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Phosphonoxins: Rational design and discovery of a potent nucleotide anti-*Giardia* agent

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Abstract—Phosphonoxins, a new class of synthetic, rationally designed anti-microbial agents, are described. From this class a sub-micromolar inhibitor of *Giardia* trophozoite growth has been identified.

Protozoa are one of the more common causes of infections and illness in humans and animals worldwide.[1,2] For example, an estimated 1 billion humans are infected with *Toxoplasma gondii*.[3] Specifically, infections caused by the flagellated protozoan *Giardia* are widespread. Yearly, there is an estimated 100 million cases of giardiasis worldwide and *Giardia* is the most commonly diagnosed waterborne cause of diarrhea in the United States.[4] *Giardia* is also commonly found in livestock and many mammals may serve as important reservoirs capable of transmitting disease to humans.[5–9] To date widely effective treatment, broad-spectrum anti-microbial agents for prophylaxis, or effective vaccines for these pathogens are not available.[10,11] In addition, clinical resistance has been reported for current anti-protozoals, including cases where both metronidazole and albendazole failed in treatment of *giardiasis*.[9–12] Diseases caused by protozoans are therefore a worldwide risk to the health of humans and animals.

Giardia species have two major stages in their life cycles. The trophozoite stage is the vegetative form that replicates in the small intestine of its host causing diarrhea and symptoms of malabsorption. Upon exposure to biliary fluid some trophozoites differentiate to the encysted form. The cysts are passed in feces, and enter the environment where they can infect another host. *Giardia* cysts are highly infective and exceptionally well adapted for survival in, and dissemination by, water. They are difficult to detect and are resistant to common treatments such as chlorine and ozone. These adaptations further complicate treatment of *Giardia* infections.[1,2,4]

While differing protozoal species have quite different life cycles the environmentally resistant and infective cyst is common to many protozoans.[13,14] Because encystation appears to occur in response to unfavorable growth conditions, it is highly likely that prevention of cyst formation would be fatal.[15] However, unlike bacterial cell wall synthesis protozoal cyst wall synthesis has not been well exploited as a drug target.[16]

While protozoal cyst walls are not as fully characterized as cell walls in other organisms, it is known that some, *e.g., Giardia, Entamoeba, and Toxoplasma*, contain chitin or a chitin-like polysaccharide.[3,15–19] Recent description of enzyme activity termed cyst wall synthase (CWS) in *Giardia*[17] inspired our discovery of a micromolar inhibitor of *Giardia* trophozoite growth.[20] We report here design, synthesis, and activity of a potent and non-toxic second generation anti-*giardial* agent that was designed as an inhibitor of CWS. Because these agents bear passing resemblance to polyoxins[21] we have termed these new synthetic agents *phosphonoxins*.

Keywords: *Giardia*; Encystation; Cyst wall synthase; Nucleotide.
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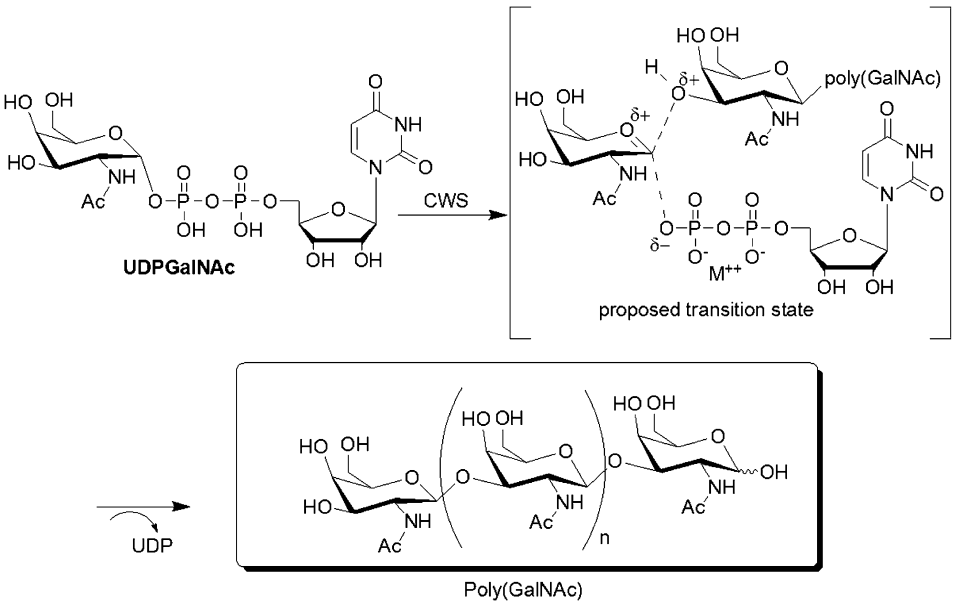


Figure 1. Synthesis of poly(GalNAc) by cyst wall synthase.

Cyst wall synthase catalyzes synthesis of the chitin-like poly β-1-3-linked *N*-acetylgalactosamine[poly(GalNAc)] that comprises about 63% of the *giardia* cyst wall. CWS has a high affinity (K_m = 0.048 mM) for its substrate and glycosyl donor, UDPGalNAc, V_{max} = 0.07 nmol/(min × mg protein). This synthase requires (in order of preference) the divalent cations Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+}. Metal chelators such as EDTA inhibit CWS. Finally, CWS is specific for UDPGalNAc. UDP-glucose, UDPGlcNAc, UDP-galactose, glucosamine, and galactosamine are not substrates.[17]

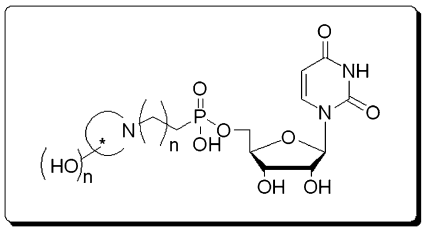
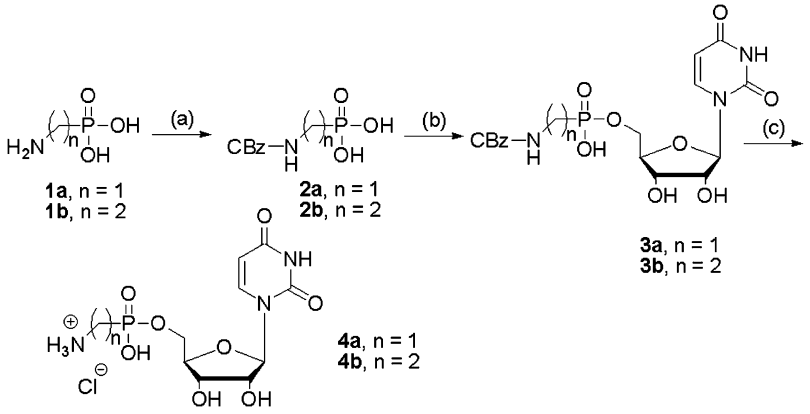


Figure 2. Proposed transition state analogs.

As with other glycosyl transferase processes, synthesis of poly(GalNAc) is proposed to proceed through a transition state with a positive charge on the hexose moiety of the UDPGalNAc substrate (Fig. 1).

Analogs containing a stable linker between uridine and a sugar transition state analog should therefore be potent inhibitors of CWS.[22] We have designed and synthesized a series of UDPGalNAc transition state analogs that contain an aza-sugar or aza-sugar analog in place of the GalNAc moiety.[23] The aza-sugar is linked by an alkylphosphonate to uridine (Fig. 2). In contrast to the



Scheme 1. Reagents and conditions: (a) CBzCl, 1 N NaOH, 12 h; (b) i—*i*PrU, DCC pyridine, Dowex-50 H^+, rt 4 d; ii—Dowex H^+; (c) 1 Atm H_2/Pd-$BaSO_4$.

natural phosphates and pyrophosphates, phosphonates are able to penetrate cell membranes.[24,25] In addition, these aminoalkylphosphonates should be efficient chelators of the metal cation proposed to be at the active site (Fig. 1).[18,26–28]

We began by synthesis of 5′-[(aminoalkyl)phosphono]uridines **4** from the commercially available aminophosphonic acids **1** (Scheme 1). The acids were protected as the CBz derivatives and the resulting intermediates **2** were coupled with 2′,3′-isopropylidinuridine (*i*PrU) using DCC in pyridine with Dowex-50 (H^+ form). Exclusion of Dowex from the reaction mixture led to slower reaction times and significantly lower yields. The protecting groups were removed by standard methods[29] and the resulting aminophosphonic acids were isolated as their hydrochloride salts **4** (Table 1).

N-substituted β-amino phosphonates were synthesized through conjugate addition of amino alcohols with diethylvinylphosphonate or diisopropylvinylphosphonate[30,31] followed by protection of the free hydroxyls, and deblocking of the phosphonate moiety with bromotrimethylsilane (Scheme 2). Carbodiimide mediated coupling of the resulting β-amino phosphonates with 2′,3′-isopropylidinuridine (iPrU) and removal of the protecting groups gave the target transition state analogs.

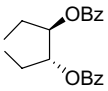
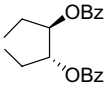
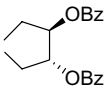
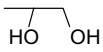
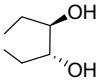
These transition state analogs were screened for activity against *Giardia lamblia* WB-6 strain. Trophozoite inhibition was determined by culturing the parasites anaerobically for 48 h in the presence of 2-fold drug dilutions in triplicate. The XTT reagent was used to measure cell viability. Cyst development was determined in a similar manner except the assay was conducted for 4 d in encysting medium glass vials. The cysts were counted, washed in deionized water, and resuspended in trophozoite medium to evaluate cyst viability. Manine Darbey Bovine Kidney (MDBK) cells were used to evaluate host toxicity using a standard method.[32]

The assays revealed that while all derivatives are nontoxic (MDBK $EC_{50} > 250$ μM), the pyrrolidine derivative **10f** is about 25-fold more active than the other derivatives, **10**, and is also the most potent inhibitor of cyst formation in this series (Table 2).

We therefore selected **10f** as a lead and investigated the importance of the length of the alkyl linker between the phosphonate moiety and the pyrrolidine group. The propyl linked derivative **15** was prepared from the pyrrolidine **11**[33] and bromopropylphosphonate **12**[34] (Scheme 3). Deblocking of the phosphonate ester of the resulting **13** followed by diimide coupling with 2′,3′-isopropylidinuridine and removal of the protecting groups using standard methods gave the desired aminopropyl derivatives.

The alkoxyaminophosphonate, **18**, was prepared in a similar manner from known phosphonate **16**[35] (Scheme 4).

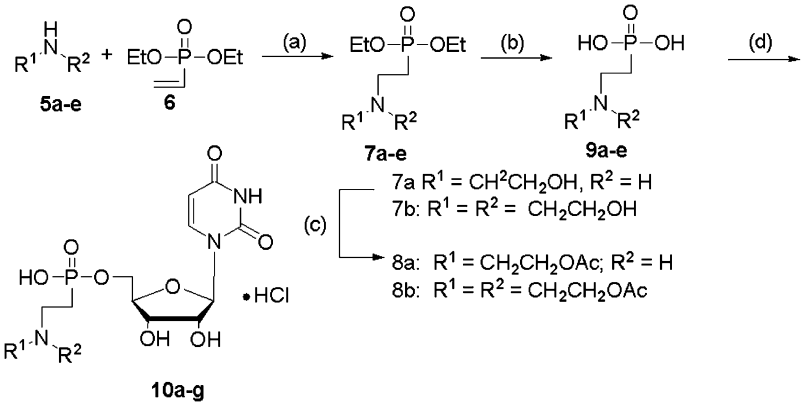
Table 1. Legend for Scheme 2

Compound	R^1	R^2
5a	$-CH_2CH_2OH$	H
5b	$-CH_2CH_2OH$	$-CH_2CH_2OH$
5c[a]		H
5d		
5e		
7a	$-CH_2CH_2OH$	H
7b	$-CH_2CH_2OH$	$-CH_2CH_2OH$
7c[a]		H
7d		
7e		
9a	$-CH_2CH_2OAc$	H
9b	$-CH_2CH_2OAc$	$-CH_2CH_2OAc$
9c[a]		H
9d		
9e		
10a	$-CH_2CH_2OH$	H
10b	$-CH_2CH_2OH$	$-CH_2CH_2OH$
10c	$-CH_2CH_2OAc$	$-CH_2CH_2OH$
10d	$-CH_2CH_2OAc$	$-CH_2CH_2OAc$
10e[b]		H
10f		
10g		

[a] Racemic.
[b] A mixture of diastereomers.

Generation of the free phosphonic acid, coupling with the protected uridine, and deprotection were performed as described above.

Condensation of **11**, formaldehyde, and diisopropylphosphite (Scheme 5) gave phosphonate **19** which was readily converted to methylene linked derivative **20**. Finally, to study the significance of the positive charge on the amino moiety, we prepared imide **22** from commercially available anhydride **21** and



Scheme 2. Reagents and conditions: (a) toluene, reflux; (b) bromotrimethylsilane, acetonitrile, rt 2 d; (c) acetic anhydride, pyridine, 14 h (d) i—*i*PrU, DCC, pyridine, Dowex-50 H^+; ii—H_2O, 0.1 N HCl.

Table 2. Anti-*Giardia* trophozoite MIC and Cyst inhibition activity

Compound	Trophozoite MIC (μM)	Toxicity: MDBK cell EC_{50} (μM)	Inhibition of Cyst formation[a]
4a	>20	>250	34.4
4b	>10	>250	14.9
8a	>20	>250	nd
8b	>20	>250	nd
10a	>20	>250	nd
10b	>10	>250	72.2
10c	>10	>250	35.6
10d	>10	>250	27.4
10e	>20	>250	nd
10f	0.48	>250	5.73
10g	>20	>250	nd
15	20	>250	22.9
18	20	>250	17.2
20	>20	>250	nd
23	>20	>250	28.7

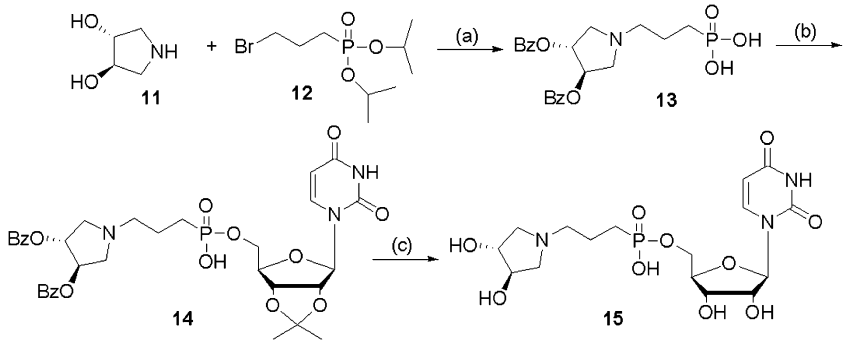
nd, not determined.

[a] Percent of control (cyst formation performed with no drug) with drug conc. = 10 μM.

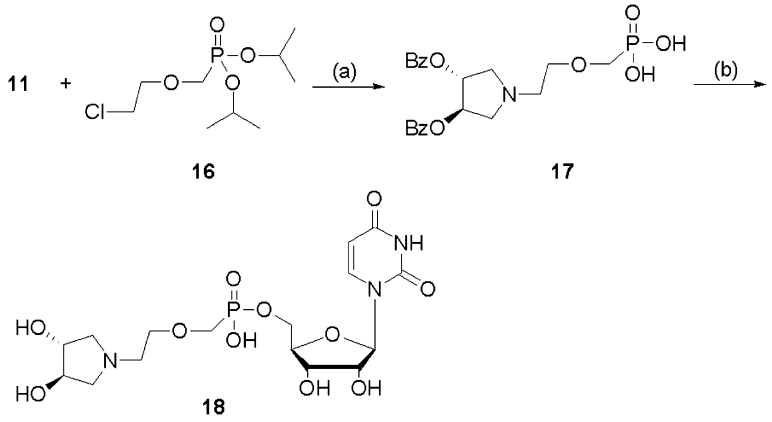
β-aminoethylphosphonate (Scheme 6). The resulting phosphonic acid **22** was coupled with protected *i*PrU and the protecting groups removed to give target **23**.

Derivatives **15**, **18**, **20**, and **23** were all considerably (at least 50-fold) less active than the lead, **10f**. We speculate that linker between the phosphonate moiety and the amino moiety in **10f** allows for optimal conformation in chelation of the metal cation (probably calcium) that is proposed at the active site in the target enzyme, possibly CWS.

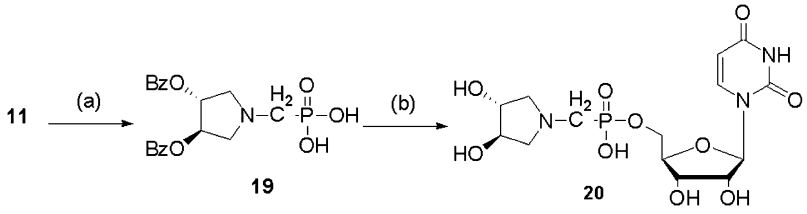
In summary we have designed and synthesized new class of anti-protozoal agents that we call *phosphonoxins*. Martin and co-workers,[36] and Schuster et al.[37] have described similar phosphonate–aza-sugar analogs as glycosyl transferase transition state analogs. However, to our knowledge this is the first report of such potential glycosyl transferase inhibitors with biological activity. Specifically we have discovered a potent inhibitor of *Giardia* trophozoite growth with activity that rivals existing therapeutics. This phosphonoxin, **10f**, is also a potent inhibitor of *Giardia* cyst formation and may have clinical potential as a new anti-*giardia* drug. Yet because **10f** is not a specific inhibitor of cyst formation, but additionally inhibits vegetative growth, it is probably not a specific inhibitor of CWS. Studies on optimization of activity, mechanism, and efficacy in animal models will be reported in due course.



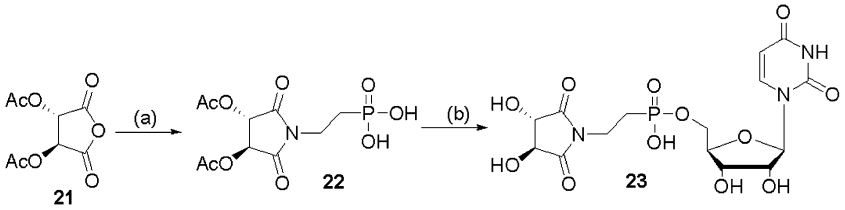
Scheme 3. Reagents and conditions: (a) i—Dioxane, K_2CO_3, rt 2 d; ii—Bz_2O, pyridine, rt 2 h; iii—bromotrimethylsilane, acetonitrile rt 2 d 74%; (b) *i*PrU, DCC, pyridine, Dowex-50 H^+; (c) i—7 N ammonia/MeOH, 14 h; ii—Dowex-50 H^+, water rt 2 d, 18% (from compound **13**).



Scheme 4. Reagents and conditions: (a) i—Dioxane, K_2CO_3, rt 2 d; ii—Bz_2O, pyridine, rt 2 h; iii—bromotrimethylsilane, acetonitrile, rt 2 d 69%; (b) i—iPrU, DCC, pyridine, Dowex-50 H^+; ii—7 N ammonia/MeOH, 14 h; iii—Dowex-50 H^+, water, rt 2 d 21%.



Scheme 5. Reagents and conditions: (a) i—Diisopropylphosphite, formaldehyde; ii—Bz_2O, pyridine, rt 2 h; iii—bromotrimethylsilane, acetonitrile, rt 2 d; (b) i—*i*PrU, DCC, pyridine, Dowex-50 H^+; ii—7 N ammonia/MeOH, 14 h; iii—Dowex-50 H^+, water, rt 2 d.



Scheme 6. Reagents and conditions: (a) i—Diisopropylaminoethyl phosphonate, rt 3 h; ii—acetic anhydride, 90 °C 16 h; iii—bromotrimethylsilane, acetonitrile, rt 3 h; (b) i—*i*PrU, DCC, pyridine, Dowex-50 H^+; ii—7 N ammonia/MeOH, 14 h; iii—Dowex-50 H^+, water, rt 2 d.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.02.063.

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