747	ND	>11 000	>11 000	>5.0	>1.6	ND
pentamidine[b]	13.9 ± 2.1	5.3 ± 0.8	54.8 ± 9.5	30.0 ± 5.0	30.4 ± 10.5	2.2	5.7	0.6
amphotericin B[c]	0.09 ± 0.02	0.095 ± 0.02	0.051 ± 0.00	12.0 ± 2.5	7.40 ± 0.40	82.2	77.9	145.1
miltefosine[b]	4.3 ± 1.1	17.3 ± 2.1	9.3 ± 2.4	20.0 ± 4	75.3 ± 9.4	17.5	4.4	8.1
paromomycin[b]	62.6 ± 9.5	24.5 ± 2.4	85.5 ± 14	>100	>100	>1.6	>4.1	>1.2
luteolin[c]	114.3 ± 20.6	89.0 ± 7.8	90.9 ± 17	43.2 ± 8.0	77.7 ± 7.7	0.7	0.9	0.9
quercetin[c]	102.9 ± 9.0	>100.0	69.6 ± 10.8	95.6 ± 2.8	>100	>1.0	>1.0	>1.4
39[c]	0.2 ± 0.03	0.21 ± 0.06	0.13 ± 0	>100	>88.0	>440	>419	>677

[a]IC_{50} values of current antileishmanials and flavonoid dimer **39** towards promastigotes (LdAG83, Ld39 and LdAG83PentR50) and macrophages (RAW 264.7 and PEM) were determined. IC_{50} values were presented as mean ± standard error of mean. N = 1-4 independent experiments. Therapeutic index was defined as the ratio of IC_{50} of antileishmanials towards PEM over promastigotes (LdAG83, Ld39 and LdAG83PentR50). ND = not determined. [b]Compounds were dissolved in sterile H_2O. [c]Compounds were dissolved in DMSO. No toxicity to the PEM cells was observed at 1% DMSO.

compound **39** was effective against SSG-resistant Ld39 and pentamidine-resistant LdAG83PentR50.

Comparison of Antipromastigotes Activity of 39 with Other Antileishmanial Agents. We have compared **39** with other current antileishmanials, namely, SSG, pentamidine, miltefosine, paromomycin, and amphotericin B (Table 3). SSG has the lowest antipromastigotes activity against LdAG83 (IC_{50} = 2216 μM) and was essentially ineffective against SSG-resistant

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Ld39 (IC_{50} = 7090 μM). Second-line antileishmanials like pentamidine and miltefosine displayed moderate antipromastigote activity (IC_{50} values ranging from 5.3 to 54.8 μM and 4.3 to 17.3 μM, respectively) and moderate cytotoxicity toward macrophage RAW 264.7 cell line (IC_{50} values of 30.0 μM and 20.0 μM, respectively). As expected, pentamidine was less effective toward pentamidine-selected strain, LdAG83PentR50, with IC_{50} 3.9-fold higher than that of wild type LdAG83. Paromomycin also displayed a poor antipromastigotes activity (IC_{50} ranging from 24.5 to 85.5 μM) although it is nontoxic to RAW 264.7 (IC_{50} > 100 μM). Amphotericin B has the highest antipromastigotes activity (IC_{50} values ranging from 0.051 to 0.095 μM). However, it was also the most toxic compound for RAW 264.7 cells (IC_{50} of 12.0 μM) (Table 3). The flavonoids luteolin and quercetin have a low antipromastigotes activity with IC_{50} ranging from 70 to 114 μM (Table 3). In comparison, compound **39** has very potent antipromastigotes activity with IC_{50} ranging from 0.13 to 0.21 μM, which is comparable to that of the most potent antileishmanial, amphotericin B (Table 3). Importantly, compound **39** was not toxic to RAW 264.7 and PEM cells (IC_{50} > 100 μM and >88 μM, respectively) (Table 3).

We have also determined the therapeutic index (ratio of IC_{50} against PEM cells to IC_{50} against promastigotes). A therapeutic index less than 10 would indicate probable nonselective cytotoxicity for the tested compounds.[11] As shown in Table 3, compound **39** has the highest therapeutic index (from greater than 419 to greater than 677), followed by amphotericin B (77.9−145.1). Other compounds displayed a significantly lower therapeutic index ranging from 0.6 to 17.5.

***In Vitro* Antiamastigotes Activity of Flavonoid Dimer 39 and Its Derivatives.** Here, we tested if compound **39** and its derivatives have antiamastigotes activity. Intracellular amastigotes of Ld39 were obtained by infecting PEM cells with late-log phase Ld39 promastigotes. A very pronounced antiamastigote activity was noted for compound **39** compared to solvent control. Numerous amastigotes were observed in host macrophages in control (Figure 1A) whereas only a few amastigotes were noted in the compound **39**-treated case (5 μM) (Figure 1B). Percentage of macrophage infected was decreased from 100% in solvent control to 20% in compound **39**-treated case (Figure 1C). Number of amastigotes per 100 macrophages was also reduced from 887 to 48 after adding 5 μM of compound **39** (Figure 1C). Similar antiamastigote activities were determined for a number of structural derivatives of compound **39** (Table 4).

We have compared compound **39** and its derivatives with other antileishmanial compounds in terms of their antiamastigotes activity and therapeutic index. As shown in Table 4, we found that antiamastigotes activity of compound **39** and its derivatives followed a similar trend as antipromastigotes, with compound **39** being the most active (IC_{50} = 0.63 ± 0.12 μM). Compound **53** (with shorter amino PEG linker than compound **39**) showed significantly lower antiamastigotes activity. Attachment position of the two flavones to the amino PEG linker is also important. Compounds **56** (linker connected to flavones at C-3′ position of B-ring), compound **57** (linker connected to flavones at C-2′ position of B-ring), and compound **58** (linker connected to flavones at C-3 position of C-ring) have an IC_{50} 3.7-fold, 11.1-fold, and 4.9-fold lower than compound **39** (linker connected at C-4′ position of B-ring). Antiamastigotes activity of compound **59** (C3′-methoxy substitution in the B-ring) cannot be measured because it was toxic to host PEM cells (IC_{50} = 2.7 ± 0.3 μM). In contrast, C3-methoxy substitution in the C-ring (compound **60**) or addition of a fluorine atom at C-6 position of A-ring

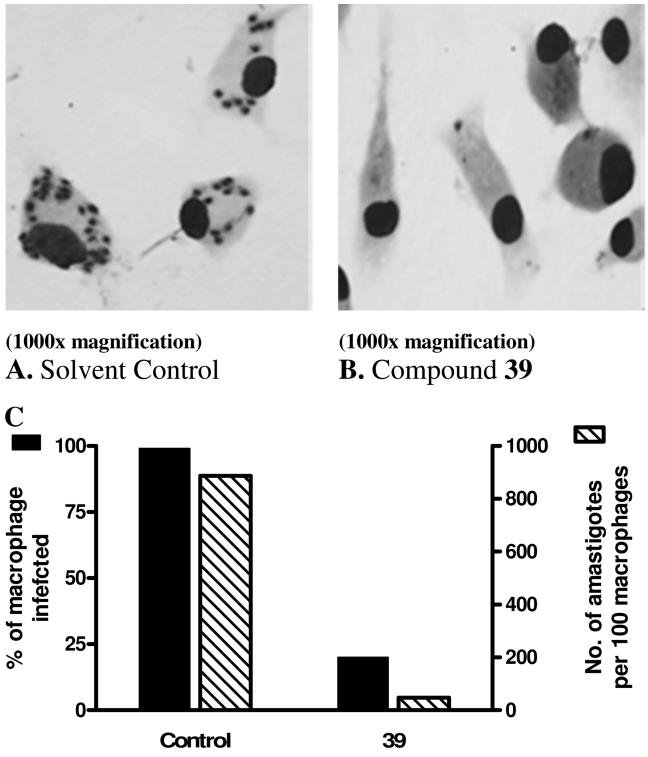

Figure 1. Antiamastigotes activity of compound **39** against SSG-resistant *L. donovani* Ld39 amastigotes grown in PEM cells. PEM cells were infected with late-log stage promastigotes of Ld39 for 24 h at 37 °C. Infected macrophages were then treated with either DMSO (A) or 5 μM compound **39** (B) for 3 days at 37 °C. Two representative microscopy pictures are shown in each treatment group. After 3 days, the coverslips were stained with Giemsa. Percentage of macrophage infected and the number of amastigotes per 100 macrophages were determined (C). The black column represents the percentage of macrophage infected, and the striped column represents the number of amastigotes per 100 macrophages.

(compound **61**) did not cause toxic effect toward the PEM cells. However, the antiamastigotes activity of compounds **60** and **61** were still 8.3-fold and 4.9-fold lower than the unsubstituted compound **39**. Therefore, substitution at two flavones did not improve antiamastigotes activity of compound **39**.

When we changed the position of the nitrogen atom in the pyridine ring, we found that the rank order of antiamastigotes activities was as follows: *para*-position (compound **39**; IC_{50} = 0.63 μM against LdAG83) ≫ *meta*-position (compound **68**; IC_{50} = 2.4 μM) > *ortho*-position (compound **40**; IC_{50} = 4.1 μM). A bromo substitution at *meta*-position (compound **69**) and *ortho*-positions (compound **71**) or a cyano group at *meta*-position (compound **70**) of the pyridine ring also dramatically reduced antiamastigotes activity. Replacement of pyridine ring by pyrimidine ring (compound **72**) exhibited about 2-fold lower antiamastigotes activity as compared to the parent compound **39**.

In terms of therapeutic index, compound **39** is still the safest compound among the synthetic derivatives because of its high antiamastigotes activities and the lower toxicity toward the PEM cells (therapeutic index >139.7). We have also determined antiamastigotes activity and therapeutic index of various antileishmanials. Although SSG, luteolin, and quercetin displayed a very poor antipromastigote activity (Table 2), they were found to possess an improved antiamastigotes activity with IC_{50} values of 675, 7.7, and 6.0 μM, respectively (Table 4). This represents 11-, 12-, and at least 17-fold increase in

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Table 4. Antiamastigotes Activities and Therapeutic Index of Flavonoid Dimer 39 and Its Derivatives Compared to Standard Antileishmanials[a]

	IC_{50} (μM)		therapeutic index
compd	amastigotes Ld39	macrophages PEM	amastigotes Ld39
39	0.63 ± 0.12	>88.0	>139.7
40	4.1 ± 2.1	>33.0	>8.0
53	5.8 ± 3.3	>58.0	>10.0
56	2.3 ± 1.0	>50.0	>21.7
57	7.0	>11.0	>1.6
58	3.1 ± 0.1	>33.0	>10.6
59	toxic	2.7 ± 0.3	ND
60	5.2 ± 1.7	>50.0	>9.6
61	3.1 ± 2.7	>100.0	>32.3
68	2.4 ± 0.1	33.1 ± 18.9	13.8
69	>10.0	>100.0	>10.0
70	7.6 ± 1.0	>92.0	>12.1
71	>7.6	>100.0	>13.1
72	1.4 ± 0.8	>33.0	>23.6
SSG	675 ± 75	>11 000	>16.3
pentamidine	>30.0	30.4 ± 10.5	<1.0
miltefosine	16.0 ± 6.4	75.3 ± 9.4	4.7
paromomycin	41.0	>100.0	>2.4
amphotericin B	0.062 ± 0.00	7.4 ± 0.4	119.4
luteolin	7.7 ± 3.6	77.7 ± 7.7	10.1
quercetin	6.0 ± 1.1	>100.0	>16.7

[a]PEM cells were infected with late-log Ld39 promastigotes of SSG-resistant Ld39 for 24 h at 37 °C. Infected macrophages were then treated with various antileishmanials and incubated for 3 days at 37 °C. After 3 days, the coverslips were stained with Giemsa. The number of amastigotes per 100 macrophages was determined and used to calculate IC_{50} values. The values were presented as mean ± standard error of mean. N = 1−3 independent experiments. Therapeutic index was determined by dividing IC_{50} towards PEM cells over IC_{50} towards Ld39 amastigotes. Therapeutic index smaller than 10 suggests probable non-selective cytotoxicity for the tested compounds.[11]

antiamastigotes activity compared to that of antipromastigotes, respectively. This stage-dependent leishmanicidal activity suggests that their targets are only present in intracellular amastigotes but not in extracellular promastigotes. Pentamidine, miltefosine, and paromomycin displayed a moderate antiamastigotes activity with IC_{50} values of >30.0, 16.0, and 41.0 μM, respectively (Table 4). Therapeutic indexes of these six compounds were smaller than 17. Amphotericin B has the highest antiamastigotes activity (0.062 μM), followed by compound **39** (0.63 ± 0.12 μM) (Table 4). Amphotericin B, however, was also the most toxic to PEM cells (IC_{50} = 7.4 μM). In contrast, compound **39** was almost nontoxic to PEM cells (IC_{50} > 88 μM). Put together, the therapeutic index of amphotericin B (therapeutic index =119.4) was slightly lower than that of compound **39** (therapeutic index >139.7), suggesting that both of them possess very high selective cytotoxicity for *Leishmania* amastigotes.

In summary, we found that flavonoid dimer **39** was highly potent against both promastigotes (IC_{50} = 0.21 μM) and amastigotes (IC_{50} = 0.63 μM) (Figure 2). Its activity was comparable to or slightly lower than that of amphotericin B (IC_{50} = 0.09 μM for promastigotes and 0.062 μM for amastigotes) and much better than SSG (Figure 2). Together with its low toxicity against macrophages, **39** is a potential candidate compound for further development into antileishmanial drug.

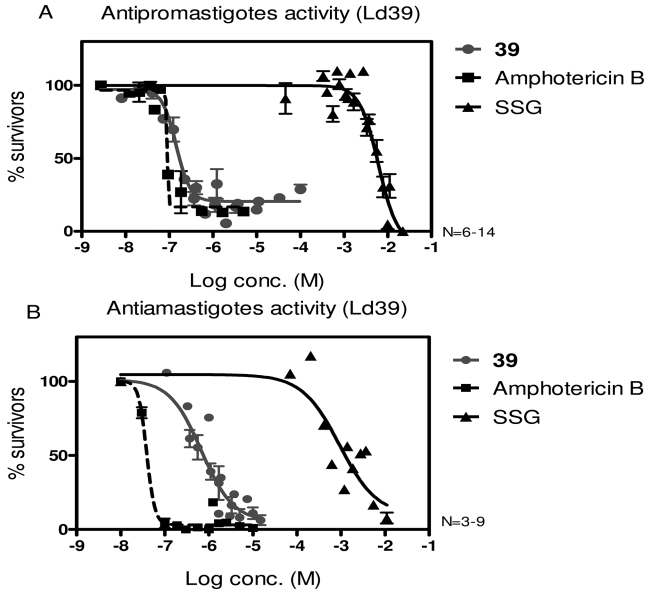

Figure 2. Dose response curve for antileishmanial activities of **39**, amphotericin B, and SSG against Ld39 parasites. Ld39 promastigotes (A) or amastigotes grown in PEM (B) were treated with different concentrations of **39**, amphotericin B, or SSG, and their percentages of survival are plotted against log concentration of compounds used (in molar). Data is presented as mean ± standard error of mean.

DISCUSSION

In the present study, we demonstrate that a group of 63 flavonoid dimers displayed novel and promising antipromastigotes and antiamastigotes activities. We are able to draw some structure−activity relationships. (1) Comparison of series **A** (with OH-group on the two flavone rings) and series **B** (removal of OH-group from the two flavone rings) reveals that the linker chain length is crucial for selective antileishmanial activity and noncytotoxicity of flavonoid dimers (Table 1). In series **A**, good antipromastigotes activity and nontoxicity toward RAW 264.7 cells were observed for PEG linker length with 3−5 EG units. On the other hand, series **B** showed that, for linker with 5−13 EG units, high toxicity toward RAW 264.7 cells were observed and that rendered them unsuitable as selective antileishmanials (Table 1). (2) Series **C** clearly demonstrates that some hydrophobic substitutions including ethyl (compound **20**) at C-6 position, methyl (compound **26**), or fluorine (compound **28** and **29**) at C-7 position of ring A of flavone would help in regaining the selective leishmanicidal activity as compared to the parent compounds **9** and **10** with 4 and 5 EG units (Table 1). (3) The amine linked flavonoid dimers reveal that an amine group in the linker has a strong influence on both leishmanicidal activity and cytotoxicity. The selectivity depends critically on the nature of the R substituent on the amine group. Substituents including Boc- (compound **35**), pyridine ring- (compound **39** and **40**), and mesylate- (compound **42**) on the amino linker of dimers markedly enhance the leishmanicidal activity (Table 2).

A good antileishmanial compound should be both potent and safe to use. Among all flavonoid dimers tested, compound **39** exhibited the strongest antipromastigotes activity with IC_{50} ranging from 0.13 to 0.21 μM (Table 2), and it is also very active against Ld39 amastigotes with IC_{50} of 0.63 μM (Table 4). It displayed no toxic effect to macrophage cell line RAW 264.7 (IC_{50} > 100 μM) (Table 2) or primary mouse PEM cells (IC_{50} > 88 μM) (Tables 4 and 5). Importantly, compound **39** displayed

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consistent efficacy against both drug-sensitive *Leishmania* as well as SSG-resistant Ld39 and pentamidine-resistant LdAG83-PentR50 (Tables 2 and 3). In comparison with other clinically used antileishmanials, compound **39** showed stronger antipromastigotes (Table 3) and antiamastigotes (Table 4) activity than most antileishmanials including SSG, pentamidine, miltefosine, and paromomycin (Table 3). Compound **39** was about 2-fold (Table 3) and 10-fold (Table 4) less potent than the second-line antileishmanial amphotericin B (Tables 3 and 4) against promastigotes and amastigotes, respectively. However, amphotericin B is also the most toxic compound toward macrophages RAW 264.7 (IC_{50} = 12.0 μM) and primary mouse PEM cells (IC_{50} = 7.4 μM) (Tables 3 and 4). Overall, therapeutic index of compound **39** (>139.7) was higher than that of amphotericin B (119.4) (Table 4), thus rendering compound **39** suitable as a potential leishmanicidal agent.

Flavonoids are widely distributed in the plant kingdom and have long been studied for their antiparasitic activities.[3,12−15] Similar to SSG, monomeric flavonoids such as luteolin and quercetin were demonstrated to solely exhibit promising leishmanicidal activity for intracellular amastigotes (IC_{50} = 7.7 and 6.0 μM), but not for extracellular promastigotes (IC_{50} = 89−114 μM and 70−103 μM) (Tables 3 and 4), suggesting that their specific targets are present only in the amastigote form. Quercitrin (quercetin 3-*O*-α-L-rhamnopyranoside), one of the constituents of the biologically active aqueous extract obtained from *Kalanchoe pinnata*, has been shown to have potent antileishmanial activity toward amastigote form of *L. amazonensis*, but not to promastigote form.[16] It has been previously reported that some polyphenols did not show selective toxicity for the *Leishmania* extracellular form, but displayed pronounced effect against *Leishmania* amastigotes.[17,18] In the present study, synthetic flavonoid dimer, **39**, was demonstrated to possess very potent antipromastigotes and antiamastigotes activity, suggesting that its target is likely different from the monomeric flavonoids. The level of that putative target is therefore predicted to be constitutively expressed in both *Leishmania* stages.

Biflavonoids are rich in many species of plant and reported to have significant antiviral and antiprotozoal activity.[11,19−22] Biflavonoids are characterized by two flavonoid monomeric units (flavone or flavanone) covalently linked either with C−C or C−O−C bonds.[11] *In vitro* studies by Weniger et al. revealed that six out of eight biflavonoids tested showed potent antiaxenic amastigote activity including lanaroflavone (IC_{50} = 7.2 μM), bilobetin (IC_{50} = 2.7 μM), ginkgetin (IC_{50} = 2.8 μM), isoginkgetin (IC_{50} = 1.9 μM), and sciadopitysin (IC_{50} = 8.2 μM).[11] In comparison, synthetic flavonoid dimer **39** (IC_{50} = 0.63 μM) is about 3-fold more active in killing amastigotes than the most active biflavonoid, isoginkgetin. However, isoginkgetin was moderately toxic to normal L6 cells (IC_{50} = 11 μM), therefore giving a relatively low therapeutic index of about 5.8. Comparatively, compound **39** showed very selective and promising leishmanicidal activity as indicated by the high value of therapeutic index (>139.7, Table 4).

The possible mechanism of action of flavonoids in killing *Leishmania* has been discussed recently.[17,23−25] Instead of direct antiparasitic activity, polyphenol and proanthcyanidins could induce nitric oxide and tumor necrosis factor-α (TNF) in macrophages.[17] Quercetin and luteolin are found to be inhibitor of topoisomerase and induce cell cycle arrest leading to apoptosis in *Leishmania*.[23−25] A biflavonoid, 2″,3″-diidroochnaflavone, isolated from the leaves of *Luxemburgia nobilis*, was cytotoxic to murine Ehrlich carcinoma and human leukemia K562 cells and found to inhibit topoisomerase I and II-α.[26] Morelloflavone, a biflavonoid, was found to inhibit tumor angiogenesis by targeting Rho GTPase and extracellular signal-regulated kinase signaling pathway.[27] However, the mode of action of antileishmanial biflavonoid remains unclear. We are currently trying to identify the potential target(s) of compound **39**.

In summary, the present study suggested that flavonoid dimers, especially those with amino linker, have marked leishmanicidal potency. Compound **39** showed very consistent and promising leishmanicidal activity irrespective of the drug-sensitivity of parasite for both extracellular promastigotes (IC_{50} ranging from 0.13 to 0.21 μM) and intracellular amastigotes (IC_{50} = 0.63 μM). Moreover, it displayed no toxicity for macrophages, and its high value of therapeutic index was comparable to other current antileishmanials like amphotericin B. With its amine and pyridine functions, compound **39** or its salts have reasonable solubility in aqueous media. Therefore, compound **39** can be potentially developed into a new, safe, and effective drug for the treatment of leishmaniasis, especially the drug refractory parasites.

EXPERIMENTAL SECTION

Chemicals. The flavonoid dimers **1**−**50** and amino diol **54** were prepared according to the reported procedures.[5,6,8,10] Amino diol **51** and 3-hydroxyflavone were commercially available. 2′-Hydroxyflavone, 3′-hydroxyflavone, 4′-hydroxyflavone, 3′-methoxy-4′-hydroxyflavone, 3-methoxy-4′-hydroxyflavone, and 6-fluoro-4′-hydroxyflavone were prepared according to the reported procedures. All NMR spectra were recorded on a Bruker MHz DPX400 spectrometer at 400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C. All NMR measurements were carried out at room temperature, and the chemical shifts are reported as parts per million (ppm) in unit relative to the resonance of $CDCl_3$ (7.26 ppm in the ^{1}H, 77.0 ppm for the central line of the triplet in the ^{13}C modes, respectively). Low-resolution and high-resolution mass spectra were obtained on a Micromass (U.K.) Q-TOF-2 by electron spray ionization (ESI) mode. Melting points were measured using Electrothermal IA9100 digital melting point apparatus and were uncorrected. All reagents and solvents were reagent grade and were used without further purification unless otherwise stated. The plates used for thin-layer chromatography (TLC) were E. Merck silica gel 60F254 (0.25-mm thickness), and they were visualized under short (254-nm) and long (365-nm) UV light. Chromatographic purifications were carried out using MN silica gel 60 (230−400 mesh). The purity of tested compounds was determined by HPLC, which was performed by using an Agilent 1100 series installed with an analytic column of Agilent Prep-Sil Scalar column (4.6 mm ×250 mm, 5 μm) at UV detection of 320 nm (reference at 450 nm) with isocratic elution of hexane (50%)/ethyl acetate (25%)/methanol (25%) at a flow rate of 1 mL/min. All tested compounds were shown to have >95% purity according to HPLC. Pentamidine, amphotericin B, paromomycin, luteolin, and quercetin were purchased from Sigma. Miltefosine was from Cayman. SSG was a generous gift from Glaxo Smith Kline.

*General Procedure I for Synthesis of Amino Diols **52**, **55**, and **62**−**67**.* To a round-bottom flask was added amino diol **51** or **54**, corresponding arylmethyl halides, excess K_2CO_3, and acetone or acetonitrile. The reaction mixture was stirred at refluxing temperature for 3 h. When TLC indicated complete consumption of starting material, the reaction mixture was cooled and filtered to remove excess K_2CO_3.The obtained filtrate was evaporated to give a crude reaction mixture, which was subjected to purification by flash column chromatography on silica gel with 3−20% MeOH in CH_2Cl_2 as eluent to furnish the desired compound.

*General Procedure II for the Synthesis of Flavonoid Dimers **53**, **56**−**61**, and **68**−**73**.* To a round-bottom flask, containing the corresponding amino diol, monohydroxyflavone, triphenylphosphine, and tetrahydrofuran (THF), was added diisopropyl azodicarboxylate (DIAD) dropwise. The reaction mixture was stirred at refluxing

temperature under nitrogen atmosphere for 12 h. When TLC indicated complete consumption of starting material, the reaction mixture was cooled and filtered through a short pad of silica gel. The obtained filtrate was evaporated to give a crude reaction mixture, which was subjected to purification by flash column chromatography on silica gel with 10−30% acetone in CH_2Cl_2 as eluent to furnish the desired compound.

*2,2′-((Pyridin-4-ylmethyl)azanediyl)diethanol (**52**).* This compound was obtained as a pale brown oil (1.6 g, 18%) from **51** (4.8 g, 45.7 mmol), 4-(chloromethyl)pyridine hydrochloride (7.5 g, 45.7 mmol), K_2CO_3 (13.0 g, 94.2 mmol), and acetonitrile (140 mL) according to the general procedure I described above. ^{1}H NMR ($CDCl_3$) δ 2.60 (t, J = 5.20 Hz, 4 H), 3.55 (t, J = 5.20 Hz, 4H), 3.64 (s, 2H), 4.25 (br, 2H), 7.25 (d, J = 6.0 Hz, 2H), 8.33 (d, J = 6.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 56.2, 58.4, 59.5, 123.9, 149.1, 149.4. LRMS (ESI) m/z 197 (M^+ + H, 100), 219 (M^+ + Na, 18). HRMS (ESI) Calcd for $C_{10}H_{17}N_2O_2$ (M^+ + H) 197.1290. Found 197.1281.

*1,7-Bis[4′-(4H-chromen-4-on-2-yl)phenyl]-4-(4-pyridylmethyl)-1,7-dioxa-4-azaheptane (**53**).* This compound was obtained as a white foam (0.81 g, 23%) from **52** (1.08 g, 5.51 mmol), 4′-hydroxyflavone (2.65 g, 11.1 mmol), triphenylphosphine (3.10 g, 11.8 mmol), THF (30 mL), and DIAD (2.40 g, 11.8 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 3.13 (t, J = 4.8 Hz, 4H), 3.93 (s, 2H), 4.16 (t, J = 4.8 Hz, 4H), 6.73 (s, 2H), 6.97 (d, J = 8.6 Hz, 4H), 7.34 (dd, J = 7.2, 8.0 Hz, 2H), 7.38 (d, J = 6.0 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H), 7.67 (dd, J = 7.2, 8.0 Hz, 2H), 7.86 (d, J = 8.6 Hz, 4H), 8.19 (dd, J = 1.2, 8.0 Hz, 2H), 8.57 (d, J = 6.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 53.6, 59.0, 67.0, 106.2, 114.9, 117.9, 123.5, 123.9, 124.2, 125.1, 125.6, 128.0, 133.6, 149.0, 149.7, 156.1, 161.4, 163.2, 178.3. LRMS (ESI) m/z 637 (M^+ + H, 100), 659 (M^+ + Na, 12). HRMS (ESI) Calcd for $C_{40}H_{33}N_2O_6$ (M^+ + H) 637.2339. Found 637.2330.

*N-(Pyridin-4′-ylmethyl)-3,9-dioxa-6-azaundecane-1,11-diol (**55**).* This compound was obtained as a pale brown oil (3.6 g, 31%) from **54** (6.0 g, 31.1 mmol), 4-(chloromethyl)pyridine hydrochloride (6.6 g, 40.2 mmol), K_2CO_3 (11.2 g, 81.2 mmol), and acetonitrile (70 mL) according to the general procedure I described above. ^{1}H NMR ($CDCl_3$) δ 2.75 (t, J = 4.8 Hz, 4H), 3.55 (t, J = 4.8 Hz, 4H), 3.61 (t, J = 4.8 Hz, 4H), 3.71 (t, J = 4.8 Hz, 4H), 3.72 (s, 2H), 4.20 (br, 2H), 7.34 (d, J = 6.0 Hz, 2H), 8.56 (d, J = 6.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 54.7, 58.7, 61.6, 68.7, 72.5, 124.0, 147.8, 149.7. LRMS (ESI) m/z 285 (M^+ + H, 100), 307 (M^+ + Na, 60). HRMS (ESI) Calcd for $C_{14}H_{24}N_2O_4Na$ (M^+ + Na) 307.1634. Found 307.1635.

*1,13-Bis[3′-(4H-chromen-4-on-2-yl)phenyl]-7-(4-pyridylmethyl)-1,4,10,13-tetraoxa-7-azatridecane (**56**).* This compound was obtained as a white foam (0.25 g, 35%) from **55** (0.28 g, 0.99 mmol), 3′-hydroxyflavone (0.48 g, 2.02 mmol), triphenylphosphine (0.54 g, 2.06 mmol), THF (20 mL), and DIAD (0.42 g, 2.08 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.87 (t, J = 5.6 Hz, 4H), 3.70 (t, J = 5.6 Hz, 4H), 3.82 (t, J = 4.4 Hz, 4H), 3.83 (s, 2H), 4.17 (t, J = 4.4 Hz, 4H), 6.78 (s, 2H), 7.05 (dd, J = 2.4, 8.0 Hz, 2H), 7.33 (d, J = 5.2 Hz, 2H), 7.37 − 7.44 (m, 6H), 7.49 (d, J = 8.0 Hz, 2H), 7.55 (d, J = 8.0 Hz, 2H), 7.69 (dd, J = 8.4, 8.4 Hz, 2H), 8.22 (dd, J = 1.2, 7.6 Hz, 2H), 8.50 (d, J = 5.6 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 54.2, 58.8, 67.7, 69.5, 70.2, 107.8, 112.5, 117.7, 118.1, 118.9, 123.6, 124.0, 125.7, 130.1, 133.1, 133.8, 149.6, 156.2, 159.2, 163.1, 178.3. LRMS (ESI) m/z 725 (M^+ + H, 100), 747 (M^+ + Na, 35). HRMS (ESI) Calcd for $C_{44}H_{41}N_2O_8$ (M^+ + H) 725.2863. Found 725.2842.

*1,13-Bis[2′-(4H-chromen-4-on-2-yl)phenyl]-7-(4-pyridylmethyl)-1,4,10,13-tetraoxa-7-azatridecane (**57**).* This compound was obtained as a white foam (0.21 g, 29%) from **55** (0.28 g, 0.99 mmol), 2′-hydroxyflavone (0.48 g, 2.02 mmol), triphenylphosphine (0.54 g, 2.06 mmol), THF (20 mL), and DIAD (0.42 g, 2.08 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.77 (t, J = 5.6 Hz, 4H), 3.60 (t, J = 5.6 Hz, 4H), 3.69 (s, 2H), 3.77 (t, J = 4.8 Hz, 4H), 4.17 (t, J = 4.8 Hz, 4H), 7.00 (d, J = 8.4 Hz, 2H), 7.08 (dd, J = 7.2, 8.0 Hz, 2H), 7.13 (s, 2H), 7.20 (d, J = 5.6 Hz, 2H), 7.36 (dd, J = 7.2, 8.0 Hz, 2H), 7.41 (dd, J = 7.2, 8.0 Hz, 2H), 7.49 (d, J = 8.0 Hz, 2H), 7.64 (dd, J = 7.2, 8.0 Hz, 2H), 7.87 (d, J = 8.0 Hz, 2H), 8.19 (d, J = 7.2 Hz, 2H), 8.35 (d, J = 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 54.1, 58.5, 68.4, 69.2, 70.2, 112.7, 113.0, 118.0, 121.0, 121.2, 123.5, 123.8, 124.8, 125.5, 129.3, 132.3, 133.5, 149.4, 156.4, 157.2, 160.8, 178.6. LRMS (ESI) m/z 725 (M^+ + H, 100), 747 (M^+ + Na, 18). HRMS (ESI) Calcd for $C_{44}H_{41}N_2O_8$ (M^+ + H) 725.2863. Found 725.2849.

*1,13-Bis[2-phenyl-4H-chromen-4-on-3-yl]-7-(4-pyridylmethyl)-1,4,10,13-tetraoxa-7-azatridecane (**58**).* This compound was obtained as a white foam (0.23 g, 32%) from **55** (0.28 g, 0.99 mmol), 3-hydroxyflavone (0.48 g, 2.02 mmol), triphenylphosphine (0.54 g, 2.06 mmol), THF (20 mL), and DIAD (0.42 g, 2.08 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.60 (t, J = 5.6 Hz, 4H), 3.40 (t, J = 5.6 Hz, 4H), 3.61 (s, 2H), 3.61 (t, J = 4.8 Hz, 4H), 4.24 (t, J = 4.8 Hz, 4H), 7.20 (d, J = 6.0 Hz, 2H), 7.33 (dd, J = 5.6, 8.0 Hz, 2H), 7.39−7.42 (m, 6H), 7.48 (d, J = 8.0 Hz, 2H), 7.62 (dd, J = 5.6, 8.0 Hz, 2H), 8.09−8.12 (m, 4H), 8.20 (dd, J = 1.2, 8.0 Hz, 2H), 8.43 (d, J = 8.0 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 54.0, 58.6, 69.6, 70.2, 71.4, 118.0, 123.5, 124.1, 124.6, 125.7, 128.3, 128.7, 130.6, 130.9, 133.4, 140.4, 149.5, 149.5, 155.2, 155.4, 175.0. LRMS (ESI) m/z 725 (M^+ + H, 100), 747 (M^+ + Na, 45). HRMS (ESI) Calcd for $C_{44}H_{41}N_2O_8$ (M^+ + H) 725.2863. Found 725.2890.

*1,13-Bis[4′-(4H-chromen-4-on-2-yl)-2-methoxyphenyl]-7-(4-pyridylmethyl)-1,4,10,13-tetraoxa-7-azatridecane (**59**).* This compound was obtained as a white foam (0.26 g, 23%) from **55** (0.42 g, 1.48 mmol), 3′-methoxy-4′-hydroxyflavone (0.80 g, 2.99 mmol), triphenylphosphine (0.82 g, 3.13 mmol), THF (20 mL), and DIAD (0.62 g, 3.07 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.78 (t, J = 5.6 Hz, 4H), 3.64 (t, J = 5.6 Hz, 4H), 3.74 (s, 2H), 3.80 (t, J = 4.8 Hz, 4H), 3.88 (s, 6H), 4.17 (t, J = 4.8 Hz, 4H), 6.68 (s, 2H), 6.95 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 6.0 Hz, 2H), 7.31−7.37 (m, 4H), 7.44−7.51 (m, 4H), 7.64 (dd, J = 3.2, 8.4 Hz, 2H), 8.15 (d, J = 8.0 Hz, 2H), 8.46 (d, J = 4.4 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 54.1, 56.0, 58.7, 68.5, 69.3, 70.2, 106.4, 109.3, 112.9, 117.9, 119.8, 123.5, 123.8, 124.5, 125.1, 125.5, 133.6, 149.5, 149.6, 151.4, 156.1, 163.2, 178.2. LRMS (ESI) m/z 785 (M^+ + H, 65), 807 (M^+ + Na, 100). HRMS (ESI) Calcd for $C_{46}H_{44}N_2O_{10}Na$ (M^+ + Na) 807.2894. Found 807.2899.

*1,13-Bis[4′-(3-methoxy-4H-chromen-4-on-2-yl)phenyl]-7-(4-pyridylmethyl)-1,4,10,13-tetraoxa-7-azatridecane (**60**).* This compound was obtained as a white foam (0.18 g, 26%) from **55** (0.25 g, 0.88 mmol), 3-methoxy-4′-hydroxyflavone (0.48 g, 1.79 mmol), triphenylphosphine (0.50 g, 1.91 mmol), THF (20 mL), and DIAD (0.38 g, 1.88 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.84 (t, J = 5.6 Hz, 4H), 3.67 (t, J = 5.6 Hz, 4H), 3.79 (s, 2H), 3.82 (t, J = 4.8 Hz, 4H), 3.88 (s, 6H), 4.16 (t, J = 4.8 Hz, 4H), 7.02 (d, J = 7.2 Hz, 4H), 7.33 (d, J = 4.8 Hz, 2H), 7.36 (dd, J = 3.2, 7.6 Hz, 2H), 7.51 (d, J = 7.6 Hz, 2H), 7.65(dd, J = 3.2, 7.6 Hz, 2H), 8.10 (d, J = 7.2 Hz, 4H), 8.24 (dd, J = 1.2, 7.2 Hz, 2H), 8.51(br, 2H). ^{13}C NMR ($CDCl_3$) δ 54.2, 58.8, 59.9, 67.5, 69.4, 70.2, 114.5, 117.9, 123.4, 123.6, 124.2, 124.6, 125.7, 130.2, 133.3, 140.8, 149.5, 149.6, 155.1, 155.4, 160.7, 174.9. LRMS (ESI) m/z 785 (M^+ + H, 100), 807 (M^+ + Na, 40). HRMS (ESI) Calcd for $C_{46}H_{45}N_2O_{10}$ (M^+ + H) 785.3074. Found 785.3063.

*1,13-Bis[4′-(6-fluoro-4H-chromen-4-on-2-yl)phenyl]-7-(4-pyridylmethyl)-1,4,10,13-tetraoxa-7-azatridecane (**61**).* This compound was obtained as a white foam (0.13 g, 12%) from **55** (0.42 g, 1.48 mmol), 6-fluoro-4′-hydroxyflavone (0.77 g, 3.01 mmol), triphenylphosphine (0.82 g, 3.13 mmol), THF (20 mL), and DIAD (0.62 g, 3.07 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.83 (t, J = 6.0 Hz, 4H), 3.66 (t, J = 6.0 Hz, 4H), 3.78 (s, 2H), 3.80 (t, J = 4.8 Hz, 4H), 4.14 (t, J = 4.8 Hz, 4H), 6.67 (s, 2H), 6.98 (d, J = 8.8 Hz, 4H), 7.32 (d, J = 5.6 Hz, 2H), 7.38 (dd, J = 3.2, 7.6 Hz, 2H), 7.38 (dd, J = 4.4, 9.2 Hz, 2H), 7.81(m, 6H), 8.49 (d, J = 5.6 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 54.3, 58.8, 67.7, 69.3, 70.2, 105.4, 110.4, 110.6, 115.0, 120.0, 120.0, 121.5, 121.8, 123.5, 123.8, 125.0, 125.1, 128.0, 149.5, 149.6, 152.3, 158.3, 160.7, 161.7, 163.5, 177.4. LRMS (ESI) m/z 761 (M^+ + H, 100), 783 (M^+ + Na, 30). HRMS (ESI) Calcd for $C_{44}H_{39}N_2O_8F$ (M^+ + H) 761.2674. Found 761.2659.

*N-(Pyridin-3′-ylmethyl)-3,9-dioxa-6-azaundecane-1,11-diol (**62**).* This compound was obtained as a pale brown oil (1.1 g, 36%) from **54** (2.0 g, 10.4 mmol), 3-(bromomethyl)pyridine hydrobromide (2.7 g, 10.7 mmol), K_2CO_3 (3.1 g, 22.5 mmol), and acetone (40 mL) according to the general procedure I described above. ^{1}H NMR ($CDCl_3$) δ 2.74 (t, J = 5.38 Hz, 4 H), 3.52−3.61 (m, 8 H), 3.67−3.74 (m, 6 H), 4.26 (br, 2 H), 7.26−7.30 (m, 1 H), 7.75−7.79 (m, 1 H), 8.49−8.54 (m, 2 H). ^{13}C NMR ($CDCl_3$) δ 54.4, 56.8, 61.6, 68.7, 72.5, 123.4, 133.7, 137.0, 148.7,

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150.4. LRMS (ESI) m/z 285 (M^+ + H, 97), 307 (M^+ + Na, 100). HRMS (ESI) Calcd for $C_{14}H_{24}N_2O_4Na$ (M^+ + Na) 307.1634. Found 307.1631.

*N-(2′-Bromopyridin-4′-ylmethyl)-3,9-dioxa-6-azaundecane-1,11-diol (**63**).* This compound was obtained as a pale brown oil (0.7 g, 38%) from **54** (1.0 g, 5.2 mmol), 4-(bromomethyl)-2-bromopyridine (1.3 g, 5.1 mmol), K_2CO_3 (0.8 g, 5.9 mmol), and acetone (40 mL) according to the general procedure I described above. ^{1}H NMR ($CDCl_3$) δ 2.77 (t, J = 5.14 Hz, 4 H), 3.55−3.64 (m, 8 H), 3.67−3.80 (m, 6 H), 7.28−7.47 (m, 1 H), 7.59 (s, 1 H), 8.31 (d, J = 4.89 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 54.6, 58.1, 61.5, 68.8, 72.4, 122.8, 127.9, 142.4, 149.9, 152.1. LRMS (ESI) m/z 363 (M^+ + H, 100), 385 (M^+ + Na, 38). HRMS (ESI) Calcd for $C_{14}H_{24}N_2O_4Br$ (M^+ + H) 363.0919. Found 363.0921.

*N-(2′-Cyanopyridin-4′-ylmethyl)-3,9-dioxa-6-azaundecane-1,11-diol (**64**).* This compound was obtained as a pale brown oil (0.9 g, 36%) from **54** (1.5 g, 7.8 mmol), 4-bromomethyl-2-pyridinecarbonitrile (1.6 g, 8.1 mmol), K_2CO_3 (1.2 g, 8.7 mmol), and acetone (50 mL) according to the general procedure I described above. ^{1}H NMR ($CDCl_3$) δ 2.75 (t, J = 5.14 Hz, 4 H), 3.50−3.61 (m, 8 H), 3.65−3.74 (m, 4 H), 3.77 (s, 2 H), 7.58−7.60 (m, 1 H), 7.82 (s, 1 H), 8.61 (d, J = 5.87 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 54.7, 58.0, 61.6, 68.8, 72.4, 117.4, 126.8, 128.5, 133.8, 150.9, 150.9. LRMS (ESI) m/z 310 (M^+ + H, 100), 332 (M^+ + Na, 18). HRMS (ESI) Calcd for $C_{15}H_{24}N_3O_4$ (M^+ + H) 310.1767. Found 310.1755.

*N-(3′-Bromopyridin-4′-ylmethyl)-3,9-dioxa-6-azaundecane-1,11-diol (**65**).* This compound was obtained as a pale brown oil (1.0 g, 43%) from **54** (1.5 g, 7.8 mmol), 4-(bromomethyl)-3-bromopyridine (1.6 g, 6.4 mmol), K_2CO_3 (1.2 g, 8.7 mmol), and acetone (40 mL) according to the general procedure I described above. ^{1}H NMR ($CDCl_3$) δ 2.75 (t, J = 5.14 Hz, 4 H), 3.44−3.66 (m, 8 H), 3.72 (s, 2 H), 7.64 (d, J = 4.89 Hz, 1 H), 8.40 (d, J = 5.38 Hz, 1 H), 8.54 (s, 1 H). ^{13}C NMR ($CDCl_3$) δ 54.9, 58.4, 61.5, 69.1, 72.5, 124.9, 126.7, 128.5, 148.2, 151.3. LRMS (ESI) m/z 363 (M^+ + H, 78), 385 (M^+ + Na, 15). HRMS (ESI) Calcd for $C_{14}H_{24}N_2O_4Br$ (M^+ + H) 363.0919. Found 363.0930.

*N-(Pyrimidin-4′-ylmethyl)-3,9-dioxa-6-azaundecane-1,11-diol (**66**).* This compound was obtained as a pale brown oil (0.44 g, 50%) from **54** (0.74 g, 3.83 mmol), 4-bromomethylpyrimidine (0.53 g, 3.07 mmol), K_2CO_3 (0.50 g, 3.62 mmol), and acetone (40 mL) according to the general procedure I described above. ^{1}H NMR ($CDCl_3$) δ 2.74 (t, J = 5.14 Hz, 4 H) 3.42−3.55 (m, 8 H), 3.56−3.70 (m, 4 H), 3.80 (s, 2 H), 7.56−7.59 (m, 1 H), 8.60 (d, J = 5.38 Hz, 1 H), 9.01 (d, J = 1.47 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 54.6, 59.9, 61.4, 68.6, 72.3, 120.4, 157.1, 158.1, 168.5. LRMS (ESI) m/z 286 (M^+ + H, 100), 308 (M^+ + Na, 81). HRMS (ESI) Calcd for $C_{13}H_{24}N_3O_4$ (M^+ + H) 286.1767. Found 286.1764.

*N-(Quinolin-4′-ylmethyl)-3,9-dioxa-6-azaundecane-1,11-diol (**67**).* This compound was obtained as a pale brown oil (1.10 g, 46%) from **54** (1.50 g, 7.78 mmol), 4-bromomethylquinoline (1.60 g, 7.21 mmol), K_2CO_3 (1.20 g, 8.70 mmol), and acetone (40 mL) according to the general procedure I described above. ^{1}H NMR ($CDCl_3$) δ 2.82−2.87 (m, 4 H), 3.46−3.63 (m, 4 H), 3.63−3.88 (m, 4 H), 4.13 (s, 2 H), 7.55−7.60 (m, 2 H), 7.69−7.74 (m, 1 H), 8.12 (dd, J = 8.31, 1.47 Hz, 1 H), 8.26−8.30 (m, 1 H), 8.85−8.88 (m, 1 H). ^{13}C NMR ($CDCl_3$) δ 54.8, 56.5, 61.4, 69.1, 72.4, 121.2, 123.9, 126.4, 127.4, 129.2, 129.6, 145.3, 147.9, 150.0. LRMS (ESI) m/z 335 (M^+ + H, 100), 357 (M^+ + Na, 45). HRMS (ESI) Calcd for $C_{18}H_{27}N_2O_4$ (M^+ + H) 335.1971. Found 335.1965.

*1,13-Bis[4′-(4H-chromen-4-on-2-yl)phenyl]-7-(3-pyridylmethyl)-1,4,10,13-tetraoxa-7-azatridecane (**68**).* This compound was obtained as a white foam (1.20 g, 36%) from **62** (1.30 g, 4.58 mmol), 4′-hydroxyflavone (2.20 g, 9.24 mmol), triphenylphosphine (2.50 g, 9.54 mmol), THF (30 mL), and DIAD (2.00 g, 9.90 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.78−2.83 (m, 4 H), 3.62−3.66 (m, 4 H), 3.75−3.79 (m, 6 H), 4.11 (t, J = 4.65 Hz, 4 H), 6.65−6.67 (m, 2 H), 6.94−6.98 (m, 4 H), 7.19 (dd, J = 7.83, 4.89 Hz, 1 H), 7.32−7.36 (m, 2 H), 7.45−7.49 (m, 2 H), 7.59−7.64 (m, 2 H), 7.68 (d, J = 7.82 Hz, 1 H), 7.76−7.80 (m, 4 H), 8.13−8.17 (m, 2 H), 8.45−8.47 (m, 1 H), 8.57 (s, 1 H). ^{13}C NMR ($CDCl_3$) δ 53.8, 57.1, 67.6, 69.3, 70.2, 106.1, 115.0, 117.9, 123.2, 123.9, 124.1, 125.0, 125.5, 127.9, 133.5, 135.1, 136.4, 148.4, 150.2, 156.1, 161.6, 163.2, 178.2. LRMS (ESI) m/z 725 (M^+ + H, 100), 747 (M^+ + Na, 29). HRMS (ESI) Calcd for $C_{44}H_{41}N_2O_8$ (M^+ + H) 725.2863. Found 725.2883.

*1,13-Bis[4′-(4H-chromen-4-on-2-yl)phenyl]-7-(4-(2-bromopyridyl)methyl)-1,4,10,13-tetraoxa-7-azatridecane (**69**).* This compound was obtained as a white foam (1.40 g, 37%) from **63** (1.70 g, 4.68 mmol), 4′-hydroxyflavone (2.23 g, 9.37 mmol), triphenylphosphine (2.65 g, 10.11 mmol), THF (30 mL), and DIAD (2.02 g, 10.00 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.77 (t, J = 5.38 Hz, 4 H), 3.60 (t, J = 5.38 Hz, 4 H), 3.68−3.77 (m, 6 H), 4.09 (t, J = 4.65 Hz, 4 H), 6.63 (s, 2 H), 6.92 (d, J = 8.80 Hz, 4 H), 7.21 (d, J = 4.89 Hz, 1 H), 7.31 (td, J = 7.46, 1.22 Hz, 2 H), 7.44 (d, J = 8.80 Hz, 2 H), 7.52 (s, 1 H), 7.59 (ddd, J = 8.56, 7.09, 1.47 Hz, 2 H), 7.73−7.78 (m, 4 H), 8.11 (dd, J = 8.07, 1.71 Hz, 2 H), 8.17 (d, J = 4.89 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 54.2, 58.1, 67.6, 69.3, 70.1, 106.0, 114.9, 117.9, 122.6, 123.8, 124.0, 125.0, 125.5, 127.5, 127.9, 133.5, 142.4, 149.8, 153.4, 156.0, 161.5, 163.1, 178.1. LRMS (ESI) m/z 803 (M^+ + H, 68), 825 (M^+ + Na, 91). HRMS (ESI) Calcd for $C_{44}H_{40}N_2O_8Br$ (M^+ + H) 803.1968. Found 803.1975.

*1,13-Bis[4′-(4H-chromen-4-on-2-yl)phenyl]-7-(4-(2-cyanopyridyl)methyl)-1,4,10,13-tetraoxa-7-azatridecane (**70**).* This compound was obtained as a white foam (0.64 g, 30%) from **64** (0.89 g, 2.88 mmol), 4′-hydroxyflavone (1.38 g, 5.80 mmol), triphenylphosphine (1.59 g, 6.07 mmol), THF (20 mL), and DIAD (1.22 g, 6.04 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.82 (t, J = 5.38 Hz, 4 H), 3.65 (t, J = 5.38 Hz, 4 H), 3.79 (t, J = 5.38 Hz, 4 H), 3.85 (s, 2 H), 4.12−4.16 (m, 4 H), 6.69 (s, 2 H), 6.89−6.99 (m, 4 H), 7.34−7.39 (m, 2 H), 7.48−7.52 (m, 2 H), 7.62−7.67 (m, 2 H), 7.77−7.84 (m, 4 H), 8.17 (dd, J = 8.07, 1.71 Hz, 2 H), 8.54 (d, J = 4.89 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 54.3, 58.1, 67.6, 69.4, 70.2, 106.1, 115.0, 117.6, 118.0, 123.9, 124.2, 125.1, 125.5, 126.5, 128.0, 128.3, 133.6, 133.8, 150.7, 152.1, 156.1, 161.5, 163.2, 178.2. LRMS (ESI) m/z 750 (M^+ + H, 100), 772 (M^+ + Na, 35). HRMS (ESI) Calcd for $C_{45}H_{40}N_3O_8$ (M^+ + H) 750.2815. Found 750.2803.

*1,13-Bis[4′-(4H-chromen-4-on-2-yl)phenyl]-7-(4-(3-bromopyridyl)methyl)-1,4,10,13-tetraoxa-7-azatridecane (**71**).* This compound was obtained as a white foam (0.39 g, 26%) from **65** (0.67 g, 1.85 mmol), 4′-hydroxyflavone (0.88 g, 3.70 mmol), triphenylphosphine (1.06 g, 4.05 mmol), THF (20 mL), and DIAD (0.82 g, 4.06 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.84 (t, J = 5.38 Hz, 4 H), 3.62 (t, J = 5.62 Hz, 4 H), 3.71−3.77 (m, 4 H), 3.81 (s, 2 H), 4.04−4.10 (m, 4 H), 6.63 (s, 2 H), 6.91 (d, J = 9.29 Hz, 4 H), 7.28−7.35 (m, 2 H), 7.44 (d, J = 8.31 Hz, 2 H), 7.56−7.65 (m, 3 H), 7.73−7.77 (m, 4 H), 8.12 (dd, J = 8.07, 1.71 Hz, 2 H), 8.37 (d, J = 4.89 Hz, 1 H), 8.54 (s, 1 H). ^{13}C NMR ($CDCl_3$) δ 54.7, 58.5, 67.6, 69.3, 70.1, 106.0, 114.9, 117.9, 121.9, 123.8, 124.0, 124.8, 125.0, 125.5, 127.9, 133.5, 148.1, 148.9, 151.3, 156.0, 161.5, 163.1, 178.1. LRMS (ESI) m/z 803 (M^+ + H, 95), 825 (M^+ + Na, 34). HRMS (ESI) Calcd for $C_{44}H_{40}N_2O_8Br$ (M^+ + H) 803.1968. Found 803.1937.

*1,13-Bis[4′-(4H-chromen-4-on-2-yl)phenyl]-7-(4-pyrimidinylmethyl)-1,4,10,13-tetraoxa-7-azatridecane (**72**).* This compound was obtained as a white foam (0.29 g, 29%) from **66** (0.39 g, 1.37 mmol), 4′-hydroxyflavone (0.65 g, 2.73 mmol), triphenylphosphine (0.74 g, 2.82 mmol), THF (20 mL), and DIAD (0.57 g, 2.82 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.91 (t, J = 5.38 Hz, 4 H), 3.66−3.87 (m, 8 H), 3.94 (s, 2 H), 4.11−4.25 (m, 4 H), 6.70−6.75 (m, 2 H), 6.97−7.06 (m, 4 H), 7.36−7.43 (m, 2 H), 7.52 (d, J = 8.31 Hz, 2 H), 7.63−7.71 (m, 3 H), 7.81−7.90 (m, 4 H), 8.20 (dd, J = 7.83, 1.47 Hz, 2 H), 8.59−8.62 (m, 1 H), 9.10 (s, 1 H). ^{13}C NMR ($CDCl_3$) δ 54.7, 60.7, 67.6, 69.3, 70.1, 106.2, 115.0, 117.9, 120.0, 123.9, 124.2, 125.1, 125.6, 128.0, 133.6, 156.1, 156.9, 158.3, 161.6, 163.2, 169.7, 178.3. LRMS (ESI) m/z 726 (M^+ + H, 100), 748 (M^+ + Na, 28). HRMS (ESI) Calcd for $C_{43}H_{40}N_3O_8$ (M^+ + H) 726.2815. Found 726.2789.

*1,13-Bis[4′-(4H-chromen-4-on-2-yl)phenyl]-7-(4-quinolinylmethyl)-1,4,10,13-tetraoxa-7-azatridecane (**73**).* This compound was obtained as a white foam (0.43 g, 26%) from **67** (0.71 g, 2.13 mmol), 4′-hydroxyflavone (1.03 g, 4.33 mmol), triphenylphosphine (1.20 g, 4.58 mmol), THF (20 mL), and DIAD (0.92 g, 4.55 mmol) according to the general procedure II described above. ^{1}H NMR ($CDCl_3$) δ 2.88 (t, J = 5.62 Hz, 4 H), 3.63−3.76 (m, 8 H), 4.03 (t, J = 5.38 Hz, 4 H), 4.20 (s, 2 H), 6.63−6.66 (m, 2 H), 6.84−6.91 (m, 4 H), 7.28−7.34 (m, 2 H), 7.43−7.48 (m, 3 H), 7.54−7.67 (m, 4 H), 7.71−7.76 (m, 4 H), 8.06−8.09 (m, 1 H), 8.14 (dd, J = 8.07, 1.71 Hz, 2 H), 8.19 (d, J = 7.82 Hz, 1

H), 8.80 (d, J = 4.40 Hz, 1 H). ^{13}C NMR ($CDCl_3$) δ 54.6, 56.8, 67.6, 69.2, 70.2, 106.0, 114.9, 117.9, 121.0, 123.8, 124.0, 125.0, 125.5, 126.1, 127.4, 127.8, 129.0, 129.9, 133.5, 145.8, 148.2, 150.2, 156.0. LRMS (ESI) m/z 775 (M^+ + H, 100), 797 (M^+ + Na, 30). HRMS (ESI) Calcd for $C_{48}H_{43}N_2O_8$ (M^+ + H) 775.3019. Found 775.3005.

Cell Lines and Cell Culture. Promastigotes of *Leishmania donovani* (LdAG83, Ld39, and LdAG83PentR50) were employed in this study. The promastigotes were cultured in Schneider's *Drosophila* Medium (Invitrogen), pH 6.9 supplemented with 10% (v/v) heat inactivated fetal bovine serum (Hyclone) with 4 mM glutamine (Sigma) and 25 μg/mL gentamicin solution (Invitrogen) at 27 °C for 4 days.[28]

Promastigotes of LdAG83PentR50 (pentamidine-resistant, IC_{50} of pentamidine = 74.7 μM) were selected by gradually increasing the pentamidine (Sigma) pressure to the wild-type promastigotes of *L. donovani* LdAG83.[9] Ld39 was SSG-resistant strain and its IC_{50} of SSG was 7090 μM.[7]

***In Vitro* Antipromastigote Activity.** Antipromastigotes activity was determined by Cell Titer 96 Aqueous Assay (Promega) that employed a tetrazolium compound (MTS, 2-(4,5-dimethylthiazol-2-yl-)-5-[3-(carboxymethoxy)phenyl]-2-(4-sulfophenyl)-2*H*-tetrazolium) and coupling reagent, phenazine methosulfate (PMS) (Sigma).[7] Promastigotes were seeded into 96-well flat bottom microtiter plate at 1×10^5 cells per well in a final volume of 100 μL medium and incubated with a series concentration of synthetic flavonoid dimers or known antileishmanials. Parasites were incubated at 27 °C for 72 h. Fresh solution of 2 mg/mL MTS and 0.92 mg/mL PMS were prepared in a ratio of 20:1 (MTS:PMS). After 72 h of incubation, 10 μL of MTS:PMS mixture was added into each well of microtiter plate. The plate was then incubated at 27 °C for 4 h for color development. After 4 h of incubation, the OD values were determined at 490 nm using automatic microtiter plate reader (Bio-Rad). The results were presented as percentage of survivors (OD value with test compound divided by that of untreated control). IC_{50} was defined as the concentration of antileishmanial compounds needed to reduce OD value by half.

***In Vitro* Antiamastigotes Activity.** Mouse peritoneal elicited macrophages (PEM) were obtained as previously described.[9] A round coverslip (12 mm in diameter) was placed into each well of 24-well culture plate. Mouse PEM were resuspended in supplemented DMEM media containing 10% heat inactivated fetal bovine serum (v/v), 100 U/mL penicillin and 100 μg/mL streptomycin and seeded into each well at a cell density of 1×10^5 cells per 500 μL. Macrophages were allowed to attach overnight. Nonadherent cells were removed by gentle washing with unsupplemented DMEM media twice. Adherent macrophages were infected with late-log promastigotes at a parasite-to-macrophage ratio of 20:1 overnight at 37 °C with 5% CO_2. Noninternalized promastigotes were removed by washing twice with unsupplemented DMEM media. Infected macrophages were further incubated in 500 μL of supplemented DMEM media in the presence or absence of flavonoid dimers or known antileishmanials for 72 h at 37 °C. After incubation, coverslips were stained with Giemsa, and the percentage of macrophages infected and number of amastigotes per 100 macrophages were enumerated.

***In Vitro* Cytotoxicity Assay of Macrophages.** Mouse macrophage cell line, RAW 264.7, or primary mouse PEM cells were grown in supplemented DMEM media in an atmosphere of 95% air with 5% CO_2 at 37 °C. RAW 264.7 or PEM cells were seeded into 96-well flat bottom microtiter plate at 1×10^4 cells and 2×10^4 cells per well in a final volume of 100 μL media, respectively. A graded dose of flavonoid dimers or antileishmanials was added into each well. The plate was incubated in an atmosphere of 95% air with 5% CO_2 at 37 °C for 72 h. Percentage of survivors was determined using the MTS assay as described previously and used to calculate IC_{50} values. Therapeutic index was calculated by dividing (IC_{50} value for cytotoxicity of the compounds toward macrophages) over (IC_{50} value for antileishmanial activity of the compounds).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Proton and carbon NMR spectra of compound **39**, **52**, **53**, **56**−**61**, **62**−**73** and HPLC chromatogram of compound **39**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: bcchanth@polyu.edu.hk (T.H.C.), bclchow@polyu.edu.hk (L.M.C.C.). Phone: (852)-34008670 (T.H.C.), (852)-34008662 (L.M.C.C.). Fax: (852)-23649932 (T.H.C.), (852)-23649932 (L.M.C.C.).

Author Contributions

[§]These authors contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): While there is no patent-licensing arrangement in place, we are in active discussion with interested parties on potential option/licensing arrangement of the intellectual property covering the results of this manuscript.

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■ ABBREVIATIONS USED

VL, visceral leishmaniasis; PEG, polyethylene glycol; EG, ethylene glycol; PEM, peritoneal elicited macrophage; SSG, sodium stibogluconate

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