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Identification of Antischistosomal Leads by Evaluating Bridged 1,2,4,5-Tetraoxanes, Alphaperoxides, and Tricyclic Monoperoxides

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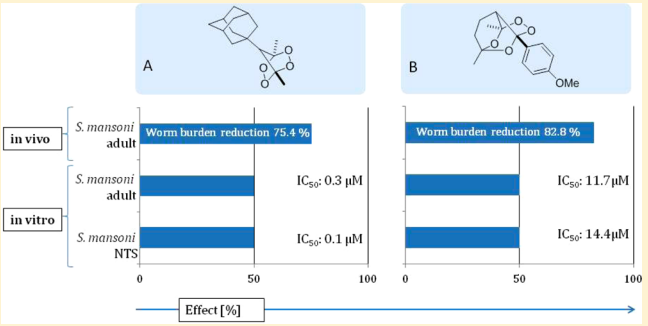
Supporting Information

ABSTRACT: Although antischistosomal properties of peroxides were studied in recent years, systematic structure–activity relationships have not been conducted. We evaluated the antischistosomal potential of 64 peroxides belonging to bridged 1,2,4,5-tetraoxanes, alphaperoxides, and tricyclic monoperoxides. Thirty-nine compounds presented IC_{50} values <15 μM on newly transformed schistosomula. Active drugs featured phenyl-, adamantane-, or alkyl residues at the methylene bridge. Lower susceptibility was documented on adult schistosomes, with most hit compounds being tricyclic monoperoxides (IC_{50}: 7.7–13.4 μM). A bridged 1,2,4,5-tetraoxane characterized by an adamantane residue showed the highest activity (IC_{50}: 0.3 μM) on adult *Schistosoma mansoni*. Studies with hemin and heme supplemented medium indicated that antischistosomal activation of peroxides is not necessarily triggered by iron porphyrins. Two compounds (tricyclic monoperoxide; bridged 1,2,4,5-tetraoxane) revealed high worm burden reductions in the chronic (WBR: 75.4–82.8%) but only moderate activity in the juvenile (WBR: 18.9–43.1%) *S. mansoni* mouse model. Our results might serve as starting point for the preparation and evaluation of related derivatives.



INTRODUCTION

Schistosomiasis remains one of the most prevalent parasitic diseases, being endemic in 76 countries worldwide with approximately 780 million people at risk of infection.[1] The infection is caused by trematodes of the genus *Schistosoma*, among which *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum* represent the most important pathogenic species for humans. During chronic infections, worms persist in the liver and hepatic portal system or urinary tract system depending on the species. Mature schistosomes start laying eggs within their habitation, which often get trapped in the tissues, resulting in inflammatory and obstructive diseases of affected organs.[2] To cure subtle morbidity and prevent the development of severe late stage morbidity, at risk populations are periodically treated with praziquantel, the drug of choice for treating schistosomiasis.[3] Following the discovery of the antischistosomal properties of the artemisinins, in recent years, various compounds characterized by a peroxidic scaffold were studied in detail for their antischistosomal activity. The overarching goal of these studies was to identify a drug with high activity against both juvenile and adult schistosomes. In contrast to praziquantel, the artemisinins revealed high activities against juvenile schistosomes but low to moderate activities on the adult worms in *S. mansoni* infected mice.[4,5] Studies with fully synthetic compounds, including ozonides,[6,7] trioxaquines,[8] and dioxolanes,[9] were undertaken. In more detail, among the aryl-ozonides, OZ418 was identified as the most promising lead candidate possessing high activity on both juvenile and adult schistosome infections in mice.[6] The trioxaquine lead candidate PA1259, a hybrid drug containing an aminoquinoline so as trioxane pharmacophore, was characterized by moderate in vivo activity on juvenile and adult *S. mansoni* in mice.[8,10] In another study, a praziquantel–ozonide hybrid was designed, which however failed to demonstrate in vivo activity.[11,12] Finally, the relationship between the peroxidic scaffold and antischistosomal activity was underscored by testing a series of alkoxydioxolanes in vitro and in vivo.[9]

However, despite the evaluation of several peroxidic compound classes for their effect on schistosomes, only little is known on the relationship between the peroxidic structures and antischistosomal activity. Therefore, we were interested in elucidating the antischistosomal potential of three practically

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Table 1. Chemical Structures of Investigated Structures Presented Regarding Their Peroxidic Class[a]

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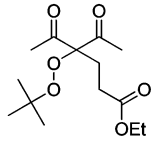
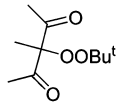
	acetyl acetone [16]					
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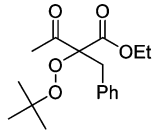
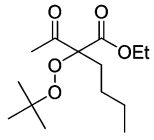
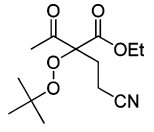
	acetoacetic ester[16]					
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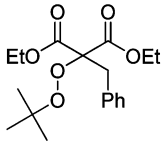
	malonic ester[16]					
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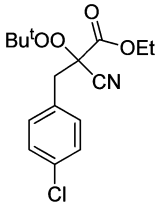
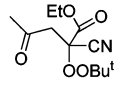
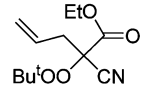
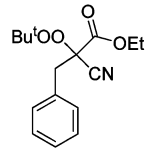
	cyanoacetic ester [15]					
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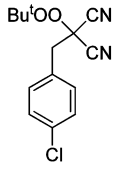
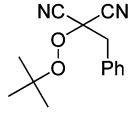
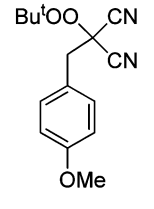
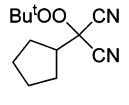
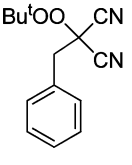
	malono nitrile[15]					
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		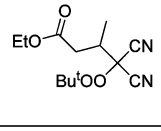	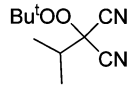			
		16	**17**			

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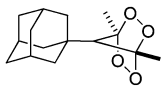
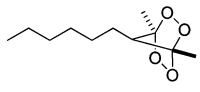
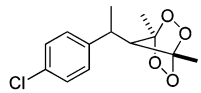
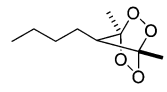
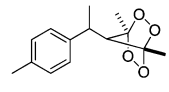
Table 1. continued

Bridged 1,2,4,5-tetraoxanes(*) [14] **18-44**					
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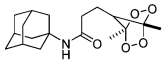	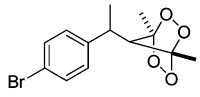	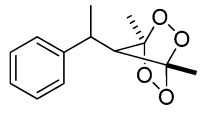	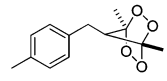	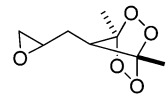	
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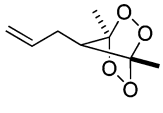	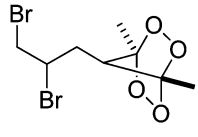	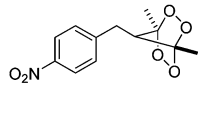	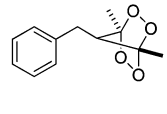	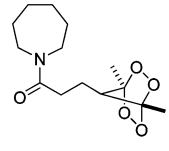	
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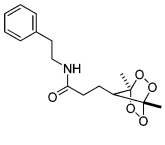	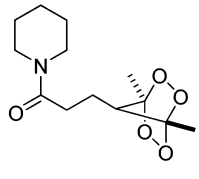	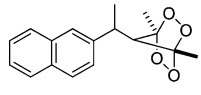	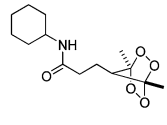	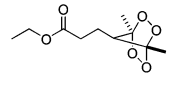	
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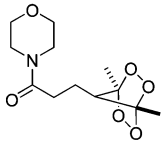	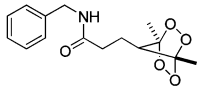	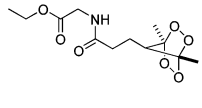	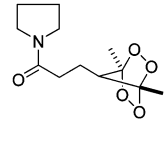	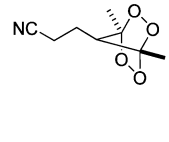	
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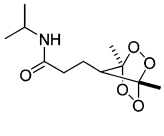	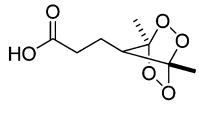				
43	**44**				

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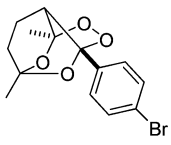
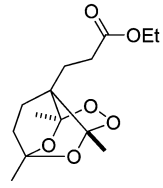
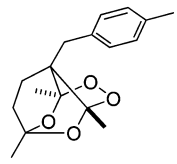
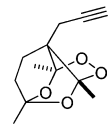
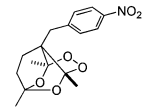
Table 1. continued

Tricyclic monoperoxides [17] **45-63**					
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	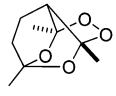	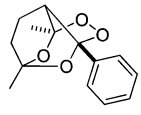	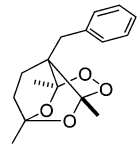	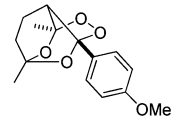	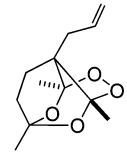
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	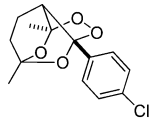	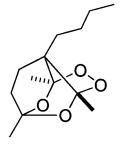	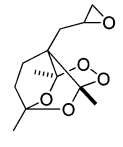	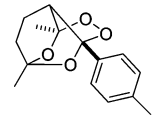	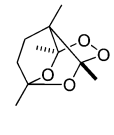
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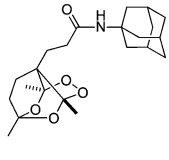
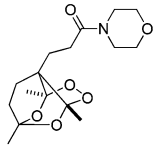
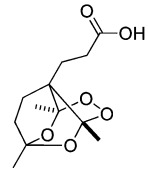
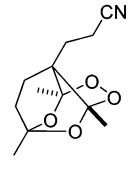
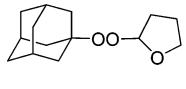
					
	60	**61**	**62**	**63**	
2- (1-adamantylperoxy) -tetrahydrofuran (*) **64**					
	64				

[a]*Detailed information can be found in Supporting Information.

new peroxide classes, namely candidates of β-dicarbonyl compounds and their heteroanalogues, bridged 1,2,4,5-tetraoxanes and alphaperoxides so as β,δ-triketones, tricyclic monoperoxides, in vitro and in vivo against *S. mansoni* for the first time. Bridged 1,2,4,5-tetraoxanes present with a methylene bridge within the pharmacophore which distinguishes them from described 1,2,4,5-tetraoxanes with known trematocidal activity.[13] The preparation of these peroxide classes has been

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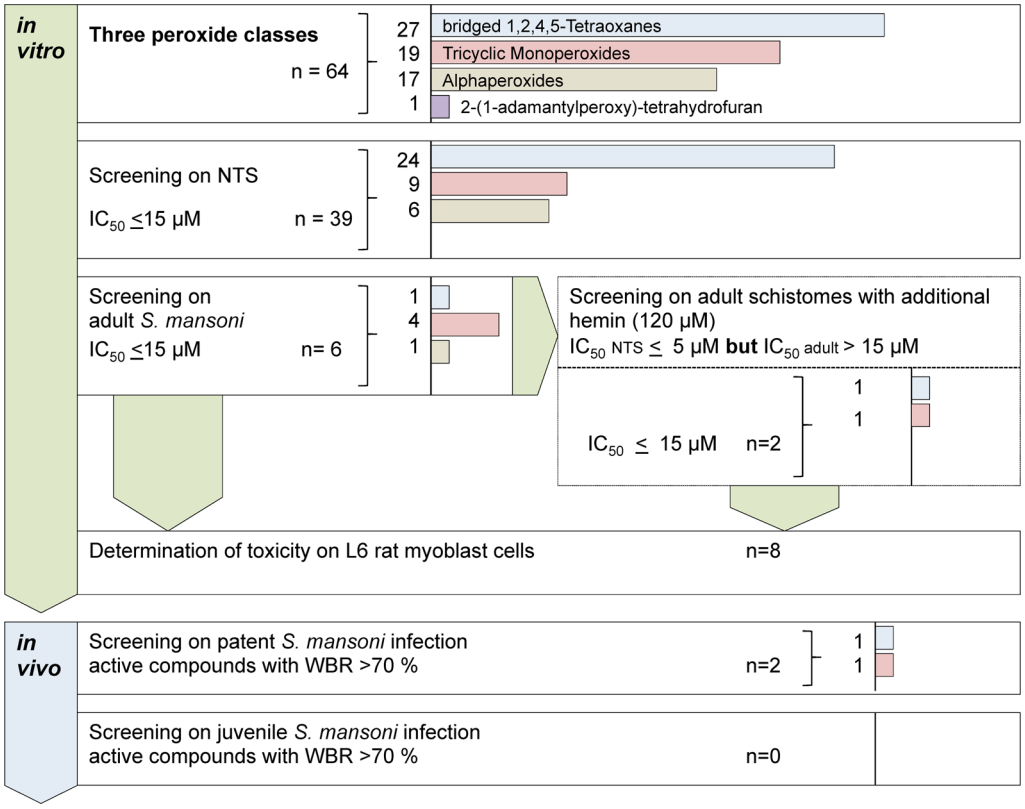

Figure 1. Flowchart of our screening procedures using three different peroxide classes and one 2-(1-adamantylperoxy)-tetrahydrofuran.

developed recently, and their syntheses imply high yields using inexpensive and accessible reagents.[14−17] A library with small druglike molecules showing high structural variability was synthesized for each class of compounds and tested in vitro on newly transformed schistosomula (NTS) and adult *S. mansoni*. The antischistosomal activity of selected compounds was additionally studied in the presence of hemin and heme, as possible activators. Compounds revealing high activity in vitro were next tested on toxicity and further characterized in vivo using a patent *S. mansoni* mouse model. Finally, compounds being active in the chronic infection model progressed into the juvenile infection model to characterize the full spectrum of activity of these peroxides.

RESULTS

In Vitro Screening on NTS. In a first step, the four compound libraries consisting of 27 tetraoxanes, 19 tricyclic monoperoxides, 17 alphaperoxides, and one 2-(1-adamantylperoxy)-tetrahydrofuran (Table 1) were tested on *S. mansoni* NTS. Our workflow is presented in Figure 1. Results are summarized in Table 2. Thirty-nine compounds (24 tetraoxanes, 9 tricyclic monoperoxides, and 6 alphaperoxides) showed high activities (as defined by half-maximal inhibitory concentrations (IC_{50}s) ≤ 15 μM) on NTS. In more detail, 24 out of 27 tetraoxanes (89%) revealed IC_{50} values ranging from 0.1 to 13.9 μM. Six of these had IC_{50} values even lower than 1 μM, with compounds **18** (IC_{50}: 0.1 μM) and **19** (IC_{50}: 0.2 μM) displaying the highest activity. Hence the most active tetraoxanes showed at least 10-fold increased activity when compared to reference drug praziquantel (IC_{50}: 2.2 μM) and 25-fold increase in activity when compared to artesunate (IC_{50}: 5.0 μM) on the schistosomular stage. The remaining three of the 27 tetraoxanes tested (compounds **42**−**44**) showed low to moderate activity (IC_{50}s: 15.3−58.1 μM). Compound **45** was the most active compound in the class of tricyclic monoperoxides (IC_{50}: 1.7 μM). Eight tricyclic monoperoxides had IC_{50} values ranging from 3.5 to 14.4 μM. The remaining compounds showed low to moderate activity (IC_{50}s: 15.6−213.2 μM **54**−**60**) or lacked activity (**61**−**63**) (motility decreased less than 50% at the highest concentration tested). Of the 17 alphaperoxides tested, six compounds (**6**, **7**, **9**, **13**, **14**, **17**) were highly active (IC_{50}s: 3.9−14.6 μM), eight molecules showed low to moderate activities (IC_{50}s 15.1−293.0 μM), and three compounds lacked activity (**1**, **2**, **5**). Finally, the tested 2-(1-adamantylperoxy)-tetrahydrofuran (**64**) lacked activity on the schistosomular stage.

In Vitro Screening on Adult Schistosomes. Thirty-nine compounds progressed into the adult *S. mansoni* screens (Table 2). Of these, six compounds (1 tetraoxane, 4 tricyclic monoperoxides, and 1 alphaperoxide) showed high activity (defined as IC_{50}s ≤ 15 μM) against adult *S. mansoni*. The highest activity was observed with tetraoxane **18** characterized by an IC_{50} of 0.3 μM, revealing comparable in vitro activity as the reference drug praziquantel (IC_{50}: 0.1 μM). The two tricyclic monoperoxides (**51** and **45**) presented IC_{50} values of 7.7 and 8.7 μM, respectively. Compounds **12** (alphaperoxide), **49**, and **53** (tricyclic monoperoxides) revealed similar IC_{50} values of 14.7, 12.2, and 11.7 μM, respectively.

Twenty-two compounds (16 tetraoxanes, 2 tricyclic monoperoxides, and 4 alphaperoxides) showed a low to moderate activity (IC_{50}s: 18.8−132.4 μM). Finally, of the 39 compounds tested, 11 revealed no decrease of motility of more than 50% on adult *S. mansoni* at the highest concentration tested.

All compounds which revealed a high antischistosomal potential on NTS (IC_{50} < 5.0 μM) but only low, moderate, or no activity against adult schistosomes (12 tetraoxanes, 2

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Table 2. In Vitro Activity of Three Different Peroxide Classes (Alphaperoxides, Bridged 1,2,4,5-Tetraoxanes, and Tricyclic Monoperoxides) and One 2-(1-Adamantylperoxy)-tetrahydrofuran on NTS and adult *S. mansoni*[a]

		NTS *S. mansoni*		adult *S. mansoni*		adult *S. mansoni* with hemin (120 μM)	
compd	no.	IC_{50} [μM]	*r*	IC_{50} [μM]	*r*	IC_{50} [μM]	*r*
artesunate		4.97	0.9	41.2	0.8	38.7	0.7
praziquantel		2.2	0.9	0.1	0.9		
alphaperoxides	**1**	>104					
	2	>795					
	3	15.1	0.9				
	4	26.5	0.9				
	5	>111					
	6	14.6	0.9	59.1	0.8		
	7	9.0	0.9	>126.8			
	8	37.8	0.8				
	9	43.2	0.9				
	10	67.7	1.0				
	11	3.9	0.9	19.3	0.8	17.9	0.9
	12	5.0	0.9	14.7	0.9		
	13	8.0	0.9	40.6	1.0		
	14	10.7	0.9	122.9	0.9		
	15	24.1	0.9				
	16	157.0	0.9				
	17	293.0	0.3				
bridged 1,2,4,5-tetraoxanes	**18**	0.1	0.9	0.3	1.0		
	19	0.2	1.0	61.5	0.9	27.3	0.9
	20	0.4	0.9	35.5	0.9	25.3	1.0
	21	0.5	1.0	37.8	1.0	>159	
	22	0.6	0.9	30.9	1.0	41.2	0.9
	23	0.8	0.8	33.3	1.0	39.3	1.0
	24	1.0	0.9	33.6	0.9	47.7	0.9
	25	1.1	0.9	18.8	1.0	12.9	0.9
	26	1.2	0.8	40.0	0.9	>127	
	27	1.2	0.8	132.4	0.9	91.9	0.9
	28	3.4	0.9	49.8	0.9	>174	
	29	3.4	0.9	>89.8		>89.8	
	30	4.4	1.0	>112		>112	
	31	5.0	0.9	41.4	0.7	134.9	0.9
	32	5.5	0.9	50.1	0.9		
	33	6.1	1.0	65.6	0.9		
	34	6.6	1.0	>110.6			
	35	7.1	0.8	35.5	1.0		
	36	8.9	0.9	66.6	0.9		
	37	9.8	0.9	>129.2			
	38	10.7	0.9	36.9	0.8		
	39	10.9	0.9	>102			
	40	11.7	0.9	>103.7			
	41	13.9	0.9	>117			
	42	15.3	1.0				
	43	25.6	0.9				
	44	58.1	0.8				
tricyclic monoperoxides	**45**	1.7	0.8	8.7	0.9		
	46	3.5	0.9	>105		92.2	0.9
	47	4.5	0.9	>103		13.4	0.9
	48	8.1	1.0	82.4	0.9		
	49	9.0	0.9	12.2	0.8		
	50	9.0	1.0	19.2	0.9		
	51	9.3	0.8	7.7	0.9		
	52	13.4	0.9	>109			
	53	14.4	0.8	11.7	0.9		

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Table 2. continued

compd	no.	NTS *S. mansoni* IC$_{50}$ [μM]	NTS *S. mansoni* r	adult *S. mansoni* IC$_{50}$ [μM]	adult *S. mansoni* r	adult *S. mansoni* with hemin (120 μM) IC$_{50}$ [μM]	adult *S. mansoni* with hemin (120 μM) r
	54	15.6	0.9				
	55	16.0	0.9				
	56	22.3	0.9				
	57	22.5	0.8				
	58	45.3	0.9				
	59	180.4	0.9				
	60	213.2	0.9				
	61	>287					
	62	>348					
	63	>376					
2-(1-adamantylperoxy)-tetrahydrofuran	**64**	>125.9					

[a]R represents the "goodness of fit," whereas >0.85 is acceptable.

tricyclic monoperoxides, and 1 alphaperoxide) were retested in the presence of Fe(III)-hemin. In addition, four of these compounds were also studied in a Fe(II)−heme supplemented media.

Selected compounds of alphaperoxides (**11**) and tricyclic monoperoxides (**46**) did not show a great difference in their IC$_{50}$ values when incubated with (**46**, 92.2 μM; **11**, 17.9 μM) or without (**46**, >123 μM; **11**, 19.3 μM) hemin. Similar patterns were also observed for the 13 tested tetraoxanes. The majority (9 compounds: **19**, **20**, **22**−**25**, **27**, **29**, **30**) showed comparable antischistosomal activity with or without hemin or slightly decreased activity (**21**, **26**, **28**, and **31**). A considerable increase of activity was detected for one of the selected tricyclic monoperoxides, compound **47**, lacking activity (>103 μM) without and showing an IC$_{50}$ value of 14.4 μM with additional hemin. Finally, in the presence of a Fe(II)−heme supplemented media, three compounds still lacked activity (**30**, **46**, **47**) and only one showed a slight increase in activity (**29**; IC$_{50}$: 33.6 μM).

Determination of Cytotoxicity. The eight compounds displaying high activity against adult *S. mansoni* in vitro were first tested on L6 cells for their cytotoxic potential. Artesunate and praziquantel were used as reference drugs, and data are summarized in Table 3. The two tricyclic monoperoxides (**47**, **49**) and praziquantel showed no cytotoxic potential at the highest concentration tested (30 μg/mL). Lowest cytotoxic potential with IC$_{50}$ values of 94.4 and 58.3 μM were observed for the alphaperoxide **12** and the tricyclic monoperoxide **53**, respectively. The tricyclic monoperoxides, **45** and **51**, revealed similar moderately cytotoxic IC$_{50}$ values from 8.2 μM (**46**) to 10.3 μM (**52**). Furthermore, both selected tetraoxanes **18** (1.7 μM) and **25** (2.2 μM) showed similar cytotoxic effects on L6 cells as observed for artesunate (IC$_{50}$: 1.5 μM). Selectivity indices ranged from 0.2 for **25** to 7.6 for **49** (Table 3). Lead candidates (**18**, **53**) (based on the in vivo activity results, see below) and artesunate were further investigated on two different human cell lines, namely HeLa and MRC-5. All compounds showed 2-fold higher effects on the cancer cell line (HeLa) than on the normal cell line (MRC-5). The lowest cytotoxicity was determined for the tricyclic monoperoxide **53** (IC$_{50}$ of 6.0 μM on HeLa and 12.4 μM on MRC-5) followed by artesunate (IC$_{50}$ of 3.2 μM on HeLa and 6.8 μM on MRC-5). Tetraoxane **18** showed cytotoxic potential on both cell lines (IC$_{50}$ of 0.4 μM on HeLa and 1.2 μM on MRC-5).

Table 3. IC$_{50}$ Values of Eight Selected Hit Compounds (**12**, **18**, **25**, **45**, **47**, **49**, **51**, and **53**) Evaluated with L6 Cells and Adult *S. mansoni* Worms[a]

compd	IC$_{50}$ [μM] L6 cells (SD)	IC$_{50}$ [μM] *S. mansoni*	SI
12	99.0 (11.0)	14.7	6.4
18	1.7 (0.3)	0.3	5.7
25	2.2 (0.4)	18.8	0.1
45	8.2 (1.9)	8.7	0.9
47	>103	13.4	>7.7
49	>93	12.2	>7.6
51	10.3 (1.8)	7.7	1.3
53	58.3 (17.9)	11.8	4.9
artesunate	1.5 (0.6)	38.7	0.04
praziquantel	>96	0.1	>960

[a]SD: standard deviation. SI: selectivity index. Selectivity indices (SI) were calculated based on evaluated IC$_{50}$ values and artesunate and praziquantel served as control compounds.

In Vivo Efficacy on Patent *S. mansoni* Infection. On the basis of their in vitro activity against adult schistosomes (IC$_{50}$ < 15 μM), two tetraoxanes (**18**, **25**), five tricyclic monoperoxides (**45**, **47**, **49**, **51**, and **53**), and one alphaperoxide (**12**) were tested in mice harboring a patent *S. mansoni* infection (Table 4). The alphaperoxide **12** and tricyclic monoperoxide **47** lacked in vivo activity. Treatment with three of the tricyclic monoperoxides and one of the tetraoxanes resulted in low total worm burden reductions (WBRs) ranging from 4.7 to 31.3% (**25**, 5.0%; **45**, 31.3%; **49**, 4.7%; **51**, 6.5%). Good antischistosomal in vivo activity was observed with tetraoxane **18** and tricyclic monoperoxide **53**. Compound **18** achieved total and female WBRs of 75.4% (p = 0.03) and 77.8% (p = 0.03), respectively. For the tricyclic monoperoxide **53**, significant total and female WBRs of 82.8% (p = 0.02) and 82.9% (p = 0.01), respectively, were determined.

In Vivo Efficacy Studies on Juvenile *S.mansoni* Infection. Compounds **18** and **53** were tested against juvenile *S. mansoni* in vivo. The tetraoxane showed moderate total and female WBRs of 43.1% and 50%, respectively. Low WBRs were observed with the tricyclic monoperoxide with 18.9% total and 27.3% female WBRs.

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Table 4. In Vivo Activity of Selected Compounds from Three Different Peroxide Classes (Bridged 1,2,4,5-Tetraoxanes, Tricyclic Monoperoxides, Alphaperoxides)[a]

		mean number of worms (SD)		adult infection [%]		juvenile infection [%]	
compd (chemical class)	no. mice investigated	total	females	TWR	FWBR	TWR	FWBR
control[a]	9	29.0 (28.5)	13.3 (12.9)				
control[b]	8	38.3 (18.1)	20.6 (8.8)				
control[c]	10	30.6 (24.7)	16.1 (13)				
control[d]	8	21.6 (12.2)	11.0 (6.4)				
12[a]	4	32.3 (21.4)	17.3 (11.6)	0	0		
18[a]	6	6.7 (2.5)	2.3 (1.2)	75.4*	77.8*	43.1	50.0
25[d]	2	20.5 (11.5)	10.5 (9.2)	5.0	4.6		
45[c]	4	21.0 (2.2)	10.3 (3.1)	31.3	36.4		
47[d]	4	22.3 (4.9)	12.0 (2.4)	0	0		
49[d]	4	23.5 (5.9)	11.8 (3.1)	4.7	9.6		
51[b]	4	35.8 (8.2)	17.5 (6.6)	6.5	15.2		
53[c]	4	5.3 (5)	2.8 (2.6)	82.8*	82.9*	18.9	27.3

[a]All tested on patent schistosoma infection (49 days post treatment) and promising candidates as well on juvenile schistosoma infections (21 days post-treatment). TWR: total worm burden reduction. FWBR: female worm burden reduction. SD: standard deviation. *p-value < 0.05 using KW test.

DISCUSSION AND CONCLUSION

In recent years, peroxides have played a prominent role in antischistosomal drug discovery and development. Various studies have been conducted ranging from preclinical in vitro and in vivo studies as well as clinical trials.[4,8] Nonetheless, to date, our knowledge is still limited with regard to the structural requirements these molecules need in order to elicit antischistosomal activity. Therefore, in the present work, three different peroxide classes were screened for their antischistosomal potential.

First, compounds were studied for activity against NTS (Figure 1). Compounds with an activity <15 μM against NTS were classified as active and progressed further. Abdulla and colleagues recently described a similar screening workflow, however, using a 15-fold lower cutoff of 1 μM to obtain an acceptable hit rate of 10%.[18] On the other hand, Mansour and Bickle noted that schistosome active drugs were best identified in their screen using concentrations of 10 μg/mL (i.e., 28−44 μM) in the primary NTS screen.[19] Given the excellent activity of peroxidic drugs against juvenile schistosomes,[4,6] as mentioned in this work, an IC_{50} value <15 μM was selected as cutoff.

Of 64 compounds tested, 39 (60%) showed high in vitro activity against NTS. For the class of tetraoxanes, we detected the highest activity against the schistosomular stage (with 89% of compounds being active), whereas within the other two peroxide classes investigated (tricyclic monoperoxides and alphaperoxides), less than half of the compounds displayed activity. Most of the active tetraoxanes elucidated activities comparable to praziquantel on this parasite stage. Structural variation was observed among active compounds on schistosomula. However, a tendency of structural features among highly active tetraoxanes and tricyclic monoperoxides could be noted. All highly active structures among these two groups presented either phenyl- (**20**, **22**, **24**−**26**, **30**, **31**, and **45**), adamantane- (**18**, **23**), or alkyl- (**19**, **21**, and **28**) residues at the methylene bridge.

In contrast to results obtained on NTS with nearly all tetraoxanes being active, adult *S. mansoni* showed a lower susceptibility to these drugs with only one tetraoxane (**18**) revealing prominent activity. This compound revealed also high worm burden reductions against *S. mansoni* in vivo. Compound **18** displays an adamantane substitute at the methylene bridge. Early studies with synthetic peroxides in the framework of a collaborative antimalarial discovery project evaluated essential characteristics for a new trioxolane antimalarial drug. It was documented that necessary pharmacokinetic characteristics could be obtained with the spiroadamantane trioxolane pharmacophore.[20] Increased lipophilicity of the adamantane substituted compounds resulted in higher antimalarial activity. In addition, using the same class of compounds as a starting point to search for a fasciocidal synthetic peroxide drug development candidate revealed that the spiroadamantane substructure is an essential part for fasciocidal activity.[21] Contrary to the known active spiroadamantane substructures, compound **18** does not contain a spiro-fragment; the adamantane and tetraoxane parts are joined directly with a C−C bond.

It is interesting to note that the tested 2-(1-adamantylperoxy)-tetrahydrofuran (**64**) did not expose any activity on the schistosomular stage which indicates that not only the presence of an adamantane part determines the antischistosomal activity. Therefore, most probably, the key fragment which determines activity is the bridged tetraoxane and adamantane bears a supporting function.

The class of tricyclic monoperoxides revealed the greatest number of hits (n = 4) on adult *S. mansoni* in vitro, with compounds **45** and **51** being most active. Three of the active tricyclic monoperoxides (**45**, **51**, and **53**) have a phenyl residue next to the peroxidic bond in common. To note, these phenyl-containing peroxides are unusual compounds from the chemical point of view; generally peroxides containing the Ar−C−O−O moiety easily decompose in accordance with heterolytic mechanism by Hock and related reactions.[22,23]

Furthermore, the substitution in the bridge of tricyclic monoperoxides seems to affect the activity as observed on the schistosomular stage likewise. Phenylic residues (as seen for **47**) seem to increase the activity, whereas a propargyl substituent results in decreased activity (as seen for **48**). However, among the three tested compounds (**45**, **51**, and **53**) in vivo, only **53** achieved a promising WBR of 82.8%. This might be explained with a higher metabolic stability of the methylether substituted compound.[24]

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It is interesting to note that, in general, adult *S. mansoni* were less affected by the peroxides than NTS. This was particularly striking for the alphaperoxides and as mentioned before for the bridged 1,2,4,5-tetraoxanes. While six alphaperoxides showed a high activity against NTS, only one was active against the adult worms. In a recent study using a random collection of 33 compounds with proven in vitro activity on adult schistosomes and 30 compounds with lacking adult activity, none of the compounds lacking activity on adult worms revealed significant activity on NTS.[19] This finding suggests a superior susceptibility of schistosomula to peroxidic compounds.

Since it was previously proposed that hemin increases the activity of peroxidic structures, as demonstrated for artemether,[25,26] additional experiments were conducted using hemin (Fe(III)) as well as heme (Fe(II)) in the incubation medium. Interestingly, only one tricyclic monoperoxide (**47**) showed increased in vitro activity in the presence of hemin, likewise, there was only one compound (**29**) which elucidated a slightly increased activity in the presence of heme. This moderately increased in vitro activity could be explained by an additional activation of the drug within the medium and not only within the parasites gut which was proposed as possible interaction site of hemin and artemether for *S. japonicum*.[26] Most of the selected tetraoxanes showed similar to decreased efficacy when incubated with hemin or heme, and no changes on the motility were observed in the supplemented media for the selected alphaperoxides. Note that slight fluctuations in IC_{50} values based on microscopical readout might be due to differences in sensitivities of worms or the subjective readout used. Nonetheless, these results indicate that an antischistosomal activation of peroxides is not necessarily triggered by hemin or heme or at least does not represent the only activator because great variations were not observed for the two tested peroxide classes (alphaperoxides and 1,2,4,5-tetraoxanes) in the different media.

Only low to moderate activity was observed for the two hit candidates, tetraoxane **18** (WBR: 43.1%) and the tricyclic monoperoxide **53** (WBR: 29.1%) against juvenile *S. mansoni* infections in mice, which is in contrary to the recently investigated ozonides or the artemisinins.[5,6] Hence, the activity profile of the investigated peroxides is different from previously studied peroxidic compounds, a finding which cannot be explained at the moment.

Interestingly, a high cytotoxic potential was observed for artesunate on all tested celllines, which is in accordance to recently shown induction of cell death by artemisinin compounds and cytotoxic observations on HepG2 cells.[27] The activity of peroxides on blood-feeding parasites is most probably dependent on the activation of the endoperoxide bridge by an iron(II) species leading to C-centered radicals, which might be responsible for cytotoxicity.[27−29] Hence, it is not surprising that some of the tested peroxides showed a higher cytotoxic potential on L6 cells than the nonperoxidic reference drug praziquantel. Furthermore, it is known that the artemisinins possess cytotoxic potential on various cancer cell lines,[30] and apoptopic processes of fast proliferating cells in prescence of iron have been described.[27] It is worthwhile stating that the class of alphaperoxides did not show cyctotoxic potential at the highest concentration tested. Most of the tricyclic monoperoxides showed none to moderate cytotoxicity, whereas the class of tetraoxanes showed a similar cytotoxic potential as artesunate. With regard to our lead compounds (**18** and **53**), the conducted in vitro cytotoxicity assay on L6 cells showed that both lead structures presented adequate selectivity indices when compared to artesunate. However, compound **18** elucidated an increased cytotoxic potential compared to artesunate on tested human cell lines, which has to be kept in mind as potential drawback.

In conclusion, the screening of three peroxides classes identified two interesting hit compounds, tetraoxane **18** and the tricyclic monoperoxide **53**, which both revealed a high activity against adult *S. mansoni* in vivo. On the other hand, no promising activity was detected within the class of alphaperoxides. Our results hint to the fact that an adamantane group represents an important feature for antischistosomal activity. Compounds **18** and **53** might serve as starting candidates for further lead modifications aiming to increase activity on juvenile schistosomes and to lower cytotoxic potential.

EXPERIMENTAL SECTION

Drugs and Media. The 63 (**1**−**63**) compounds belonging to three types of peroxide classes (bridged 1,2,4,5-tetraoxanes, tricyclic monoperoxides, and alphaperoxides) illustrated in Table 1 were prepared based upon methods described by Terent'ev and colleagues.[14−17] Additionally, a 2-(1-adamantylperoxy)-tetrahydrofuran (**64**) was prepared from 1-adamantylhydroperoxide and 2,3-dihydrofuran as described in the Supporting Information. 1-Adamantylhydroperoxide was prepared from 1,3-dehydroadamantane and H_2O_2 in accordance with Son and colleagues.[31] A hemin solution (1.5 mM) was prepared as follows: 50 mg of hemin-chloride (Fluka Analytical, Netherlands) was dissolved in 10 mL of 0.1 M NaOH and 39.5 mL of PBS (pH = 7.4). A Fe(II) heme solution (1.5 mM) was prepared by addition of 5 mM dithionite (Sigma Aldrich) to the prepared hemin solution, adapted from Barr et al.[32] Praziquantel and artesunate were purchased from Sigma-Aldrich GmbH.

Synthesis and Analytical Data for Key Compounds. *7-(1-Adamantyl)-1,4-dimethyl-2,3,5,6-tetraoxabicyclo[2.2.1]heptane* ***18.***[13] A 37% aqueous H_2O_2 solution (0.353 g, 3.84 mmol) was added to a solution of 3-(1-adamantyl)pentane-2,4-dione (0.3 g, 1.28 mmol) in EtOH (3 mL), the reaction mixture was cooled to 10 °C, and a solution of H_2SO_4 (2 g, 0.02 mol) in EtOH (2 mL) was added with stirring. The reaction mixture was stirred at 20−25 °C for 1 h. Then CH_2Cl_2 (30 mL) was added. The organic layer was washed with water (2 × 10 mL), a 5% aqueous $NaHCO_3$ solution (2 × 10 mL), and again with water (2 × 10 mL), dried with Na_2SO_4, and filtered. The solvent was removed using a water-jet vacuum pump. Product 7-(1-adamantyl)-1,4-dimethyl-2,3,5,6-tetraoxabicyclo[2.2.1]heptane **18** was isolated by silica gel chromatography with elution by a hexane−ethyl acetate (EA) mixture using the gradient of the latter from 0 to 30%. Product **18** was obtained in 68% yield (0.231 g, 0.87 mmol). White crystals; mp = 130−131 °C (partially decomposed); R_f = 0.60 (TLC, hexane−EA, 5:1). ^{1}H NMR (300.13 MHz, $CDCl_3$), δ: 1.63−2.04 (m, 21H), 2.37 (s, 1H). ^{13}C NMR (75.48 MHz, $CDCl_3$), δ: 12.7, 28.3, 33.0, 36.7, 40.6, 66.8, 110.6. Anal. Calcd for $C_{15}H_{22}O_4$: C, 67.64; H, 8.33. Found: 67.37; H, 8.61.

3-(4-Methoxyphenyl)-6,7a-dimethyltetrahydro-3H,4H-3,6-epoxy-[1,2]dioxolo[3,4-b]pyran ***53.***[16] A 37% aqueous H_2O_2 solution (0.158 g, 1.72 mmol) and a solution of H_2SO_4 (1.0 g, 0.01 mol) in EtOH (1 mL) were added with stirring to a solution of 3-(4-methoxybenzoyl)-heptane-2,6-dione (0.30 g, 1.14 mmol) in EtOH (4 mL) at 10−15 °C. The reaction mixture was stirred at 20−25 °C for 1 h, and a mixture of CH_2Cl_2− hexane = 1:1 (10 mL) was added. Then $NaHCO_3$ was added to the reaction mixture with stirring until the pH reached 7.0. The precipitate was filtered off. The filtrate was dried over Na_2SO_4, the precipitate was filtered off, and the solvent was removed in a water jet vacuum. Product 3-(4-methoxyphenyl)-6,7a-dimethyltetrahydro-3*H*,4*H*-3,6-epoxy[1,2]dioxolo[3,4-*b*]pyran **53** was isolated by chromatography on SiO_2 using a hexane−ethyl acetate mixture as the eluent with a gradient of ethyl acetate from 5 to 50 vol %. Product **53** was obtained in 41% yield (0.131 g, 0.47 mmol). White crystals; mp = 89−90 °C; R_f = 0.52 (TLC, hexane−EA, 2:1). ^{1}H NMR (300.13 MHz,

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$CDCl_3$): δ 1.55 (s, 3H), 1.61 (s, 3H), 1.68−1.80 (m, 4H), 2.62−2.66 (m, H), 3.80 (s, 3H), 6.90 (d, 2H, J = 8.8 Hz), 7.49 (d, 2H, J = 8.8 Hz). ^{13}C NMR (75.48 MHz, $CDCl_3$): δ 12.5, 17.9, 24.8, 29.3, 50.5, 55.3, 95.8, 105.6, 106.5, 113.9, 124.7, 128.1, 160.5. Anal. Calcd for $C_{15}H_{18}O_5$: C, 64.74; H, 6.52. Found: C, 64.73; H, 6.75. HRMS (ESI) m/z $[M + H]^+$ calcd for $[C_{15}H_{19}O_5]^+$ 279.1227; found 279.1227.

Instrumentation and Methods. NMR spectra of novel compounds were recorded on a commercial instrument (300.13 MHz for ^{1}H, 75.48 MHz for ^{13}C) in $CDCl_3$. The TLC analysis was carried out on standard silica gel chromatography plates. The melting points were determined on a Kofler hot-stage apparatus. Chromatography was performed on silica gel (63−200 mesh). Elemental analysis on carbon, hydrogen, and nitrogen was carried out using a 2400 Perkin-Elmer CHN analyzer. Determination of purity of all peroxides was executed by elemental (combustion) analysis. For all peroxides, deviation from the theoretical values for C, H, and N content was less than 0.4%. These data confirm >95% purity of compounds **1**−**64**. Structures of all compounds were confirmed using ^{1}H and ^{13}C NMR spectra.

Maintenance of Mice and Infection with *S. mansoni*. The in vivo studies were approved by the veterinary authorities of the Canton Basel-Stadt. Female NMRI mice (3-week old, weight ca. 14 g) were purchased from Charles River (Sulzfeld, Germany) or Harlan Laboratories (Horst, The Netherlands). Prior to infection, animals were allowed to adapt for one week under controlled conditions (temperature ca. 22 °C, humidity ca. 50%, 12 h light and 12 h dark cycle, free access to rodent diet and water). Mice were infected with *S. mansoni* (Liberian strain) by subcutaneous injection of ∼100 cercariae. Cercariae were harvested from infected intermediate host snails *Biomphalaria glabrata* by exposure to light for 3 h, following standard procedures of our laboratory.

In Vitro Compound Screening on *S. mansoni* NTS. Harvested *S. mansoni* cercariae were mechanically transformed to NTS following standard procedures.[33,34] The obtained NTS suspension was adjusted to a concentration of 100 NTS per 50 μL using Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal calf serum (iFCS), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). NTS suspensions were incubated (37 °C, 5% CO_2 in ambient air) for a minimum of 12−24 h until usage to ensure completed conversion into schistosomula.[35] On the following day, drug dilution series were prepared in 96-flat bottom well-plates (BD Falcon, USA) with concentrations ranging from 0.37 to 90 μg/mL (0.37, 1.1, 3.3, 10, 30, 90 μg/mL) using supplemented (iFCS and antibiotics) Medium 199. The prepared NTS suspension was then added to each well, and plates were incubated at 37 °C, 5% CO_2. NTS incubated in the presence of the highest DMSO concentration served as control. NTS were evaluated by microscopical readout (Carl Zeiss, Germany, magnification 80×) with regard to death, changes in motility, viability, and morphological alterations 72 h post drug exposure. Drug effects were evaluated using a viability scale as described recently.[33,34] Each concentration was tested in duplicate, and experiments were performed at least three times. IC_{50} values of test compounds were determined as described before.[36] Compounds were defined as highly active with IC_{50} values ≤15 μM, moderate activity was defined as IC_{50} 16−40 μM, and low activity for values >40 μM.

In Vitro Compound Screening on Adult *S. mansoni*. Highly active compounds (IC_{50} ≤ 15 μM) on NTS were studied on adult schistosomes (workflow presented in Figure 1). Adult flukes were harvested from the hepatic portal veins and mesenteric veins of infected NMRI mice (7−8 weeks post infection) following standard procedures.[7] Schistosomes were placed in RPMI 1640 culture medium supplemented with 5% iFCS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C, 5% CO_2 until usage. Supplemented RPMI 1640 medium and drug stock solutions (10 mg/mL) were used to obtain final test concentrations of 1.1−30 μg/mL (1.1, 3.3, 10, 30 μg/mL) in 24-flat bottom well-plates (BD Falcon, USA) with a final volume of 2 mL. At least three schistosomes of both sexes were next added to each well. Schistosomes incubated in the presence of blank medium supplemented with the highest concentration of DMSO used in the assay served as control. Then 24, 48, and 72 h post drug exposure, schistosomes were examined phenotypically using the motility scale described before[37] and an inverse microscope (Carl Zeiss, Germany, magnification 80×). Experiments were repeated at least three times and IC_{50} values determined.[36]

The IC_{50} determination (72 h post drug exposure) for selected compounds was repeated with the addition of hemin (120 μM). Compounds showing high antischistosomal potential on the schistosomular stage (IC_{50} ≤ 5 μM) but only moderate, low, or no activity on adult schistosomes (without hemin supplementation) were selected for these additional experiments. Experiments were performed and repeated as described above with exception of hemin supplementation (120 μM) during the entire drug exposure time. Compounds which showed very good activities on NTS (IC_{50} ≤ 5 μM) and lacked activity on adult worms were furthermore tested in Fe(II)-heme (120 μM) supplemented media.

Determination of Cytotoxicity. The determination of cytotoxicity was performed with L-6 cells according to a previously reported procedure.[38] Briefly, L-6 cells were seeded in 96-well microtiter plates at a density of 4×10^4 cells/mL in RPMI 1640 medium with 10% fetal bovine serum and L-glutamine (2 mM). Drugs serially diluted 3-fold ranging from 0.123 to 30 μg/mL in test medium were added. The plates were incubated at 37 °C at an atmosphere of 5% CO_2. After 70 h, Alamar Blue (10 μL) was added to each well, and incubation was continued for another 2 h. The plate was then read using a SpectraMax M2 (Molecular Devices) instrument by use of an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Fluorescence development was expressed as percentage of the control, and the IC_{50} values were determined. Experiments were performed at least three times and IC_{50} values calculated as averages. The selectivity indices (SI) of tested compounds were calculated by dividing IC_{50} values obtained on L6 cells with IC_{50} values determined on adult *S. mansoni*.

The cytotoxic potential of two lead candidates was additionally determined on human cervical carcinoma cells (HeLa) cells and human fetal Lung fibroblast cells (MRC-5) (see Supporting Information).

In Vivo Testing. Compounds revealing an activity of ≤15 μM on adult worms post 72 h drug exposure were tested in vivo. Groups of four infected NMRI mice characterized by a patent schistosome infection (49 days post infection) were treated orally with the test drug using single oral doses of 400 mg of compound per kg body weight. Eight to ten untreated mice served as controls. Fourteen days post-treatment, animals were killed by the CO_2 method, dissected, and worms were sexed and counted.[7] Worm burdens of treated mice were compared to untreated animals and reductions of worm burden calculated.

Compounds displaying high activities against adult *S. mansoni* in vivo were also tested in the juvenile *S. mansoni* mouse model. For that purpose, mice were treated with the test compounds 21 days post infection. Mice were killed and dissected four weeks post treatment. Worm burden reductions were calculated as described above.

Statistics. Parasite viability values of treated and untreated NTS and adult schistosomes obtained from sextuplicate evaluation were averaged (means (± standard deviation)) using Microsoft Excel software. IC_{50} values of test compounds were determined using the CompuSyn software (version 3.0.1, 2007; ComboSyn, Inc.). The Kruskal−Wallis test was applied for in vivo studies, comparing the medians of the worm burden reductions of the treatment and control groups. A difference in median was considered to be significant at a significance level of 5% (StatsDirect statistical software, version 2.7.2.; StatsDirect Ltd., United Kingdom).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra, data of elemental analysis, and physical state of newly introduced tetraoxanes, structure **64** and cytotoxic potential of lead candidates on human cell lines. 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

NTS, newly transformed schistosomula; WBR, worm burden reduction; TWBR, total worm burden reduction; FWBR, female worm burden reduction; SI, selectivity index

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