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A Class of 5-Nitro-2-furancarboxylamides with Potent Trypanocidal Activity against *Trypanosoma brucei* in Vitro

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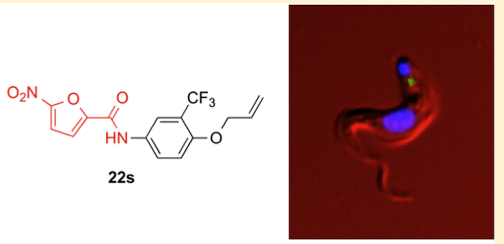
Supporting Information

ABSTRACT: Recently, the World Health Organization approved the nifurtimox−eflornithine combination therapy for the treatment of human African trypanosomiasis, renewing interest in nitroheterocycle therapies for this and associated diseases. In this study, we have synthesized a series of novel 5-nitro-2-furancarboxylamides that show potent trypanocidal activity, ∼1000-fold more potent than nifurtimox against in vitro *Trypanosoma brucei* with very low cytotoxicity against human HeLa cells. More importantly, the most potent analogue showed very limited cross-resistance to nifurtimox-resistant cells and vice versa. This implies that our novel, relatively easy to synthesize and therefore cheap, 5-nitro-2-furancarboxylamides are targeting a different, but still essential, biochemical process to those targeted by nifurtimox or its metabolites in the parasites. The significant increase in potency (smaller dose probably required) has the potential for greatly reducing unwanted side effects and also reducing the likelihood of drug resistance. Collectively, these findings have important implications for the future therapeutic treatment of African sleeping sickness.



	22s	Nifurtimox
T. brucei brucei	2.4 ± 0.3 nM	2397 ± 127 nM
T. brucei rhodesiense	2.9 ± 0.4 nM	1708 ± 197 nM

INTRODUCTION

Infectious diseases caused by parasitic protozoa affect ca. 15% of the global population and more than 65% of the population in the Third and developing world, yet current drug therapies for protozoan infections are woefully inadequate. As protozoan infections take their toll predominantly in the developing world, market forces are insufficient to promote the development of novel antiprotozoan drugs. In 2000, only ca. 0.1% of global investment in health research was spent on drug discovery for tropical diseases.

One such neglected parasitic disease is human African trypanosomiasis (HAT) or African sleeping sickness, which is caused by the protozoan parasite *Trypanosoma brucei*. The World Health Organization (WHO) estimates that HAT constitutes a serious health risk to 60 million people in sub-Saharan Africa. The WHO also estimates that there are <30000 new cases per year in sub-Saharan Africa and at present an annual death toll of ∼8000.[1]

The related disease in cattle, cattle trypanosomiasis or Nagana, also represents a major health concern due to its devastating economic (estimated by the WHO to cause an annual economic loss of ∼US$4 billion), social, and nutritional impact on African families. As such, the total burden of trypanosomiasis translates into 1598000 disability-adjusted life years (DALY). This is on a par with big killers such as tuberculosis and malaria.[2,3]

Treatment of HAT is solely dependent upon a repertoire of four drugs: suramin (**1**), pentamidine (**2**), melarsoprol (**3**), and eflornithine (**4**) (Figure 1). These therapies are often toxic, difficult to administer, and increasingly have an acquired drug resistance, highlighting the urgent need for new, more effective drug therapies. Developed before the 1950s, suramin (**1**) and

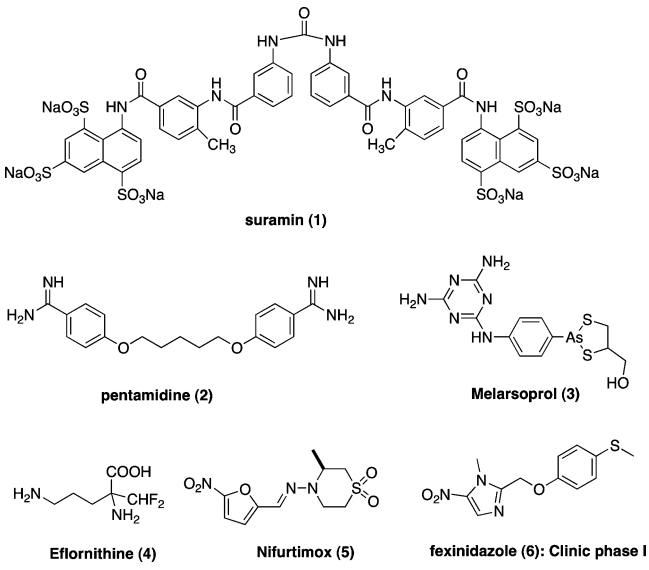


Figure 1. Structures of clinic drugs **1**−**5** for sleeping sickness and clinic phase I drug Fexinidazole (**6**).

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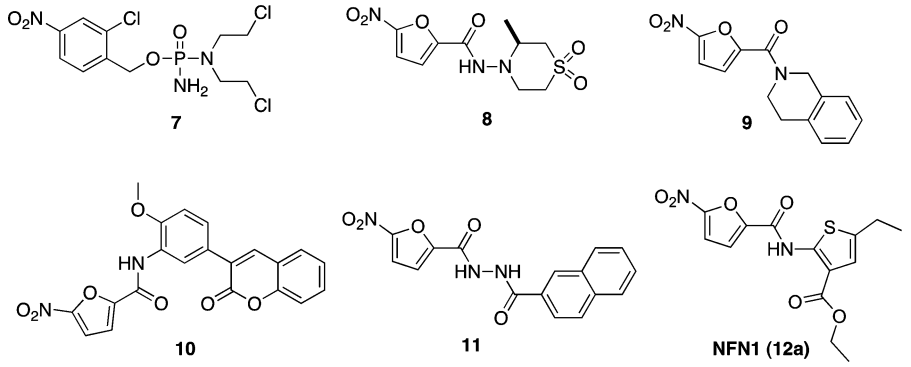

Figure 2. Structures of some reported bioactive nitroaromatic compounds **7**−**12a**.

melarsoprol (**3**) are used for chemotherapy of the early stage of the disease (*Trypanosoma brucei rhodesiense*), as is pentamidine (**2**) (*Trypanosoma brucei gambiense*). The arsenical melarsoprol (**3**) is extremely toxic, with death in ∼6% of cases and treatment failure rates of as high as 30% in certain areas. Treatment of the second stage of the disease, where the parasites cross the blood−brain barrier and invade the central nervous system, is limited to melarsoprol (**3**) and eflornithine (**4**), the ornithine decarboxylase inhibitor (difluoromethylornithine).[4,5]

Nifurtimox (**5**) (Figure 1), is often used to treat Chagas' disease, caused by *Trypanosoma cruzi*, and has been used as a monotherapy for melarsoprol-refractory HAT on compassionate grounds despite its low efficacy and severe toxicity. The recent introduction of a nifurtimox−eflornithine combination therapy (NECT) by WHO in the Model Lists of Essential Medicines has been seen as highly advantageous in terms of cost, logistics, and human resources in areas of poverty. Patients given NECT, consisting of oral nifurtimox over 10 days with eflornithine (**4**) infusions for 7 days, were found to fair just as well as those given the eflornithine monotherapy, with cure rates of around 97%.[2]

The use of NECT has renewed general interest in the use of nitroheterocyclic compounds to treat a wide range of infectious disease, including tuberculosis and hepatitis C.[6−8] This increased awareness of the potential of nitroheterocyclic compounds has led to the reinvestigation of fexinidazole (**6**) (Figure 1), which is presently in clinical trials against both early- and late-stage African sleeping sickness.[6−8]

Alarmingly, it has been shown that *T. brucei* nifurtimox-resistant cells show cross-resistance to other nitro-containing drugs, including fexinidazole, that are currently in clinical trials.[9,10] Several different types of studies on nitroheterocycles have shed some light on this observed drug resistance. Genome-wide RNAi screens of nifurtimox and benznidazole (another trypanocidal nitroheterocyclic drug) resistant *T. brucei* have identified that a decrease in nitroreductase activity is linked to nifurtimox resistance.[9,10] This was confirmed by showing that genetic deletion of one allele of the *T. brucei*'s nitroreductase led to nifurtimox resistance. Likewise, the overexpression of this nitroreductase, but not the alternative proposed prostaglandin F2α synthase and cytochrome P450 reductase, in *T. brucei* resulted in increased susceptibility to nifurtimox.[11] Collectively, it is clear that this type I *T. brucei* nitroreductase, a NAD(P)H dependent, flavin binding protein, is involved in the reductive activation of nifurtimox. The nitroreductases mediate a series of two-electron reductions of the nitro group to a nitroso intermediate, then to a hydroxylamine, and eventually to the corresponding amine. However, the true function of the *T. brucei* mitochondrial nitroreductase is unknown, but its essentiality may explain why only a single allele deletion/mutation is associated with nifurtimox resistance.[10,11]

As well as the development of nifurtimox-resistance and its cross-resistance to other nitro-containing drugs, side effects are also a major problem for nitro-containing drugs. For example, the adverse off-target side effects with nifurtimox lead to treatment cessation in over 30% of patients with Chagas' disease.[12] The most common side effects are anorexia, loss of weight, psychic alterations, excitability or sleepiness, and digestive manifestations such as nausea or vomiting and occasionally intestinal colic and diarrhea.[13] Various human enzymes have been identified to be capable of 5-nitrofuran reduction in vitro, in cells, or tissues.[14−16] Recently, we have elucidated in collaboration with the Patton Laboratory a possible role of aldehyde dehydrogenase 2 (ALDH2) in the toxicity caused by 5-nitrofuran containing drugs such as nifurtimox.[17]

While considerable effort has gone into the synthesis of nitroaromatic containing compounds over the years,[18−23] there remains several unexplored areas of chemical space. For example, in the context of nifurtimox, most of the efforts have been focused on changing either the furan ring to other heterocycles (such as imidazole) or changing the substituent in the hydrazone motif,[18−23] with limited increases in activity compared to nifurtimox. Recently, two new types of typanocidal compounds, nitrobenzylphosphoramide mustards (for example see compound **7**, Figure 2)[24] and aziridinyl nitrobenzamides, have been developed to target specifically *T. brucei* type I nitroreductase, the best having an EC_{50} ∼ 1 μM against both *T. brucei* and *T. cruzi*.[25,26] Several 5-nitro-2-furancarboxylamide containing compounds have been reported to have potent antituberculosis and antimicrobial activity,[27−30] however, very few 5-nitro-2-furancarboxylamides are reported to have typanocidal activity, primarily low activity against *T. cruzi*.[31,32] A nifurtimox analogue (**8**) (Figure 2) showed trace activity against *T. cruzi* in a mouse model.[33] The best 5-nitro-2-furancarboxylamides identified thus far, **9** and **10,** have EC_{50} values of 42 and 262 nM, respectively, against *T. cruzi* amastigotes (Figure 2) but have not been tested against *T. brucei* to the best of our knowledge.[34] Several 5-nitro-2-furancarbohydrazides possess activity against *T. brucei* with the best, compound **11**, having an EC_{50} of 0.13 μM but low selectivity against human cells.[35]

In this study, we report that our recently discovered nitrofuran NFN1 (**12a**)[17] also shows promising activity against *T. brucei* in vitro. We have therefore synthesized a series of 5-nitro-2-furancarboxylamide analogues of **12a**. A lack of cross-resistance with nifurtimox and significantly increased (2−3 orders of magnitude) trypanocidal activity has important implications for the future therapeutic use of these nitrofuran containing

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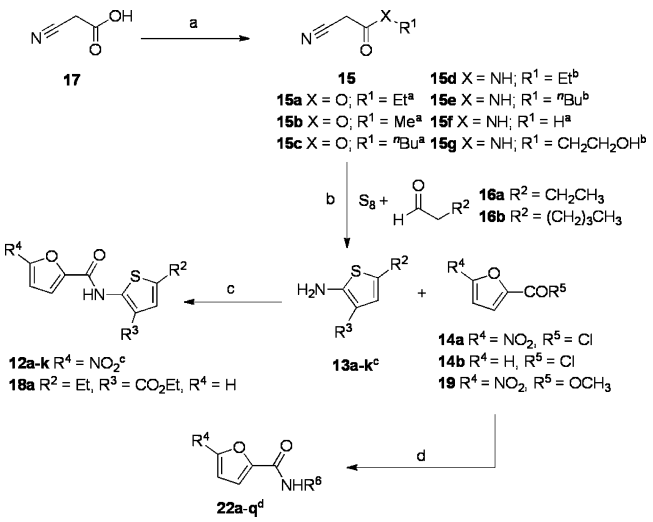
compounds in the treatment of HAT, instead of, or in combination with, nifurtimox.

RESULTS AND DISCUSSION

Chemistry. 5-Nitrofurans **12a–k** were prepared by acylation of the corresponding 2-amino-thiophenes **13a–k** with 5-nitrofurancarboxylic acid chloride **14a** (Scheme 1, Table 1 for substituents). The required 2-amino-thiophenes **13a–h** were prepared using the Gewald multicomponent reaction with various combinations of the 2-cyanoacetyl esters or amides (**15a–g**) with aldehydes (**16a** or **16b**) and elemental sulfur (see Supporting Information Table S1 for details).[36,37] **13h** was *O*-silyl-protected before coupling to **14a**. This reaction gave **12h** directly as a result of in situ removal of the silyl-protecting group. Thiophene **13i** was prepared by hydrolysis of **13a** and **13j** prepared by catalytic amination of 2-iodothiophene using CuI and L-proline.[38] **13k** and **13l** (Table 2) were commercially available. Reaction of **13a** (R^2 = Et, R^3 = CO_2Et) with **14b** gave control compound **18a**. An analogous reaction of **14b** with aniline gave **18b** (Table 2). Furan ester **19** was commercially available.

Scheme 1. Synthetic Route to 5-Nitro-2-furancarboxylamide Analogues 12a–k[a]



[a]Reagents and conditions: (a) (i) $(COCl)_2$, DCM/DMF, RT, 2 h, (ii) R^1NH_2, Et_3N, DCM, 52% for **15d**, 49% (**15e**), 37% (**15g**); (b) S_8, **16a** or **16b**, Et_2NH, DMF, RT, o/n, 66% for **13a**, 59% (**13b**), 76% (**13c**), 53% (**13d**), 49% (**13e**), 46% (**13f**), 48% (**13g**), 46% (**13h**), for synthesis of **13i–j** see ESI, **13k** and **13l** were commercially available; (c) **13a–k**, Et_3N, DCM, RT with **14a**, 68% for **12a**, 62% (**12b**), 65% (**12c**), 58% (**12d**), 57% (**12e**), 57% (**12f**), 54% (**12g**), 47% (**12h**), 25% (**12i**), 41% (**12j**), 51% (**12k**), with **14b**, 56% for **18a**; (d) RNH_2, Et_3N, DCM, RT with **14a**, 65% for **22a**, 61% (**22b**), 72% (**22c**), 67% (**22d**), 37% (**22e**), 50% (**22f**), 45% (**22g**), 41% (**22h**), 66% (**22i**), 39% (**22j**), 51% (**22k**), 73% (**22l**), 51% (**22m**), 68% (**22n**), 67% (**22o**), 69% (**22p**), 48% (**22q**), with **14b** 73% for **18b** (see Table 2 for structure of **18b**). (a) **15a–c** and **15f** were commercially available; (b) **15d**, **15e**, and **15g** were prepared from **17**; (c) for R^2 and R^3 substituents, see Table 1; (c) for R^6 substituents, see Tables 2 and 3.

Analogues **20a–c** and **21** (Table 2) based on **12g** but containing alternative nitroaromatic rings were prepared by coupling the corresponding acid chlorides with **13g** (see ESI for details). Similarly, reaction of **14a** with a range of amines and anilines led to the synthesis of **22a–q** (Scheme 1). Demethylation of **22o** to give **22r** was followed by selective *O*-allylation to give **22s** (Scheme 2), the structure of which was confirmed by X-ray crystallographic analysis (data not shown). When a longer reaction time and an excess amount of allyl bromide was used in the *O*-allylation of **22r**, the diallylated analogue **22t** was formed (Scheme 2). Mono-*O*-allylation of **22p** gave **22u** (Scheme 2). Methylation of **22q** to give **22v** was achieved using MeI under basic conditions in moderate yield (Scheme 2). Conversion of 5-nitrofuran aldehyde **23** to imines **24a** or **24b** enabled the synthesis of the corresponding amines **25a** and **25b** using standard reductive amination conditions (Scheme 2).

Structure–Activity Relationships. In comparison to nifurtimox, NFN1 was 2 orders of magnitude more potent with an EC_{50} of 31.3 ± 3.1 nM (Table 1, cf. entries 1 and 2). The nitro group in NFN1 was found to be essential with no activity being observed up to the solubility limit of **18a** (entry 3). Removal, or worse, elongation of the alkyl substituent in NFN1 led to reduced activity (cf. entry 2 and entries 4 and 5, respectively), and while removal of the ester group initially appeared to be tolerated in the R^2 = H series (cf. entries 4 and 6), it was found that the methyl and *n*-butyl ester analogues (**12c** and **2d**) of NFN1 had similar activity to NFN1 (cf. entry 2 and entries 7 and 8, respectively). Conversion of the ester groups in NFN1 and **12d** to the corresponding amides in **12e** and **12f** led to a significant loss in activity (cf. entries 2 and 8 with entries 9 and 10, respectively). This observation, coupled with the decrease in activity associated with amide **12h** (entry 11), may be explained by the fact that hydrolysis of the ester groups in **12a–d** and **12k** in the parasite gives the corresponding carboxylic acid-containing bioactive metabolites, something that is unlikely to occur for the amides **12e** and **12f**. However, acid **12i** showed much less activity with an EC_{50} of 959.0 ± 33.9 nM (entry 12), which may be due to a lack of cell permeability. The primary amide **12g** was found to be the most active thiophene-containing analogue with an EC_{50} of 17.3 ± 2.4 nM (entry 13). Furthermore, analogues **12a–k** showed no significant toxicity against the human HeLa cell line even at a concentration above 20 μM. The highly selective toxicity of these analogues against the parasite suggests these nitrofuran analogues have real potential for therapeutic applications.

Encouraged by the potent activity of **12g** (Table 1), it was decided to prepare more analogues based on this structure. Fragments **19** and **13g** along with **13l** were shown to be inactive against *T. brucei*, suggesting that both the 5-nitrofuran and thiophene fragments are required (Table 2, cf. entry 1 and entries 2–4, respectively). Changing the nitrofuran motif in **12g** to either a nitrophenyl or nitropyrazole ring also led to a 100-fold or more drop in activity (cf. entry 1 and entries 5–8, respectively). Interestingly, the position of the nitro group in the nitrophenyl ring had an influence as **20b**, an analogue with the nitro group in the *meta*-position, was more active than either **20a** (*ortho*-NO_2) or **20c** (*para*-NO_2). Studies next focused on replacing the thiophene motif in **12a–k**. By incorporating a cyclohexyl ring to give **22a**, a 10-fold decrease in activity was observed compared to **12g** (entry 9), while the phenyl (**22b**) and benzyl (**22c**) analogues were essentially equipotent with **12g** (cf. entry 1 and entries 10 and 11, respectively). Incorporation of a nitrogen atom into the 2-position of the phenyl ring in **22c** to give **22d** led to a 10-fold drop in activity (entry 12). The nitrofuran group in **22b** was shown to be essential (cf. entries 10 and 13), and the amide linkage in **22b** and **12g** was also shown to be important, as the

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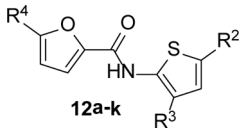
Table 1. SAR Study of Analogues 12a−12k



12a-k

Entry	Compound	R^2	R^3	R^4	*T. brucei* EC_{50} (nM)	HeLa EC_{50} (μM)	Selectivity Index
1	Nifurtimox (**5**)[a]	-[a]	-[a]	-[a]	2400 ± 100	91.2±11.3	~38
2	NFN1 (**12a**)[b]	Et	CO_2Et	NO_2	31.3±3.1	>20	>640
3	**18a**	Et	CO_2Et	H	>20000	>20	N/A[c]
4	**12k**	H	CO_2Et	NO_2	72.3 ± 6.1	>20	>278
5	**12b**	^{n}Bu	CO_2Et	NO_2	125.3±6.0	>20	>160
6	**12j**	H	H	NO_2	62.6 ± 2.7	>20	>322
7	**12c**	Et	CO_2Me	NO_2	21.0±1.2	>20	>950
8	**12d**	Et	$CO_2{}^nBu$	NO_2	32.6±0.7	>20	>625
9	**12e**	Et	CONHEt	NO_2	147.0±10.1	>20	>136
10	**12f**	Et	CONH^{n}Bu	NO_2	336.3±18.8	>20	>60
11	**12h**	Et	$CONH(CH_2)_2OH$	NO_2	79.6 ± 6.7	>20	>253
12	**12i**	Et	CO_2H	NO_2	959.0 ± 33.9	>20	N/A[c]
13	**12g**	Et	$CONH_2$	NO_2	17.3±2.4	>20	>1155

[a]For structure, see Figure 1. [b]See ref 17. [c]Not applicable as essentially no activity against *T. brucei* was observed for **12i**.

amine analogues **25a** and **25b** showed a dramatic decrease in activity (entries 14 and 15, respectively).

On the basis of the potent activity of **22b**, the effect of incorporating additional substituents into the phenyl ring was investigated (Table 3). Substitution at the *para*-(R^3) and *meta*-(R^2 or R^4)-positions with either electron-withdrawing (entries 3 and 5−7, respectively) or electron-donating groups (entries 4 and 8, respectively) proved detrimental, with the exception of the incorporation of the *meta*-CF_3 group in **22e** (EC_{50} of **22e** = 4.69 ± 0.14 nM, entry 2). Interestingly, incorporation of a *meta*-CF_3 into the phenyl ring of the benzyl analogue **22c** (Table 2) gave analogue **22l**, which had an EC_{50} = 17.4 ± 3.0 nM, suggesting that the use of a phenyl substituent (as in **22e**) may be preferred to the benzyl substituent in **22l**. Combining the *meta*-CF_3 substituent with either an additional *meta*-methoxy- or a second *meta*-CF_3 substituent led to a decrease in activity (entries 9 and 11, respectively), whereas the *meta*-CF_3 group was able to override the effect of a *para*-methoxy substituent (cf. entries 8 and 12). Incorporation of a *para*-hydroxy in both the *meta*-CF_3 and unsubstituted series led to a significant reduction in activity (entries 13 and 14, respectively), but extending the *para*-alkoxy chain from methoxy in **22o** to allyloxy led to the most active compound prepared, analogue **22s**, which had an EC_{50} against *T. brucei* of 2.4 ± 0.3 nM (entry 15). The positive role of the *para*-allyloxy substituent was also observed in the unsubstituted series (cf. entries 8 and 16). The incorporation of an additional allyloxy substituent on the amide nitrogen in **22s** led to a significant loss in activity for **22t** (cf. entries 15 and 17).

Interestingly, the lack of significant biological activity displayed by the 2-trifluoromethylphenol **22r**, e.g., EC_{50} value of 2.2 ± 0.1 μM versus 2.4 ± 0.3 nM for **22s** or 7.8 ± 0.3 nM for **22o**, might be due to the high instability of **22r** in the tested conditions as the result of fluorine elimination generating a quinone methide, as previously reported.[39,40] Masking the phenol as allyl or methyl ethers, e.g., as in **22s** or **22o**, respectively, restored the trypanocidal activity against *T. brucei*.

Finally, the special role played by the *meta*-CF_3 substituent was further highlighted by a more than 5-fold drop in activity observed on its replacement by a *meta*-CH_3 substituent (cf. entries 12 and 18). As was the case for analogues **12a−k**, all the analogues shown in Tables 2 and 3 showed low toxicity against the human HeLa cell line.

CROSS-RESISTANCE STUDIES

The striking increase in potency of the 5-nitro-2-furancarboxylamide analogues compared to nifurtimox against bloodstream *T. brucei* suggests they have a different or possibly additional mode of action to that of nifurtimox, i.e., in addition to the activation by the *T. brucei* nitroreductase. To investigate this possibility, it was decided to generate drug resistant cell lines to nifurtimox and the most potent of the 5-nitro-2-furancarboxylamide analogues, **22s** (EC_{50} = 2.4 ± 0.3 nM). This would allow subsequent cross-resistance studies to be conducted. Previous lab-generated nifurtimox-resistant strains have shown that deletion or mutation of one of the alleles of the nitroreductase confers resistance.[9,10]

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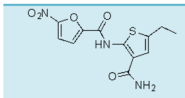
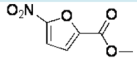
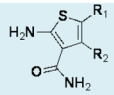
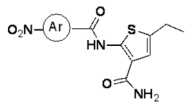
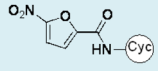
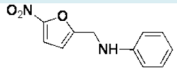
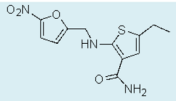
Table 2. SAR Study of Series 2 Analogues

Structure	Substituent	Entry	Compound	*T. brucei* EC_{50} (μM)	HeLa EC_{50} (μM)	Selectivity Index
	-	**1**	**12g**	0.0173 ± 0.0024	>20	>1155
	-	**2**	**19**	67.0 ± 2.6	>20	N/A[a]
	R_1=Et, R_2= H	**3**	**13g**	32.6 ± 1.9	>20	N/A[a]
	R_1=H, R_2= Me	**4**	**13l**	65.1 ± 3.2	>20	N/A[a]
	*ortho*-Ph	**5**	**20a**	26.1 ± 2.5	>20	N/A[a]
	meta-Ph	**6**	**20b**	2.6 ± 0.2	>20	>8
	para-Ph	**7**	**20c**	>20	>20	N/A[a]
	Ortho-Pyrazole	**8**	**21**	>300	>20	N/A[a]
	Cyclohexyl	**9**	**22a**	0.2310 ± 0.0313	>20	>86
	Ph	**10**	**22b**	0.0281 ± 0.0015	>20	>710
	Bn	**11**	**22c**	0.0273 ± 0.0018	>20	>732
	CH_2-2-Py	**12**	**22d**	0.2910 ± 0.0315	>20	>68
	No NO_2, Ph	**13**	**18b**	>20	>20	N/A[a]
	-	**14**	**25a**	113.3 ± 14.7	>20	N/A[a]
	-	**15**	**25b**	5.3 ± 0.8	>20	>4

[a]Not applicable as no activity or low activity against *T. brucei* was observed for **18b** and **25a**.

As with these previous studies, nifurtimox-resistant parasites were generated easily by culturing bloodstream *T. brucei* in the continuous presence of nifurtimox. A stepwise increase in the concentrations of nifurtimox, initially starting at 1.5 μM, increasing to 2.5, 4, 5, 7.5, 10, 15, 20, 25, 30, 40, 45, and 50 μM on days 2, 5, 9, 14, 23, 39, 55, 65, 76, 90, 109, and 120, respectively, was used (Figure 3A). At 143 days, these nifurtimox-resistant parasites were cloned by serial dilution while maintaining the 50 μM nifurtimox.

Compound **22s**-resistant parasites were initially harder to generate. Selection studies started at 1 nM of **22s**, increasing to 2, 3, 5, 7.5, 10, 15, 20, 25, 30, 35 40, and 50 μM on days 2, 5, 9, 26, 36, 47, 58, 70, 76, 103, 130, and 148, respectively (Figure 3B). At 160 days, the **22s**-resistant parasites were cloned by serial dilution while maintaining a 50 nM concentration of **22s**. Both of the drug-resistant cell lines were stable for 30 days in the absence of their respective drugs. The nifurtimox-resistant parasites had a doubling time of ~11.5 ± 0.6 h compared to wild-type ~7.8 ± 0.4 h, while **22s**-resistant parasites had a doubling time of ~13.3 ± 1.1 h.

The sensitivities of the cloned nifurtimox-resistant and **22s**-resistant parasites to nifurtimox, **22s**, and the diamidine pentamidine (**2**) were determined and compared to wild-type cells. The nifurtimox-resistant cells did not greatly alter (~2-fold increase) the sensitivity to pentamidine (**2**) compared to wild-type cells (Table 4), in accordance with previous lab generated nifurtimox-resistant cell lines.[9,10] **22s**-resistant parasites also showed very little alteration in the EC_{50} (1.1 ± 0.1 nM) of pentamidine (**2**) compared to that of wild-type cells (Table 4).

The nifurtimox-resistant cells were found to be 9−10-fold less sensitive to nifurtimox than wild-type cells, with EC_{50}s of 20.9 ± 1.7 and 2.1 ± 0.2 μM, respectively (Table 4), in accordance with previous findings.[9] **22s**-resistant cells were found to be ~14-fold less sensitive to **22s** than wild-type cells, with EC_{50}s of 29.3 ± 2.0 and 2.4 ± 0.3 nM, respectively (Table 4). Even at this level of resistance, **22s** is still ~70 times more potent than nifurtimox in wild-type *T. brucei*.

To investigate cross-resistance between nifurtimox and **22s**, the EC_{50}s of the drug-resistant cell lines versus the alternative nitrofuran containing compounds were determined (Table 4). The results showed that nifurtimox and **22s** showed a low level of cross-resistance. Nifurtimox-resistant cells showed a ~3-fold increase in the EC_{50} of **22s**, while nifurtimox also showed ~3-fold increase in the EC_{50} against **22s**-resistant cells. These findings

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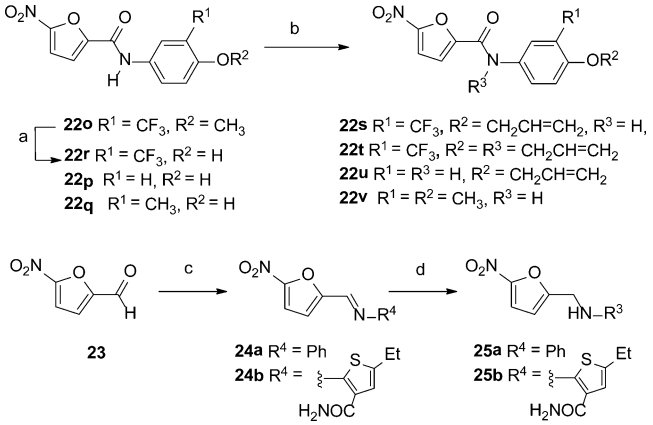
Scheme 2. Synthetic Routes to Series 2 and Series 3 Nitrofurancarboxylamides[a]



[a]Reagents and conditions: (a) pyridine hydrochloride, 160 °C, MW, 5 min, 52%; (b) **22r** or **22p**, allyl bromide (2 or 10 equiv), K_2CO_3, acetone, RT, 2 h for **22s** (75%), 24 h for **22t** (55%), 24 h for **22u** (72%), **22q**, MeI, K_2CO_3, acetone, 3 h, for **22v** (51%); (c) aniline or **13g**, DCM, RT, 89% for **24a**, 63% for **24b**; (d) $NaBH_4$, DCM, RT, 79% for **25a**, 87% for **25b**.

imply that these two nitrofuran compounds, nifurtimox and **22s**, are trypanocidal as a result of primarily targeting different biochemical process within the parasites. However, the low level of cross-resistance suggests there may be some minor overlap in the mode of action. This issue is discussed in more detail later. These findings have important implications for the therapeutic use of this new generation of nitrofuran compounds as part of novel combination therapies or more importantly potentially replacing nifurtimox in a clinical setting.

As nifurtimox and **22s** apparently act through primarily different modes of action, this suggests that their combined action against the parasite may be synergistic. As such, a series of EC_{50}s were determined for **22s** in the presence of various concentrations of nifurtimox (Figure 4). At low concentrations of nifurtimox (<700 nM), synergy can be observed as the EC_{50}s are below the straight diagonal line that represents what would be expected for an additive trypanocidal effect of the two compounds. Above concentrations of 700 nM nifurtimox, there is a distinct lack of synergy; if anything, competition occurs between the two inhibitors. This, taken together with the observed low level of cross-resistance (Table 4), may indicate that nifurtimox at high concentrations is competing with the same biochemical activation process which is normally targeted by **22s**. This further highlights the possible multitarget nature for nifurtimox that has been suggested previously.[9−14] This type of information is relevant for the potential pharmacological applications of these novel nitro-furancarboxylamides as a combinational therapy would likely lead to the use of lower nifurtimox doses.

It was decided to test some of the 5-nitro-2-furancarboxylamides from this study against cultured *T. b. rhodesiense*, one of the human-infective subspecies (Table 5). The potency of a selection of analogues against *T. b. rhodesiense* were similar to those observed for *T. b. brucei,* suggesting that these novel nitrofurans could be considered lead candidates for the next step toward a novel treatment for HAT, as well as the related animal disease, Nagana.

CONCLUSIONS

In this work, we designed and synthesized a series of nitrofuran-containing analogues that were tested for trypanocidal and cytotoxicity activity against cultured bloodstream form *T. b. brucei* and human HeLa cells, respectively. The analogue **22s** showed an EC_{50} of 2.4 ± 0.3 nM, 3 orders of magnitude more potent than nifurtimox with a selectivity index >8000. SAR studies showed that all fragments of the compounds were required for activity. In addition, both the nitrofuran and amide functional groups and the amide NH were necessary. The thiophene ring in the starting analogue **12a** can be replaced by a range of other substituents. However, in the thiophene series, an ester group was required and is likely converted to the corresponding carboxylic acid in the parasite. Several of our analogues were also tested against *T. b. rhodesiense*, showing analogous activity to that in *T. b. brucei*. This exciting result demonstrates that this series of analogues is worthy of further study as a potential therapy for HAT.

Importantly, the cross-resistance studies showed that the 5-nitro-2-furancarboxylamide analogues in this study have low levels of resistance to nifurtimox-resistant cells and vice versa while showing no resistance to pentamidine (**2**). These results indicate that the trypanocidal mode-of-action of the 5-nitro-2-furancarboxylamide analogues in this study does not rely upon nitroreductase activation unlike nifurtimox and other nitro-heterocyclic compounds that are presently in clinical trials against HAT. However, the lack of expected synergy at high concentrations of nifurtimox suggests it may be interacting with the novel mode of action which is primarily targeted by our novel 5-nitro-2-furancarboxylamide analogues.

Our data suggest that these new 5-nitro-2-furancarboxylamide analogues have a potential therapeutic implication as a single reagent in regard to potency and selectivity. Also, combination therapies can be considered due to their low cross-resistance to other trypanocidal drugs. This idea is supported by our results that show there is a synergistic relationship between nifurtimox and **22s** at concentrations of nifurtimox of <700 nM (Figure 4). While there remain many more challenges to the development of a HAT therapeutic that have not been addressed here (see Supporting Information Table S6 for a detailed discussion of drug-likeness parameters associated with our analogues), we believe that the novel mechanistic and high in vitro potency of the antitrypanosomal compounds presented here renders them worthy of further study in the drug discovery context.

EXPERIMENTAL SECTION

Biology. *Materials.* All materials unless stated were purchased from either Sigma-Aldrich or Invitrogen.

Trypanocidal Studies. The trypanocidal activity over a 72 h period was determined using the Alamar Blue viability, as described previously.[41] Nifurtimox, pentamidine, melarsoprol, and various novel nitrofuran analogues were tested against *Trypanosoma brucei brucei* bloodstream-form (strain 427) or drug-resistant strains were cultured at 37 °C in HMI9 medium supplemented with 10% fetal calf serum and 2.5 $\mu g\ mL^{-1}$ G418 as described previously.[42] *T. brucei rhodesiense* (strain Z310) were cultured in a similar manner but in the absence of G418. For the synergistic experiments, various concentrations of nifurtimox (0, 200, 400, 600, 800, 1000, 1500, 2000, and 2400 nM) were used in conjunction with a serial dilution of **22s**. Experiments were conducted in replicates of four; the data was fitted using GraFit software to obtain EC_{50} ± standard deviations and slope factors.

Cytotoxicity Studies. Cytotoxic affects against HeLa cells were determined in a similar manner. Briefly, the cells were cultured in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine.

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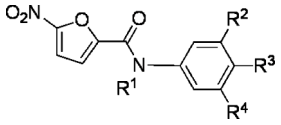
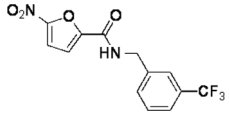
Table 3. SAR Study of Series 3 Analogues



Entry	Compound	R^1	R^2	R^3	R^4	*T.brucei* EC_{50} (nM)	HeLa EC_{50} (μM)	Selectivity Index
1	**22b**	H	H	H	H	28.1 ± 1.5	>20	>710
2	**22e**	H	CF_3	H	H	4.69 ± 0.14	>20	>4454
3	**22f**	H	Br	H	H	103.8 ± 7.7	>20	>192
4	**22g**	H	OCH_3	H	H	58.6 ± 2.9	>20	>345
5	**22h**	H	H	CF_3	H	126.3 ± 10.2	>20	>158
6	**22i**	H	H	Cl	H	411.9 ± 41.6	>20	>48
7	**22j**	H	H	$COOCH_3$	H	680.0 ± 6.1	>20	>29
8	**22k**	H	H	OCH_3	H	76.5 ± 5.5	>20	>261
9	**22m**	H	CF_3	H	OCH_3	26.6 ± 2.6	>20	>752
10	**22l**[a]	-	-	-	-	17.4 ± 3.0	>20	>1149
11	**22n**	H	CF_3	H	CF_3	128.0 ± 5.1	>10	>78
12	**22o**	H	CF_3	OCH_3	H	7.8 ± 0.3	>20	>2565
13	**22r**	H	CF_3	OH	H	2200 ± 100	>20	>9
14	**22p**	H	H	OH	H	394.4 ± 21.0	>20	>50
15	**22s**	H	CF_3	*O*-allyl	H	2.4 ± 0.3	>20	>8330
16	**22u**	H	H	*O*-allyl	H	7.73 ± 0.72	>20	>258
17	**22t**	*O*-allyl	CF_3	*O*-allyl	H	2200 ± 100	>20	>9
18	**22v**	H	CH_3	OCH_3	H	38.0 ± 3.7	>20	>525

[a]Structure of 5-nitro-*N*-(3-(trifluoromethyl)benzyl)furan-2-carboxamide (**22l**).



Cells were plated at initial cell concentration of 2×10^4 cells/well and incubated with the compounds for ~65 h prior to addition of Alamar Blue solution for a further 5 h.

Generation of Drug-Resistant T. brucei Cell Lines. Drug-resistant *T. brucei* cell lines were generated by subculturing bloodstream trypanosomes in the continuous presence of either nifurtimox or compound **22s**. Parasites were exposed to stepwise-increased concentrations of drug, starting at appropriate sublethal concentrations, until they were routinely growing between 20 and 25 times their respective original EC_{50}s. After 150−160 days in culture, drug-resistant parasites were cloned by limiting dilution and used for further study. Cell doubling times were determined in replicates of 4 over a 96 h period.

Chemistry. *General.* Chemicals and reagents were obtained from either Aldrich or Alfa-Aesar, except nifurtimox (**5**) from Bayer Argentina. All reactions involving moisture sensitive reagents were performed in oven-dried glassware under a positive pressure of argon. Dichloromethane (DCM) was obtained dry from a solvent purification system (MBraun, SPS-800). Melting points were recorded in open capillaries using an Electrothermal 9100 melting point apparatus. Infrared spectra were recorded on a Perkin-Elmer Spectrum GX FT-IR spectrometer using thin films on KBr (for solids) discs or Nujol (for liquids). Low resolution (LR) and high resolution (HR) electrospray mass spectral (ES-MS) analyses were acquired by electrospray ionization (ESI) within the School of Chemistry, University of St. Andrews. Low and high resolution ESI MS were carried out on a Micromass LCT spectrometer or at a high performance orthogonal acceleration reflecting TOF mass spectrometer coupled to a Waters 2975 HPLC. Nuclear magnetic resonance (NMR) spectra were acquired either on a Bruker Avance 300 (^{1}H, 300.1 MHz; ^{13}C, 75.5 MHz) or on a Bruker Avance 400 (^{1}H, 400 MHz; ^{13}C, 100.1 MHz) spectrometer and in the deuterated solvent stated. ^{13}C NMR spectra were acquired using the PENDANT or DEPTQ pulse sequences.

For characterization of 2-aminothiophenes **13a−k**, compounds **20a−c**, **21**, intermediates, and other known compounds, please see Supporting Information. The prepared analogues were analyzed by HPLC with purity above 95% (Supporting Information).

*General Procedure for the Synthesis of Furancarboxyl Amides **12a−k**, **18a−b**, and **22a−q**.* The furoic acid chlorides **14a** and **14b** were prepared in situ: thionyl chloride (1.10 equiv) was added dropwise to a mixture of 5-nitrofuran-2-carboxylic acid or 2-furoic acid (1.10 equiv) and triethylamine (1.50 equiv) in DCM (0.4 M) under a N_2 atmosphere. The reaction mixture was stirred at room temperature for 5

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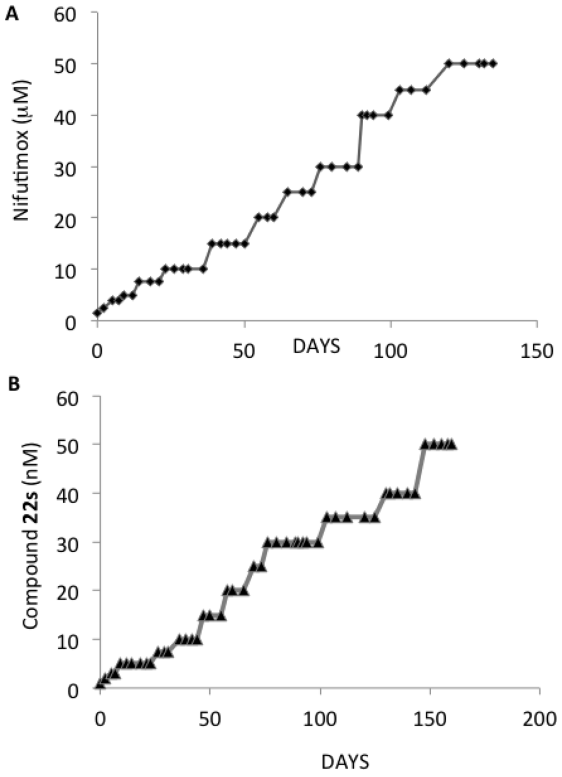

Figure 3. Generation of nifurtimox and **22s** resistance cell-lines: schematic representation of the generation of a (A) nifurtimox-resistant and (B) **22s**-resistant cell lines in *T. brucei*. Each point represents the concentration at that time point when cells were checked and divided if required.

h. Then crude **14a** or **14b** was added to another flask containing the corresponding amine or aniline (1.00 equiv) and triethylamine (2.00 equiv) in DCM (0.4 M). The reaction mixture was stirred at room temperature for 5 h. The solvent was then removed under reduced pressure, and the crude reaction mixture was purified by column chromatography.

Details of the synthesis of furancarboxyl amides **12a**, **12g**, **22e**, **22h**, and **22s** are given here in the main text of the paper; the remainder (**12b**−**f**, **12h**−**k**, **22a**−**d**, **22f**, **22g**, **22i**−**t**, and **22u**) are described in the Supporting Information.

*Ethyl 5-Ethyl-2-(5-nitrofuran-2-carboxamido)thiophene-3-carboxylate (**12a**).* The general procedure was followed using 2-amiothiophene **13a** (3.99 g, 20.0 mmol). The crude reaction mixture was purified by column chromatography on silica gel (5:1, hexane/ethyl

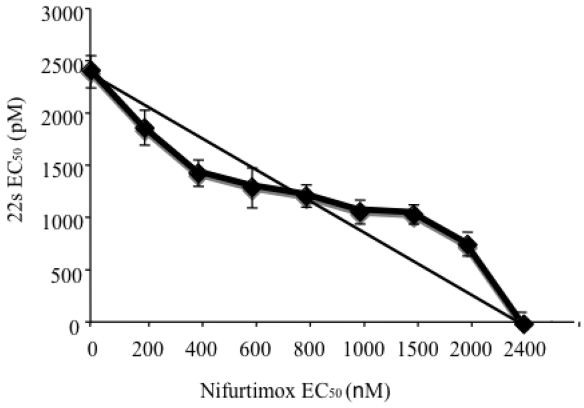

Figure 4. Studies of possible synergy between nifurtimox (**5**) and **22s** in *T. brucei*.

Table 5. EC_{50}s of Typical Series 1 and Series 2 Analogues against *T. b. rhodesiense*

Entry	Compound	*T. brucei rhodesiense* EC_{50} (nM)
1	nifurtimox (**5**)[a]	1708 ± 197
2	NFN1 (**12a**)	44.2 ± 4.5
3	**12e**	201.8 ± 18.7
4	**12g**	23.1 ± 2.9
5	**22e**	9.4 ± 1.8
6	**22h**	159.0 ± 12.1
7	**22s**	2.9 ± 0.4
8	Melarsoprol (**3**)[b]	7.8 ± 0.9

[a]EC_{50} literature value of nifurtimox in *T. b. rhodesiense* 1.5 μM.[43] [b]EC_{50} literature value of melarsoprol against *T. b. rhodesiense* 6 nM .[43]

Table 4. Cross-Resistance Studies, EC_{50}s of Wild-Type and Cloned Drug-Resistant Cell Lines

Cell Type	Nifurtimox (μM)[a]	Compound 22s (nM)	Pentamidine (nM)[b]
Wild-Type	2.1 ± 0.2	2.4 ± 0.3	1.0 ± 0.1
Nifurtimox Resistant	20.9 ± 1.7	7.8 ± 0.4	2.4 ± 0.2
Compound **22s** Resistant	7.3 ± 0.5	29.3 ± 2.0	1.1 ± 0.1

[a]EC_{50} literature value of nifurtimox in wild-type *T. brucei* 2.4 ± 0.1 μM and in nifurtimox resistant cells 20.1 ± 0.9 μM.[9] [b]EC_{50} literature value of pentamidine (**2**) in wild-type *T. brucei* 0.95 ± 0.02 nM and in nifurtimox resistant cells 2.6 ± 0.1 nM.[9]

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acetate) to afford the product as an orange solid (4.60 g, 13.6 mmol, 68%); mp 127−128 °C. IR (KBr) ν_{max} = 3120 (s) (NH), 2958 (m) (C−H), 1667 (s) (C═O), 1569 (s), 1531 (s) (NO_2), 1350 (s) (NO_2), 1280 (s) (C−O), 1266 (s) (C−O) cm^{-1}. ^1H NMR (400 MHz, acetone-d_6): δ 11.75 (br s, 1H), 7.58 (d, ^3J = 3.9 Hz, 1H), 7.42 (d, ^3J = 3.9 Hz, 1H), 6.85 (t, ^4J = 1.1 Hz, 1H), 4.28 (q, ^3J = 7.1 Hz, 2H), 2.67 (dq, ^3J = 7.5 Hz, ^4J = 1.1 Hz, 2H), 1.27 (t, ^3J = 7.1 Hz, 3H), 1.17 (t, ^3J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, acetone-d_6): δ 165.8 (C), 153.6 (C), 153.1 (C), 147.4 (C), 145.6 (C), 139.2 (C), 120.6 (CH), 118.6 (CH), 115.0 (C), 113.8 (CH), 61.7 (CH_2), 23.2 (CH_2), 15.9 (CH_3), 14.6 (CH_3). LRMS (ES^+): m/z (%) 360.87 (100) $[M + Na]^+$. HRMS (ES^+): m/z calcd for $C_{14}H_{14}N_2O_6NaS$ $[M + Na]^+$, 361.0470; found, 361.0469.

*N-(3-Carbamoyl-5-ethylthiophen-2-yl)-5-nitrofuran-2-carboxamide (**12g**).* The general procedure was followed using 2-amiothiophene **13g** (170 mg, 1.00 mmol). The crude reaction mixture was purified by column chromatography on silica gel (1:1, hexane/ethyl acetate) to afford the product as a yellow solid (167 mg, 0.54 mmol, 54%); mp (dec.) > 224 °C. IR (KBr) ν_{max} = 3476 (m), 3341 (m) (NH), 3211 (m), 3143 (m), 1659 (s) (C═O), 1591 (s), 1565 (s), 1534 (s) (NO_2), 1349 (s) (NO_2), 739 (m) cm^{-1}. ^1H NMR (400 MHz, acetone-d_6): δ 7.56 (d, ^3J = 3.9 Hz, 1H), 7.36 (d, ^3J = 3.9 Hz, 1H), 7.05 (t, ^4J = 1.1 Hz, 1H), 6.80 (br s, 1H), 2.66 (dq, ^3J = 7.5 Hz, ^4J = 1.1 Hz, 2H), 1.16 (t, ^3J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, acetone-d_6): δ 168.3 (C), 153.4 (C), 152.9 (C), 148.0 (C), 144.2 (C), 138.9 (C), 119.4 (CH), 117.9 (CH), 117.0 (C), 113.7 (CH), 23.4 (CH_2), 15.8 (CH_3). LRMS (ES^+): m/z (%) 331.91 (100) $[M + Na]^+$. HRMS (ES^+): m/z calcd for $C_{12}H_{11}N_3O_5NaS$ $[M + Na]^+$, 332.0317; found, 332.0323.

*5-Nitro-N-(3-(trifluoromethyl)phenyl)furan-2-carboxamide (**22e**).* The general procedure was followed using 3-(trifluoromethyl)aniline (622 μL, 5.00 mmol). The crude reaction mixture was purified by column chromatography on silica gel (2:1, hexane/ethyl acetate) to afford the product as a yellow solid (550 mg, 1.83 mmol, 37%); mp 188−189 °C. IR (KBr) ν_{max} = 3271 (m) (NH), 1668 (s) (C═O), 1496 (s) (NO_2), 1334 (s) (NO_2), 1266 (s), 1157 (s) (CF_3), 1119 (s) (CF_3) cm^{-1}. ^1H NMR (300 MHz, acetone-d_6): δ 10.37 (br s, 1H), 8.30 (s, 1H), 8.07 (d, ^3J = 8.3 Hz, 1H), 7.66 (d, ^3J = 3.8 Hz, 1H), 7.61 (d, ^3J = 7.9 Hz, 1H), 7.53−7.50 (m, 2H). ^{13}C NMR (75 MHz, acetone-d_6): δ 156.2 (C), 152.3 (C), 149.2 (C), 140.2 (C), 132.0 (C), 131.2 (CH), 127.3 (C), 125.2 (CH), 122.3 (CH), 118.3 (CH), 118.2 (CH), 114.1 (CH). LRMS (ES^+): m/z (%) 301.05 (100) $[M + H]^+$. HRMS (ES^+): m/z calcd for $C_{12}H_8N_2O_4F_3$ $[M + H]^+$, 301.0436; found, 301.0428. Anal. Calcd for $C_{12}H_7F_3N_2O_4$: C, 48.01; H, 2.35; N, 9.33. Found: C, 47.57; H, 2.27; N, 9.05.

*5-Nitro-N-(4-(trifluoromethyl)phenyl)furan-2-carboxamide (**22h**).* The general procedure was followed using 4-(trifluoromethyl)aniline (622 μL, 5.00 mmol). The crude reaction mixture was purified by column chromatography on silica gel (3:1, hexane/ethyl acetate) to afford the product a yellow solid (615 mg, 2.01 mmol, 41%); mp 235−236 °C. IR (KBr) ν_{max} = 3406 (m) (NH), 1697 (s) (C═O), 1538 (s), 1528 (s) (NO_2), 1323 (s) (NO_2), 1129 (s) (CF_3), 1115 (s) (CF_3), 1069 (s), 855 (s) cm^{-1}. ^1H NMR (300 MHz, acetone-d_6): δ 10.29 (br s, 1H), 7.94 (d, ^3J = 8.6 Hz, 2H), 7.61 (d, ^3J = 8.6 Hz, 2H), 7.54 (d, ^3J = 3.8 Hz, 1H), 7.40 (d, ^3J = 3.8 Hz, 1H). ^{13}C NMR (100 MHz, acetone-d_6): δ 155.8 (C), 152.8 (C), 148.8 (C), 142.5 (C), 127.0 (2CH), 126.9 (C), 124.0 (C), 121.3 (2CH), 117.9 (CH), 113.7 (CH). LRMS (ES^+): m/z (%) 301.04 (100) $[M + H]^+$. HRMS (ES^+): m/z calcd for $C_{12}H_8N_2O_4F_3$ $[M + H]^+$, 301.0436; found, 301.0432.

*N-(4-(Allyloxy)-3-(trifluoromethyl)phenyl)-5-nitrofuran-2- carboxamide (**22s**).* A mixture of **22r** (64 mg, 0.20 mmol, 1.00 equiv), K_2CO_3 (55 mg, 0.40 mmol, 2.00 equiv), and allyl bromide (0.03 mL, 0.40 mmol, 2.00 equiv) in acetone (5 mL) was stirred at room temperature for 3 h. The reaction mixture was then diluted in ethyl acetate (20 mL), washed with water (10 mL), dried (Na_2SO_4), and concentrated under vacuum. The crude reaction mixture was purified by column chromatography (3:1, hexane/ethyl acetate) to afford the product as a yellow solid (53 mg, 0.15 mmol, 75%); mp 155−156 °C. IR (KBr) ν_{max} = 3350 (s), 3115 (m) (NH), 1681 (s) (C═O), 1611 (m), 1504 (s) (NO_2), 1331 (s) (NO_2), 1250 (s) (C−O), 1135 (s) (CF_3), 1113 (s) (CF_3) cm^{-1}. ^1H NMR (400 MHz, acetone-d_6): δ 10.07 (br s), 8.03 (d, ^4J = 2.5 Hz, 1H), 7.89 (dd, ^3J = 9.1 Hz, ^4J = 2.5 Hz, 1H), 7.52 (d, ^3J = 3.9 Hz, 1H), 7.35 (d, ^3J = 3.9 Hz, 1H,), 7.13 (d, ^3J = 9.1 Hz, 1H), 6.02−5.87 (m, 1H), 5.35 (d, ^3J = 17.5 Hz, 1H), 5.15 (d, ^3J = 10.7 Hz, 1H), 4.64−4.59 (m, 2H). ^{13}C NMR (100 MHz, acetone-d_6): δ 155.5 (C), 153.9 (C), 152.6 (C), 149.1 (C), 133.8 (CH), 131.8 (C), 126.7 (CH), 125.9 (C), 123.2 (C), 120.3 (CH), 117.5 (CH), 117.4 (CH_2), 115.1 (CH), 113.7 (CH), 70.08 (CH_2). LRMS (ES^+): m/z (%) 378.91 (100) $[M + Na]^+$. HRMS (ES^+): m/z calcd for $C_{15}H_{11}N_2O_5NaF_3$ $[M + Na]^+$, 379.0518; found, 379.0526.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental for 2-aminothiophenes **13a−k**, compounds **20a−c**, **21**, intermediates, and other known compounds and the unknown compounds **12b−f**, **12h−k**, **22a−d**, **22f**, **22g**, **22i−t**, and **22u**. HPLC purity analysis and calculated chemical properties. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

HAT, human african trypanosomiasis; WHO, World Health Organization; NECT, nifurtimox−eflornithine combination therapy; DALY, disability-adjusted life years

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