Efficacy of a series of alpha-pyrone derivatives against *Leishmania (L.) infantum* and *Trypanosoma cruzi*

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Keywords: alpha-pyrones, *Leishmania*, *Trypanosoma cruzi,* SAR, phenotypic screening

Abstract

The neglected tropical diseases Chagas disease and leishmaniasis affect together more than 20 million people living mainly in developing countries. The mainstay of treatment is chemotherapy, however the drugs of choice, which include benznidazole and miltefosine, are toxic and have numerous side effects. Safe and effective therapies are urgently needed. Marine alpha-pyrones have been previously identified as scaffolds with potential antiprotozoan activities. In this work, using a phenotypic screen, twenty-seven examples of 3-substituted 4-hydroxy-6-methyl alpha-pyrones were synthesized and their antiparasitic efficacy evaluated against *Leishmania* (*L.*) *infantum* and *Trypanosoma cruzi* in order to evaluate structure-activity relationships within the series. The mechanism of action and the *in vivo* efficacy of the most selective compound against *T. cruzi* were evaluated using different techniques. *In vitro* data indicated that compounds **8**, **15**, **25**, **26** and **28** presented IC_{50} values in the range between 13 to 53 µM against *L. infantum* intracellular amastigotes. Among them, hexanoyl substituted pyrone **8** was the most selective and potent, with a Selectivity Index (SI) >14. Fifteen of the alpha-pyrones were effective against *T. cruzi* trypomastigotes, with 3-undecanoyl (**11**) and 3-tetradecanoyl (**12**) substituted pyrones being the most potent against trypomastigotes, with IC_{50} values of 1 and 2 µM, respectively, and SI higher than 70. Using flow cytometry and fluorescent-based assays, pyrone **12** was found to induce hyperpolarization of the mitochondrial membrane potential of *T. cruzi*, without affecting plasma membrane permeability. An experimental acute phase-murine model, demonstrated that *in vivo* dosing of **12** (30 mg/kg/day; 5 days), had no efficacy at the first parasitemia onset of *T. cruzi*, but reduced the second onset by 55% ($p<0.05$), suggesting a delayed action in BALB/c mice. Additionally, a histopathology study demonstrated no toxic effects to the treated mice. The finding that several 3-substituted alpha-pyrones have *in vitro* efficacy against both *L. infantum* and *T. cruzi*, and that one analogue exhibited moderate and non-toxic

in vivo efficacy against *T. cruzi* is encouraging, and suggests that this compound class should be explored as long-term treatments in experimental Chagas disease.

1. Introduction

Neglected diseases affect mainly developing countries and are usually associated with poverty conditions.[1] Visceral Leishmaniasis (VL) is a fatal protozoan disease transmitted by the bite of infected female sand flies. About 350 million people are at risk and 12 million people are affected worldwide. It is estimated that 500,000 new cases of VL occur annually, with more than 90% of cases concentrated in six countries, namely Bangladesh, Brazil, India, Nepal and Sudan and South Sudan.[1] Considering the lack of vaccines, therapeutic treatment with drugs represents the main intervention. Such drugs include pentavalent antimony and amphotericin B and the oral drug miltefosine in India. However, their use is limited by the need for prolonged administration of toxic drugs, hospitalization and the occurrence of moderate to severe adverse effects. More recently, efforts to discover classes of antileishmanial whole organism growth inhibitors and leishmanial target inhibitors have been reported.[2–5]

Trypanosoma cruzi is the etiologic agent of Chagas disease, an important cause of mortality and morbidity in Latin America. About 30% of patients progress to chagasic cardiomyopathy, a debilitating heart condition associated with conduction disturbances and heart failure, leading to sudden death. There are about 9.4 million people living with Chagas disease especially in Latin America and the US, leading to untreatable heart disease and cardiomyopathy.[6] A recent study evaluated the clinical efficacy of benznidazole (BZ), the only available drug; despite the reduction of the parasitemia, the study concluded that BZ induced no significant improvement in cardiac clinical outcomes. According to the Drugs for Neglected Diseases initiative (DNDi), in the next five years, 200,000 people living with Chagas disease will die from heart disease and related complications.[7] The DNDi also pointed out "*We urgently need to redouble our efforts to identify and treat young people who are still in the early stages of their illness, but ultimately we need to find better treatments and new cures*".[8]

The pyrone ring system is present in a diverse array of natural products isolated from a wide variety of sources, collected from both marine and terrestrial environments.

Of the two pyrone isomers, alpha (2)-pyrone-bearing natural products[9] are more common than their gamma (4)-pyrone counterparts.[10] Both alpha- and gamma-pyrones have been reported to exhibit biological properties including antitumor, antimicrobial and anti-HIV activities.[9–11] There are only a few reports describing the evaluation of the pyrone class of natural products for activity towards infectious diseases such as leishmaniasis and Chagas disease. For example, gamma-pyrones **1** and **2**, isolated from the seeds of *Podolepsis hieracioides* (Asteraceae), exhibit *in vitro* antileishmanial activity in the micromolar range while kojic acid (**3**), isolated from *Aspergillus* fungi, demonstrates *in vitro* and *in vivo* antileishmanial efficacy (**Figure 1**).[12,13] With regard to alpha-pyrones, styryl pyrone,11-methoxyyangonin (**4**) exhibits activity towards *Trypanosoma brucei rhodesiense* (IC_{50} 31 µM) and *Plasmodium falciparum* (IC_{50} 3.0 µM) while the marine microbial pyrones pseudopyronine A (**5**) and B (**6**) were found to be growth inhibitors of *Trypanosoma brucei rhodesiense* (IC_{50} 13.09 and 12.46 µg/mL, respectively)*, Leishmania* (*L.*) *donovani* (IC_{50} 2.63 and 1.38 µg/mL, respectively) and *Plasmodium falciparum* (IC_{50} 14.89 and 14.2 µg/mL, respectively).[14,15]

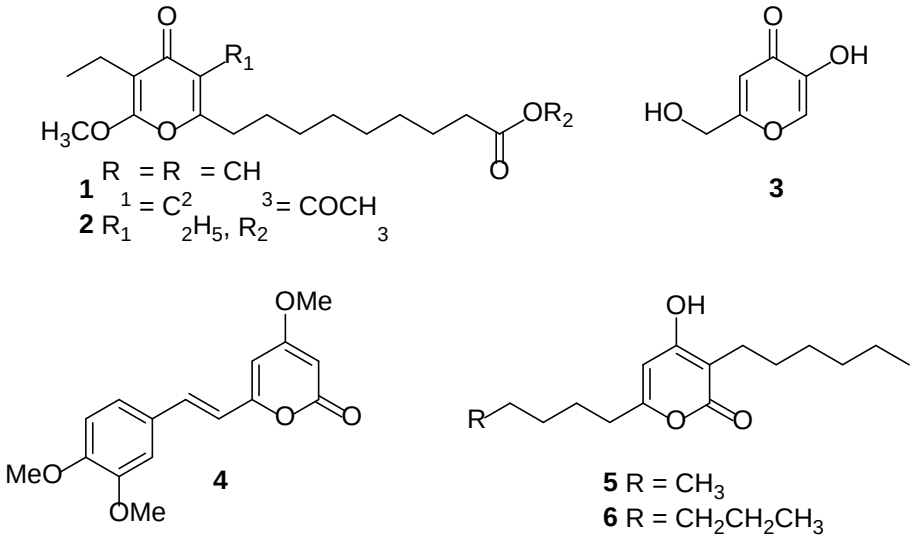


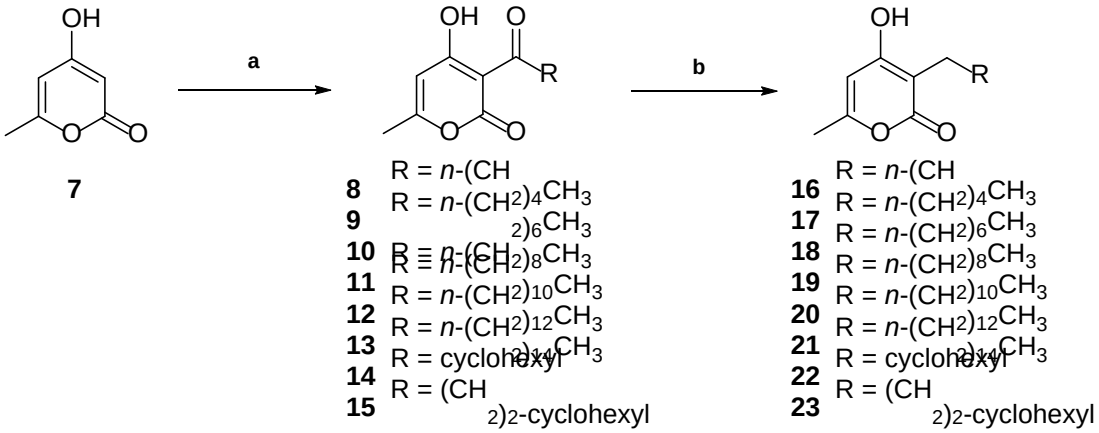
Figure 1. Structures of alpha and gamma pyrone natural products (**1**–**6**).

Structure-activity relationship studies were carried out on this series with a set of twenty-seven alpha-pyrone derivatives related to the pseudopyronines being

synthesized and their antiparasitic efficacy evaluated against *L. infantum* and *T. cruzi.* The efficacy of the most selective compound against *T. cruzi* was investigated in an acute phase *in vivo* murine model. Additionally, using flow cytometry analysis and fluorescent-based assays, the lethal action of the most selective compound in *T. cruzi* parasites was evaluated.

2. CHEMISTRY

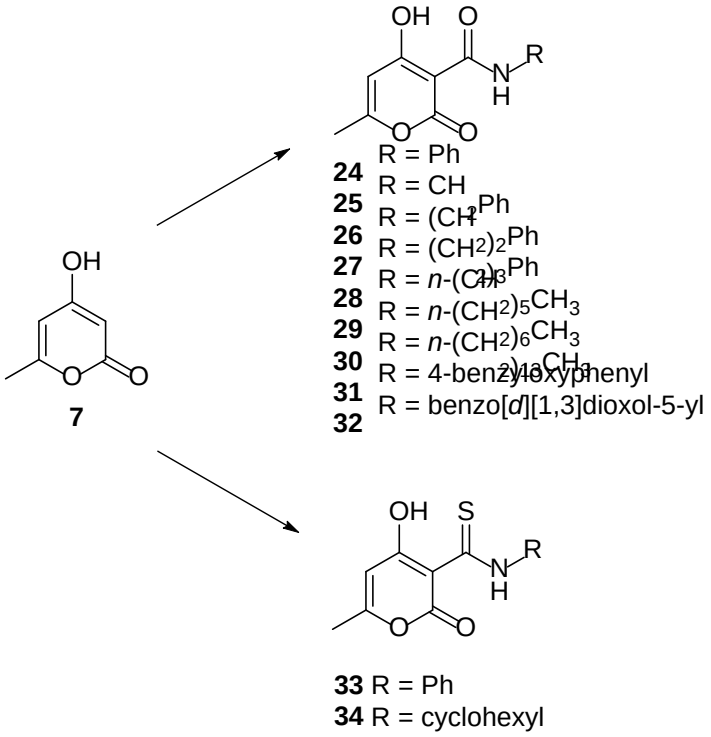
Target alpha-pyrones were chosen to explore the effects of chain length, steric bulk and lipophilicity on bioactivity. To this end, the pyrones were prepared by electrophilic substitution of 4-hydroxy-6-methyl-2-pyrone (**7**) with a variety of acyl chlorides in TFA and heated under reflux for 5 hours (**Scheme 1**). Solvent removal under reduced pressure followed by purification of the crude product by silica gel column chromatography afforded alkanoyl pyrones **8**–**15** in yields ranging from 43% to 63%. Subsequently, reaction of alkanoyl pyrones **8**–**15** with sodium cyanoborohydride and HCl in THF for 2–5 hours afforded alkyl pyrones **16**–**23** in yields ranging from 63% to 76% after purification.



Scheme 1. Synthesis of alkanoyl and alkyl pyrones (**8**–**23**).
Reagents and conditions: a) Acyl chloride (2.1 eq.), TFA, reflux, 5 h, 53% (**8**), 48% (**9**), 59% (**10**), 52% (**11**), 49% (**12**), 43% (**13**), 62% (**14**), 53% (**15**); b) sodium cyanoborohydride (2.5 eq.), HCl, THF, 2–5 h, 76% (**16**), 75% (**17**), 70% (**18**), 71% (**19**), 63% (**20**), 75% (**21**), 68% (**22**), 70% (**23**).

Further variation at the pyrone C-3 position was then explored by the introduction of amido or thioamido substituents. To achieve this, pyrone **7** was reacted

with the appropriate isocyanate or isothiocyanate and TEA in toluene at 120 ºC for 6 hours (**Scheme 2**). Purification of the crude product by column chromatography afforded amido pyrones **24**–**32** and thioamido pyrones **33** and **34** in yields ranging from 6% to 70%.



Scheme 2. Synthesis of amido and thioamido pyrones (**24**–**34**).
Reagents and conditions: Isocyanate/isothiocyanate (5 eq.), TEA (0.25 eq.), toluene, 120 ºC, 6 h, 67% (**24**), 70% (**25**), 53% (**26**), 42% (**27**), 46% (**28**), 37% (**29**), 34% (**30**), 25% (**31**), 50% (**32**), 45% (**33**), 6% (**34**).

The synthesis and characterization of alpha-pyrones **8**–**11**, **13**, **14**, **17** and **18** have been previously reported and in all cases the ^{1}H NMR spectroscopic and mass spectrometric data from the current study were in agreement with literature values.[16–20] Novel compounds **12**, **15**, **16** and **19**–**34** were characterized utilizing a combination of 1D and 2D NMR and HRMS.

3. RESULTS

3.1. *In vitro* Efficacy and Cytotoxicity

Among the twenty seven synthesized alpha-pyrones, eighteen compounds demonstrated activity against *Leishmania* and *T. cruzi* parasites (**Table 1**). The *in vitro* data indicated that compounds **8**, **15**, **25**, **26** and **28** presented activity in the range of 13 to 53 µM against *Leishmania* intracellular amastigotes. Among them, hexanoyl analogue **8** was the most selective and potent, with an IC_{50} value of 13 µM and a Selectivity Index (SI) higher than 14. Miltefosine was used as standard drug and resulted in an IC_{50} value of 16.2 µM against the promastigotes and 7.4 µM against the intracellular amastigotes. Fifteen of the tested alpha-pyrones were effective against *T. cruzi* trypomastigotes, with IC_{50} values in the range of 1 to 90 µM. Compounds **11** and **12** were the most potent against trypomastigotes, with IC_{50} values of 1.3 and 2.6 µM, respectively, and with SI values >70. The most selective compound (**12**) was tested against intracellular amastigotes of *T. cruzi*, but demonstrated limited selectivity, eliminating 14% ±3 of amastigotes at 25 µM. Benznidazole was used as standard drug and resulted in an IC_{50} value of 16.4 µM against the trypomastigotes. The series demonstrated low mammalian cytotoxicity (NCTC); among the 27 compounds, 10 (37%) presented no mammalian cytotoxicity to the highest tested concentration (200 µM), and only compounds **20** and **21** presented 50% Cytotoxic Concentration (CC_{50}) values below 50 µM (**Table 1**).

Table 1. Antiparasitic and mammalian cytotoxicity of alpha-pyrone derivatives**.**

Nº	**IC_{50} (µM)[a] (CI 95%)[b]**				**Selectivity Index**	
	***L. inf* pro[c]**	***L. inf* ama[d]**	***T. cruzi*[e]**	**NCTC**	***L. inf* ama[d]**	***T. cruzi*[e]**
8	NA[f]	13.8 (10.32 – 18.39)	NA	>200	>14.5	–
9	NA	NA	25.7 (22.6 – 29.3)	140.1 (106.7 – 184.0)	–	5.4
10	NA	NA	17.5 (13.4 – 22.8)	134.1 (107.4 – 167.4)	–	7.6
11	NA	NA	1.3 (0.6 – 2.5)	90.3 (59.7 – 136.5)	–	71.1
12	NA	NA	2.6 (1.0 – 6.6)	>200	–	>77.5
13	NA	NA	66.3	164.5	–	2.5

			(48.0 – 91.7)	(114.2 – 237.0)		
14	NA	NA	NA	>200	–	–
15	NA	39.2 (20.7 – 74.4)	4.4 (2.7 – 7.12)	117.2 (90.1 – 152.4)	2.9	26.8
16	NA	NA	NA	>200	–	–
17	NA	NA	40.0 (36.1 – 44.3)	144.7 (123.9 – 168.8)	–	3.6
18	117.7 (88.9 – 155.7)	NA	NA	56.0 (41.9 – 74.9)	–	–
19	137.5 (119.7 – 157.9)	NA	16.1 (13.4 – 19.4)	57.2 (44.4 – 73.6)	–	3.5
20	NA	NA	4.9 (3.3 – 7.3)	24.1 (17.4 – 33.4)	–	4.9
21	NA	NA	26.6 (16.2 – 43.4)	38.2 (32.2 – 45.4)	–	1.4
22	NA	NA	NA	>200	–	
23	NA	NA	32.2 (26.3 – 39.5)	109.1 (71.5 – 166.4)	–	3.4
24	NA	NA	NA	> 200	–	–
25	NA	52.8 (34.7 – 80.4)	NA	> 200 (182.5 – 389.2)	5.0	–
26	NA	46.8 (31.8 – 69.1)	NA	178.3 (115.3 – 275.6)	3.8	–
27	NA	NA	81.4 (75.8 – 87.4)	142.9 (110.8 – 184.2)	–	1.7
28	NA	54.0 (42.7 – 68.2)	72.6 (56.8 – 92.7)	195.5 (168.8 – 226.4)	3.6	2.7
29	NA	NA	78.1 (64.4 – 94.6)	186.6 (148.8 – 234.1)	–	2.4
30	NA	NA	NA	> 200	–	–
31	NA	NA	NA	> 200	–	–
32	NA	NA	NA	> 200	–	–
33	NA	>100	NA	> 200	–	–
34	NA	NA	90.5 (60.4 – 135.5)	176.7 (153.3 – 203.6)	–	1.9
BZ	–	–	16.2 (12.9 – 20.3)	>200		>36
MT	16.2 (14.6 – 16.9)	7.4 (6.7 – 7.6)		241.4 (236.7 – 254.1)		32.6

[a] 50% inhibitory concentration; [b] 95% Confidence interval ; [c] *Leishmania infantum* promastigotes; [d] *Leishmania Infantum* amastigotes; [e] *Trypanosoma cruzi* trypomastigotes; [f] Not active up to 200 µM; BZ-benznidazole, MT- miltefosine.

3.2. Lethal Action studies in *T cruzi*

The ability of **12** to compromise the plasma membrane of *T. cruzi* was assessed using the fluorescent probe SYTOX green method.[21] Compound **12** induced no alteration in plasma membrane permeability within 7 hours of incubation (**Figure 2**). Triton X-100 was used as the positive control.

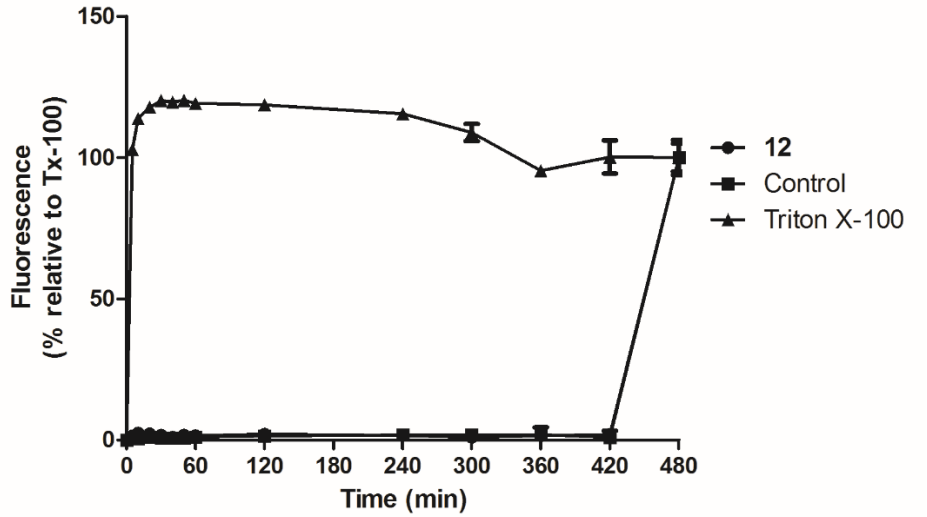


Figure 2. Permeability of *T. cruzi* trypomastigotes, assessed with the vital dye SYTOX green. Trypomastigotes were treated at the IC_{99} value of compound **12** and also with Triton X-100 (used as the 100 % permeabilization positive control). A control group (untreated) was also included.

Using flow cytometry analysis, the action of **12** on the mitochondrial function of living *T. cruzi* parasites was also investigated, using two fluorescent probes, propidium iodide and rhodamine 123.[21] At the IC_{50} and IC_{99} values, pyrone **12** induced hyperpolarization of the mitochondrial membrane potential, increasing the fluorescent levels by 25% and 26% ($p<0.05$), respectively, when compared to untreated parasites (**Figure 3A**). Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was used as a positive control and caused an intense depolarization of the mitochondrial membrane potential, as observed by the reduced fluorescence levels after 2 h incubation (**Figure 3B**).

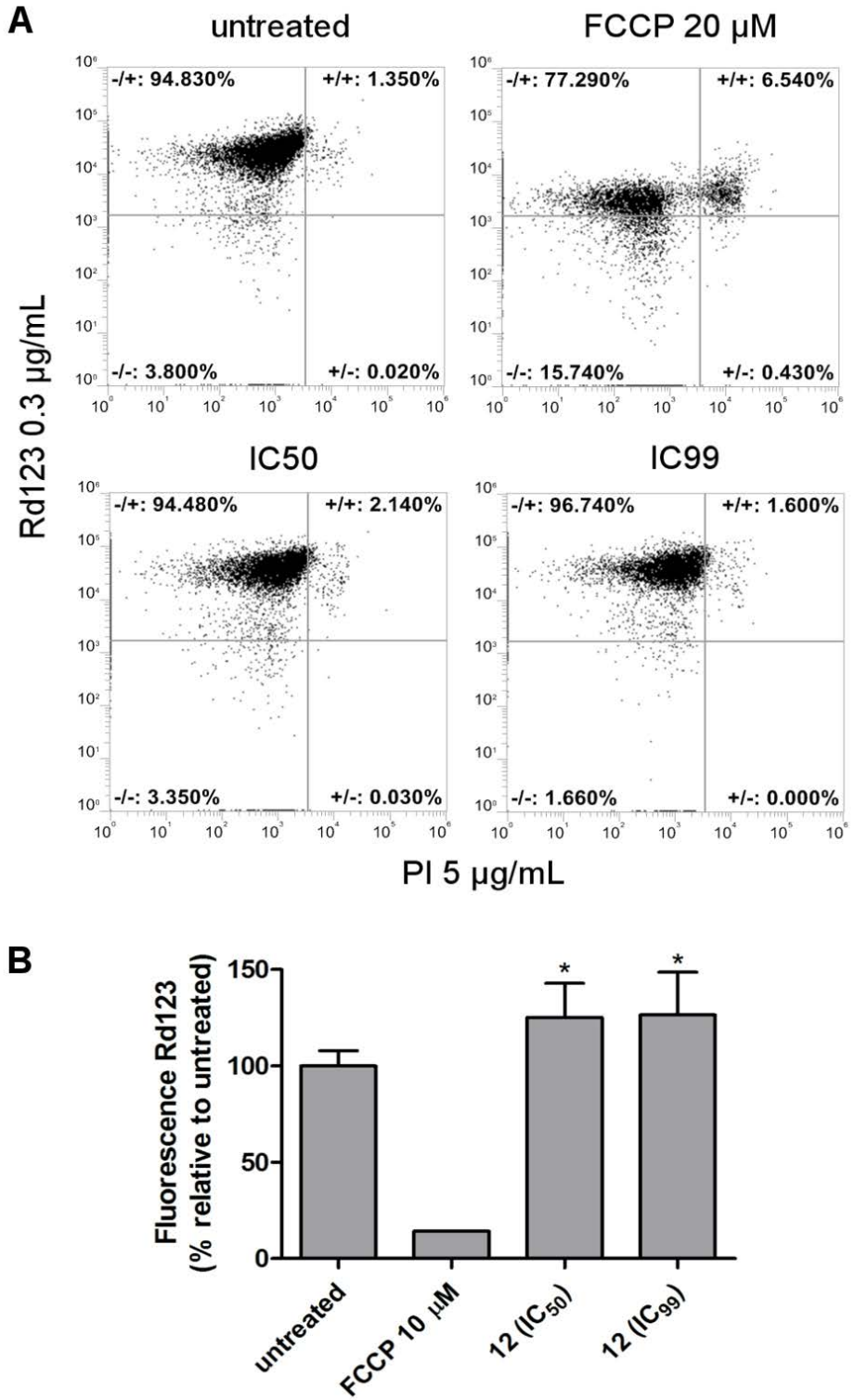


Figure 3. Evaluation of the mitochondrial membrane potential of *T. cruzi* trypomastigotes incubated with compound **12**. **(A)** Flow cytometry graphs showing in y axis Rd123 fluorescence and x axis PI fluorescence. Changes of Rd123 fluorescence determined by flow cytometry and reported as percentage relative to untreated parasites. **(B)** Maximal and minimal fluorescence of Rd123 were achieved by non-treatment and treatment with FCCP (10 µM), respectively. Trypomastigotes were treated at the IC_{50} and IC_{99} values of compound **12** for 2 h. * $p<0.05$ related to positive control (FCCP).

3.3. *In vivo* efficacy studies with *T. cruzi*

Five days post infection (5 d.p.i), BALB/c mice were treated with compound **12** at 30 mg/kg/day via intraperitoneal route for 10 consecutive days. Parasitemia was determined in blood by light microscopy counting. Eight days post infection (8 d.p.i.), at the first parasitemia onset, the compound showed no efficacy, demonstrating similar number of parasites to the untreated group. After 14 d.p.i., at the second and last parasitemia onset, compound **12** reduced the number of parasites in blood by 55% ($p<0.05$) when compared to untreated animals (**Figure 4A**). Benznidazole was used as a standard drug at 100 mg/kg via oral route and reduced parasite numbers by almost 100% at both parasitemia onsets. The survival curve demonstrated no death rate at compound **12**-treated and benznidazole-treated groups. Non-treated animals resulted in a 20% death rate after 14 days (**Figure 4B**).

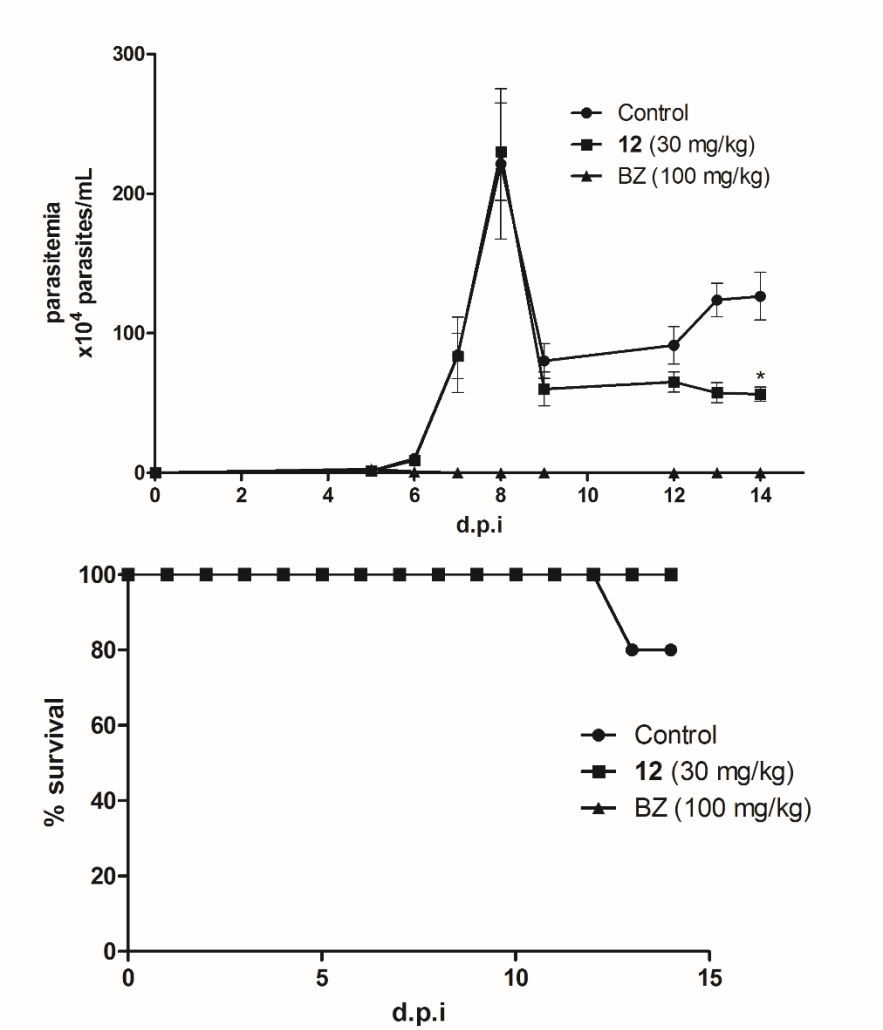


Figure 4. *In vivo* efficacy of compound **12** in experimental acute-phase mice model of *T. cruzi* (Y strain).The compound was administered intraperitoneally at 30 mg/kg/day and benznidazole (per oral gavage) at 100 mg/kg/day at the same conditions. The parasitemia (blood) was determined by light microscopy counting (**A**) and the survival rate was observed during 14 days (**B**). *p<0.05

3.4. Histopathological Examination

Healthy animals were intraperitoneally treated with compound **12** for 10 consecutive days at 30 mg/kg/day, and a histopathological examination of the organs was performed. **Figure 5A** shows the liver of a representative animal from the untreated group (control), demonstrating a healthy organ. The liver image of a treated animal (**Figure 5B**) showed minimum hydropic degeneration (vacuolation) of hepatocytes when compared to control. The other organs revealed normal architecture

(data not presented), suggesting no detrimental changes and morphological disturbances.

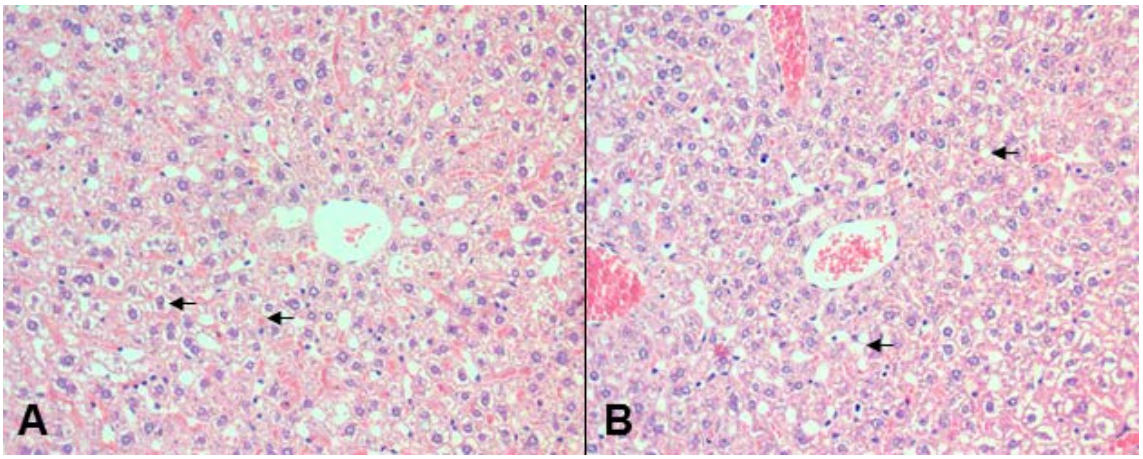


Figure 5. Histological sections of liver stained with hematoxylin and eosin demonstrating the vacuolization of liver cells. Untreated animal (**A**); animal treated intraperitoneally with compound **12** at 30 mg/kg/day for 10 consecutive days (**B**). Vacuolization of liver cells (indicated by arrows).

4. DISCUSSION

2-Pyrone derivatives show promising antimicrobial and antiparasitic activities. Previous studies have identified the 3,6-dialkyl-4-hydroxy-pyran-2-one marine microbial metabolites pseudopyronines A and B as inhibitors of protozoan parasites *Plasmodium falciparum* and *Leishmania donovani.*[14] In the search for new anti-parasitic hit compounds, we now report the synthesis, antileishmanial and the antitrypanosomal activities and a structure-activity relationship (SAR) study of twenty-seven alpha-pyrones. The biological activities of these compounds were evaluated against extracellular and intracellular amastigote forms of *L. infantum* and *T. cruzi* and for cytotoxicity towards murine fibroblasts.

Among the twenty seven alpha-pyrones, over half (55%) exhibited *in vitro* efficacy against *T. cruzi* parasites, with two compounds (**11** and **12**) demonstrating superior antiparasitic potency and no toxicity to mammalian cells. Although compound **12** was effective against trypomastigotes, it failed to eliminate adequately the intracellular amastigotes of *T. cruzi*. This fact might be ascribed to poor internalization

into the macrophages (lack of receptors), intracellular degradation or even to the resistance of the amastigote forms, which differ metabolically from trypomastigotes. When compared to the standard drug benznidazole (BZ), pyrone **12** was approximately 6-fold more potent. The intracellular amastigotes are clinically relevant forms of *T. cruzi*, but trypomastigote forms are also found in mammalians mainly in the acute phase of the disease, resulting in recrudescence of the parasitemia, especially when complete elimination of parasites is not achieved during treatment. Finding new drugs to selectively affect trypomastigotes is considered a relevant therapeutic approach.[22]

Leishmania parasites were also susceptible to the alpha-pyrones. In the present study, four compounds were effective against the intracellular amastigotes of *Leishmania*; among them, hexanoyl pyrone **8** showed similar potency to the standard drug miltefosine. Natural alpha-pyrones isolated from *Raimondia cf. monoica* showed *in vitro* activities against *Plasmodium falciparum* and *Leishmania panamensis*, with IC_{50} values in a range between 0.4 to 10 µg/mL, but these compounds presented an elevated mammalian cytotoxicity (CC_{50} values between 1 and 2 µg/mL).[23] Considering the need for new hit compounds with wide-ranging antiparasitic activity, the present study has identified two compounds (**15** and **28**) that inhibit the growth of both *L. infantum* intracellular amastigotes and *T. cruzi* trypomastigotes. Although the compounds activities were not considered very promising (e.g. IC_{50}>10 µM), these compounds could serve as future scaffolds for the synthesis of new derivatives. Overall, limited cytotoxicity towards murine NCTC fibroblasts was observed for the alpha-pyrones in this study - 22 compounds (81%) demonstrated cytotoxicity (CC_{50} values) higher than 100 µM and a total of 10 compounds completely lacked mammalian toxicity up to 200 µM. This is very encouraging and suggests that a reasonable therapeutic window already exists for the compounds, given the *T. cruzi* activity is < 5 µM for the leading examples.

Pyrone **12** was selected for further analysis of two properties relevant to its potential mechanism of antiparasitic action. Alteration of membrane permeability is

recognized as one method that small molecules can exhibit antiparasitic activity. For example, antimicrobial peptides[21] and marine guanidine alkaloids[24] demonstrate the ability to increase the permeability of *Trypanosoma* and *Leishmania* parasite membranes, respectively. In both cases, permeabilization leads to cell death. Also relevant to the current study, was the report by Singh *et al*.[25] that pseudopyronine A (**5**) alters bacterial membrane permeability. In the present study, pyrone **12** induced no alteration in the permeability of the plasma membrane of *T. cruzi* trypomastigotes after 7 hours of incubation.

Differing from mammalian cells, the mitochondria of *T. cruzi* is a unique organelle, and consequently, has been considered a promising target for new drugs. For example, alpha-pyrones brevipolides A-F, isolated from the plant *Hyptis brevipes*, were found to alter the mitochondrial transmembrane potential of mammalian cells.[26] In our assays, we investigated the mitochondrial membrane potential of *T. cruzi* after incubation with compound **12**. By using flow cytometry analysis, we separated living from dead cells using the fluorescent probe propidium iodide, and assessed trypanosomal mitochondrial activity using the fluorescent probe rhodamine 123. When compared to positive controls and untreated cells, hyperpolarization of the mitochondrial membrane potential of living parasites in a dose-independent manner after 1 h incubation was observed. Mitochondrial membrane potential ($\Delta\Psi m$) is critical for maintaining the physiological function of the respiratory chain to generate ATP. A significant loss of $\Delta\Psi m$ renders cells depleted of energy with subsequent death.[27] The mitochondrial transmembrane potential ($\Delta\Psi m$; negative inside and positive outside) is the result of an electrochemical gradient maintained by two transport systems. In mammalian cells, mitochondrial hyperpolarization (MHP) is an early event of death, and appears to be mediated through inhibition of F_0F_1- ATPase or dephosphorylation of cytochrome c oxidase.[28] In addition, MHP appears to be the earliest change associated with several apoptosis pathways. Considering that apoptosis-like or programmed cell death has been proposed in *Trypanosoma*

cruzi,[29–31] future studies could investigate the apoptosis-like induction of compound **12** in *T. cruzi*. These results would direct the synthesis of more potent and selective compounds.

Considering the need for new effective compounds against the trypomastigote forms of *T. cruzi*, which causes recrudescence of the disease in benznidazole-treated patients,[22] we investigated the *in vivo* efficacy of pyrone **12** in an acute-phase experimental model of Chagas disease. Compound **12** was administered at a dose (30 mg/kg/day) for five consecutive days, and compared to the standard drug benznidazole (100 mg/kg/day). At the first parasitemia onset [observed at the 8^{th} day post-infection (dpi)], **12** showed no efficacy (**Figure 4**). However by the second and last parasitemia onset (14 dpi), the compound significantly reduced parasitemia by 55% ($p<0.05$). The results suggest a delayed action of the pyrone, perhaps due to drug penetration issues. Pharmacokinetic and pharmacodynamic (PK/PD) parameters, such as a short half-life ($T_{1/2}$), or a reduced maximal concentration in blood (Cmax), or even rapid metabolism, could also be possible explanations for the moderate efficacy of **12** in *T. cruzi*-infected animals. Future experimental PK studies will be performed in an effort to optimize efficacy in animals.

Analysis of structure activity relationships (SAR) was next conducted. The main features considered in the analyses were atom chain length / steric bulk and various molecular properties such as lipophilicity, polar surface area and the presence or absence of hydrogen bond donor / acceptor groups. An important caveat was that since a phenotypic screening approach was being used certain assumptions were necessarily made in this SAR analysis, for example, that all compounds were binding to the same biological target or inhibiting the same pathway, and that permeability of the various analogues was broadly similar. Compound properties were calculated using DataWarrior.[32]

Compound activity seemed to be strongly affected by the length of the alkyl chain (n atoms), with a linear arrangement of 12–14 atoms being favored. Longer chain

examples appeared to be disfavored, being up to 50 fold less active, for example compare **21** (n=16, IC_{50} = 26.6 µM) with **20** (n=14, IC_{50} = 4.9 µM); also **13** (n=16, IC_{50} = 66.3 µM) with **11** (n=12, IC_{50} = 1.3 µM) and **12** (n=14, IC_{50} = 2.6 µM). Shorter chain examples also appeared less active, for example compare **11** (n=12, IC_{50} = 1.3 µM) with **9** (n=8, IC_{50} = 25.5 µM). This suggests the presence of a hydrophobic binding site having a defined depth, with the shorter chain analogues still being able to bind, but having reduced potency due to decreased hydrophobic interactions.[33]

Steric bulk close to the pyranone core did not appear to be tolerated; for example, compounds **14**, **24** and **32** were all inactive, although interestingly as the chain is extended, activity was recovered with examples **27** (IC_{50} = 81.4 µM), and strikingly so with alkyl-cyclohexyl analogue **15** (IC_{50} = 4.4 µM). This suggests that the binding site for the molecule can accommodate lipophilic bulk at certain points along the alkyl chain. This information could prove important when designing a new set of analogues for synthesis.

The introduction of hydrogen bond acceptor (HBA) and donor (HBD) groups was also explored. For example, replacing a methylene unit with a keto group appeared favorable, giving approximately a ten-fold improvement in activity, compare **19** (IC_{50} = 16.1 µM) with **11** (IC_{50} = 1.3 µM). Interestingly, amide derivatives seemed to generally be much less favored. Although the direct analogues of compounds with comparable chain lengths to **11** or **12** were not prepared, examples were seen where the matched pairs of amides had significantly lower activity than the corresponding keto analogues; compare **30** (inactive) with **13** (IC_{50} = 66.3 µM), and **28** (IC_{50} = 72.6 µM) with **9** (IC_{50} = 25.7 µM).

Looking at predicted molecular characteristics, it seemed important that polar surface area of the compounds be less than around 65$Å^2$ in order to maintain activity. This may reflect the permeability of the molecules or some characteristic of the binding site – at this stage it is not possible to draw any more specific conclusions. Lipophilicity does not seem to be directly relevant, with compounds having similar cLogP values

ranging from very active (**11**, cLogP = 4.3, IC_{50} 1.3 μM) to inactive (**18**, cLogP = 4.5, and **30** cLogP = 4.9; both IA).

Whilst the compounds currently have encouraging potencies against *T. cruzi* trypomastigotes, the most active analogues such as **12** are rather lipophilic and likely to have modest solubilities and limited metabolic stability. This would be expected to provide challenges when conducting extended *in vivo* dosing experiments. Having some preliminary structure-activity data in hand for the series allows us to identify areas where modification of structure with other motifs could be contemplated to mitigate these potential liabilities and improve their overall drug-like properties.[34] For example, synthesis of derivatives of **12** contain polyether-type chains would be expected to maintain activity, yet have much improved physicochemical and metabolic properties than the parent compound.

The alpha-pyrone analogues described in the present study represent new hit compounds with *in vitro* efficacy against *T. cruzi* and *L. infantum*. Encouragingly, pyrone **12** demonstrated moderate efficacy in an acute-phase experimental model of *T. cruzi*. These preliminary results are being used to direct the synthesis of new and more potent derivatives against *Leishmania* and *Trypanosoma cruzi* parasites.

5. Material and Methods

5.1. General experimental procedures

Infrared spectra were recorded using a Perkin-Elmer spectrum One Fourier-transform IR spectrometer as a dry film. NMR spectra were recorded on a Bruker Avance DRX-400 spectrometer operating at 400 MHz for ^{1}H nuclei and 100 MHz for ^{13}C nuclei. Proto-deutero solvent signals were used as internal references (DMSO-d_6: δ_H 2.50, δ_C 39.52; $CDCl_3$: δ_H 7.26, δ_C 77.16). Standard Bruker pulse sequences were utilized. HRMS data were acquired on either a VG-7070 or a Bruker micrOTOF Q II mass spectrometer. Elemental analysis was performed at the Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand. Column chromatography was

performed using Davisil® silica gel. Thin layer chromatography was carried out on 0.2 mm thick plates of Kiesegel F_{254} (Merck).

5.2. General procedures for synthesis of compounds

5.2.1. General procedure A: synthesis of alkanoyl pyrones

4-Hydroxy-6-methyl-2-pyrone (**7**) (1 eq.) was dissolved in TFA (2 mL), the appropriate acyl chloride (2.1 eq.) added, reaction vessel purged with nitrogen and heated under reflux for 5 h. TFA was removed under reduced pressure to afford a yellow-brown oil which was dissolved in Et_2O or CH_2Cl_2, washed twice with brine and dried over anhydrous $MgSO_4$. Solvent removal under reduced pressure afforded the crude product as a yellow-brown oil which was purified by silica gel column chromatography eluting with CH_2Cl_2 to afford target compounds **8**–**15**.

5.2.2. General procedure B: synthesis of alkyl pyrones

To a solution of acylated pyrone (**8**–**15**) (1 eq.) in THF (4 mL) was added HCl (2 N, 3.5 mL) followed by sodium cyanoborohydride (2.5 eq.). The mixture was stirred for 2–5 h and monitored by TLC. If reaction had not gone to completion after 2 h, an additional 2 eq. of sodium cyanoborohydride was added and the reaction stirred for another 3 h. Water (10 mL) was then added and the aqueous layer extracted with CH_2Cl_2. The combined organic layers were washed twice with brine, dried over anhydrous $MgSO_4$ and the solvent removed under reduced pressure. Purification by silica gel column chromatography (CH_2Cl_2/MeOH 95:5) afforded target compounds **16**–**23**.

5.2.3. General procedure C: synthesis of amido and thioamido pyrones

To a solution of 4-hydroxy-6-methyl-2-pyrone (**7**) (1 eq.) in toluene (5 mL) was added isocyanate or isothiocyanate (5 eq.). Triethylamine (0.25 eq.) was then added and the reaction mixture heated at 120 ºC for 6 h under nitrogen. The reaction was

then cooled to room temperature followed by solvent removal under reduced pressure. The solid was then dissolved in CH_2Cl_2 (10 mL) and purified by silica gel column chromatography eluting with hexane followed by increments of CH_2Cl_2 to afford the target compounds **24**–**34**.

5.3. Synthesis of compounds

5.3.1. 3-Hexanoyl-4-hydroxy-6-methyl-2-pyrone (8)

Using general procedure A, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.404 g, 3.2 mmol) with hexanoyl chloride (0.916 g, 6.8 mmol) followed by purification afforded the title compound as white crystals (0.380 g, 53%). m.p. 64.0–65.0 ^{o}C (lit.66.0–66.5 ^{o}C[16]); R_f (*n*-hexane/CH_2Cl_2, 1:1) 0.3; IR(ATR) ν_{max} 3088, 2941, 1717, 1560, 1451 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.93 (1H, s, H-5), 3.07 (2H, t, *J* = 7.4 Hz, H_2-9), 2.27 (3H, d, *J* = 1.0 Hz, H_3-7), 1.70–1.62 (2H, m, H_2-10), 1.38–1.34 (4H, m, H_2-11, H_2-12), 0.91 (3H, t, *J* = 7.1 Hz, H_3 -13); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 208.2 (C-8), 181.4 (C-4), 168.9 (C-6), 161.1 (C-2), 101.7 (C-5), 99.6 (C-3), 41.7 (C-9), 31.5 (C-11), 23.8 (C-10), 22.6 (C-12), 20.8 (C-7), 14.1 (C-13); (+)-HRESIMS *m/z* 247.0937 $[M+Na]^+$ (calcd for $C_{12}H_{16}NaO_4$, 247.0941); Anal calcd for $C_{12}H_{16}O_4$: C, 64.27; H, 7.19. Found: C, 64.46; H, 7.19.

5.3.2. 4-Hydroxy-6-methyl-3-octanoyl-2-pyrone (9)

Using general procedure A, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.202 g, 1.6 mmol) with octanoyl chloride (0.553 g, 3.4 mmol) followed by purification afforded the title compound as pale yellow crystals (0.192 g, 48%). m.p. 67.5–68.5 °C (lit. 67.5–68.0^{o}C[16]); R_f (*n*-hexane/CH_2Cl_2, 1:1) 0.3; IR (ATR) ν_{max} 3094, 2922, 1719, 1560, 1453 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.93 (1H, s, H-5), 3.07 (2H, t, *J* = 7.5 Hz, H_2-9), 2.27 (3H, *J* = 1.0 Hz, H_3-7), 1.69–1.62 (2H, m, H_2-10), 1.39–1.29 (8H, m, H_2-11, H_2-12, H_2-13, H_2-14), 0.88 (3H, t, *J* = 7.3Hz, H_3 -15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 208.2 (C-8), 181.4 (C-4), 168.9 (C-6), 161.1 (C-2), 101.7 (C-5), 99.6 (C-3), 41.8 (C-9),

31.8 (C-13), 29.3 (C-11, C-12), 24.1 (C-10), 22.8 (C-14), 20.8 (C-7), 14.2 (C-15); (+)-HRESIMS *m/z* 275.1254 $[M+Na]^+$ (calcd for $C_{14}H_{20}NaO_4$, 275.1254); Anal calcd for $C_{14}H_{20}O_4$: C, 66.65; H, 7.99. Found: C, 66.86; H, 8.01.

5.3.3. 3-Decanoyl-4-hydroxy-6-methyl-2-pyrone (10)

Using general procedure A, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.202 g, 1.6 mmol) with decanoyl chloride (0.648 g, 3.4 mmol) followed by purification afforded the title compound as white crystals (0.266 g, 59%). m.p. 73.5–74.5 °C (lit. 74.0–74.5ºC[16]); IR (KBr) 3094, 2915, 1713, 1555, 1452 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.93 (1H, s, H-5), 3.07 (2H, t, *J* = 7.3 Hz, H_2-9), 2.27 (3H, s, H_3-7), 1.69–1.61 (2H, m, H_2-10), 1.39–1.27 (12H, m, H_2-11, H_2-12, H_2-13, H_2-14, H_2-15, H_2-16), 0.88 (3H, t, *J* = 6.5 Hz, H_3-17); ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.2 (C-8), 181.4 (C-2), 168.9 (C-4), 161.1 (C-6), 101.7 (C-5), 99.7 (C-3), 41.8 (C-9), 32.0 (C-15), 29.6 (C-12, C-13), 29.4 (C-11, C-14), 24.1 (C-10), 22.8 (C-16), 20.8 (C-7), 14.2 (C-17); (+)-HREIMS *m/z* 280.1672 $[M+H]^+$ (calcd for $C_{16}H_{24}O_4$, 280.1675); Anal calcd for $C_{16}H_{24}O_4$: C, 68.54; H, 8.63. Found: C, 68.79; H, 8.55.

5.3.4. 3-Dodecanoyl-4-hydroxy-6-methyl-2*H*-pyran-2-one (11)

Using general procedure A, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.337 g, 2.7 mmol) with dodecanoyl chloride (1.269 g, 5.8 mmol) followed by purification afforded the title compound as white crystals (0.434 g, 52%). m.p. 80.0–81.0 °C (lit. 82.0–82.5ºC[16]); R_f (*n*-hexane/CH_2Cl_2, 1:1) 0.3; IR (ATR) ν_{max} 3094, 2921, 1722, 1555, 1449 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.93 (1H, s, H-5), 3.07 (2H, t, *J* = 7.3 Hz, H_2-9), 2.27 (3H, s, H_3-7), 1.69–1.61 (2H, m, H_2-10), 1.39–1.26 (16H, m, H_2-11, H_2-12, H_2-13, H_2-14, H_2-15, H_2-16, H_2-17, H_2-18), 0.88 (3H, t, *J* = 7.0 Hz, H_3 -17); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 208.2 (C-8), 181.4 (C-4), 168.9 (C-6), 161.1 (C-2), 101.7 (C-5), 99.6 (C-3), 41.8 (C-9), 32.1 (C-17), 29.8 (C-13, C-14), 29.6 (C-12, C-15), 29.5 (C-16), 29.4 (C-11), 24.1 (C-10), 22.8 (C-18), 20.8 (C-7), 14.3 (C-19); (+)-HRESIMS *m/z*

331.1876 $[M+Na]^+$ (calcd for $C_{18}H_{28}NaO_4$, 331.1880); Anal calcd for $C_{18}H_{28}O_4$: C, 70.10; H, 9.15. Found: C, 70.20; H, 9.03.

5.3.5. 4-Hydroxy-6-methyl-3-tetradecanoyl-2-pyrone (12)

Using general procedure A, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.337 g, 2.7 mmol) with tetradecanoyl chloride (1.431 g, 5.8 mmol) followed by purification afforded the title compound as white needles (0.440 g, 49%). m.p. 84.5–85.0 °C; R_f (*n*-hexane/CH_2Cl_2, 1:1) 0.3; IR (ATR) ν_{max} 3093, 2915, 1713, 1562, 1471 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.93 (1H, d, *J* = 1.0 Hz, H-5), 3.07 (2H, t, *J* = 7.2 Hz, H_2-9), 2.26 (3H, s, H_3-7), 1.69–1.61 (2H, m, H_2-10), 1.39–1.26 (20H, m, H_2-11, H_2-12, H_2-13, H_2-14, H_2-15, H_2-16, H_2-17, H_2-18, H_2-19, H_2-20), 0.88 (3H, t, *J* = 7.0 Hz, H_3-21); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 208.2 (C-8), 181.4 (C-4), 168.9 (C-6), 161.1 (C-2), 101.7 (C-5), 99.6 (C-3), 41.8 (C-9), 32.1 (C-19), 29.8 (C-13, C-14, C-15, C-16), 29.6 (C-12, C-17), 29.5 (C-18), 29.4 (C-11), 24.1 (C-10), 22.8 (C-20), 20.8 (C-7), 14.3 (C-21); (+)-HRESIMS *m/z* 359.2183 $[M+Na]^+$ (calcd for $C_{20}H_{32}NaO_4$, 359.2193); Anal calcd for $C_{20}H_{32}O_4$: C, 71.39; H, 9.59. Found: C, 71.45; H, 9.62.

5.3.6. 4-Hydroxy-6-methyl-3-palmitoyl-2-pyrone (13)

Using general procedure A, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.337 g, 2.7 mmol) with palmitoyl chloride (1.594 g, 5.8 mmol) followed by purification afforded the title compound as pale yellow crystals (0.422 g, 43%). m.p. 87.0–88.0 °C (lit. 87.0–88.0ºC[17]); R_f (*n*-hexane/CH_2Cl_2, 1:1) 0.3; IR (ATR) ν_{max} 3092, 2915, 1713, 1557, 1471 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.93 (1H, s, H-5), 3.07 (2H, t, *J* = 7.3 Hz, H_2-9), 2.26 (3H, s, H_3-7), 1.69–1.61 (2H, m, H_2-10), 1.39–1.26 (24H, m, H_2-11, H_2-12, H_2-13, H_2-14, H_2-15, H_2-16, H_2-17, H_2-18, H_2-19, H_2-20, H_2-21, H_2-22), 0.88 (3H,t, *J* = 7.0 Hz, H_3 -23); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 208.2 (C-8), 181.4 (C-4), 168.9 (C-6), 161.1 (C-2), 101.7 (C-5), 99.7 (C-3), 41.8 (C-9), 32.1 (C-21), 29.8 (C-13, C-14, C-15, C-16, C-17, C-18), 29.7 (C-19), 29.6 (C-12), 29.5 (C-20), 29.4 (C-11), 24.1 (C-10), 22.8 (C-22), 20.8 (C-7), 14.3 (C-23); (+)-HRESIMS *m/z* 387.2515 $[M+Na]^+$ (calcd for

$C_{22}H_{36}NaO_4$, 359.2506); Anal calcd for $C_{22}H_{36}O_4$: C, 72.49; H, 9.95. Found: C, 72.71; H, 9.89.

5.3.7. 3-(Cyclohexanecarbonyl)-4-hydroxy-6-methyl-2-pyrone (14)

Using general procedure A, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.534 g, 4.2 mmol) with cyclohexanecarbonyl chloride (1.232 g, 8.4 mmol) followed by purification afforded the title compound as white crystals (0.615 g, 62%). m.p. 71.0–73.0 °C (lit. 72.0ºC[18]); R_f (*n*-hexane/CH_2Cl_2, 1:1) 0.3; IR (ATR) ν_{max} 3093, 2916, 1713, 1558, 1472 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.92 (1H, s, H-5), 3.70–3.65 (1H, m, H-9), 2.26 (3H, d, *J* = 1.0 Hz, H_3-7), 1.88–1.86 (2H, m, H_2-10_A, H_2-14_A), 1.83–1.80 (2H, m, H_2-11_A, H_2-13_A), 1.75–1.69 (1H, m, H_2-12_A), 1.47–1.33 (4H, m, H_2-10_B, H_2-11_B, H_2-13_B, H_2-14_B), 1.27–1.20 (1H, m, H_2 -12_B); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 211.3 (C-8), 182.2 (C-4), 168.8 (C-6), 160.8 (C-2), 101.8 (C-5), 98.9 (C-3), 47.3 (C-9), 28.8 (C-10, C-14), 26.1 (C-12), 25.8 (C-11, C-13), 20.7 (C-7); (+)-HRESIMS *m/z* 259.0941 $[M+Na]^+$ (calcd for $C_{13}H_{16}NaO_4$, 259.0941); Anal calcd for $C_{13}H_{16}O_4$: C, 66.09; H, 6.83. Found: C, 66.06; H, 6.96.

5.3.8. 3-(3-Cyclohexylpropanoyl)-4-hydroxy-6-methyl-2-pyrone (15)

Using general procedure A, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.337 g, 2.7 mmol) with 3-cyclohexylpropanoyl chloride (1.013 g, 5.8 mmol) followed by purification afforded the title compound as white crystals (0.375 g, 53%). m.p. 93–95 °C; R_f (*n*-hexane/CH_2Cl_2, 1:1) 0.3; IR (ATR) ν_{max} 3097, 2921, 1722, 1555, 1450 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.93 (1H, s, H-5), 3.07 (2H, t, *J* = 7.5 Hz, H_2-9), 2.26 (3H, d, *J* = 1.0 Hz, H_3-7), 1.77–1.66 (6H, m, H_2-12, H_2-14, H_2-15), 1.58–1.51 (2H, m, H_2-10), 1.36–1.27 (1H, m, H-11), 1.25–1.13 (2H, m, H_2-13), 0.98–0.88 (2H, m, H_2-16); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 208.6 (C-8), 181.4 (C-4), 168.9 (C-6), 161.1 (C-2), 101.7 (C-5), 99.6 (C-3), 39.5 (C-9), 37.5 (C-11), 33.3 (C-12, C-16), 31.5 (C-10), 26.7 (C-14), 26.4 (C-13, C-15), 20.8 (C-7); (+)-HRESIMS *m/z* 287.1250 $[M+Na]^+$ (calcd for

$C_{15}H_{20}NaO_4$, 287.1254); Anal calcd for $C_{15}H_{20}O_4$: C, 68.16; H, 7.63. Found: C, 68.29; H, 7.74.

5.3.9. 3-Hexyl-4-hydroxy-6-methyl-2-pyrone (16)

Using general procedure B, reaction of **8** (0.050 g, 0.22 mmol) with sodium cyanoborohydride (0.035 g, 0.56 mmol) followed by purification afforded the title compound as white crystals (0.035 g, 76%). m.p. 114.0–115.5 °C; R_f (CH_2Cl_2/MeOH, 97:3) 0.3; IR (ATR) ν_{max} 2923, 2664, 1571, 1443 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 10.02 (1H, br s, OH), 6.21 (1H, s, H-5), 2.45 (2H, t, *J* = 7.6 Hz, H_2-8), 2.21 (3H, d, *J* = 1.0 Hz, H_3-7), 1.53–1.45 (2H, m, H_2-9), 1.37–1.29 (6H, m, H_2-10, H_2-11, H_2-12), 0.86 (3H, t, *J* = 6.5 Hz, H_3-13); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.4 (C-2), 167.2 (C-4), 159.9 (C-6), 103.5 (C-3), 101.8 (C-5), 31.9 (C-11), 29.4 (C-10), 28.2 (C-9), 23.2 (C-8), 22.8 (C-12), 19.8 (C-7), 14.2 (C-13); (+)-HRESIMS *m/z* 233.1154 $[M+Na]^+$ (calcd for $C_{12}H_{18}NaO_3$, 233.1148); Anal calcd for $C_{12}H_{18}O_3$: C, 68.54; H, 8.63. Found: C, 68.79; H, 8.68.

5.3.10. 4-Hydroxy-6-methyl-3-octyl-2-pyrone (17)

Using general procedure B, reaction of **9** (0.050 g, 0.20 mmol) with sodium cyanoborohydride (0.031 g, 0.50 mmol) followed by purification afforded the title compound as pale pink crystals (0.035 g, 75%). m.p. 103.0–104.5 °C (lit. 100–102°C[19]); R_f (CH_2Cl_2/MeOH, 97:3) 0.3; IR (ATR) ν_{max} 2921, 2662, 1571, 1445 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.31 (1H, br s, OH), 6.12 (1H, d, *J* = 1.0 Hz, H-5), 2.43 (2H, t, *J* = 7.5 Hz, H_2-8), 2.21 (3H, d, *J* = 1.0 Hz, H_3-7), 1.53–1.46 (2H, m, H_2-9), 1.35–1.25 (10H, m, H_2-10, H_2-11, H_2-12, H_2-13, H_2-14), 0.86 (3H, t, *J* = 6.7 Hz, H_3-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.7 (C-2), 166.3 (C-4), 159.8 (C-6), 103.4 (C-3), 101.3 (C-5), 31.9 (C-13), 29.7 (C-12), 29.6 (C-11), 29.4 (C-10), 28.1 (C-9), 23.1 (C-8), 22.7 (C-14), 19.7 (C-7), 14.1 (C-15); (+)-HRESIMS *m/z* 261.1457 $[M+Na]^+$ (calcd for $C_{14}H_{22}NaO_3$, 261.1461); Anal calcd for $C_{14}H_{22}O_3$: C, 70.56; H, 9.30. Found: C, 70.80; H, 9.38.

5.3.11. 3-Decyl-4-hydroxy-6-methyl-2-pyrone (18)

Using general procedure B, reaction of **10** (0.050 g, 0.18 mmol) with sodium cyanoborohydride (0.028 g, 0.45 mmol) followed by purification afforded the title compound as white crystals (0.033 g, 70%). m.p. 101.5–102.5 °C (lit.103–104°C[20]); R_f (CH_2Cl_2/MeOH, 97:3) 0.3; IR (ATR) ν_{max} 2919, 2644, 1571, 1444 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.69 (1H, br s, OH), 6.18 (1H, d, *J* = 1.0 Hz, H-5), 2.44 (2H, t, *J* = 7.4 Hz, H_2-8), 2.21 (3H, s, H_3-7), 1.53–1.46 (2H, m, H_2-9), 1.36–1.24 (14H, m, H_2-10, H_2-11, H_2-12, H_2-13, H_2-14, H_2-15, H_2-16), 0.87 (3H, t, *J* = 6.8 Hz, H_3-17); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.2 (C-2), 166.9 (C-4), 159.9 (C-6), 103.5 (C-3), 101.7 (C-5), 32.1 (C-15), 29.9 (C-10), 29.8 (C-11, C-12, C-13), 29.5 (C-14), 28.3 (C-9), 23.2 (C-8), 22.8 (C-16), 19.8 (C-7), 14.2 (C-17); (+)-HRESIMS *m/z* 289.1769 $[M+Na]^+$ (calcd for $C_{16}H_{26}NaO_3$, 289.1774); Anal calcd for $C_{16}H_{26}O_3$: C, 72.14; H, 9.84. Found: C, 72.28; H, 9.60.

5.3.12. 4-Hydroxy-6-methyl-3-undecyl-2-pyrone (19)

Using general procedure B, reaction of **11** (0.050 g, 0.17 mmol) with sodium cyanoborohydride (0.026 g, 0.41 mmol) followed by purification afforded the title compound as white crystals (0.034 g, 71%). m.p. 98.0–99.0 °C; R_f (CH_2Cl_2/MeOH, 97:3) 0.3; IR (ATR) ν_{max} 2918, 2662, 1571, 1445 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.35 (1H, br s, OH), 6.15 (1H, d, *J* = 1.0 Hz, H-5), 2.44 (2H, t, *J* = 7.5 Hz, H_2-8), 2.21 (3H, d, *J* = 1.0 Hz, H_3-7), 1.53–1.43 (2H, m, H_2-9), 1.35–1.24 (18H, m, H_2-10, H_2-11, H_2-12, H_2-13, H_2-14, H_2-15, H_2-16, H_2-17, H_2-18), 0.87 (3H, t, *J* = 6.8 Hz, H_3-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.0 (C-2), 166.6 (C-4), 160.0 (C-6), 103.5 (C-3), 101.5 (C-5), 32.1 (C-17), 29.9 (C-11, C-12, C-13, C-14), 29.8 (C-15, C-16), 29.5 (C-10), 28.2 (C-9), 23.2 (C-8), 22.8 (C-18), 19.8 (C-7), 14.3 (C-19); (+)-HRESIMS *m/z* 317.208 $[M+Na]^+$ (calcd for $C_{18}H_{30}NaO_3$, 317.2087); Anal calcd for $C_{18}H_{30}O_3$: C, 73.43; H, 10.27. Found: C, 73.62; H, 10.01.

5.3.13. 4-Hydroxy-6-methyl-3-tetradecyl-2-pyrone (20)

Using general procedure B, reaction of **12** (0.050 g, 0.15 mmol) with sodium cyanoborohydride (0.024 g, 0.37 mmol) followed by purification afforded the title compound as pale yellow crystals (0.030 g, 63%). m.p. 98.0–99.0 °C; R_f (CH_2Cl_2/MeOH, 97:3) 0.3; IR (ATR) ν_{max} 2918, 2662, 1571, 1445 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.33 (1H, br s, OH), 6.14 (1H, d, *J* = 1.0 Hz, H-5), 2.44 (2H, t, *J* = 7.5 Hz, H_2-8), 2.21 (3H, d, *J* = 1.0 Hz, H_3-7), 1.53–1.46 (2H, m, H_2-9), 1.35–1.24 (22H, m, H_2-10, H_2-11, H_2-12, H_2-13, H_2-14, H_2-15, H_2-16, H_2-17, H_2-18, H_2-19, H_2-20), 0.87 (3H, t, *J* = 6.8 Hz, H_3-21); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.9 (C-2), 166.6 (C-4), 160.0 (C-6), 103.5 (C-3), 101.5 (C-5), 32.1 (C-19), 29.9 (C-10, C-11, C-12, C-13, C-14), 29.8 (C-15, C-16, C-17), 29.5 (C-18), 28.3 (C-9), 23.2 (C-8), 22.8 (C-20), 19.8 (C-7), 14.3 (C-21); (+)-HRESIMS *m/z* 317.208 $[M+Na]^+$ (calcd for $C_{18}H_{30}NaO_3$, 317.2087); Anal calcd for $C_{20}H_{34}O_3.0.3H_2O$: C, 73.13; H, 10.64. Found: C, 72.78; H, 10.40.

5.3.14. 3-Hexadecyl-4-hydroxy-6-methyl-2-pyrone (21)

Using general procedure B, reaction of **13** (0.050 g, 0.14 mmol) with sodium cyanoborohydride (0.022 g, 0.34 mmol) followed by purification afforded the title compound as white solid (0.036 g, 75%). m.p. 107–109°C; R_f (CH_2Cl_2/MeOH, 97:3) 0.3; IR (ATR) ν_{max} 2918, 2662, 1571, 1445 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.45 (1H, br s, OH), 6.15 (1H, d, *J* = 1.0 Hz, H-5), 2.44 (2H, t, *J* = 7.5 Hz, H_2-8), 2.21 (3H, s, H_3-7), 1.53–1.46 (2H, m, H_2-9), 1.35–1.24 (26H, m, H_2-10, H_2-11, H_2-12, H_2-13, H_2-14, H_2-15, H_2-16, H_2-17, H_2-18, H_2-19, H_2-20, H_2-21, H_2-22), 0.88 (3H, t, *J* = 6.8 Hz, H_3-23); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.0 (C-2), 166.7 (C-4), 159.9 (C-6), 103.5 (C-3), 101.5 (C-5), 32.1 (C-21), 29.9 (C-10, C-11, C-12, C-13, C-14, C-15, C-16, C-17), 29.8 (C-18, C-19), 29.5 (C-20), 28.3 (C-9), 23.2 (C-8), 22.8 (C-22), 19.8 (C-7), 14.3 (C-23); (+)-HRESIMS *m/z* 317.208 $[M+Na]^+$ (calcd for $C_{18}H_{30}NaO_3$, 317.2087); Anal calcd for $C_{22}H_{38}O_3.0.6H_2O$: C, 72.88; H, 10.94. Found: C, 72.82; H, 10.57.

5.3.15. 3-(Cyclohexylmethyl)-4-hydroxy-6-methyl-2-pyrone (22)

Using general procedure B, reaction of **14** (0.050 g, 0.21 mmol) with sodium cyanoborohydride (0.033 g, 0.53 mmol) followed by purification afforded the title compound as white crystals (0.032 g, 68%). m.p. 196.5–198.0 °C; R_f (CH_2Cl_2/MeOH, 98:2) 0.3; IR (ATR) ν_{max} 2920, 2652, 1561, 1448 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 5.96 (1H, d, *J* = 1.0 Hz, H-5), 2.14 (2H, d, *J* = 6.8 Hz, H_2-8), 2.13 (3H, s, H_3-7), 1.65–1.60 (2H, m, H_2-11), 1.57–1.52 (4H, m, H_2-12, H_2-14), 1.51–1.41 (1H, m, H-9), 1.16–1.09 (2H, m, H_2-13), 0.94–0.85 (2H, m, H_2 -10); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 165.4 (C-2), 165.0 (C-4), 159.6 (C-6), 99.8 (C-3, C-5), 36.3 (C-9), 32.7 (C-10, C-14), 30.1 (C-8), 26.1 (C-12), 25.8 (C-11, C-13), 19.2 (C-7); (+)-HRESIMS *m/z* 245.1143 $[M+Na]^+$ (calcd for $C_{13}H_{18}NaO_3$, 245.1148); Anal calcd for $C_{13}H_{18}O_3$: C, 70.24; H, 8.16. Found: C, 70.30; H, 8.29.

5.3.16. 3-(3-Cyclohexylpropyl)-4-hydroxy-6-methyl-2-pyrone (23)

Using general procedure B, reaction of **15** (0.050 g, 0.19 mmol) with sodium cyanoborohydride (0.030 g, 0.47 mmol) followed by purification afforded the title compound as pale yellow needles (0.034 g, 70%). m.p. 140.0–141.5 °C; R_f (CH_2Cl_2/MeOH, 97:3) 0.2; IR (ATR) ν_{max} 2921, 2659, 1574, 1445 cm^{-1}; ^1H NMR (DMSO-d_6 ,400 MHz) δ 5.96 (1H, *J* = 1.0 Hz, H-5), 2.21 (2H, t, *J* = 7.5 Hz, H_2-8), 2.12 (3H, *J* = 1.0 Hz, H_3-7), 1.65–1.55 (4H, m, H_2-13, H_2-16), 1.40–1.33 (2H, m, H_2-9), 1.22–1.07 (7H, m, H_2-10, H-11, H_2-14, H_2-15), 0.86–0.76 (2H, m, H_2-12); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 164.9 (C-2), 164.8 (C-4), 159.6 (C-6), 101.2 (C-3), 99.8 (C-5), 36.9 (C-10), 36.7 (C-11), 32.9 (C-12, C-16), 26.2 (C-14), 25.9 (C-13, C-15), 24.8 (C-9), 22.9 (C-8), 19.2 (C-7); (+)-HRESIMS *m/z* 273.1454 $[M+Na]^+$ (calcd for $C_{15}H_{22}NaO_3$, 273.1461); Anal calcd for $C_{15}H_{22}O_3$: C, 71.97; H, 8.86. Found: C, 71.83; H, 8.93.

5.3.17. 4-Hydroxy-6-methyl-2-oxo-*N*-phenyl-2-pyran-3-carboxamide (24)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with isocyanatobenzene (0.238 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with

CH_2Cl_2, afforded the title compound as a white crystalline solid (0.066 g, 67%). m.p. 153–154 ºC; R_f (CH_2Cl_2) 0.66; IR (ATR) ν_{max} 3063, 1698, 1544, 1493, 1441 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 10.93 (1H, br s, NH-9), 7.61 (2H, dd, *J* = 7.4, 1.2 Hz, H-11, H-15), 7.37 (2H, td, *J* = 7.4, 1.2 Hz, H-12, H-14), 7.18 (1H, tt, *J* = 7.4, 1.2 Hz, H-13), 6.04 (1H, d, *J* = 1.0 Hz, H-5), 2.31 (3H, s, H_3-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.5 (C-4), 168.4 (C-8), 166.3 (C-6), 164.0 (C-2), 136.6 (C-10), 129.2 (C-12, C-14), 125.4 (C-13), 121.3 (C-11, C-15), 102.6 (C-5), 91.7 (C-3), 20.5 (C-7); (+)-HRESIMS *m/z* 246.0759 $[M+H]^+$ (calcd for $C_{13}H_{12}NO_4$, 246.0761); Anal calcd for $C_{13}H_{11}NO_4$: C, 63.67; H, 4.52; N: 5.71. Found: C, 63.37; H, 4.68; N, 5.72.

5.3.18. *N*-Benzyl-4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-carboxamide (25)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with (isocyanatomethyl)benzene (0.266 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with *n*-hexane/CH_2Cl_2 (3:7), afforded the title compound as a light yellow crystalline solid (0.072 g, 70%). m.p. 123–124 ºC; R_f (*n*-hexane/CH_2Cl_2, 1:3) 0.59; IR (ATR) ν_{max} 3301, 3068, 1687, 1555, 1448 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.23 (1H, br s, NH-9), 7.28–7.17 (5H, m, H-12, H-13, H-14, H-15, H-16), 5.88 (1H, s, H-5), 4.49 (2H, d, *J* = 6.0 Hz, H_2-10), 2.16 (3H, d, *J* = 1.0 Hz, H_3-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.4 (C-4), 170.0 (C-8), 165.9 (C-6), 163.7 (C-2), 137.4 (C-11), 128.8 (C-13, C-15), 127.7 (C-12, C-16), 127.6 (C-14), 102.4 (C-5), 91.1 (C-3), 43.0 (C-10), 20.3 (C-7); (+)-HRESIMS *m/z* 260.0913 $[M+H]^+$ (calcd for $C_{14}H_{14}NO_4$, 260.0917).

5.3.19. 4-Hydroxy-6-methyl-2-oxo-*N*-phenethyl-2*H*-pyran-3-carboxamide (26)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with (2-isocyanatoethyl)benzene (0.294 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with *n*-hexane/CH_2Cl_2 (2:8), afforded the title compound as white needles (0.058 g,

53%). m.p. 121–123 ºC; R_f (*n*-hexane/CH_2Cl_2, 3:7) 0.77; IR (ATR) ν_{max} 3307, 3085, 2947, 1693, 1539, 1453 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.00 (1H, br s, NH-9), 7.33–7.29 (2H, m, H-13, H-17), 7.24–7.21 (3H, m, H-14, H-15, H-16), 5.94 (1H, s, H-5), 3.63 (2H, dt, *J* = 7.2, 6.8 Hz, H_2-10), 2.90 (2H, t, *J* = 7.2 Hz, H_2-11), 2.24 (3H, s H_3-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.4 (C-4), 170.1 (C-8), 165.8 (C-6), 163.7 (C-2), 138.5 (C-12), 128.9 (C-14/C-16), 128.8 (C-14/C-16), 128.7 (C-13/C-17), 128.6 (C-13/C-17), 126.7 (C-15), 102.5 (C-5), 91.0 (C-3), 40.7 (C-10), 35.6 (C-11), 20.3 (C-7); (+)-HRESIMS *m/z* 274.1071 $[M+H]^+$ (calcd for $C_{15}H_{16}NO_4$, 274.1074).

5.3.20. 4-Hydroxy-6-methyl-2-oxo-*N*-(3-phenylpropyl)-2*H*-pyran-3-carboxamide (27)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with (3-isocyanatopropyl)benzene (0.322 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with *n*-hexane/CH_2Cl_2 (2:8), afforded the title compound as a light yellow crystalline solid (0.048 g, 42%). m.p. 63–65 ºC; R_f (*n*-hexane/CH_2Cl_2, 2:8) 0.64; IR (ATR) ν_{max} 3317, 2951, 2847, 1697, 1557, 1453 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.00 (1H, br s, NH-9), 7.28–7.26 (2H, m, H-14, H-18), 7.20–7.17 (3H, m, H-15, H-16, H-17), 5.96 (1H, s, H-5), 3.40 (2H, dt, *J* = 6.8, 6.8 Hz, H_2-10), 2.70 (2H, t, *J* = 7.6 Hz, H_2-12), 2.25 (3H, s, H_3-7), 1.94 (2H, tt, *J* = 7.6, 6.8 Hz, H_2-11); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.5 (C-4), 170.1 (C-8), 165.7 (C-6), 163.8 (C-2), 141.1 (C-13), 128.6 (C-14, C-18), 128.5 (C-15, C-17), 126.1 (C-16), 102.6 (C-5), 91.0 (C-3), 38.7 (C-10), 33.1 (C-12), 30.8 (C-11), 20.4 (C-7); (+)-HRESIMS *m/z* 288.1223 $[M+H]^+$ (calcd for $C_{16}H_{18}NO_4$, 288.1230).

5.3.21. *N*-Hexyl-4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-carboxamide (28)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with 1-isocyanatohexane (0.254 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with

CH_2Cl_2, afforded the title compound as light yellow needles (0.047 g, 46%). m.p. 45–47 ºC; R_f (CH_2Cl_2) 0.77; IR (ATR) ν_{max} 3298, 2929, 2857, 1698, 1538, cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.95 (1H, br s, NH-9), 5.96 (1H, s, H-5), 3.37 (2H, dt, *J* = 5.9, 5.9 Hz, H_2-10), 2.26 (3H, d, *J* = 1.0 Hz, H_3-7), 1.60 (2H, tt, *J* = 7.3, 7.3 Hz, H_2-11), 1.35–1.31 (6H, m, H_2-12, H_2-13, H_2-14), 0.89 (3H, t, *J* = 6.7 Hz, H_3-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.6 (C-4), 170.0 (C-8), 165.6 (C-6), 163.8 (C-2), 102.6 (C-5), 90.9 (C-3), 39.3 (C-10), 31.5 (C-13), 29.3 (C-11), 26.7 (C-12), 22.6 (C-14), 20.4 (C-7), 14.1 (C-15); (+)-HRESIMS *m/z* 254.1386 $[M+H]^+$ (calcd for $C_{13}H_{20}NO_4$, 254.1387); Anal calcd for $C_{13}H_{19}NO_4$: C, 61.64; H, 7.56; N, 5.53. Found: C, 61.53; H, 7.76; N, 5.53.

5.3.22. *N*-Heptyl-4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-carboxamide (29)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with 1-isocyanatoheptane (0.282 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with *n*-hexane/CH_2Cl_2 (3:7), afforded the title compound as a white crystalline solid (0.047 g, 37%). m.p. 51–52 ºC; R_f (*n*-hexane/CH_2Cl_2, 1:1) 0.44; IR (ATR) ν_{max} 3291, 2926, 2852, 1698, 1536, 1467 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.95 (1H, br s, NH-9), 5.96 (1H, s, H-5), 3.38 (2H, dt, *J* = 6.4, 6.4 Hz, H_2-10), 2.26 (3H, s, H_3-7), 1.60 (2H, tt, *J* = 7.2, 7.2 Hz, H_2-11), 1.34–1.29 (8H, m, H_2-12, H_2-13, H_2-14, H_2-15), 0.88 (3H, t, *J* = 7.0 Hz, H_3-16); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.6 (C-4), 170.0 (C-8), 165.6 (C-6), 163.8 (C-2), 102.6 (C-5), 90.9 (C-3), 39.3 (C-10), 31.8 (C-14), 29.3 (C-11), 29.0 (C-13), 26.9 (C-12), 22.7 (C-15), 20.4 (C-7), 14.1 (C-16); (+)-HRESIMS *m/z* 268.1541 $[M+H]^+$ (calcd for $C_{14}H_{22}NO_4$, 264.1543).

5.3.23. 4-Hydroxy-6-methyl-2-oxo-*N*-tetradecyl-2*H*-pyran-3-carboxamide (30)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with 1-isocyanatotetradecane (0.479 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with

n-hexane/CH_2Cl_2 (45:55), afforded the title compound as a white crystalline solid (0.050 g, 34%). m.p. 68–69 ºC; R_f (*n*-hexane/CH_2Cl_2, 1:3) 0.33; IR (ATR) ν_{max} 3296, 3093, 2917, 2849, 1684, 1565, 1472 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.94 (1H, br s, NH-9), 5.96 (1H, s, H-5), 3.37 (2H, dt, *J* = 6.6, 6.6 Hz, H_2-10), 2.26 (3H, s, H_3-7), 1.62–1.56 (2H, tt, *J* = 7.0, 7.0 Hz, H_2-11), 1.39–1.26 (22H, m, H_2-12, H_2-13, H_2-14, H_2-15, H_2-16, H_2-17, H_2-18, H_2-19, H_2-20, H_2-21, H_2-22), 0.88 (3H, t, *J* = 6.7 Hz, H_3-23); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.6 (C-4), 170.1 (C-8), 165.7 (C-6), 163.9 (C-2), 102.7 (C-5), 91.0 (C-3), 39.4 (C-10), 32.1 (C-21), 29.8 (C-11, C-15, C-16, C-17), 29.7 (C-18), 29.6 (C-14), 29.5 (C-19), 29.4 (C-13, C-20), 27.0 (C-12), 22.8 (C-22), 20.4 (C-7), 14.2 (C-23); (+)-HRESIMS *m/z* 366.2644 $[M+H]^+$ (calcd for $C_{21}H_{36}NO_4$, 366.2639).

5.3.24. *N*-(4-(Benzyloxy)phenyl)-4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3 - carboxamide (31)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with 1-(benzyloxy)-4-isocyanatobenzene (0.450 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with *n*-hexane/CH_2Cl_2 (1:9), afforded the title compound as a brown crystalline solid (0.035 g, 25%). m.p. 185–186 ºC; R_f (CH_2Cl_2) 0.67; IR (ATR) ν_{max} 3109, 2935, 1702, 1610, 1555, 1456 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 10.80 (1H, br s, NH-9), 7.50 (2H, d, *J* = 8.7 Hz, H-11, H-15), 7.44–7.30 (5H, m, H-19, H-20, H-21, H-22, H-23), 6.97 (2H, d, *J* = 8.7 Hz, H-12, H-14), 6.02 (1H, s, H-5), 5.07 (2H, s, H_2-17), 2.30 (3H, s, H_3-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.5 (C-4), 168.1 (C-8), 166.1 (C-6), 164.0 (C-2), 156.4 (C-13), 137.0 (C-18), 129.9 (C-10), 128.8 (C-20, C-22), 128.2 (C-21), 127.6 (C-19, C-23), 123.0 (C-11, C-15), 115.4 (C-12, C-14), 102.7 (C-5), 93.3 (C-3), 70.4 (C-17), 20.5 (C-7); (+)-HRESIMS *m/z* 352.1189 $[M+H]^+$ (calcd for $C_{20}H_{18}NO_5$, 352.1179).

5.3.25. *N*-(Benzo[*d*][1,3]dioxol-5-yl)-4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-carboxamide (32)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with 5-isocyanatobenzo[*d*][1,3]dioxole (0.326 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with *n*-hexane/CH_2Cl_2 (1:9), afforded the title compound as a yellow crystalline solid (0.058 g, 50%). m.p. 183–185 ºC; R_f (CH_2Cl_2) 0.59; IR (ATR) ν_{max} 3247, 3134, 2925, 1703, 1560 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 10.82 (1H, br s, NH-9), 7.27 (1H, d, *J* = 6.0 Hz, H-15), 6.93 (1H, dd, *J* = 8.5, 1.3 Hz, H-11), 6.79 (1H, d, *J* = 8.4 Hz, H-12), 6.03 (1H, s, H-5), 5.97 (2H, s, H_2-17), 2.31 (3H, s, H_3-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.4 (C-4), 168.1 (C-8), 166.2 (C-6), 164.0 (C-2), 148.0 (C-14), 145.2 (C-13), 130.7 (C-10), 114.8 (C-11), 108.3 (C-12), 103.8 (C-15), 102.7 (C-5), 101.6 (C-17), 91.7 (C-3), 20.5 (C-7); (+)-HRESIMS *m/z* 290.0656 $[M+H]^+$ (calcd for $C_{14}H_{12}NO_6$, 290.0659).

5.3.26. 4-Hydroxy-6-methyl-2-oxo-*N*-phenyl-2*H*-pyran-3-carbothioamide (33)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with isothiocyanatobenzene (0.270 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with CH_2Cl_2, afforded the title compound as yellow needles (0.047 g, 45%). m.p. 197–199 ºC; R_f (*n*-hexane/CH_2Cl_2, 1:1) 0.44; IR (ATR) ν_{max} 3100, 1672, 1642, 1552, 1512, 1436 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 13.14 (1H, br s, NH-9), 7.50 (2H, dd, *J* = 7.6, 1.6 Hz, H-11, H-15), 7.44 (2H, td, *J* = 7.6, 1.6 Hz, H-12, H-14), 7.33 (1H, tt, *J* = 7.6, 1.6 Hz, H-13), 6.09 (1H, d, *J* = 1.0 Hz, H-5), 2.30 (3H, s, H_3-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 188.5 (C-4), 178.0 (C-8), 164.6 (C-6), 164.3 (C-2), 137.4 (C-10), 129.2 (C-12, C-14), 127.7 (C-13), 125.9 (C-11, C-15), 104.1 (C-5), 97.3 (C-3), 20.2 (C-7); (+)-HRESIMS *m/z* 262.0536 $[M+H]^+$ (calcd for $C_{13}H_{12}NO_3S$, 262.0532).

5.3.27. *N*-Cyclohexyl-4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-carbothioamide (34)

Using general procedure C, reaction of 4-hydroxy-6-methyl-2-pyrone (**7**) (0.050 g, 0.4 mmol) with isothiocyanatocyclohexane (0.282 g, 2.0 mmol) and triethylamine (22 μL, 0.1 mmol) followed by purification by silica gel column chromatography, eluting with *n*-hexane/CH_2Cl_2 (6:4), afforded the title compound as a light yellow crystalline solid (0.006 g, 6%). m.p. 44–46 ºC; R_f (*n*-hexane/CH_2Cl_2, 1:1) 0.54; IR (ATR) ν_{max} 3253, 2929, 2855, 2170, 1682, 1531, 1454 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 11.59 (1H, br s, NH-9), 6.01 (1H, s, H-5), 4.38 (1H, br s, H-10), 2.25 (3H, s, H_3-7), 2.04–2.02 (2H, m, H-11_A, H-15_A), 1.77–1.74 (2H, m, H-12_A, H-14_A), 1.64–1.61 (1H, m, H-13_A), 1.46–1.40 (4H, m, H-11_B, H-12_B, H-14_B, H-15_B), 0.93–0.87 (1H, m, H-13_B); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 185.7 (C-4), 177.3 (C-8), 164.2 (C-6), 164.1 (C-2), 103.9 (C-5), 97.0 (C-3), 52.6 (C-10), 31.2 (C-11, C-15), 25.6 (C-13), 24.5 (C-12, C-14), 20.1 (C-7); (+)-HRESIMS *m/z* 268.0998 $[M+H]^+$ (calcd for $C_{13}H_{17}NO_3S$, 268.1002).

5.4. General Bioassays Procedures

Golden hamsters and BALB/c mice were obtained from the animal breeding facility at the Adolfo Lutz Institute-SP, Brazil. The animals were maintained in sterilized cages under a controlled environment, and received water and food *ad libitum*. Animal procedures were performed with the approval of the Research Ethics Commission (project number CEUA IAL/Pasteur 04/2016), in agreement with the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences.

5.4.1. Parasites and Mammalian Cell Maintenance

Leishmania (*L.*) *infantum* (MHOM/BR/1972/LD) was maintained in Golden hamsters (*Mesocricetus auratus*) up to approximately 60–70 days post-infection. Promastigotes were maintained in M-199 medium supplemented with 10% fetal calf serum and 0.25% hemin at 24 ºC. Amastigotes were obtained from the spleen of previously infected hamsters and purified by differential centrifugation. Macrophages were collected from the peritoneal cavity of BALB/c mice by washing them with RPMI-

1640 medium supplemented with 10% fetal calf serum, and were maintained at 37 ^{o}C in a 5% CO_2-humidified incubator. Trypomastigotes of *T. cruzi* (Y strain) were maintained in Rhesus-monkey kidney cells (LLC-MK2 - ATCC CCL 7), cultivated in RPMI-1640 medium supplemented with 2% fetal calf serum at 37 ^{o}C in 5% CO_2-humidified incubator. The murine conjunctive cells (NCTC clone 929, ATCC) were maintained in RPMI-1640 supplemented with 10 % FBS at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. NCTC - clone 929 cells were cultivated in M-199 medium, supplemented with 10% fetal calf serum at 37 ^{o}C in 5% CO_2-humidified incubator.

5.4.2. Determination of the 50% Inhibitory Concentration (IC_{50})

Leishmania: Promastigotes in late growth-phase (non-stationary at 3 x 10^7/mL, passage 5) were counted in a hemocytometer chamber and seeded at 1 x 10^6/well, with a final volume of 150 µL. The compounds were dissolved in DMSO and diluted in M-199 medium in 96-well microplates, with the highest concentration of 100 µM for 48 h at 24 ^{o}C. The parasite viability was determined using the MTT colorimetric assay.[35] The optical density was read at 570 nm (FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices) using control wells without drugs (100% viability) and without cells (blank). The control group consisted of promastigotes incubated with 0.5% DMSO. Miltefosine was used as a standard drug. Compounds were tested to the highest concentration of 100 µM and were reported as NA (not active) when the IC_{50} value was above this concentration. *Amastigotes*: Peritoneal macrophages were collected from the peritoneal cavity of BALB/c mice, and the macrophages were seeded at 1 x 10^5/well for 24 h in a 16-well slides (NUNC®- ThermoFisher Scientific). Amastigotes were prepared as described previously in a 1:10 ratio of macrophages to amastigotes and incubated in RPMI-1640 for 24 h at 37 ^{o}C 5% CO_2-humidified incubator. The compounds were incubated with infected macrophages for 72 h. Miltefosine was used as a positive control. Subsequently, the cells were fixed with MeOH, stained with

Giemsa and observed using a light microscope. The parasite burden was determined by the number of infected macrophages out of 400 cells.[36]

Trypanosoma cruzi: Trypomastigotes were counted in a hemocytometer chamber and seeded at 1 x 10^6 cells per well in 96-well microplates. The compounds were diluted in RPMI-1640 medium and incubated for 24 h at 37 ^oC in a 5% CO_2-humidified incubator. The parasite viability was determined using the resazurin (0.011% in PBS). The optical density was read at 570 nm using control wells without drugs (100% viability) and without cells (blank). The control group consisted of trypomastigotes incubated with 0.5% DMSO. Benznidazole was used as a positive control. Compounds were tested to the highest concentration of 100 µM and were reported as NA (not active) when the IC_{50} value was above this concentration.[24] Intracellular amastigotes: Peritoneal macrophages were dispensed in 16-well chamber slides (NUNC, Thermo, USA) and maintained for 24 h in the same medium at 37 °C in a 5% CO_2 humidified incubator for attachment. Non-adherent cells were removed by two step washings with medium. After 24 h, these cells were infected with 1 x 10^6 culture trypomastigote forms for 4 h. Subsequently, infected cells were incubated with the compounds for 48 h. Finally, the slides were fixed with methanol, stained with Giemsa, and observed in light microscopy. The parasite load was defined by counting 400 macrophages/well by evaluating the number of infected macrophages. Benznidazole was used as the standard drug.

5.4.3. Cytotoxicity in Mammalian Cells

NCTC cells were counted in a hemocytometer chamber, seeded at 6 x 10^4/well and incubated in highest concentrations to 200 µM for 72 h at 37 ^oC in a 5% CO_2-humidified incubator. The cell viability was determined using the MTT assay.[35] Miltefosine was used as a positive control. The selectivity index (SI) was determined using the relationship, CC_{50} against NCTC/IC_{50} against parasites.

5.4.4. Evaluation of Plasma Membrane Permeability

Late growth-phase trypomastigotes of *T. cruzi* (2 x 10^6/well) were washed and incubated in the dark with 1 µM Sytox Green probe (Molecular Probes) in HANKS' balanced salts solution (HBSS; Sigma-Aldrich) supplemented with 10 mM D-Glucose (HBSS+Glu). The test compound was added (t=0) at IC_{50} and IC_{90} and fluorescence was measured every 1 hour for up to 7 h. The maximum permeabilization was obtained with 0.5% Triton X-100. Fluorescence intensity was determined using a fluorimetric microplate reader (FilterMax F5 Multi-Mode Microplate Reader-Molecular Devices) with excitation and emission wavelengths of 485 and 520 nm, respectively. The following internal controls were used in the evaluation: i) the background fluorescence of the compound at the respective wavelengths, ii) the possible interference of DMSO. Samples were tested in duplicate.[37]

5.4.5. Evaluation of Mitochondrial Membrane Potential

Late growth-phase trypomastigotes of *T. cruzi* (2 x 10^6/well) were treated with the test compound at IC_{50} and IC_{99} in HBSS+Glu during 2 h incubation at 26 °C. After incubation, parasites were washed and co-stained with Rhodamine 123 (Rd123 0.3 µg/mL) and propidium iodate (PI 5 µg/mL) for 10 minutes under the absence of light at 37 °C in order to monitor the mitochondrial membrane potential ($\Delta\psi m$) and cell viability, respectively. Flow cytometry was performed using an Attune NxT Acoustic Focusing Cytometer (ThermoFisher Scientific) by analyzing 10,000 gated events using forward/side scatter (FSC/SSC), Rd123 fluorescence (BL1-A, filter 530/30 nm), PI fluorescence (BL2-A, filter 574/26 nm) and Attune Nxt® software included with the equipment. Unstained, Rd123-stained (untreated) and PI-stained (amphotericin B 1 µg/mL-treated) parasites were used to set background fluorescence and compensation. Trypomastigotes treated with FCCP (10 µM) and untreated were used to obtain maximal and minimal mitochondrial depolarization, respectively.[38]

5.4.6. *In vivo Efficacy Studies*

The *in vivo* efficacy of compound **12** was investigated in the acute-phase mice model of *T. cruzi*. Male BALB/c mice (n=5/group) were infected with 5 x 10^3 trypomastigotes and the parasitemia was individually checked by direct light microscopy counting of parasites (5 µL of blood), according to de Souza *et al*..[39] Compound **12** was dissolved in a solution of ethanol:cremophor:PBS (20:15:65 v/v ratio). After 5 days post infection (5º d.p.i), compound **12** was administered for 10 consecutive days (intraperitoneally) at 30 mg/kg/day and the parasitemia was evaluated daily for 14 days. Beznidazole was administered as standard drug at 100 mg/kg/day for 10 consecutive days (orally). Body weight was evaluated weekly, and mortality was checked daily until 14 days.

5.4.7. Histopathology

Samples of major tissues, including lungs, liver, adrenal glands, heart, kidneys, spleen, liver, pancreas, stomach and intestine, were carefully removed during the necropsy of treated (compound **12** at 30 mg/kg/day) and untreated animals. Sections of each tissue were fixed in 10% neutral buffered formalin, for at least 24 h. Tissues were processed routinely and histological sections were stained with hematoxylin and eosin. All organs from all animals were examined under light microscopy.

5.5. Statistical Analysis

The data obtained represent the mean of duplicate or triplicate samples from at least two independent assays. IC_{50} and IC_{99} values were calculated using sigmoid dose–response curves in GraphPad Prism 5.0 software, and the 95% confidence intervals are included in parentheses. One-way ANOVA of variance with Tukey's Multiple Comparison Test was used for significance test (P value).

Acknowledgments

This work was funded by grants and fellowships provided from São Paulo State Research Foundation (FAPESP 2015/23403-9, 2015/50075-2) and scholarship 2013/07275-5). We also thank Conselho Nacional de Pesquisa e Desenvolvimento scientific research award (CNPq to AGT) and CAPES for the student's scholarships.

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