Journal of Medicinal Chemistry Article

J. Med. Chem. **2010,** *53,* 6071–6078 **6071**
DOI: 10.1021/jm1004754

Aryl Phosphoramidates of 5-Phospho Erythronohydroxamic Acid, A New Class of Potent Trypanocidal Compounds

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Received April 19, 2010

RNAi and enzymatic studies have shown the importance of 6-phosphogluconate dehydrogenase (6-PGDH) in *Trypanosoma brucei* for the parasite survival and make it an attractive drug target for the development of new treatments against human African trypanosomiasis. 2,3-*O*-Isopropylidene-4-erythrono hydroxamate is a potent inhibitor of parasite *Trypanosoma brucei* 6-phosphogluconate dehydrogenase (6-PGDH), the third enzyme of the pentose phosphate pathway. However, this compound does not have trypanocidal activity due to its poor membrane permeability. Consequently, we have previously reported a prodrug approach to improve the antiparasitic activity of this inhibitor by converting the phosphate group into a less charged phosphate prodrug. The activity of prodrugs appeared to be dependent on their stability in phosphate buffer. Here we have successfully further extended the development of the aryl phosphoramidate prodrugs of 2,3-*O*-isopropylidene-4-erythrono hydroxamate by synthesizing a small library of phosphoramidates and evaluating their biological activity and stability in a variety of assays. Some of the compounds showed high trypanocidal activity and good correlation of activity with their stability in fresh mouse blood.

Introduction

Trypanosoma brucei is a parasite belonging to the order kinetoplastida. Two subspecies of this parasite, *T. b. gambiense* and *T. b. rhodesiense*, are responsible for the infection called human African trypanosomiasis (HAT[a]), which is one of the most lethal neglected diseases in the developing world. Currently, only five drugs are available to treat HAT, three of which were developed more than 50 years ago (suramin, pentamidine, and melarsoprol); the fourth drug, eflornithine (D,L-α-difluoromethylornithine, DFMO) is active only against *T. b. gambiense*,[1] and recently a new combination, nifurtimox/eflornithine, has been introduced.[2] However, these treatments are far from satisfactory because of issues that include toxicity, mode of administration, and efficacy (suramin and pentamidine are only active against the first stage of the disease). We are therefore interested in developing new drugs to treat HAT.

We have been working on inhibitors of 6-phosphogluconate dehydrogenase (6-PGDH) as a potential way to treat HAT. 6-PGDH is involved in the third step of the pentose phosphate pathway and is believed to be a good drug target for the chemotherapy of HAT.[3] The bloodstream form of the parasite relies exclusively on glycolysis as a source of energy; therefore, the parasite should be exquisitely sensitive to interference in glycolysis. Inhibition of 6-PGDH should increase the cellular level of 6-phosphogluconate, a potent inhibitor of glycolysis. Moreover, it will reduce the cellular pool of NADPH, which is produced by 6-PGDH along with glucose-6-phosphate dehydrogenase in the pentose phosphate pathway. Diminished NADPH production would reduce the parasite's ability to withstand oxidative stress and lower its capability to carry out reductive biosyntheses. We have discovered some potent inhibitors of the 6-PGDH,[3] typified by compound **1** (Figure 1), with good selectivity toward the *T. brucei* enzyme over the mammalian orthologue. Unfortunately these compounds are phosphates and as such do not readily enter cells. The lack of bioavailability has been a general hindrance to development of phosphorylated enzyme inhibitors as drugs due to the inability of these molecules to cross cellular membranes and reach the desired site of action/target at an efficacious concentration.[4] To circumvent this, phosphate-prodrugs have been developed. The design of phosphate-prodrugs is a relatively new field[5] which has been applied in antivirals, anticancer, and signaling regulators research.[6−10] A number of phosphate-prodrugs have recently progressed to clinical trials,[11,12] which proves the applicability of this approach. In our previous work, we reported the synthesis and the biological evaluation of several classes of prodrug of compound **1**, compounds **2**−**6**[13] (Figure 1). These prodrugs showed antiparasitic activity in vitro against cell culture of trypanosomes with EC_{50} values in the micromolar range. We also investigated their stability in pseudophysiological conditions measuring their half-life (phosphate buffer at pH 7.4 and 37 °C). Yet despite some good trypanocidal

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[a] Abbreviations: 6-PGDH, 6-phosphogluconate dehydrogenase; HAT, human African trypanosomiasis; PHLM, pooled human liver microsomal assay.

Published on Web 07/28/2010

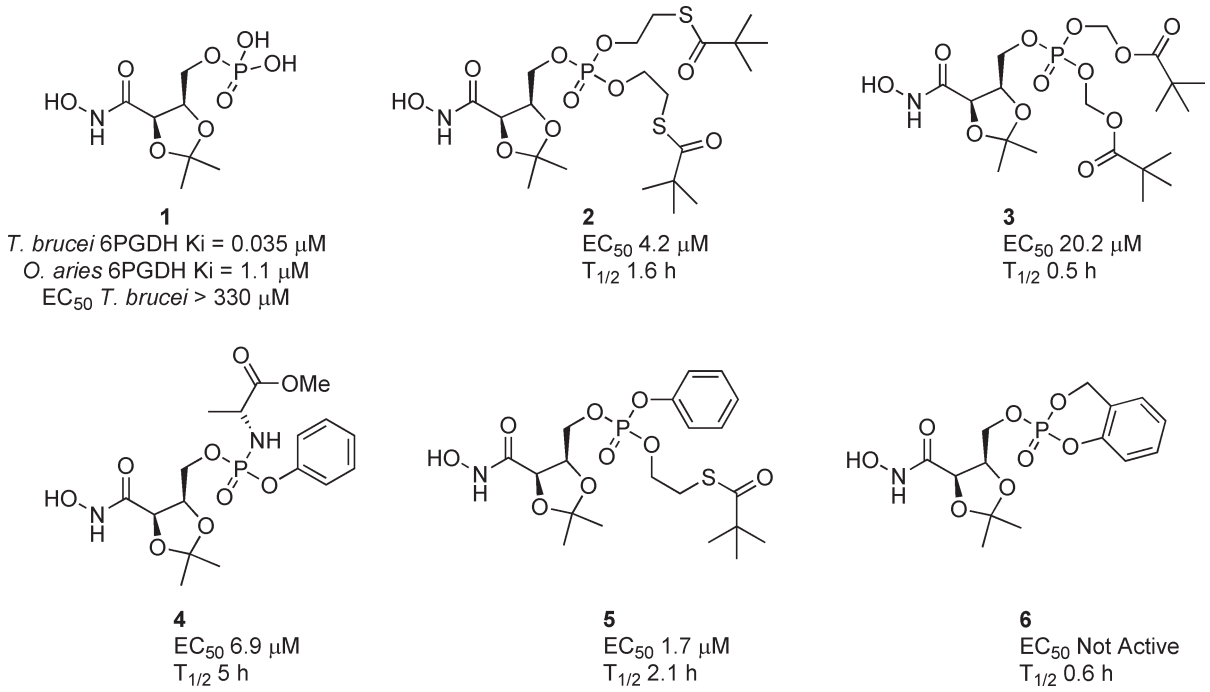


Figure 1. Lead compound **1** and the corresponding phosphate prodrugs synthesized.

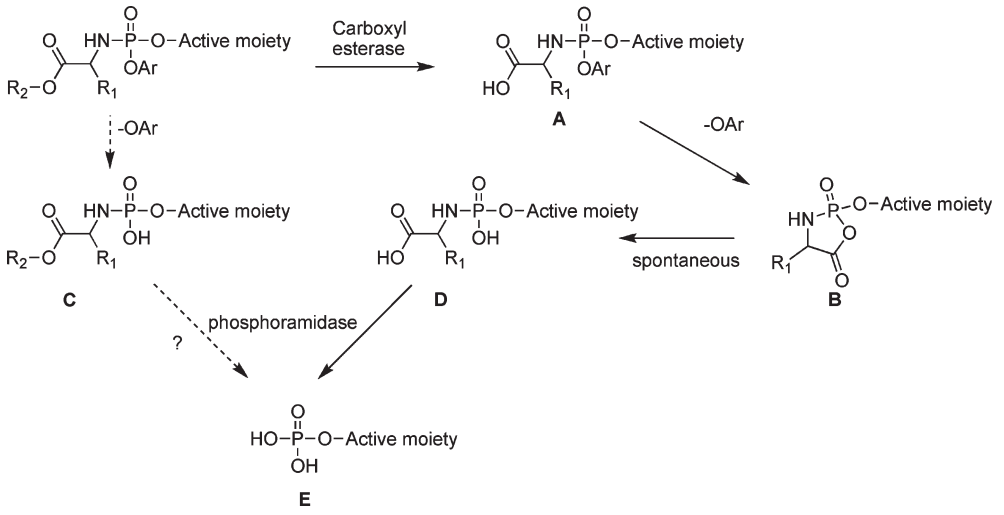


Figure 2. Mechanism of cleavage of phosphoramidates as proposed by McGuigan et al.[14]

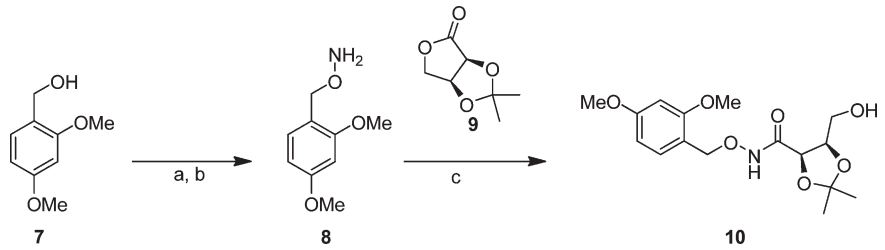
activity, most of the phosphate masking groups showed low stability and very short half-life in phosphate buffer, with the exception of the aryl phosphoramidate **4**, which had both reasonable stability and good activity. We therefore took the phosphoramidate **4** and worked on novel chemistry to probe stability in phosphate buffer in fresh whole blood and with hepatic microsomes and related this to trypanocidal activity.

Work by McGuigan and co-workers has shown that it is possible to optimize the aryl phosphoramidate stability by combining variations of the amino acid side chain, the amino acid ester, and the aryl group. The proposed mechanism of cleavage for aryl phosphoramidate suggested by Mcguigan et al.[14] is shown in Figure 2. The cleavage can follow two main pathways. The first route requires the action of a carboxyl esterase, which hydrolyses the amino acid ester to form the free acid carboxylate (compound **A**). The oxygen of the carboxylate then attacks the phosphorus atom and displaces the aryl ester to form a 5-membered intermediate **B**, which then rearranges, forming compound **D**. The final step is proposed to be catalyzed by a "phosphoramidase" enzyme that produces the final product **E**. The second pathway, which can be predominant in the presence of electron-withdrawing groups on the phenyl ester, follows a simple chemical hydrolysis with release of the aromatic ester and then proceeds to the final product by enzymatic cleavage of the amino acid group.

Consequently, optimization of the chemical and enzymatic stability of the aryl phosphoramidates can be achieved by alterations to the amino acid side chain, the amino acid ester, and the substituent on the aryl ester group, as illustrated in Figure 3. To increase the stability of the prodrugs we pursued the following modifications:

- Increasing the steric bulk of the amino acid side chain (aimed at reducing interaction with esterases thus slowing down the formation of intermediate **A**).
- Increasing the steric bulk of the ester (aimed at reducing interaction with esterases and also increasing the chemical

Scheme 1[a]



[a] (a) DIAD, PPh_3, *N*-hydroxyphthalimide, DCM; (b) Me-$NHNH_2$, EtOH, reflux; (c) $AlMe_3$, DCM.

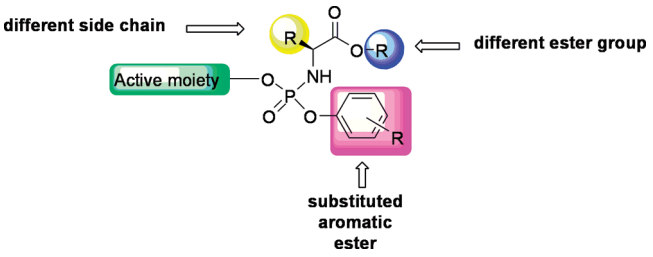


Figure 3. Planned modifications on the aryl-phosphoramidates.

stability of the esters, also slowing down the formation of intermediate **A**).

- Introduction of electron donating groups on the phenyl ring, aimed at destabilizing the anion generated during the hydrolysis step, thus making the aryl phenolate a poorer leaving group and increasing the stability against chemical hydrolysis. This should slow down conversion of **A** to **B** (mechanism 1) or formation of **C** (mechanism 2).

Chemistry

The phosphoramidates were prepared according to previously reported methodology. The 2,4-dimethoxybenzyl erythrono hydroxamate was obtained in three steps from 2,4-dimethoxy benzyl alcohol and 2,3-*O*-isopropylidene erythrono lactone (Scheme 1). The product **10** was crystallized in 40% yield from EtOAc/hexane.[13]

The phosphorochloridates **12a**−**e** were synthesized from the corresponding substituted phenols, phosphorus oxychloride and the corresponding amino acid ester hydrochloride (Scheme 2). The phosphochloridates were then coupled with the protected erythronohydroxamate **10** following conditions developed by McGuigan et al.[7] The intermediates **14a**−**q** were obtained in moderate to good yields.

The final step of the synthesis was the cleavage of the protecting group from the hydroxamate moiety by trifluoroacetic acid in dichloromethane. The small library was obtained in reasonable yields and in good purity as mixtures of diastereoisomers due to chirality of the phosphorus atom.

Biological Stability

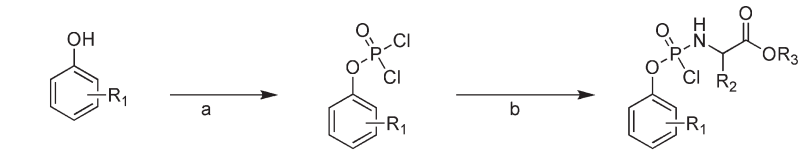
The prodrug approach for the treatment of a parasitic disease such as HAT is challenging because of the complexity of the system (host + parasite) and the particular requirements that the prodrug must fulfill in order to achieve the desired effect. In the case of *Trypanosoma brucei*, the ideal prodrug must have a good stability in the bloodstream of the mammalian host and should be quickly converted into the active form (drug) only inside the parasite (Figure 4). Once converted to the active form, the charged compound should not be able to diffuse back out of the parasite. Whether this ideal situation may be achieved is not clear, particularly the specific activation of the prodrug within the parasite, as we have only minimal information relating to metabolic capabilities of blood and the parasites. However, by developing compounds whose conversion from prodrug to drug in blood is delayed will improve the prospects of those compounds entering the parasites and achieving bioconversion within the target cell. Therefore we focused our efforts on increasing the stability of prodrugs in blood on the assumption that prodrug activation would occur in parasites at an enhanced rate (Figure 4).

To investigate the stability of the prodrugs, compounds were incubated in buffer at pH 7.4 and 2.0 in order to investigate the chemical hydrolysis, and in mouse blood to determine stability in the presence of esterases. Finally some of them were evaluated in a pooled human liver microsomal assay (PHLM) to determine metabolic stability. The data for these experiments is shown in Tables 1 and 2. The stability data was compared to the activity of compounds against bloodstream form *T. b. brucei* BS427.

Trypanocidal Activity and Chemical Stability in Phosphate Buffer at pH 7.4. Compounds were assessed for activity against *T. brucei*. Most of the compounds showed submicromolar EC_{50} values; four compounds were particularly potent: **15g**, EC_{50} 90 nM; **15l**, EC_{50} 40 nM; **15p**, EC_{50} 50 nM; **15q**, EC_{50} 8 nM.

The chemical stability effects of the different substituents can be analyzed by comparing compounds where there are stepwise changes. First, we consider the effect of changing the aryl substituent. In general, electron donating groups increase the buffer (chemical) stability of the compounds. In analyzing and comparing the data for compounds **15a** (4-NO_2), **15c** (no substituent), **15h** (4-Me), **15m** (4-MeO), and **15o** (2,3-diMe) (where the amino acid moiety is alanine methyl ester), it is evident that the introduction of electron-donating groups improves the buffer stability at pH 7.4 (half-life increases from 0.85 h for **15a** up to 30 h for compound **15o**), and this is translated, in the same manner, into a higher in vitro activity with compound **15o** (IC_{50} 0.27 μM) being the most active in vitro for the alanine methyl ester series. There are several exceptions. The 4-methoxy derivative **15m** demonstrated neither increased buffer stability nor better in vitro activity compared to the analogue **15c**. This data follows a similar trend to that of McGuigan et al.,[15] who also reported that the 4-methoxyphenyl phosphoramidate of the nucleoside d4T was slightly less stable at pH 7.4 than the corresponding phenyl analogue. Also for some reason, compounds with valine as the amino acid and esterified as an ethyl ester (**15f**) showed a small decrease in stability on substituting the phenyl ring with either the 4-methyl (**15l**) or 2,3-dimethyl (**15p**) substitutents. 2,3-Dimethylphenyl esters and 4-methylphenyl esters showed comparable activity and stability (see pairs **15l**−**15p** and **15h**−**15o**), and they are

Scheme 2[a]



11a: R = 4-CH_3
11b: R = 4-OCH_3
11c: R = 2,3-CH_3

12a: R_1 = 4-NO_2
12b: R_1 = H
12c: R_1 = 4-CH_3
12d: R_1 = 4-OCH_3
12e: R_1 = 2,3-CH_3

13a: R_1 = 4-NO_2, R_2 = CH_3, R_3 = CH_3
13b: R_1 = H, R_2 = H, R_3 = CH_3
13c: R_1 = H, R_2 = CH_3, R_3 = CH_3
13d: R_1 = H, R_2 = CH_3, R_3 = CH_2CH_3
13e: R_1 = H, R_2 = *i*-Pr, R_3 = CH_3
13f: R_1 = H, R_2 = *i*-Pr, R_3 = CH_2CH_3
13g: R_1 = H, R_2 = *i*-Pr, R_3 = *t*-Bu
13h: R_1 = 4-CH_3, R_2 = CH_3, R_3 = CH_3
13i: R_1 = 4-CH_3, R_2 = CH_3, R_3 = CH_2CH_3
13j: R_1 = 4-CH_3, R_2 = *i*-Pr, R_3 = CH_3
13l: R_1 = 4-CH_3, R_2 = *i*-Pr, R_3 = CH_2CH_3
13m: R_1 = 4-OCH_3, R_2 = CH_3, R_3 = CH_3
13n: R_1 = 4-OCH_3, R_2 = *i*-Pr, R_3 = CH_2CH_3
13o: R_1 = 2,3-CH_3, R_2 = CH_3, R_3 = CH_3
13p: R_1 = 2,3-CH_3, R_2 = *i*-Pr, R_3 = CH_2CH_3
13q: R_1 = 2,3-CH_3, R_2 = *i*-Pr, R_3 = *t*-Bu

15a: R_1 = 4-NO_2, R_2 = CH_3, R_3 = CH_3, 22%
15b: R_1 = H, R_2 = H, R_3 = CH_3, 73%
15c: R_1 = H, R_2 = CH_3, R_3 = CH_3, 11%
15d: R_1 = H, R_2 = CH_3, R_3 = CH_2CH_3, 62%
15e: R_1 = H, R_2 = *i*-Pr, R_3 = CH_3, 43%
15f: R_1 = H, R_2 = *i*-Pr, R_3 = CH_2CH_3, 47%
15g: R_1 = H, R_2 = *i*-Pr, R_3 = *t*-Bu, 53%
15h: R_1 = 4-CH_3, R_2 = CH_3, R_3 = CH_3, 40%
15i: R_1 = 4-CH_3, R_2 = CH_3, R_3 = CH_2CH_3, 60%
15j: R_1 = 4-CH_3, R_2 = *i*-Pr, R_3 = CH_3, 53%
15l: R_1 = 4-CH_3, R_2 = *i*-Pr, R_3 = CH_2CH_3, 66%
15m: R_1 = 4-OCH_3, R_2 = CH_3, R_3 = CH_3, 50%
15n: R_1 = 4-OCH_3, R_2 = *i*-Pr, R_3 = CH_2CH_3, 37%
15o: R_1 = 2,3-CH_3, R_2 = CH_3, R_3 = CH_3, 75%
15p: R_1 = 2,3-CH_3, R_2 = *i*-Pr, R_3 = CH_2CH_3, 59%
15q: R_1 = 2,3-CH_3, R_2 = *i*-Pr, R_3 = *t*-Bu, 62%

14a: R_1 = 4-NO_2, R_2 = CH_3, R_3 = CH_3, 33%
14b: R_1 = H, R_2 = H, R_3 = CH_3, 12%
14c: R_1 = H, R_2 = CH_3, R_3 = CH_3, 29%
14d: R_1 = H, R_2 = CH_3, R_3 = CH_2CH_3, 61%
14e: R_1 = H, R_2 = *i*-Pr, R_3 = CH_3, 43%
14f: R_1 = H, R_2 = *i*-Pr, R_3 = CH_2CH_3, 61%
14g: R_1 = H, R_2 = *i*-Pr, R_3 = *t*-Bu, 47%
14h: R_1 = 4-CH_3, R_2 = CH_3, R_3 = CH_3, 36%
14i: R_1 = 4-CH_3, R_2 = CH_3, R_3 = CH_2CH_3, 26%
14j: R_1 = 4-CH_3, R_2 = *i*-Pr, R_3 = CH_3, 10%
14l: R_1 = 4-CH_3, R_2 = *i*-Pr, R_3 = CH_2CH_3, 25%
14m: R_1 = 4-OCH_3, R_2 = CH_3, R_3 = CH_3, 28%
14n: R_1 = 4-OCH_3, R_2 = *i*-Pr, R_3 = CH_2CH_3, 12%
14o: R_1 = 2,3-CH_3, R_2 = CH_3, R_3 = CH_3, 5%
14p: R_1 = 2,3-CH_3, R_2 = *i*-Pr, R_3 = CH_2CH_3, 41%
14q: R_1 = 2,3-CH_3, R_2 = *i*-Pr, R_3 = *t*-Bu, 18%

[a] (a) $POCl_3$, TEA, Et_2O; (b) amino acid ester hydrochloride, TEA, DCM, rt; (c) NMI, DCM, (2,4-dimethoxybenzyl)-*O*-erythronoydroxic alcohol; (d) TFA, DCM, rt 15 min.

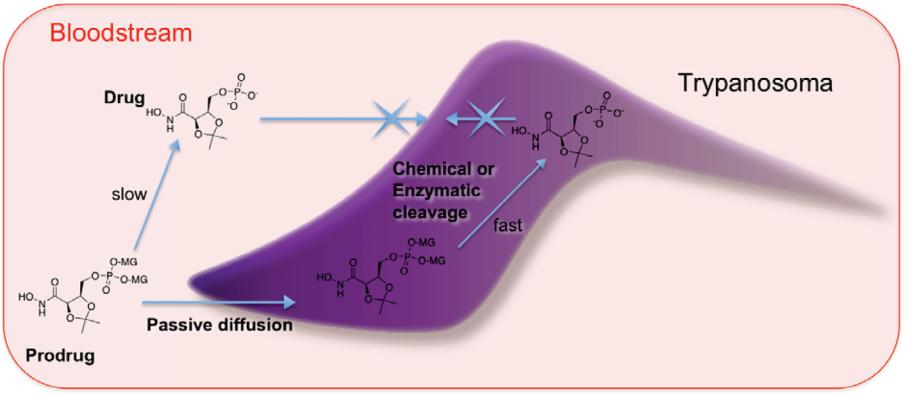
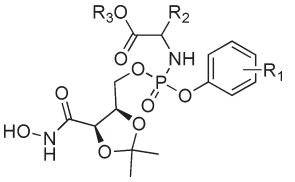


Figure 4. Schematic representation of selective delivery of prodrugs into trypanosomes (MG: masking group).

generally more active than the corresponding phenyl, 4-methoxyphenyl and 4-nitrophenylesters.

The importance of amino acid side chain can be seen by analyzing the pairs **15c**−**15e**, **15h**−**15j**, **15i**−**15l**, **15d**−**15f**,

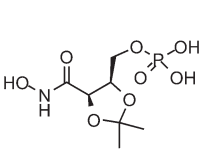
Table 1. Biological Activity and Stability for Phosphoramidates



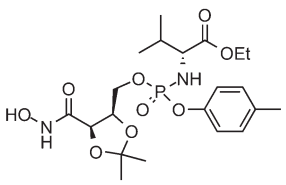
compd	R_1	R_2	R_3	$t_{1/2}$ pH 7.4 h	$t_{1/2}$ pH 2 h	$t_{1/2}$ mouse blood (min)	PHLM (mL/min/g)	EC_{50} (μM)
15a[a]	4-NO_2	Me	Me	0.85				43.8
15b	H	H	Me			< 15		11.4
15c[a]	H	Me	Me	5		< 15		6.95
15d	H	Me	Et	29		< 15		0.78
15e	H	*i*-Pr	Me	21		45	< 0.5	0.36
15f	H	*i*-Pr	Et	61		120	< 0.5	0.24
15g	H	*i*-Pr	*t*-Bu	68	28	120	5.0	0.09
15h	4-Me	Me	Me	30		< 15		0.31
15i	4-Me	Me	Et	34				0.18
15j	4-Me	*i*-Pr	Me	45	28			0.15
15l	4-Me	*i*-Pr	Et	25	15	45	< 0.5	0.04
15m	4-OMe	Me	Me		8.5		< 0.5	3.79
15n	4-OMe	*i*-Pr	Et				< 0.5	0.11
15o	2,3-Me	Me	Me	30				0.27
15p	2,3-Me	*i*-Pr	Et	48	10	60	< 0.5	0.05
15q	2,3-Me	*i*-Pr	*t*-Bu	> 24		> 480	10	0.008

[a] Compounds **15a** and **15c** have been reported previously.[13]

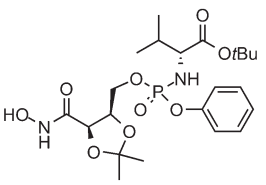
Table 2. Biological Properties for the Most Potent Trypanocidal Compounds



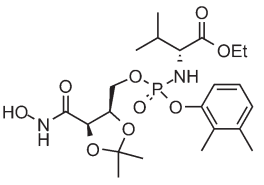
1



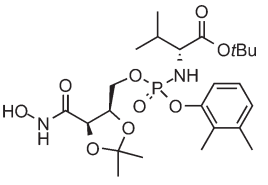
15l



15g



15p



15q

	1	**15l**	**15g**	**15p**	**15q**
EC_{50} (μM)	> 330	0.04	0.09	0.05	0.008
$T_{1/2}$ mouse blood (min)		45	120	60	480
$T_{1/2}$ ph 7.4 (h)		25	30	48	24
PHLM Cli mL/min/g		< 0.5	5.0	< 0.5	10
clogD		2.4	2.6	2.9	3.6

and the three phenyl esters **15b**, **15c**, and **15e**. Changes from glycine to alanine or alanine to valine increased the buffer stability in every case, with the exception of **15i** (Ala)−**15l** (Val); in this latter case, the compounds had reasonably comparable buffer stability with half-lives of 34 and 25 h, respectively. The less hindered amino acid glycine **15d** showed much lower buffer stability, which made it difficult to measure the half-life (data not reported in the table). The poor stability for this aryl phosphoramidate was also observed during its synthesis when the coupling of the phosphochoridate **13b** with the erythronohydroxamate **10** produced the prodrug **14b** with one of the lowest yields (12%), due to a degree of decomposition that occurred during the purification. With all of these compounds, the increase in buffer stability also corresponded to an increase in activity against the parasites, with valine being better than alanine which is in turn better than glycine.

The other point of variation is with the amino acid ester (R_3). In terms of the antiparasitic activity, the *tert*-butyl esters have more potent EC_{50} values than the ethyl and methyl ester as shown by compounds **15e** (Me), **15f** (Et), and **15g** (*t*-Bu) (EC_{50} 0.36, 0.24, and 0.09 μM, respectively) and also **15p** (Et) and **15q** (*t*-Bu) (EC_{50} 0.050 and 0.008 μM, respectively). A relationship between the buffer stability at pH 7.4 and antiparasitic activity is less obvious, although there are fewer data points. The *tert*-butyl esters have similar half-lives to the ethyl analogues (see pair **15p** (Et) and **15q** (*t*-Bu) and pair **15f** (Et) and **15g** (*t*-Bu)). Comparison of the ethyl esters **15d**, **15i**, and **15l** with the corresponding methyl ester analogues **15c** and **15h** and **15j** shows how the ethyl ester improves both the trypanocidal activity and the buffer stability.

Overall, there is a general correlation between buffer stability and activity of the compounds, with those compounds showing longer half-life in phosphate buffer also the most active against the parasite (Figure 5). Thus moving from compound **15a**, which has a half-life of about 50 min, to **15q**, which has a half-life of > 24 h, the potency changes from 44 to 0.008 μM, an increase in activity of more than 5000 times. The relationship between in vitro antiparasitic data and buffer stability is almost certainly complicated by the presence of esterases (see below) which have a role in removal of the ester.

We also looked at the buffer (chemical) stability of several compounds at pH 2, to see if the compounds were likely to survive in the stomach. The compounds showed a small

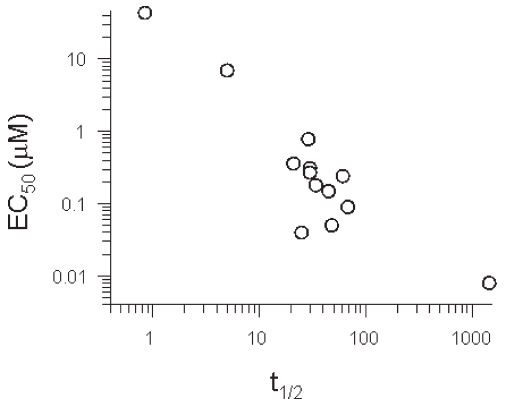


Figure 5. Correlation between EC_{50} and half-life of the prodrugs in phosphate buffer pH 7.4.

reduction (approximately 2-fold) in stability at more acidic conditions, which should not preclude them being used orally.

Mouse Blood Stability. The stability in fresh mouse blood was investigated for key compounds. This would give an indication of stability to esterases found in the blood. Hydrolysis of the prodrug by esterases is probably a key step in the conversion of the prodrugs into the active species (Figure 2). Many of the compounds showed a very short half-life, probably due to hydrolysis of the amino acid ester by various esterases in the blood, leading to loss of the masking groups.

Looking at series **15e**−**g** and **15p**−**q** indicates that as the bulk of the ester increases, so does blood stability. The order of blood stability appears to be *tert*-butyl > ethyl > methyl. We hypothesize that this is the order of stability of the esters to esterases. Similarly, an increase in bulk at the α-position appears to increase the blood stability, as can be seen by comparison of **15c** (R_2 = Me) and **15e** (R_2 = *i*-Pr) and **15d** (R_2 = Me) and **15f** (R_2 = *i*-Pr). Comparison of **15c** and **15h** and of **15f**, **15l**, and **15p** indicates lack of correlation between blood stability and the substituent on the phenyl ring. Therefore, compounds with bulky esters or α-substituents are more stable in blood, which is presumably a measure of increased stability to esterases. However, the substituent on the aromatic ring has a much smaller effect on blood (esterase) stability. Again, this is consistent with the mechanism shown in Figure 2.

It is interesting to compare our experience with that reported in the literature for prodrugs of the nucleotides.[15,16] In the case of the nucleotide prodrugs, the stability of the prodrug moiety, at least in human plasma, appears much greater than we have observed here. Possibly this is due to some intramolecular general acid/base catalysis in our system causing a more rapid hydrolysis of the compounds. In the case of the nucleotide prodrugs, there also was different correlation between substituents on the prodrugs and antiviral activity compared to what we have seen here. According to McGuigan and co-workers, in the case of phosphoramidates of d4T, the higher esterase stability was not correlated to higher potency of the compounds. The reported data showed that the *t*-butyl ester phosphoramidate of d4T was not degraded after 21 h of incubation in the presence of PLE.[16] In our case, however, *t*-butyl esters are converted to potent trypanocidal compounds in a reasonable time frame (2−8 h).

Stability in Pooled Human Liver Microsomes (PHLM). Compounds were also investigated for their metabolic stability, using human liver microsomes (Table 2). Most compounds assayed had good metabolic stability (< 0.5 mL/min/g). However, the *tert*-butyl esters appeared to be very susceptible to metabolism. This may be due to the extra liphophilicity of this group. Liability of the *tert*-butyl derivatives to hepatic metabolism may mitigate against their utility as drug candidates in spite of their other stability characteristics being favorable.

Conclusions

Compound **1** is a potent and selective inhibitor of *T. brucei* 6-PGDH; however, it is inactive against the parasite, presumably due to the presence of the phosphate group. By making prodrugs through masking of the phosphate group, it is possible to prepare compounds that have activity against the parasite and the activity of these prodrugs appears to correlate to their buffer stability. In particular, the aryl phosphoramidate prodrugs appeared to have the best balance of stability and activity. In this article, we describe the optimization of these prodrugs and their evaluation. By variation of the ester, the α-amino substituent, and the substituent on the phenyl ester on the phosphate prodrug, it is possible to fine-tune the buffer and blood stability of the compounds. Thus, for the carboxylic ester, increasing the bulk of the ester increases the buffer and blood stability (presumably chemical and esterase stability); for the α-amino substituent, increasing the bulk also increases the buffer and blood stability. For the phenyl substituent, electron-donating substituents increase the buffer (chemical) stability without revealing consistent effects on blood stability. The prodrugs with the greatest buffer and blood stability thus have a *tert*-butyl ester, an isopropyl substituent at the α-position, and a 2,3-dimethyl substituent on the phenyl ring. By these modifications, we have improved trypanocidal activity of the compounds from 6.9 to 0.008 μM and the stability in mouse blood from less than 15 min to greater than 480 min.

The biological data show how important the chemical stability in phosphate buffer at pH 7.4 is for the in vitro trypanocidal activity of these compounds, thus we can possibly assume that in the Alamar Blue assay the main decomposition pathway of the prodrugs in the assay media is via chemical hydrolysis.

Even though we have not proved that the mode of action of these compounds is through inhibition of the enzyme 6-PGDH, we have shown the aryl phosphoramidate prodrugs derived from the inhibitor **1** have a higher antitrypanosomal activity compared to parent compound. thus phosphoramidates show correlation between the trypanocidal activity and the stability in pseudophysiological conditions, with EC_{50} values over 5000-fold lower than compound **1**. However, some microsomal (metabolic) instability was observed for the *tert*-butyl-valinyl phosphoramidates (**15g** and **15q**), indicating these compounds are probably susceptible to hepatic clearance.

This article shows that it is possible to improve these compounds by optimizing the prodrug moiety. Further optimization will require retaining the good buffer and blood (chemical and esterase) stability of the prodrug moiety while optimizing key developability properties of the molecule (such as solubility and metabolic stability).

Experimental Section

Parasite Testing. *T. b. brucei* (s427), wild-type cells were cultured to the optimum density of (1−2) × 10^6 cells mL^{-1} in HMI-9 supplemented with 10% fetal calf serum (FCS) under

environmental conditions of 37 °C and 5% CO_2. Solutions of test compounds were prepared in culture media at stock concentration of 200 μM and diluted serially (1:2) across the 96-well, flat-bottom solid white plates to give a total of 11 decreasing concentrations (100 μL well^{-1}). The last well of each series was left blank, i.e., "drug-free" (negative control). Cells were prepared at the concentration of 4×10^4 cells mL^{-1} and added to each of the respective compound series (100 μL well^{-1}). Plates were incubated at 37 °C/5% CO_2 for 48 h prior to the addition of Alamar Blue solution (20 μL well^{-1}, 0.49 mM in 1X PBS, pH 7.4) followed by a further 24 h incubation. Assay end points were measured fluorimetrically with the fluorescence spectrometer (FluoStar, BMG LabTech, Germany) and Optima program set at $\lambda_{excitation}$ of 544 nm and $\lambda_{emission}$ of 590 nm. Data was analyzed using Prism 5.0 software to obtain EC_{50} values. Experiment was performed in duplicate and repeated three times.

Microsomal Stability. Each test compound (0.5 μM) was incubated with pooled human liver microsomes (Tebu-Bio, UK; 0.5 mg/mL 50 mM potassium phosphate buffer, pH7.4) and the reaction started with addition of excess NADPH (8 mg/mL 50 mM potassium phosphate buffer, pH7.4). Immediately, at time zero, then at 3, 6, 9, 15, and 30 min, an aliquot (50 μL) of the incubation mixture was removed and mixed with acetonitrile (100 μL) to stop the reaction. Internal standard was added to all samples, the samples centrifuged to sediment precipitated protein, and the plates were then sealed prior to UPLCMSMS analysis using a Quattro Premier XE (Waters).

XLfit (IDBS, UK) was used to calculate the exponential decay and consequently the rate constant (k) from the ratio of peak area of test compound to internal standard at each time point. The rate of intrinsic clearance (CLi) of each compound was then calculated using the following calculation:

$$\text{CLi (mL/min/g liver)} = k \times V \times \text{microsomal protein yield}$$

where V (mL/mg protein) is the incubation volume/mg protein added and microsomal protein yield is taken as 52.5 mg protein/g liver

Verapamil was used as a positive control to confirm acceptable assay performance.

General Methods. ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, and 2D-NMR spectra were recorded either on a Bruker Avance DPX 300 spectrometer or on a Bruker Avance DPX 500 spectrometer. Chemical shifts (δ) are expressed in ppm. Signal splitting patterns are described as singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q), multiplet (m), or combination thereof.

LCMS analyses were performed with an Agilent HPLC 1100 (Waters XBridge Column) and diode array detector in series with a Bruker MicroTof mass spectrometer. Compounds were eluted using either method A (methanol, methanol/water (95:5) or water/acetonitrile (1:1) + 0.1% formic acid as the mobile phase on Phenomenex Gemini Column) or with method B (water/acetonitrile 95:5 to 5:95 on a Water Xbridge column). High-resolution electrospray measurements were performed on Bruker MicroTof mass spectrometer.

Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F254 plates using UV light and/or PMA or $KMnO_4$ for visualization. TLC data are given as the R_f value with the corresponding eluent system specified in brackets. Column chromatography was performed using Fluka silica gel 60. All reactions were carried out under dry and inert conditions (Ar atmosphere) unless otherwise stated. Reactions using microwave irradiation were carried in a Biotage InitiatorTM microwave.

The purity/identity of compounds was determined by a combination of NMR (^{1}H, ^{13}C, ^{31}P), LCMS, and HRMS. The purity of all compounds was determined by LCMS.

General Procedure A: Synthesis of Phosphorodichloridates. A solution of phosphorus oxychloride (1 mol) and the appropriate phenol (1 mol) in dry diethyl ether was cooled at −78 °C, followed by the addition of triethyl amine (1 mol). The white suspension was left to warm to room temperature and stirred for 12 h. The triethylamine salt was filtered off, and the filtrate was concentrated under reduced pressure to an oil, which was used for the next step without further purification.

General Procedure B: Synthesis of Phosphorochloridates. The appropriate phosphorodichloridate (1.2 mol) and the amino acid esters hydrochloride (1 mol) were stirred in dry DCM with at −78 °C. TEA was added dropwise with a syringe. The mixture was stirred for 15 min at −78 °C and then warmed to room temperature. The reaction was monitored by ^{31}P NMR, and when the analysis showed the completed disappearance of the starting material, the reaction was concentrated under reduced pressure. The residue was taken in Et_2O, and the precipitated triethylamine salt was filtered off. The filtrate was concentrated in vacuo and used for the next step without further purification.

General Procedure C: Synthesis of Phosphoramidates. The protected (2,4dimethoxybenzyl)erythrono hydroxamate (1 mol) was stirred with the appropriate phosphorochloridate phosphoramidate (1.2−2.1 mol) in DCM (10 mol) and cooled to −78 °C under argon atmosphere. *N*-Methyl imidazole (4 mol) was added dropwise with a syringe to the solution. The mixture was stirred for 15 min at −78 °C and then was left warm to room temperature and stirred for 4−12 h. The reaction was monitored by ^{31}P NMR and quenched with MeOH (1 mL). The organic phase was washed with 0.5 M HCl (3 × 10 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude oil was purified by column chromatography with 100% chloroform → 4% MeOH/chloroform.

General Procedure D: Cleavage of the 2,4-Dimethoxybenzyl Group with TFA. The protected hydroxamate was stirred in DCM at room temperature, 2% of TFA was added with syringe. The reaction was stirred at room temperature until the complete disappearance of the starting material was observed by TLC (5% MeOH/DCM) 15−20 min. The white suspension was diluted with Et_2O, and the white precipitate was filtered off. The filtrate was concentrated under reduced pressure and purified by chromatography eluting the silica with 100% DCM → 4% MeOH/DCM.

Examples of three final compounds are given below. Details of the remainder of the compounds is found in the Supporting Information.

2,3-*O*-Isopropylidene Erythronocarboxamide-4-[phenyl(methoxyglycinyl)phosphoramidate] (15b). The title compound was synthesized following the general procedure D from compound **15b** (41 mg, 0.07 mmol), TFA (40 μL), and DCM (5 mL). Slightly orange hygroscopic foam, 22 mg (73%). ^{1}NMR (500 MHz, $CDCl_3$) δ: 1.27 (s, 3H, 1 of C(C$\underline{H}_3$)$_2$), 1.43 (d, 3H, J = 5.34 Hz, C(C$\underline{H}_3$)$_2$), 3.64 (d, 3H, J = 4.70 Hz, OC$\underline{H}_3$), 3.68−3.80 (m, 2H, NHC$\underline{H}_2$), 4.08−4.25 (m, 2H, C$\underline{H}_2$OP), 4.49−4.52 (m, 1H, C$\underline{H}$CH$_2$OP), 4.66 (d, 1H, J = 7.47 Hz, C(O)C$\underline{H}$), 7.06−7.09 (m, 1H, Ar$\underline{H}$), 7.12−7.14 (m, 2H, Ar$\underline{H}$), 7.21−7.25 (m, 2H, Ar$\underline{H}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 24.50, 24.60 (C($\underline{C}$H$_3$)$_2$), 26.35, 26.67 (C($\underline{C}$H$_3$)$_2$), 42.80, 42.86 (NH$\underline{C}$H$_2$), 52.41, 52.46 (O$\underline{C}$H$_3$), 65.04, 65.08, 65.24, 65.28 ($\underline{C}$H$_2$OP), 74.78, 75.27 ($\underline{C}$HCH$_2$OP), 75.76, 75.84 (C(O)$\underline{C}$H), 110.63, 110.64 (C($\underline{C}$H$_3$)$_2$), 120.16, 120.20, 120.25 (Ar$\underline{C}$H), 125.02, 125.08 (Ar$\underline{C}$H), 129.69, 129.70 (Ar$\underline{C}$H), 150.54, 150.60 (Ar$\underline{C}$-OP), 165.76, 165.17 (HN$\underline{C}$(O)), 171.44, 171.50, 171.60 ($\underline{C}$O$_2$Me). ^{31}P NMR (121 MHz, $CDCl_3$) δ: 3.68, 3.50. LCMS (ES+): m/z 419.12 ([M + H]$^+$, 100%); m/z 436.14 ([M + NH$_4$]$^+$, 20%). R_t 3.0 min, (purity >99% by UV). HRMS: Found 419.1223; $C_{16}H_{24}N_2O_9P$ [M + H$^+$] requires 419.1214.

2,3-*O*-Isopropylidene Erythronocarboxamide-4-[phenyl(ethoxyalaninyl) phosphoramidate] (15d). Compound **14d** (200 mg, 0.33 mmol) was deprotected according to the general procedure D using TFA (89 μL) and DCM (9 mL). Orange foam, 93 mg (62%). The compound contains traces of TFA. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.16−1.28 (m, 9H, CH$_2$C$\underline{H}_3$ + CHC$\underline{H}_3$ + 1 of C(C$\underline{H}_3$)$_2$), 1.43, 1.42 (2s, 3H, 1 of C(C$\underline{H}_3$), 3.86−4.04 (m, 2H, C$\underline{H}$CH$_3$ + O$\underline{H}$), 4.09 (q, 2H, J = 6.9 Hz, OC$\underline{H}_2$CH$_3$), 4.12−4.19

(m, 2H, CH_2OP), 4.32 (bs, 0.5H, PN*H*), 7.08 (q, 1H, J = 7.4 Hz, Ar*H*), 7.10−7.15 (m, 2H, Ar*H*), 7.22−7.26 (m, 2H, Ar*H*), 8.89, 9.20 (2bs, 1H, ON*H*). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.07, 14.10 (CH_2CH_3), 20.82, 20.95 ($CHCH_3$), 24.35, 24.52, 26.25, 26.64 ($C(CH_3)_2$), 50.01, 50.29 ($CHCH_3$), 61.82 (OCH_2CH_3), 64.84 (CH_2OP), 75.40, 75.75 ($CHCH_2OP$), 75.84, 76.08 (C(O)*C*HCH), 110.46, 110.54 ($C(CH_3)_2$), 120.15, 120.33, 120.37, (Ar*C*H), 125.02, 125.08 (Ar*C*H), 129.73 (Ar*C*H), 150.49, 150.52 (Ar*C*-OP), 165.12 (HN*C*(O)), 173.49, 173.72 (CO_2Et). ^{31}P NMR (121 MHz, $CDCl_3$) δ: 2.56, 2.30. LCMS (ES+): m/z 447.18 ($[M + H]^+$, 100%). R_t 3.4 min, (purity 97.6% UV). HRMS: Found 447.1532; $C_{18}H_{28}N_2O_9P$ $[M + H^+]$ requires 447.1527.

2,3-*O*-Isopropylidene Eerythronocarboxamide-4-[phenyl(methoxyvanlinyl)phosphoramidate] (15e). The title compound was synthesized following the general procedure D from **14e** (191 mg, 0.31 mmol), TFA (40 μL), and CM (5 mL). Orange foam, 62 mg (43%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.76−0.85 (m, 6H, $CH(CH_3)_2$), 1.29-(d, 3H, J = 3.0 Hz, 1 of $C(CH_3)_2$), 1.44 (s, 3H, 1 of $C(CH_3)_2$), 1.95−2.02 (m, 1H, $CH(CH_3)_2$), 3.64 (d, 3H, J = 10.6 Hz, OCH_3), 3.85−3.87 (m, 1H, NHC*H*), 3.85−3.87 (d, 1H, J = 11.2 Hz, PN*H*), 4.14 (t, 1H, J = 4.3 Hz, C*H*HOP), 4.18−4.35 (m, 1H, CH*H*OP), 4.49−4.53 (m, 1H, C*H*CH_2OP), 4.69 (dd, 1H, J_1 = 3.0 Hz, J_2 = 7.5 Hz, C(O)C*H*CH), 7.06−7.10 (m, 1H, Ar*H*), 7.14−7.16 (m, 2H, Ar*H*), 7.22−7.27 (m, 2H, Ar*H*), 8.85 (bs, 1H, ON*H*). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 17.12, 18.93 ($CH(CH_3)_2$), 24.49, 26.63 ($C(CH_3)_2$), 31.97, 32.02 ($CH(CH_3)_2$), 52.43 (CO_2CH_3), 59.94 (PNH*C*H), 64.84, 64.89 (CH_2OP), 75.49, 75.80 ($CHCH_2OP$), 75.89 (C(O)*C*HCH), 110.51 ($C(CH_3)_2$), 120.30, 120.34 (Ar*C*H), 125.00 (Ar*C*H), 129.64 (Ar*C*H), 150.68, 150.74 (Ar*C*-OP), 166.10 (HN*C*(O)), 174.54, 174.57 (CO_2Me). ^{31}P NMR (121 MHz, $CDCl_3$) δ: 3.49, 3.32. LCMS (ES+): m/z 461.18 ($[M + H]^+$, 100%). R_t 3.1 min, (purity 92.5% by UV). HRMS: Found 461.1680; $C_{19}H_{30}N_2O_9P$ $[M + H^+]$ requires 461.1683.

Acknowledgment. We acknowledge the Wellcome Trust (075277 and 083481) for financial support.

Supporting Information Available: Experimental procedures and spectral data for all intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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