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Synthesis of Marine-Derived 3-Alkylpyridinium Alkaloids with Potent Antiprotozoal Activity

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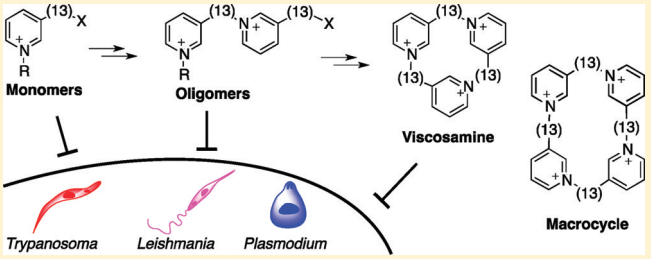
Supporting Information

ABSTRACT: Given the pressing need for new antiprotozoal drugs without cross-resistance with current (failing) chemotherapy, we have explored 3-tridecylpyridinium alkaloids (3TPAs), derivatives of viscosamine, as antiparasitic agents. We have developed a simple synthetic route toward viscosamine and related cyclic and linear monomers and oligomers. Evaluation for cytotoxicity on the protozoan parasites *Trypanosoma brucei*, *Leishmania* spp., and *Plasmodium falciparum* revealed several 3TPAs with antiprotozoal activity in the nanomolar range. Their promising selectivity index in vitro prompted us to study the dynamics of cytotoxicity on trypanosomes in more detail. Parasites were killed relatively slowly at therapeutically safe concentrations, in a process that did not target the cell cycle. Clearance of *T. brucei* cultures was observed at drug concentrations of 1−10 μM.



KEYWORDS: *Alkylpyridinium alkaloids, synthesis, antitrypanosomal activity, antileishmanial activity, antiplasmodial activity, cytotoxicity*

About half of the world's population lives in areas where seriously pathogenic protozoan parasites, including *Trypanosoma*, *Leishmania*, and *Plasmodium* sp., are endemic and cause significant morbidity, mortality, and economic hardship (Supporting Information, text 1).[1] Treatment of these parasitic infections relies solely on chemotherapy. As these parasites have evolved intricate immune evasion strategies, effective antiparasite vaccines are not expected in the near future, despite considerable efforts in this field.[2,3] Severe adverse effects and resistance to current drugs[4−6] articulate the urgent demand for novel, safe, and effective drugs.

We aim to develop antiprotozoal lead compounds that lack cross-resistance with current chemotherapy. Marine organisms are an abundant source of bioactive molecules, and the 3-alkylpyridinium alkaloids isolated from sponges of the order Haplosclerida display antibacterial[7] and anticancer[8−10] activity. However, there have been no reports on their antiprotozoal potential. Here, we focus on the synthesis and antiprotozoal evaluation of 3-tridecylpyridinium alkaloids (3TPAs) of the viscosamine family, consisting of *N*-alkylated pyridinium units and saturated C_{13} alkyl chains (Supporting Information, text 2). Naturally occurring 3TPAs isolated to date include linear and cyclic oligomers such as cyclostelletamine C (dimer)[11] or viscosamine (trimer).[12] The first total synthesis of viscosamine was based on the Zincke reaction.[13] We present a simpler route toward viscosamine and related analogues. We have evaluated their activity against a panel of parasites and studied aspects of their effect on *Trypanosoma brucei* in particular.

We based the synthesis of 3TPAs on a versatile protection strategy of the pyridine nitrogen with a *p*-methoxybenzyl (PMB) group.[14] Pyridyl alkanol **1** is readily converted to its PMB-protected iodide **3** (Scheme 1). Dimerization of 3-alkylpyridine **1** with **3** in refluxing acetonitrile gave alcohol **4**, which was similarly converted to corresponding iodide **5** and condensed with a third molecule of **1** to yield trimeric alcohol **6**. After transfer-deprotection of the PMB group in refluxing pyridine macrocyclization, precursor **7** was obtained in pure form by crystallization (57% yield starting from **4**). The corresponding iodide **8** was cyclized by refluxing in acetonitrile (1 mM), which gave viscosamine **9** in 43% yield over two steps. Unlike all of the dimeric bis-3-alkylpyridinium macrocycles that we prepared before,[14] trimeric iodide salt **9** refused to crystallize and was purified by chromatography over Al_2O_3. Ion exchange of the triiodide salt with Dowex 1X2-200 gave the better water-soluble trichloride, also as an amorphous solid. A comparable sequence was used to prepare cyclic tetramer **13**.

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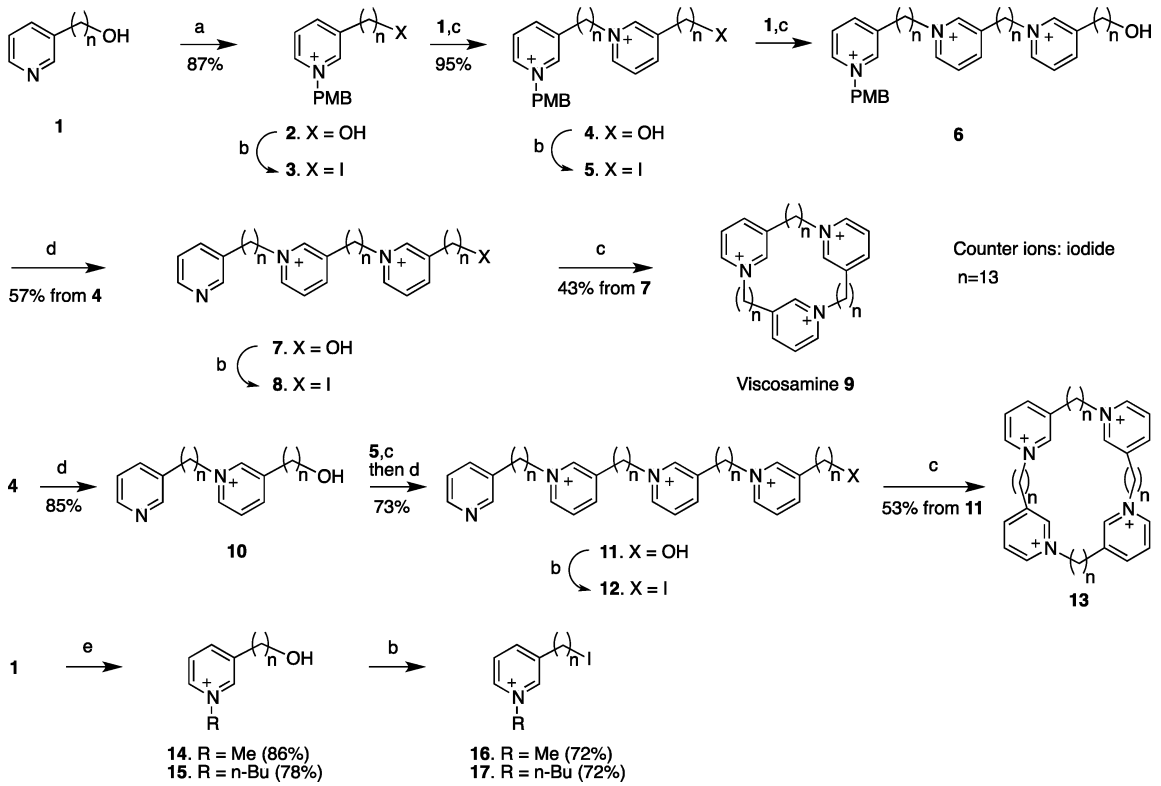
Scheme 1. Synthesis of 3-TPAs[a]



[a]Reagents and conditions: (a) PBMCl, KI, acetonitrile. (b) I_2, TPP, imidazole, toluene-acetonitrile. (c) Acetonitrile, reflux. (d) Pyridine, reflux. (e) R-I, ethyl acetate (for **14**) or acetonitrile (for **15**), reflux. TPP, triphenylphosphine.

PMB-alcohol **4** was deprotected to **10** in refluxing pyridine and dimerized with iodide **5** to the corresponding tetrapyridine **11**. Deprotection, iodination, and macrocyclization gave the novel, 64-membered macrocycle **13** in crystalline form. Monomeric 3TPAs containing C1 or C4 alkyl chains on the pyridinium nitrogen were easily synthesized by N-alkylation of pyridine **1**, yielding alcohols **14** and **15**, and, following iodination, their corresponding iodides **16** and **17**, respectively. In short, we generated viscosamine **9** in 21% yield in eight steps starting from pyridyl alcohol **1** employing only four reaction types. The convergent route reported before required nine steps and eight different reaction types yielding viscosamine in 3% yield starting from pyridyl alcohol **1**.[13] Our strategy constitutes an easy entry toward the controlled synthesis of higher order (cyclic) oligomers, for example, the novel tetrameric macrocycle **13**.

The antitrypanosomal activity was tested against bloodstream form (BF) *T. brucei* (Table 1). Cationic analogues alkylated on the pyridine nitrogen all displayed submicromolar EC_{50} values, except **14**, which displayed an EC_{50} value of just over 2 μM. Pyridyl alcohol **1**, lacking a substituent on the pyridine nitrogen, appeared to be much less toxic to these parasites. Cyclic oligomers (**9** and **13**) displayed similar antitrypanosomal activity as linear oligomers (**7**, **10**, and **11**) with EC_{50} values between 0.22 and 0.56 μM. Monomers **2**, **3**, **15**, **16**, and **17** were even more active than the reference drug diminazene aceturate; the most active compounds were **2** (EC_{50} = 50 nM) and **17** (EC_{50} = 14 nM); reference drugs pentamidine and cymelarsan displayed at least an order of magnitude higher activity.

To assess potential cross-resistance with the current first-line trypanocidal drugs, the 3TPAs were also tested against two drug-resistant clonal lines, derived from *T. brucei* s427: (a) Δ*Tbat1*, lacking the TbAT1/P2 aminopurine transporter[15] and consequently displaying reduced sensitivity to diamidines and melaminophenyl arsenicals,[16,17] and (b) B48, derived from Δ*Tbat1*, lacking activity of a second drug transporter, HAPT1, and consequently highly resistant to diminazene, pentamidine, and the melaminophenyl arsenicals.[18,19] The trypanocidal activity of the 3TPAs was not, as a class, substantially different between wild-type and the two drug-resistant lines, suggesting that uptake of this compound class is not dependent on the known drug transporters and that cross-resistance with the diamidine and melaminophenyl arsenical drugs does not occur.

The antileishmanial activity was tested against *Leishmania major* promastigotes and *Leishmania mexicana* axenic amastigotes. The data on *L. major* promastigotes revealed similar trends as those with the African trypanosomes: monomers (**2** and **15**−**17**) generally show higher activity than oligomers (**7**, **9**−**11**, and **13**). On amastigotes, linear 3TPAs were more active than cyclic derivatives. Among oligomers, the presence of three heterocycles, as in **7**, appeared optimal for toxicity against all kinetoplastids studied. Cationic 3TPAs showed higher leishmanicidal activity than the reference drug pentamidine, currently in clinical use to treat leishmaniasis. The strong correlation between the activities of the 3TPAs against *Trypanosoma* and *Leishmania* sp. (r^2 = 0.86, linear regression using *T. brucei* wt and *L. mexicana* amastigote EC_{50} values) suggests a similar mode of action against the different kinetoplastids.

Screening against the apicomplexan parasite *Plasmodium falciparum* revealed viscosamine **9** and its linear precursor **7** as the alkaloids with the highest antiplasmodial activity, with EC_{50} values of 53 and 68 nM, respectively—2 orders of magnitude less active than reference drug chloroquine. The trend observed

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Table 1. Antiprotozoal and Cytotoxic Activities of 3-TPAs[a]

compd	*T. brucei* wt	*T. brucei* Δ*T*bAt1	*T. brucei* clone B48	*L. major* promastigotes	*L. mexicana* amastigotes	*P. falciparum*	HEK293
1	16 ± 3	34 ± 4	35 ± 1	>50	>50	7.5 ± 2.6	204 ± 10
2	0.049 ± 0.005	0.084 ± 0.011	0.062 ± 0.010	0.32 ± 0.01	0.65 ± 0.03	0.24 ± 0.05	21 ± 1
3	0.19 ± 0.04	0.48 ± 0.10	0.20 ± 0.01	1.0 ± 0.04	0.40 ± 0.06	0.22 ± 0.04	41 ± 1
7	0.22 ± 0.03	0.27 ± 0.003	0.25 ± 0.01	0.85 ± 0.08	0.19 ± 0.04	0.068 ± 0.015	16 ± 2
9	0.41 ± 0.02	0.49 ± 0.03	0.28 ± 0.02	0.72 ± 0.04	0.81 ± 0.03	0.053 ± 0.012	26 ± 2
10	0.40 ± 0.06	0.62 ± 0.04	0.30 ± 0.02	1.6 ± 0.08	0.34 ± 0.19	0.14 ± 0.04	25 ± 1
11	0.31 ± 0.04	0.41 ± 0.03	0.33 ± 0.004	1.1 ± 0.04	0.23 ± 0.03	0.16 ± 0.06	11 ± 2
13	0.56 ± 0.04	0.66 ± 0.05	0.42 ± 0.05	0.64 ± 0.04	1.1 ± 0.02	0.19 ± 0.02	14 ± 3
14	2.3 ± 0.1	2.3 ± 0.1	2.7 ± 0.5	2.7 ± 0.2	4.8 ± 0.2	1.5 ± 0.4	183 ± 4
15	0.16 ± 0.01	0.26 ± 0.01	0.20 ± 0.02	0.33 ± 0.04	1.7 ± 0.08	0.51 ± 0.06	48 ± 2
16	0.086 ± 0.019	0.18 ± 0.03	0.11 ± 0.02	0.31 ± 0.04	0.28 ± 0.01	0.26 ± 0.06	47 ± 3
17	0.014 ± 0.002	0.063 ± 0.008	0.034 ± 0.010	0.36 ± 0.002	0.29 ± 0.04	0.31 ± 0.03	26 ± 2
diminazene	0.26 ± 0.05	7.9 ± 0.7			24 ± 4		
pentamidine	0.0014 ± 0.0005	0.0032 ± 0.0007	0.066 ± 0.018	4.3 ± 0.02	8.8 ± 0.9		
phenylarsine oxide	0.00066 ± 0.00013	0.00045 ± 0.00004	0.0013 ± 0.003				6.4 ± 2.3
cymelarsan	0.0039 ± 0.0009	0.0078 ± 0.0014	0.21 ± 0.04				
chloroquine						0.00067 ± 0.00018	

[a]Data are EC_{50} values in μM ± standard error of means ($n \geq 3$). EC_{50} values were determined after at least 72 h of drug treatment.

for the kinetoplastids that monomers in general displayed higher activity than oligomers is not seen with *P. falciparum*. Yet, as also seen for the kinetoplastid parasites, linear trimer **7** was the most active compound among the oligomeric 3TPAs (**7**, **10**, and **11**). The data suggest that either the 3TPAs act on a different target in kinetoplastid and apicomplexan parasites or that these compounds act on similar targets but that these are differentially essential to either phylum. Nevertheless, the strong in vitro activity seen against these evolutionary distinct phyla is remarkable.

Without exception, the cytotoxicity of 3TPAs on human HEK293 cells was much lower than their antiprotozoal activity. Monomers showed the most favorable selectivity index ($EC_{50(HEK)}/EC_{50(parasite)}$ ratio) related to antitrypanosomal activity: **2** (437-fold), **15** (299-fold), **16** (540-fold), and **17** (>1000-fold). The selectivity index for antileishmanial activity was lower than for antitrypanosomal activity, yet with reasonable values remaining for monomers **15** (145-fold) and **16** (149-fold). The antiplasmodial selectivity was found to be promising for viscosamine **9** (493-fold) and its linear precursor **7** (237-fold).

Given the promising selectivity indices of 3TPAs on *T. brucei*, we investigated their trypanocidal dynamics in more detail. The EC_{50} values in Table 1 were determined after 72 h of drug treatment; to study the early toxic effects of 3TPAs on trypanosomes, we used a real-time cell lysis assay based on propidium iodide (PI) fluorescence.[20] PI is unable to cross the intact cell membrane; hence, it can only bind to DNA and RNA (and thus fluoresce) when the cell membrane is compromised (indicative of cell death). At high 3TPA concentrations (50−100 μM), all trypanosomes died within the first 30 min (Figure 1). We found that for monomers (**2**, **3**, and **15**−**17**), only concentrations ≥100 × EC_{50} caused cell lysis within the 8 h of duration of the experiment. In contrast, as little as 780 nM dimer **10** (2 × EC_{50}) caused full cell lysis within 3 h, whereas higher order oligomers (**7**, **9**, **11**, and **13**) showed rapid lysis at 10−15 × EC_{50}.

It appears that monomeric 3TPAs (**2**, **3**, and **15**−**17**), even at concentrations well above their EC_{50} values (Table 1; determined after 72 h of drug incubation), kill trypanosomes slowly. This could be due to induction of apoptosis or because the drug induces growth arrest rather than direct cell lysis. We investigated this by performing a series of flow cytometry experiments scoring for total DNA content (as a cell cycle indicator) and cell lysis and DNA fragmentation (as a marker for apoptosis). Cultures were incubated for up to 48 h with various drug concentrations, and at 24 and 48 h, duplicate samples were taken. In one sample, incubated directly with PI, the fluorescence correlated to the amount of DNA but only in cells permeable to the dye (Figure 2, "lysis" panel). The other sample was fixed and permeabilized with digitonin before PI incubation so that all cells revealed their DNA content ("DNA content" panel). The drug-free controls show a normal distribution of DNA content,[21] with the majority of cells having one diploid set of chromosomes (2C) and those undergoing mitosis two sets (4C), representing an unperturbed cell cycle. At 24 h, the drug-free controls show no permeability to PI, and even at 48 h, only a small number of cells had become permeable, reflecting normal aging of a newly passaged culture ("lysis" panel).[21] Treatment with 0.3 μM monomer **17**, shown as a representative example, had almost no measurable effect at 24 h, but a significant proportion of cells had become PI-permeable at 48 h ("lysis" panel). Some of the lysed cells display fluorescence below 100 units, indicating some DNA degradation,[21] but this likely occurred following cell death, rather than causing it. The "DNA content" panel showed little

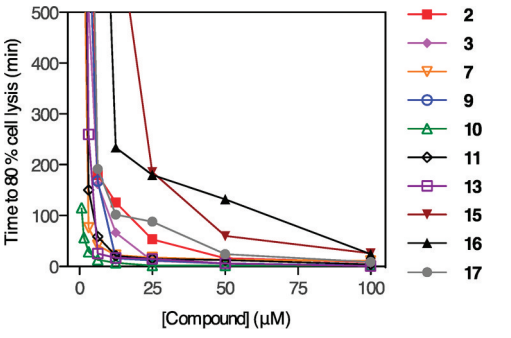

Figure 1. Time to reach 80% lysis of BF *T. brucei* vs concentration of 3TPA as determined by the PI-based rapid lysis assay.

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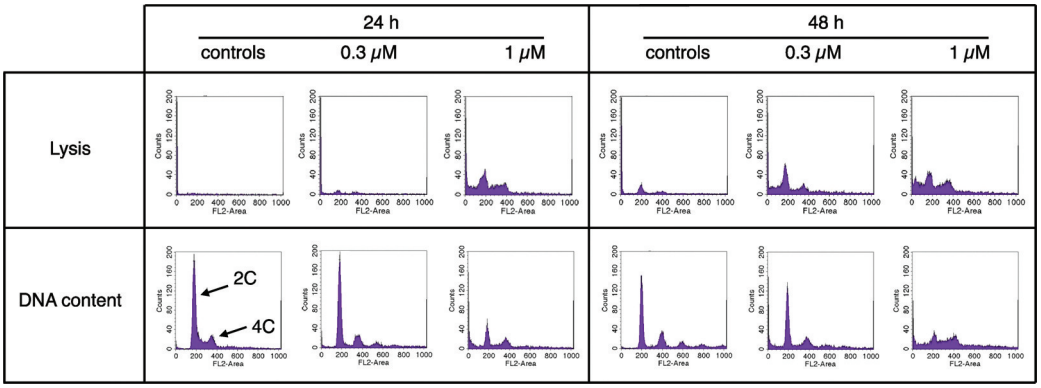

Figure 2. Flow cytometry analysis of BF *T. brucei* cultures incubated in the presence or absence (controls) of monomer **17**. "Lysis" panel: Cells were incubated with PI for 10 min after exposure for 24 or 48 h to **17**. "DNA content" panel: Cells were fixed with 70% methanol after incubation with **17** and treated with RNase prior to staining with PI and analysis by flow cytometry. Samples for DNA content and for lysis analysis were taken from the same culture. Each panel represents the counting of 10000 cells. The experiment shown is representative of three similar experiments.

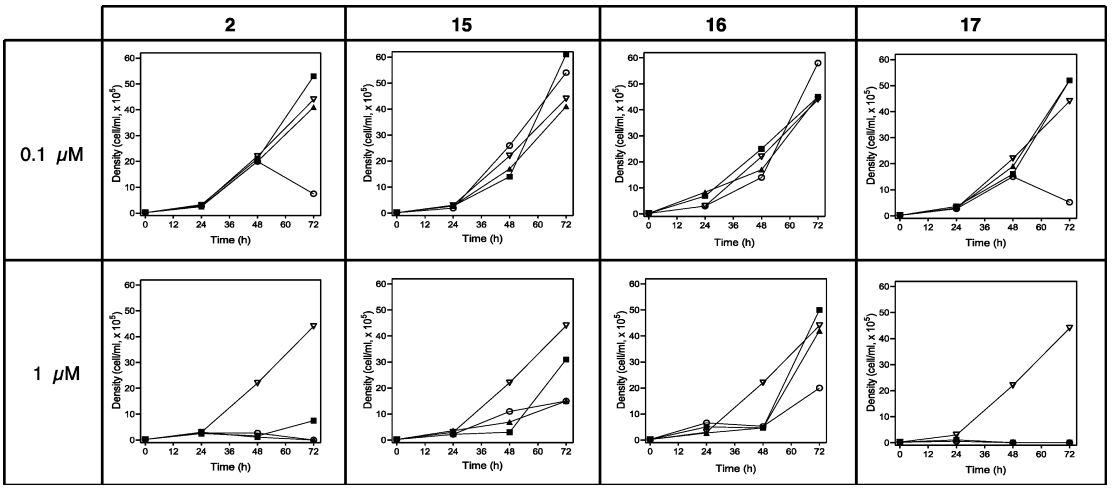

Figure 3. Reversibility of the effect of various concentrations of monomers **2** and **15**−**17** on the growth of BF *T. brucei*. Cultures (seeded at 2×10^4 cells) exposed to test compounds at the indicated concentrations were monitored over 72 h. Compounds were added at $t = 0$ h and removed by washing cultures after 24 h (■), 48 h (▲), or not at all (○). Controls are drug-free cultures (▽).

evidence of DNA fragmentation, indicating that only the dead cells had less than 2C DNA content and that no significant DNA degradation occurred prior to cell death. This conclusion is much strengthened by analysis of the culture incubated with 1 μM **17**: Even at 24 h, most of the cells had become PI-permeable, and indeed, the lower panel shows that there are relatively few cells left with 2C or 4C DNA content but that nonetheless very few cells with fragmented DNA are identifiable. As DNA fragmentation is believed to be an inherent consequence of apoptosis, it is unlikely that the 3TPAs exert their antikinetoplastid actions in this way.

The same experiments were performed with various other alkaloids used in this study (see Figures S1 and S2 in the Supporting Information). In no case was evidence for DNA fragmentation observed after incubation with the 3TPAs, in contrast to a series of choline-derived compounds that we recently reported.[21] Nor did the flow cytometry provide any indication of specific cell cycle arrest, as the proportion of cells 2C or 4C DNA content remained constant throughout a 48 h of incubation with concentrations of 3 × or 10 × EC_{50} (Figures 2 and S1 and S2 in the Supporting Information). This result was confirmed using fluorescence microscopy after DAPI staining (not shown). We conclude that unlike some antitrypanosomal compounds,[22] 3TPAs do not cause growth arrest at a particular check point in the cell cycle.

Slow-acting drugs may still induce a rapid cellular effect that eventually leads to cell death. Diamidines, for example, accumulate rapidly to high concentrations in the cell, leading to cell death many hours later.[18] Pentamidine, with an EC_{50} value of 1.4 nM on wild type *T. brucei*, has been shown to act slowly on the parasite at therapeutic concentrations (i.e., $\leq 10 \times EC_{50}$).[20] Whether an agent needs only relatively short contact at effective concentrations with the target cell or must be maintained at effective levels until the entire cell population has died has obvious consequences for the therapeutic efficacy of the compound class in vivo. To investigate whether the 3TPAs require continuous exposure, growth of BF *T. brucei* treated with various concentrations of monomers was monitored over 72 h, while drugs were washed out after 24 and 48 h. Treatment with **2** and **15**−**17** at 10 μM for 24 h showed complete trypanocidal action (not shown), while at 1 μM only **17** and **2** cleared a trypanosome culture completely, requiring 24 and 48 h of exposure, respectively (Figure 3). At lower concentrations, treatment with **2** and **17** was incomplete even at 72 h of drug incubation. These results seem to confirm that the effect of monomeric 3TPAs was reversible at low concentrations, as in

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all cases the parasites returned to normal growth after removal of the drug. It is not clear at present whether the apparent reversibility at low concentrations represents the recovery and resumed growth of affected organisms or whether the increase in the number of parasites on withdrawal of the alkaloids is solely due to replication of unaffected cells within the population.

Summarizing, we have developed an efficient route toward 3TPA monomers and oligomers, including viscosamine **9** and the novel, cyclic tetramer **13**. Cheap starting materials, easy crystallization (except **9**), and the absence of expensive chromatographic steps make this method an attractive entry to novel antiprotozoal agents. The 3TPAs showed submicromolar and several, even nanomolar, antiprotozoal activity. The evolutionary distance between *Plasmodium* en Kinetoplastida is enormous, and it is very rare that one class of compounds has such promising effects on all three parasite species studied, rendering these compounds interesting entries for development of broad spectrum antiprotozoal chemotherapy.

The similar activities found on both drug sensitive and resistant *T. brucei* lines established the absence of cross-resistance from the outset, which is encouraging, as current drugs are going out of use because of resistance problems.[4,15,18] Like most current antitrypanosomal agents, quaternary ammonium salts display limited oral availability. Nevertheless, their lack of cross-resistance may form a valuable addition to combating drug-resistant parasites. We have established that monomeric 3TPAs have a favorable selectivity window and act potently on trypanosome populations, while killing the parasites relatively slowly at therapeutically safe concentrations in a process that does not target the cell cycle. Sterilization of a trypanosome culture required exposure to 1−10 μM 3TPA. Given the small number of compounds tested here, the hit rate is remarkable, and we are confident that further chemical modification and systematic structure−activity relationships will lead to even higher levels of activity and selectivity and will expand our understanding of the mode of action of these marine-derived alkaloids.

■ ASSOCIATED CONTENT

Supporting Information

Additional text, compound synthesis, and characterization; biological assays; and FACS histograms. This material is available free of charge via the Internet at http://pubs.acs.org.

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