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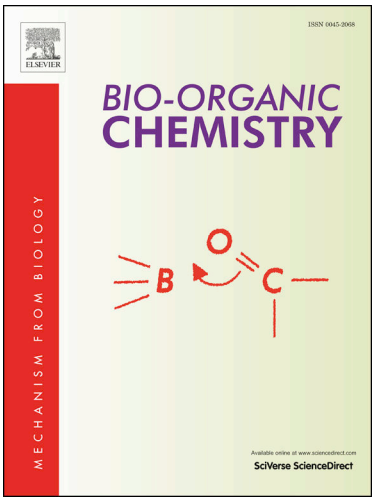

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(+)-(*R*)- and (-)-(*S*)-Perhexiline maleate: enantioselective synthesis and functional studies on *Schistosoma mansoni* larval and adult stages

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Abstract

Schistosomiasis is a neglected tropical disease mainly affecting the poorest tropical and subtropical areas of the world with the impressive number of roughly 200 million infections per year. Schistosomes are blood trematode flukes of the genus *Schistosoma* causing symptoms in humans and animals. Organ morbidity is caused by the accumulation of parasite eggs and subsequent development of fibrosis. If left untreated, schistosomiasis can result in substantial morbidity and even mortality. Praziquantel (PZQ) is the most effective and widely used compound for the treatment of the disease and in prevention control programs in the last 30 years. Unfortunately, it has no effect on juvenile immature schistosomes and cannot prevent re-infection or interfere with the schistosome life cycle; moreover drug-resistance represents a serious threat. The search for an alternative or complementary treatment is urgent and drug repurposing could accelerate a solution. The anti-anginal drug perhexiline maleate (PHX) has been previously shown to be effective on larval, juvenile, and adult stages of *S. mansoni* and to impact egg production *in vitro*. Since PHX is a racemic mixture of *R*-(+)- and *S*-(-)-enantiomers, we designed and realized a stereoselective synthesis of both PHX enantiomers and developed an analytical procedure for the direct quantification of the enantiomeric excess also suitable for semipreparative separation of PHX enantiomers. We next investigated the impact of each enantiomer on viability of newly transformed schistosomula (NTS) and worm pairs of *S. mansoni* as well as on egg production and vitellarium morphology by *in vitro* studies. Our results indicate that the *R*-(+)-PHX is mainly driving the anti-schistosomal activity but that also the *S*-(-)-PHX possesses a significant activity towards *S. mansoni in vitro*.

KEYWORDS: PHX maleate enantiomers; Stereoselective synthesis; Michael initiated ring closure reactions; *Schistosoma mansoni*

1. Introduction

Schistosomiasis is a neglected tropical parasitic disease affecting more than 200 million people in the poorest regions of the world, mainly in sub-Saharan Africa (85%) [1]. Schistosomiasis is caused by a parasitic trematode of the genus *Schistosoma* with different species accounting for the majority of human infections in different world regions, namely *S. haematobium* in Africa and the Middle East, *S. mansoni* in Africa and South America, and *S. japonicum* in China and the Philippines; *S. intercalatum* in Africa and *S. mekongi* in Southeast Asia are less common [2]. Recent outbreaks revealed the re-emergence of urogenital schistosomiasis in southern Europe [3]. Importantly, *S. haematobium* is a biological carcinogen as classified by the International Agency for Research in Cancer of the World Health Organization (WHO) [4].

A vaccine is unavailable to date; in addition, the treatment and most control initiatives rely on a single drug, praziquantel (PZQ) that has high efficacy, few and transient side effects, competitive cost. However, while very active on adult parasites, PZQ is poorly effective against juvenile worms both *in vitro* and *in vivo*. Therefore, in endemic areas, where patients are likely to harbor juvenile and adult parasites concurrently and reinfection is common, a complete cure is not reliably achieved with a single dose of PZQ [5]. Moreover, the extensive use of PZQ has brought concern about the emergence of PZQ-resistance/tolerance by schistosomes, also for the isolation of field and laboratory isolates less responsive to treatment, thus reaffirming an urge for the development of new alternative treatments [5].

In the search for novel schistosomicidal multi-stage compounds, and taking into account drug-repositioning as a key aspect of drug discovery trajectories [6], we have identified perhexiline maleate (PHX, **1**, Figure 1) as a novel promising multi-stage lead compound [7]. This drug, introduced to clinical use over 30 years ago as an efficacious monotherapy for the treatment of angina [8], showed activity against schistosomula (NTS), juvenile, and adult worms *in vitro* [7]. In addition, at sub-lethal doses, PHX impairs egg-laying *in vitro*, impact the morphology of the parasite reproductive systems, and increases lipids accumulation in the vitellarium [7]. The *in vivo* study in *S. mansoni*-infected mice showed variability in worm burdens and an overall minimal effect upon PHX treatment. The short half-life in mice, due to very high plasma clearance [9] together with a very high plasma protein binding, could be a possible explanation for the modest efficacy of PHX in a schistosomiasis murine model [7]. In mammalian cells, PHX appears to act on several molecular targets [10], with carnitine plamitoyltransferase 1 (CPT-1) and 2 (CPT-2), enzymes involved in the metabolism of fatty acids, being among the most investigated [10-14]. On the contrary, the molecular mechanisms of action of PHX in

Schistosoma is still unknown and it should be considered that the parasite lacks CPT-1 and CPT-2 orthologues and that fatty acid oxidation still remains controversial in schistosomes [15-18].

PHX is formulated for therapeutic use as a racemic mixture of the (+) and (−) enantiomers ((+)-(*R*)-**2** and (-)-(*S*)-**2**, Figure 1). Reports of severe side effects upon chronic treatment, including peripheral neuropathy and fatal hepatotoxicity, firstly prompted the restriction of its use and then the worldwide withdraw from the market, with the exception of Australia and New Zealand where is still used for the treatment of refractory angina [11]. The major determinant of the oral clearance of PHX is hepatic metabolism mediated by the cytochrome P450 2D6 (CYP2D6) [19] and polymorphism of CYP2D6 is the primary contributor to the highly variable clinical pharmacokinetics of racemic PHX, thus limiting its therapeutic application [20-23]. Interestingly, it has been reported that in human liver microsomes, the intrinsic clearance of (-)-(*S*)-**2** is greater than that of the (+)-(*R*)-**2** [22], while differential toxicity of each enantiomer in rat has been observed [21].

In order to understand whether the anti-schistosomal activity of PHX is mediated by a single enantiomer or both are involved in the observed phenotypic effects, we developed a straightforward stereoselective synthesis of this piperidine-based drug. In particular, we exploited a stereoselective Michael initiated ring closing reaction as the key step for the construction of the chiral piperidine ring (Figure 1) [24]. The enantiomeric purity of the single PHX enantiomers was established on the basis of a newly developed chiral HPLC method that can be extended to the semi-preparative scale. The *in vitro* activities of (+)-(*R*)-**2** and (-)-(*S*)-**2**, both prepared as maleate salts, and the racemic PHX were then comparatively assessed against NTS and adult worm pairs. Drug effects were evaluated by using viability assays and confocal microscopy studies on adult parasites.

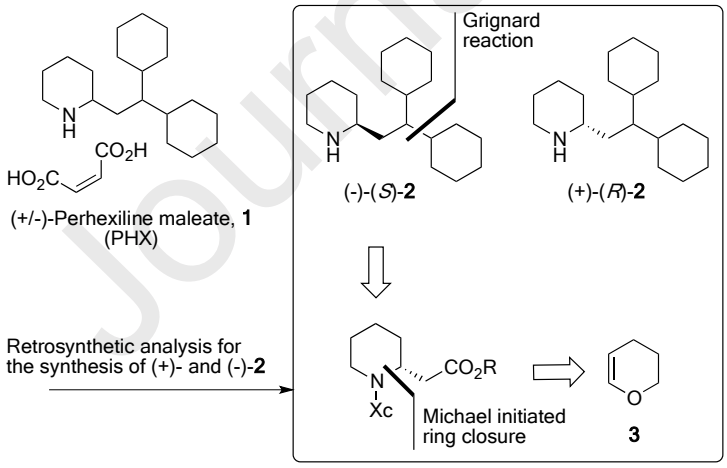


Figure 1. Structure of PHX maleate (**1**), of enantiomers (+)-(*R*)-**2** and (-)-(*S*)-**2**, and retrosynthetic analysis for their stereoselective synthesis.

2. Results and discussion

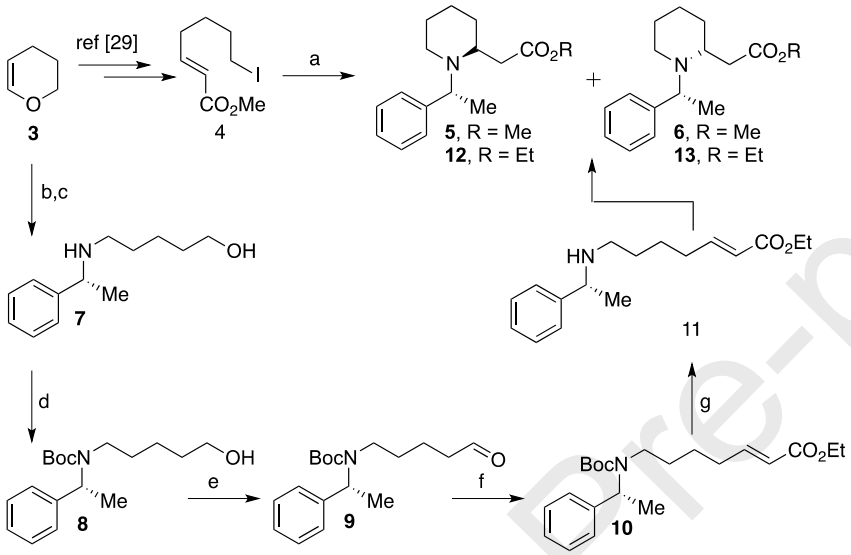
2.1. Stereoselective synthetic approach and chiral HPLC

In the previous years, several attempts have been made to separate or synthesize both enantiomers of **1** in high enantiomeric excess. Davies et al. [25] employed fractional crystallization processes to obtain optically enriched perhexiline by resolving 1,1'-binaphthyl-2,2'-diyl(hydrogen)phosphate (BNPA) salts of PHX. In this process, the racemic PHX was treated with (*S*)-(+)-BNPA to obtain (*S*)-(-)-**2**, followed by crystallization using methanol-acetone mixture. Consequently, (*R*)-(+)-**2** was obtained by the resolution with (*R*)-(-)-BNPA. Even though both the enantiomers obtained were of high optical purity, the tedious crystallization operation involving the diastereomeric salts limits the availability of the enantiomeric forms. To this end, Tseng et al. [26] recently reported the synthesis and absolute configuration of both (*S*)-(-)-**2** and (*R*)-(+)-**2** using a protocol that parallels the synthesis of racemic **1**. The limit of this strategy is highlighted by the reduction step: in fact the catalytic hydrogenation of a pyridine ring appropriately functionalized at C2 and bearing an Evans chiral auxiliary at C5, afforded both enantiomers with enantiomeric excess of 93% for the (S)-(-)-2 and 88% for the (R)-(+)-2. In our pursuit to improve upon the enantiomeric purity of the PHX enantiomers, and to develop a more versatile synthetic approach also suitable for structure-activity relationship investigations, we prepared the stereochemically defined 2-piperidineacetate reported in Figure 1 as the key intermediate, whose ester group could be used for the introduction of a variety of substituents beside the dicyclohexylmethyl moiety of PHX. Among the described procedures for the synthesis of this key building block, we applied a stereoselective Michael initiated ring closing reaction as described in Scheme 1. Accordingly, 3,4-dihydropyran (**3**) was converted to the unsaturated iodide (**4**) through a described 3-step protocol [27]. The key precursors **5** (major product) and **6** were obtained by the reaction of intermediate **4** with (*R*)-α-methylbenzylamine in the presence of triethylamine as a base in ethanol [28].

Due to the limited 6:5 diastereomeric ratio obtained, we investigated if an improvement of the diastereomeric ratio of this reaction could be achieved by isolating intermediate **11** (Scheme 1) and submitting it to an *n*-butyllithium-promoted cyclization reaction. Accordingly, dihyropyran **3** was converted into the corresponding ϖ-hydroxyaldehyde by treatment with HCl, followed by conversion to aminoalcohol **7** through a reductive amination protocol using $NaBH_3CN$ as the reducing agent [29]. The free secondary amine **7** was then protected as the corresponding *N-tert*-butoxycarbonyl-derivative **8** in presence of triethylamine and this latter intermediated was then converted to the corresponding aldehyde **9** using Dess-Martin Periodinane. Aldehyde **9** was subjected to the Horner-Wadsworth-Emmons olefination reaction to obtain the α,β-unsaturated ethyl

ester **10** employing triethylphosphonoacetate (TEPA) and sodium hydride as the base. Compound **10** was deprotected using trifluoroacetic acid and the resulting amine was treated with *n*-butyllithium to obtain diastereomers **12** and **13**, respectively. As expected, **12** was the major product of the reaction but no improvement of the overall diastereomeric ratio was achieved. Interestingly, it was observed that the deprotected amine was able to spontaneously cyclize intramolecularly to obtain low amounts of piperidine products at room temperature before the cyclization step with *n*-butyllithium.

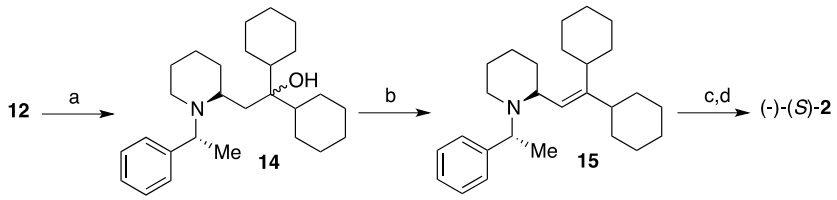
Scheme 1.[a]



[a]Reagents and conditions: (a) (*R*)-α-methylbenzylamine, Et_3N, EtOH, reflux, 16 h (b) 1 N HCl, 0 to 25 °C, 30 mins (c) (*R*)-α-methylbenzylamine, acetic acid, $NaCNBH_3$, EtOH, 1 h, 25 °C (d) $(Boc)_2O$, Et_3N, THF, 12 h (e) Dess-Martin Periodinane, DCM, 25 °C, 4 h (f) TEPA, NaH, THF, 25 °C, 30 min (g) Trifluoroacetic acid, DCM, (1:1), 25 °C (h) *n*-butyl lithium, -78 °C, 2 h.

The final steps for the synthesis of *S*-(-)-**2** are described in Scheme 2. The key precursor (**12**) was subjected to a Grignard-reaction using 2 M cyclohexylmagnesium chloride in THF to afford the alcohol intermediate **14**. Subsequent treatment with thionyl chloride afforded the chlorination/elimination product **15** which on catalytic hydrogenation with $Pd(OH)_2$ in ethanol yielded the debenzylated intermediate which was further reduced using catalytic Pd/C in H_2 atmosphere to afford the desired enantiomer. (*R*)-(+)-**2** was prepared using the similar procedure as discussed above, starting from (*S*)-α-methylbenzylamine and dihydropyran **3**. The obtained enantiomers were converted to their maleate salts using maleic acid.

Scheme 2.[a]



[a]Reagents and conditions: (a) Cyclohexylmagnesiumchloride (2 M), THF, reflux, 3 h (b) $SOCl_2$, THF, reflux, 5 h (c) $Pd(OH)_2$, ammonium formate, EtOH, reflux, 4 h (d) H_2, Pd/C, MeOH, 25 °C, 8 h.

The chiral purity of synthesized piperidines was assessed through the development of a chiral HPLC methodology. After considerable experimentation on several stationary phases, better results were obtained on a Chirex® (*S*)-Val/(*R*)-NEA column (stationary phase including *S*-valine and *R*-1-(alpha-naphthyl)ethylamine), with mobile phase a mixture of *n*-hexane 85%/isopropanol 10%/methanol 5%. A representative chromatogram is reported in Figure 2.

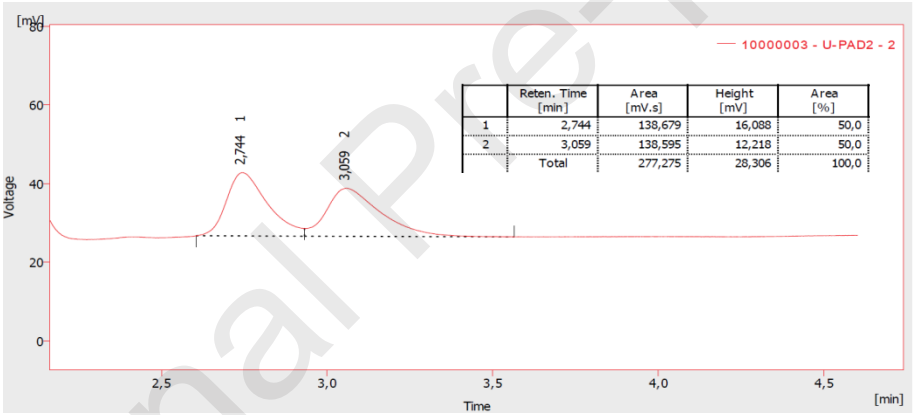


Figure 2. Representative Chiral HPLC chromatogram of PHX racemic mixture. Optical rotation was used as simple tool to assign the separated enantiomers. Thus, peak eluting at T = 2,74 min was identified as (*S*)-(-)-**2** and peak eluting at T = 3,06 min was identified as (*R*)-(+)-**2**.

*2.2. Antischistosomal properties of PHX and enantiomers (S)-(-)-**2** and (R)-(+)-**2***

Each PHX maleate enantiomer, together with the racemic mixture, was tested *in vitro* for its relative schistosomicidal properties. The *in vitro* assay on NTS, was based on ATP quantitation by a luminescence assay at 24 h, as previously described [30]. DMSO (vehicle) and gambogic acid (positive control) that induces complete death of NTS, were respectively used as 100% and 0% viability reference values. A dose-response curve ranging from 0.78 µM up to 100 µM was performed, and the LC_{50} value was estimated for each compound. In this setting, *(R)-(+)-**2*** appears to be more effective (LC_{50} = 9 µM) than the (*S*)-(-) enantiomer

(LC_{50} =16 μM) and overall comparable to the LC_{50} value of the racemic PHX preparation (LC_{50} = 8 μM) (Figure 2).

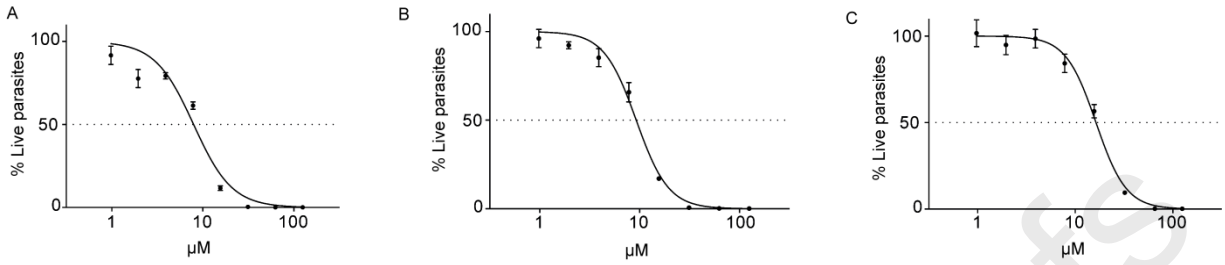


Figure 3. Effects of PHX maleate, (*R*)-(+) **, and** (*S*)-(-) **PHX maleate enantiomers on NTS.** Dose-response curves of racemic PHX (A), (*R*)-(+) PHX (B), and (*S*)-(-) PHX (C) on NTS. For each compound the LC_{50} value was estimated 24 h upon incubation by means of the % ATP reduction (death of NTS) plotted on the y-axis and normalized with 50 μM GA-treated control (0% survival) and DMSO treated-schistosomula (100% survival).

The PHX enantiomers and the racemic PHX maleate were then tested against adult worm pairs *in vitro*. Viability was recorded by assigning a multi-parametric score as previously reported [7]. In these experiments the concentration of racemic PHX was doubled in order to match that of the two enantiomers, under the assumption that: (i) only one of the two enantiomers might be active and (ii) that the racemate would contain only 50% of active substance, as already proposed for the anti-schistosomal drug oxamniquine (OXA) enantiomer studies [31].

For the treatment 3 different concentrations (5, 10, and 20 μM) of racemic PHX together with the equivalent halved dose of each PHX enantiomers were tested (Table 1).

Our results indicate that on adult worm pairs, the (*R*)-(+)-**2** enantiomer was markedly more active than the (*S*)-(-)-enantiomer (Table 1). In particular the dose range of 10 μM of (*R*)-(+)**-2** reduced worm vitality to 20% at 72 h, while (*S*)-(-)**-2** had only a modest effect, lowering worm vitality to 80% at 72 h and 50% at day 7. The racemic mixture used at 20 μM showed a strong reduction on worm's vitality with about 10% survival 72 h after treatment. Thus, under these conditions the (*R*)-(+)-**2** enantiomer and the racemic mixture had a comparable effect on adult parasites as observed for NTS. On the other hand, the (*S*)-(-)-enantiomer resulted to be about four time less active than the (*R*)-(+)-**2** enantiomer. When lower concentrations of both enantiomers were tested (2,5 and 5 μM) the overall activity of both compounds appeared to be quite similar with the (*R*)-(+)-2 resulting always slightly more active than (*S*)-(-)-2. It is worth to point out that in these conditions the racemic

mixture used at 5 and 10 μM showed a strong reduction in worm's vitality to 50% and 10% respectively upon 72 h incubation (Table 1).

When higher drug concentrations (20 μM) of both enantiomers was tested, the effect on worms was very pronounced for both enantiomers, and even (*S*)-(-)**-2** led to a complete worm death. These results suggest that the bulk of anti-schistosomal activity is mainly exerted by (*R*)-(+)**-2**, with (*S*)-(-)-**2** retaining a considerable activity when used at higher concentration.

Table 1 Effects of PHX and PHX enantiomers on *Schistosoma mansoni* adult pairs.

Treatment (μM)	% Viability ± SEM 3d	% Pairing	% Relative egg reduction	% Viability ± SEM 7d
DMSO	100±0	100	100	100±0
PHX 5	47±7	60-100	20±3 ***	52±10
R-(+)-PHX 2.5	97±3	100	89±12	92±8
S-(-)-PHX 2.5	100±0	100	132±28	100±0
DMSO	100±0	100	100	100±0
PHX 10	18±2	0	/	/
R-(+)-PHX 5	79±6	100	60±9 *	83±3
S-(-)-PHX 5	100±0	100	86±10	97±3
DMSO	98±2		100	98±2
PHX 20	11±6	0	/	/
R-(+)-PHX 10	20±3	80-100	12±3 ****	13±2
S-(-)-PHX 10	75±8	100	47±5 **	50±3
DMSO	98±2	100	100	98±2
R-(+)-PHX 20	0±0	0	/	/
S-(-)-PHX 20	16±1	0	/	/

Viability and egg reduction are reported as residual percentage with respect to the DMSO-treated samples. Each point represents the average ± SEM of three independent experiments. *P <0.05, **P <0.01 or ****P <0.0001, Student's t test.

Due to the high relevance of eggs for both disease transmission and pathology, the effect of the PHX enantiomers was also investigated for their impact on egg-laying *in vitro* (Table 1). Eggs released by worm pairs treated with 2,5, 5 and 10 μM of each compound were counted after 72 h from treatment and normalized to the number of couples. At both concentrations (*R*)-(+)**-2** had a significant effect in lowering eggs laid by treated female parasites while the (*S*)-(-)**-2** had a substantial effect only when used at 10 μM. No significant differences were observed at 2.5 μM for both compounds.

Adult worm couples treated with 10 μM of PHX enantiomers for 72 h, the condition at which differences were observed for both viability and egg production, were considered for morphological analysis by means of carmine red-staining, followed by confocal laser scanning microscopy (CLSM). In particular, vitellaria of female parasites treated with (*R*)-(+)**-2**, showed high levels of damage with numerous unstained, dark, hole-like areas, representing lipid droplets. Also, the loss of gut epithelial cells in 4 of 6 male worms was observed. (*S*)-(-)-**2**

had a less severe effect on the overall tissue morphology: only a small number of dark, hole-like areas were present in the vitellocytes and subtle damage of gut endothelium was observed only in 1 out of 8 worms analyzed.

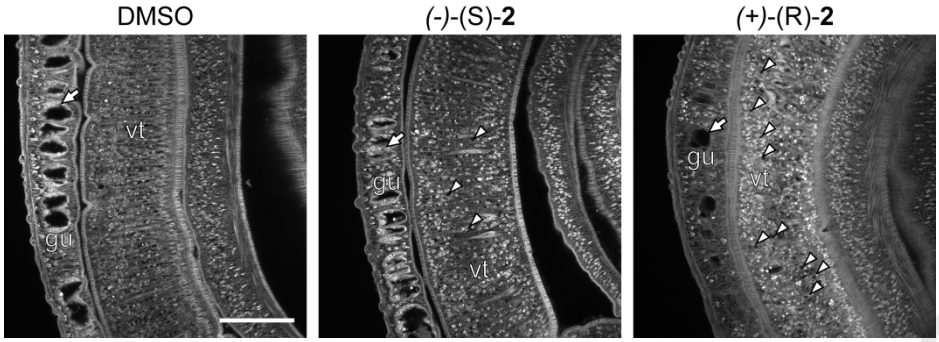


Figure 4. Confocal microscopy of carmine red-stained adult worm couples. Confocal microscopy of carmine red-stained worm couples treated with DMSO and PHX enantiomers (10 μM). Images represent female vitellarium and male gut. Abbreviations: vt, vitellarium; g, gut; white arrows indicate gut epithelium, white triangles indicate black areas round shaped in vitellocytes. Scale bar 50 μm.

Data presented here show that the (*R*)-(+)**-2** enantiomer accounts for the majority of the anti-schistosomal activity in particular on adult parasites at 10 μM, while the (*S*)-(-)-**2** is capable of exerting activity only at higher concentration (20 μM). This suggests that when schistosomes are exposed to the racemic PHX mixture, the (*R*)-(+)**-2** component is likely to be the more active, though (*S*)-(-)-2 is not devoid of any activity. How the two enantiomers behave remains to be unveiled.

3. Conclusions

We here developed a strategy for the synthesis and enantiomeric analysis of PHX. The synthetic strategy here presented is versatile and will be useful for the exploration of the structure-activity relationships at the pendant aliphatic chain on the single enantiomers. The separated enantiomers and the racemic mixture have been comparatively tested on various life cycle stages of *S. mansoni* with the aim of assessing the relative contribution of each enantiomer to the anti-schistosomal activity. Due to the reported difference in pharmacology, toxicology, pharmacokinetics, and metabolism of PHX enantiomers, our studies are important in the frame of designing novel PHX derivatives with improved pharmacokinetics properties for *in vivo* efficacy studies as well as for the identification of PHX targets in schistosomes.

4. Experimental Section

4.1. Chemistry.

Reactions were carried out under nitrogen atmosphere in oven-dried (120 °C) glassware. All reagents and chemicals were purchased from commercial suppliers and used without further purification. Anhydrous solvents were obtained as follows: dichloromethane from calcium hydride diethyl ether and tetrahydrofuran from sodium benzophenone. All purification procedures were carried out with reagent grade solvents (purchased form Fisher Scientific) in air. TLC analysis was conducted using aluminium foil supported thin-layer silica gel chromatography plates (F254 indicator). Column chromatography was performed using 230–400 mesh, 60 Å pore diameter silica gel. Chiral chromatography was performed on a Chirex® (*S*)-Val/(R)-NEA 5 µm column (Phenomenex, Torrance, CA, USA) using a mxiture *n*-hexane 85%/isopropanol 10%/methanol 5%. as eluent at a flow rate of 0.5 mL/min. Samples were solubilized in the mobile phase. ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Varian 300 or a Bruker ARX-400. Chemical shifts (d values) are reported in parts per million and are referenced to the deuterated residual solvent peak. NMR data are reported as: d value (chemical shift, J-value (Hz), integration, where s = singlet, d = doublet, t = triplet, q = quartet, br = broad peak). Low-resolution mass analyses were performed on an Agilent 1100 spectrometer.

4.1.1.(E)-Methyl 7-iodohept-2-enoate (4).

To a solution of Ph_3P (160 mg, 0.60 mmol) in anhydrous DCM (3 mL) were added imidazole (41 mg, 0.60 mmol), iodine (154 mg, 0.60 mmol), and a solution of the (*E*)-methyl 7-hydroxyhept-2-enoate (80 mg, 0.50 mmol) in anhydrous DCM (2 mL). The mixture was stirred at room temperature for 30 min, concentrated *in vacuo*, and chromatographed on silica gel (PE : EtOAc 20:1) to afford the title compound **4** as a clear oil (85 mg, 63%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00 – 6.86 (m, 1H), 5.83 (d, *J* = 15.7,Hz, 1H), 3.72 (s, 3H), 3.17 (t, *J* = 6.9, 2H), 2.22 (q, *J* = 7.2, 2H), 1.93 – 1.75 (m, 2H), 1.66 – 1.45 (m, 2H). ESI MS: 269 $[M+H]^+$

4.1.2.Methyl 2-((*S*)-1-((*R*)-1-phenylethyl)piperidin-2-yl)acetate (5).

(*R*)-*α*-Methylbenzylamine (40 µL, 0.30 mmol) was added dropwise to a stirred solution of **4** (75 mg, 0.28 mmol) and triethylamine (80 µL, 0.56 mmol) in EtOH (4 mL) at 25 °C under nitrogen. The resulting solution was heated at reflux for 16 h. After cooling to 25 °C, the solvent was evaporated under reduced pressure. Water (5 mL) was added and the mixture was extracted with Et_2O (2 × 10 mL). The combined organic extracts were washed with water (5 mL), 5% aqueous sodium thiosulfate solution (5 mL) and brine (5 mL), then dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by flash column

chromatography on silica gel (PE: EtOAc: Et_3N 30:10:0.1) affording the amino ester **5** (31 mg, 42%) as a pale-yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41 – 7.10 (m, 5H), 3.76 – 3.59 (m, 4H), 3.53 – 3.41 (m, *J* = 9.1, 4.6 Hz, 1H), 2.64 – 2.45 (m, 2H), 2.38 – 2.10 (m, 2H), 1.83 – 1.66 (m, 1H), 1.62 – 1.32 (m, 5H), 1.29 (d, *J* = 6.7 Hz, 3H). ESI MS: 262 $[M+H]^+$

4.1.3.Methyl 2-((*R*)-1-((*R*)-1-phenylethyl)piperidin-2-yl)acetate (6).

Pale-yellow oil (17 mg, 23%) ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 – 7.08 (m, 5H), 3.82 (q, *J* = 6.7 Hz, 1H), 3.59 (s, 3H), 3.24 – 3.06 (m, 1H), 2.69 (dd, *J* = 14.0, 4.9 Hz, 1H), 2.61 – 2.53 (m, 2H), 2.53 – 2.33 (m, 1H), 1.76 – 1.36 (m, 6H), 1.32 (d, *J* = 6.7 Hz, 3H). ESI MS: 262 $[M+H]^+$..

4.1.4.(*R*)-5-((1-Phenylethyl)amino)pentan-1-ol (7).

A suspension of 3,4-dihydropyran (1000 mg, 1084 µL, 11.88 mmol) in 1 M aqueous solution of HCl (3 mL) was heated to reflux for 1 h. Successively, the reaction was cooled to 25 °C, neutralized with a 0.5 M solution of NaOH and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with brine (10 mL), dried over Na_2SO_4 and evaporated *in vacuo*. The residue was dissolved in a mixture of ethanol (4 mL) and acetic acid (30 µL), then (*R*)-α-methylbenzylamine (484 mg, 363 µL, 4.00 mmol) was added. The mixture was stirred at 25 °C for 1 h then sodium cyanoborohydride (251 mg, 4.00 mmol) was added and the reaction was stirred at 25 °C for 1 h. After this time a 1 M solution of NaOH in water was added and the mixture was extracted with diethyl ether (3 x 10 mL), the combined organic layers were dried over Na_2SO_4 and evaporated *in vacuo*, affording **7** (788 mg, 32%). as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 – 7.13 (m, 5H), 3.74 (q, *J* = 6.6 Hz, 1H), 3.61 (t, *J* = 6.4, 2H), 2.60 – 2.30 (m, 2H), 1.69 (brs, 2H), 1.58 – 1.44 (m, 4H), 1.41 – 1.30 (m, 5H). ESI MS: 208 $[M+H]^+$

4.1.5.(*R*)-*tert*-Butyl (5-hydroxypentyl)(1-phenylethyl)carbamate (8).

To a stirred solution of **7** (310 mg, 1.49 mmol) in anhydrous THF (5 mL) cooled to 0 °C, triethylamine (625 µL, 4.48 mmol) and Boc_2O (652 mg, 2.99 mmol) were added. The reaction was stirred for 12 h at 25 °C, then it was diluted with DCM (10 mL) and extracted 3 times with a saturated solution of $NaHCO_3$ (10 mL). The combined organic layers were dried by over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The crude mixture was purified by flash column chromatography on silica gel (3:1 PE : EtOAc) to obtain the **8** (288 mg, 63%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 – 7.11 (m, 5H), 5.40 (brs, 1H), 3.54 (t, *J* = 6.5 Hz, 2H), 3.13 – 2.70 (m, 2H), 1.53 – 1.31 (m, 18H). ESI MS: 308 $[M+H]^+$

4.1.6.(*R*)-*tert*-Butyl (5-oxopentyl)(1-phenylethyl)carbamate (9).

To a stirred solution of **8** (115 mg, 0.37 mmol) in anhydrous DCM (4 mL) cooled to 0 °C, Dess-Martin Periodinane (364 mg, 0.86 mmol) was added. The reaction was allowed to reach 25 °C and it was stirred until complete consumption of the starting material monitored by TLC. On completion, the reaction was neutralized with a saturated solution of $NaHCO_3$ and extracted with DCM (3 x 5 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The crude mixture was purified by flash column chromatography on silica gel (4:1 PE : EtOAc) to obtain the ntermediate **9** (65 mg, 57%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.67 (s, 1H), 7.39 – 7.13 (m, 5H), 5.41 (brs, 1H), 3.23 – 2.66 (m, 2H), 2.31 (s, 2H), 1.57 – 1.45 (m, 16H). ESI MS: 306 $[M+H]^+$

4.1.7.(*R,E*)-Ethyl 7-((*tert*-butoxycarbonyl)(1-phenylethyl)amino)hept-2-enoate (10).

To a stirred suspension of NaH (60% dispersion in mineral oil, 42 mg, 1.06 mmol) in anhydrous THF (3 mL) cooled to 0 °C, was added TEPA (210 µL, 1.06 mmol) dropwise. After 30 min, a solution **9** (65 mg, 0.21 mmol) in anhydrous THF (2 mL) was added dropwise. The reaction was allowed to reach 25°C and stirred for 2 h until the complete consumption of the starting material monitored by TLC. On completion, water (5 mL) was added to the reaction mixture and the mixture was extracted with EtOAc (3 x 5 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The crude mixture was purified by flash column chromatography on silica gel (4:1 PE : EtOAc) to obtain intermediate **10** (56 mg, 70%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 – 7.15 (m, 5H), 6.92 - 6.79 (m, 1H), 5.74 (d, J = 15.6, 1H), 5.40 (br, 1H), 4.18 (q, J = 7.1 Hz, 2H), 3.12 – 2.77 (m 2H), 2.18 – 1.95 (m, 2H), 1.57 – 1.39 (m, 16H), 1.28 (t, J = 7.1 Hz, 3H). ESI MS: 376 $[M+H]^+$.

4.1.8.(*R,E*)-Ethyl 7-((1-phenylethyl)amino)hept-2-enoate (11).

Intermediate **10** (56 mg, 0.15 mmol) was dissolved in anhydrous DCM (3 ml) and cooled to 0 °C. To this cold solution, TFA (610 µL) was added dropwise and the reaction was allowed to stir for 2 h until the complete consumption of the starting material monitored by TLC. The volatiles were removed *in vacuo* and the crude was diluted with DCM (5 mL) and extracted 3 times with a saturated solution of $NaHCO_3$ (5 mL). The combined organic layers were dried by passage through anhydrous Na_2SO_4 and the solvent was removed in vacuo to obtain the pure product as a pale-yellow oil (38 mg, 93%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43 – 7.16 (m, 5H), 7.02 – 6.83 (m 1H), 5.78 (d, J = 15.6, 1H), 4.17 (q, J = 7.1 Hz, 2H), 3.77 (q, J = 6.6 Hz, 1H), 2.78 – 2.27 (m, 3H), 2.28 – 1.92 (m, 2H), 1.48 (m, 4H), 1.37 (d, J = 6.5 Hz, 3H), 1.29 (t, J = 7.1, 3H). ESI MS: 276 $[M+H]^+$

4.1.9.Ethyl 2-((*S*)-1-((*R*)-1-phenylethyl)piperidin-2-yl)acetate (12).

n-Butyllithium (49 µL, 0.12 mmol) was added dropwise to a solution of **11** (34 mg, 0.12 mmol) in anhydrous THF (4 mL) at -78 °C and the resultant mixture was stirred for 2 h at the same temperature. A saturated solution of NH_4Cl was then added and the aqueous layer was extracted with EtOAc (3 x 5 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The resulting mixture was purified by flash column chromatography on silica gel (50:1:0.1 PE : EtOAc : Et_3N) to obtain **12** (11 mg, 32%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 – 7.11 (m, 5H), 4.25 – 4.00 (m, 2H), 3.68 (q, *J* = 6.6 Hz, 1H), 3.58-3.40 (m, 1H), 2.64 – 2.41 (m, 2H), 2.38 – 2.11 (m, 2H), 1.85 – 1.68 (m, 1H), 1.63 – 1.32 (m, 5H), 1.32 – 1.21 (m, 6H). ESI MS: 276 $[M+H]^+$

4.1.10. Ethyl 2-((*R*)-1-((*R*)-1-phenylethyl)piperidin-2-yl)acetate (13).

Colorless oil (7 mg, 21%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 – 7.16 (m, 5H), 4.06 (q, *J* = 7.1 Hz, 2H), 3.84 (q, *J* = 6.7 Hz, 1H), 3.21 – 3.12 (m, 1H), 2.74 – 2.36 (m, 4H), 1.78 – 1.37 (m, 5H), 1.34 (d, *J* = 3.1 Hz, 3H), 1.29 – 1.00 (m, 4H). ESI MS: 276 $[M+H]^+$

4.1.11. 1,1-Dicyclohexyl-2-((*S*)-1-((*R*)-1-phenylethyl)piperidin-2-yl)ethanol (14).

To a stirred 2.0 M solution of cyclohexylmagnesium chloride (306 µL, 0.61 mmol) in anhydrous THF (3 mL) intermediate **12** (40 mg, 0.15 mmol) dissolved in anhydrous THF was added. The reaction was heated at reflux for 2 h until the complete consumption of the starting material monitored using TLC. A saturated solution of NH_4Cl (5 mL)) was added to the reaction mixture that was successively extracted with EtOAC (3 x 5 mL). The combined organic extracts were collected, dried over anhydrous Na_2SO_4 and concentrated *in vacuo.* The crude was purified by flash column chromatography on silica gel (PE: EtOAc 5:1) obtaining intermediate **14** (38 mg, 62%) as a pale-yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 – 7.23 (m, 5H), 4.09 (q, *J* = 6.6 Hz, 1H), 3.37 – 2.85 (m, 3H), 2.38 - 2.23 (m 1H), 2.21 – 1.89 (m, 2H), 1.85 – 1.48 (m, 10H), 1.49 – 0.59 (m, 20H). ESI MS: 398 $[M+H]^+$

4.1.12. (*S*)-2-(2,2-Dicyclohexylvinyl)-1-((*R*)-1-phenylethyl)piperidine (15).

To a stirred solution of **10** (30 mg, 0.08 mmol) in anhydrous DCM (3 mL) cooled at 0 °C, $SOCl_2$ (18 µL, 0.15 mmol) was added dropwise. The reaction was heated at reflux for 2 h until the complete disappearance of the starting material monitored using TLC. Then a saturated solution of $NaHCO_3$ (5 mL) was added to the reaction mixture and extracted with DCM (3 x 5 mL). The combined organic extracts were collected, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude was purified by flash column chromatography on silica gel (PE: EtOAc 6:1) obtaining intermediate **15** (22 mg, 77%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$)

δ 7.51 – 7.39 (m, 2H), 7.35 – 7.11 (m, 3H), 5.17 (d, *J* = 9.2 Hz, 1H), 4.19 (q, *J* = 6.8 Hz, 1H), 3.38 (td, *J* = 9.3, 3.1 Hz, 1H), 2.65 – 2.50 (m, 1H), 2.49 – 2.37 (m, 1H), 2.12 (td, *J* = 11.0, 2.9 Hz, 1H), 1.92 – 1.04 (m, 30H). ESI MS: 398 $[M+H]^+$

4.1.13. (*S*)-2-(2,2-dicyclohexylethyl)piperidine (*S*-(-)-Perhexiline, 2).

To a stirred solution of **15** (15 mg, 0.04 mmol) in EtOH (2 mL) ammonium formate (8 mg, 0.12 mmol) and a catalytic amount of $Pd(OH)_2$ on carbon were added. The reaction mixture was refluxed at 85 °C under nitrogen for 2 h. Reaction was monitored by TLC for the complete consumption of starting material, upon which it was filtered through a pad of Celite™. The filtrate was concentrated *in vacuo* and the crude obtained (debenzylated product) (7 mg, 64%) was used in the next step without further purification. ESI MS: 276 $[M+H]^+$. To a stirred solution of the above mentioned intermediate (10 mg, 0.04 mmol) dissolved in MeOH (2 mL), a catalytic amount of Pd/C was added under nitrogen atmosphere. The nitrogen was replaced by hydrogen and the reaction was stirred for 8 h until complete disappearance of the starting material was observed. The reaction mixture was filtered through a pad of Celite™ and the filtrate was concentrated. The crude was purified by flash column chromatography on alumina (DCM: MeOH 150:1) to obtain the desired product (5 mg, 50%) as a pale-yellow oil. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 3.09 – 3.00 (m, 1H), 2.59 (td, *J* = 11.7, 2.9 Hz, 1H), 2.43 – 2.32 (m, 1H), 1.84 – 0.77 (m, 32H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 57.0, 47.4, 45.3, 40.1, 39.9, 36.6, 33.2, 31.9 (2C), 30.0, 29.8, 27.1(2C), 26.9, 26.8, 26.7, 26.6, 25.1. ESI MS: 278 $[M+H]^+$

4.1.14. (*R*)-2-(2,2-dicyclohexylethyl)piperidine (*R*-(+)-Perhexiline, 2).

The title compound was prepared by the same method as described above for preparing *S*-(-)-Perhexiline, **2**, but by using (*R*)-2-(2,2-dicyclohexylvinyl)piperidine (11 mg, 0.04 mmol), to obtain a pale-yellow oil (6 mg, 55%). The analytical and spectroscopic data were identical to those for *S*-(-)-Perhexiline.

4.2. Antischistosomal activity

4.2.1.Reagents

Dimethyl sulfoxide (DMSO), percoll, foetal bovine serum (FBS), gambogic acid, perhexiline maleate (1:1 racemic mixture of (R) and (S) enantiomers (PHX), carmine-red, and Canada balsam were purchased from Sigma-Aldrich (Saint Louis, USA); CellTiter-Glo (CTG) reagent from Promega (Madison, USA); Dulbecco-Modified Eagle's Medium (DMEM) with or without phenol red, Hepes, L-glutamine from Lonza (Basel, Switzerland); antibiotic-antimycotic reagent (100×) from Thermo Fisher Scientific (Waltham, USA); Mowiol 4–88 from Calbiochem was used to prepare Calbiochem mounting medium using a standard protocol.

4.2.2.Ethical statement

The experimental protocols involving the use of animals were reviewed and approved by the Public Veterinary Health Department of the Italian Ministry of Health (Authorization N. 25/2014-PR and no. 336/2018-PR). All experiments were conducted in respect to the 3R rules according to the ethical and safety rules and guidelines for the use of animals in biomedical research provided by the relevant Italian law and European Union Directive (Italian Legislative Decree 26/2014 and 2010/63/EU) and the international guiding principles for biomedical research involving animals (Council for the International Organizations of Medical Sciences, Geneva, Switzerland).

4.2.3.Parasite maintenance

A Puerto Rican strain of *S. mansoni,* that has been maintained in the laboratory for several decades, was used throughout this study. An albino strain of *Biomphalaria glabrata* served as the intermediate host, while ICR (CD-1) outbred female mice (Envigo, Italy) were used for the mammalian stage. Mice were infected by tail immersion with 200-300 double sex cercariae and adult worms recovered after 7 weeks by perfusion as previously described [30].

4.2.4.Schistosomes' viability assay and egg count

Schistosomula were obtained by mechanical transformation of cercariae and percoll gradient as previously described [30]. The viability assay of NTS was performed in 96-well black plates with 150-200 NTS/well cultured in Dulbecco modified Minimum Eagle's Medium (DMEM) (w/o phenol red) complete medium, containing 4500 mg/L glucose, 1 mM Hepes pH 6.98 –7.30, 2 mM L-glutamine, 1x antibiotic-antimycotic reagent and 10% heat inactivated FBS, by CellTiterGlo as previously described [30]. Sample luminescence levels (proportional to ATP levels) was quantified as RLU (Relative Luminescence Unit) and ATP signal percentage normalization (% live parasites) was calculated as previously described [30].

Worm pairs, isolated from infected mice, were used for drug assay as previously described [7]. Briefly, 5 worm pairs were distributed in tissue culture dishes (3.5 cm) in DMEM complete medium (with phenol red). After 24 h parasites were exposed to racemic PHX maleate or the single enantiomers, and cultured for 7 days at 37°C in an atmosphere of 5% CO_2. Vitality was assessed daily under a Leica MZ12.5 stereomicroscope, as previously described [7, 32] by using the following phenotypical score: 3, plate-attached, good movements, clear; 2, slower or diminished movements, darkening, minor tegumental damage; 1, movements heavily lowered, darkening, tegument heavily damaged; 0, dead, lack of any movement. For each sample the scores of all worms divided by the number of worms present in the dish represented the average scores. For each

compound, three experiments were carried out and compounds were added only once without medium addition and/or replacement. DMSO (vehicle) treated worms were used as control.

For egg count, the number of eggs laid *in vitro* by worm couples was assessed three days upon compound treatment using an inverted Leica DM IL microscope (Leica Microsystems, Wetzlar, Germany) with a gridded-plate as supporting tool [7, 32].

4.2.5. Carmine red staining and Laser scanning confocal microscopy analysis.

Carmine red staining was performed as previously described [32]. Images were acquired on an Olympus FV1200 confocal laser scanning microscope using a UplanFLN 40x immersion oil lens (NA = 1.30) with optical pinhole at 1 AU and a multiline argon laser focused. Excitation was at 488 nm and fluorescence was recovered in the range 500 nm to 700 nm. Images were collected as single stack. Data analysis was performed using GraphPad Prism v7.0 software

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Abbreviations

World Health Organization (WHO); praziquantel (PZQ); schistosomula (NTS);carnitine plamitoyltransferase 1 (CPT-1); carnitine plamitoyltransferase 2 (CPT-2); 1,1'-binaphthyl-2,2'-diyl(hydrogen)phosphate (BNPA); triethylphosphonoacetate (TEPA).

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Highlights

- Both enantiomers of perhexiline were prepared through an enantioselective synthesis
- Their antischistosomal activity was assessed in vitro in comparison to the racemate
- (R)-(+)-Perhexiline accounts for the majority of the antischistosomal activity