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TOWARD AN UNDERSTANDING OF THE SCHISTOSOMICIDAL EFFECT OF 4-METHYL-5-(2-PYRAZINYL)-1,2-DITHIOLE-3-THIONE (OLTIPRAZ)

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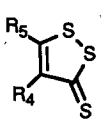
Abstract—In order to gain an interpretation of the schistosomicidal effect of 4-methyl 5-(2-pyrazinyl)-1,2-dithiole-3-thione (oltipraz), chemical, electrochemical and enzymatic hypotheses are discussed from a pharmacological standpoint. The enzymatic hypothesis is in good agreement with experimental results which ascertain that oltipraz behaves as a prodrug.

1,2-Dithiole-3-thiones substituted at the C-4 and C-5 positions are endowed with pharmacological properties. 5-Anethole-1,2-dithiole-3-thione has been administered to large numbers of people as choleretic agent and to counteract the inhibition of salivation caused by antidepressant drugs [1]. According to recent findings dealing with the mechanism of protection against aflatoxin tumorigenicity in rats, selectivity of enzyme inductive effects may render some of the 1,2-dithiole-3-thiones as excellent compounds for chemoprotection in humans [2].

The compounds considered in this work are:



1: R_5 = 2-pyrazinyl, $R_4 = CH_3$ (oltipraz).
2: R_5 = 2-pyridyl, $R_4 = CO_2C_2H_5$.
3: R_5 = 2-pyridyl, $R_4 = CH_3$.
4: R_5 = 5-pyrimidinyl, $R_4 = CH_3$.

4-Methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione, **1**, (35972 RP, oltipraz) was synthesized by Barreau *et al.* [3]. The schistosomicidal activity of this compound has been assessed in many clinical trials [4–8]. Oltipraz has an extremely slow onset of action. In mice given high doses of the drug (250 mg/kg p.o.), worms begin to die after 10 days, and all schistosomes are dead by 14 days post treatment [9]. An early manifestation of activity of the drug is the loss of attachment of the schistosomes to the internal wall of the mesenteric veins. As a result, the worms are carried out to the liver by the mesenteric venous blood flow. The movement of the parasite towards the liver was preceded by the lowering of glutathione (GSH) level in *Schistosoma mansoni*. In the 1,2-dithiole-3-thione series, it is of considerable interest that administration of only those compounds which have antischistosomal properties resulted in the lowering of the GSH level of the worm. None of the inactive analogues had any significant effect on schistosome GSH levels [9].

Oltipraz is metabolized in mice to pyrrolo[1,2-*a*]pyrazine derivatives [10]. When [^{14}C]oltipraz was orally administered, the majority of the radioactivity was excreted over the first 48 hr. The amount of unchanged oltipraz accounted for only 17% of the radioactive products [11].

In schistosomes, the maximum levels of total radioactivity in both sexes occurred at 18 hr post dose (250 mg/kg p.o.). Comparison of the percentages of oltipraz and its metabolites present in female and male schistosomes at 24 hr post dose showed a large percentage (70%) of unchanged oltipraz present in the female schistosomes. In male *S. mansoni*, the percentage of the parent compound recovered was about four times less [11].

The results presented in our preceding papers [12–17] point to the fact that the same pyrrolo[1,2-*a*]pyrazine species can be obtained both by chemical (use of nucleophiles RS^-) and electrochemical methods. Thus, it is possible to isolate disulphide intermediates that are endowed with schistosomicidal activity *in vitro*. A recent work [18] provides evidence for a metabolism pathway involving these disulphides as inhibitors of the schistosome glutathione (GSSG) reductase. According to this work, these disulphides are active on the worm enzyme provided that they derive from an active parent dithiole-thione.

In this paper, an attempt is made to carry out the investigation of the schistosomicidal effect of oltipraz. The two following points are reviewed successively: metabolism pathway; *in vitro*, enzymatic and *in vivo* assays. Chemical, electrochemical and enzymatic hypotheses are discussed from a pharmacological standpoint.

MATERIALS AND METHODS

Substituted 1,2-dithiole-3-thiones **1–4** were supplied by Rhone-Poulenc Sante.

The apparatus, as well as chemical and electrochemical procedures, have been described elsewhere [14, 16].

In vitro and *in vivo* tests were performed in the Rhone-Poulenc Sante laboratories. Unseparated pairs of worms were picked up manually from the mesenteric and portal veins and the livers of CD_1

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female mice (Charles River, France) infected 2 months previously with cercariae of a Brazilian strain of *S. mansoni* bred for 30 years in the Rhone-Poulenc laboratory.

Test No. 1. Four unseparated pairs of worms were suspended in a survival medium (50% tyrode and 50% calf serum) to which variable concentrations of the products to be assayed were added. Inactivation of the worms was observed at 37° and 72 hr after addition of oltipraz, analogues or disulphide intermediates. The schistosomicidal activity was estimated from the mobility of the worms which were observed under a binocular lens. Then, still worms were picked up manually and placed at 37° in a survival medium devoid of test compound. After 4 hr, the remaining still worms were counted and considered as dead.

Compounds to be assayed were dissolved in water–dimethylformamide (9:1, v/v) medium. One part of this solution was added to nine parts of the survival medium. The concentration of the stock solution was chosen so that increasing concentrations from 3 to 100 μg/mL were used in the assay.

Test No. 2. Oltipraz was orally administered to five uninfected mice which were killed 6 hr post-dose (250 mg/kg).

Test No. 3. Oltipraz was orally administered to a lot of 20 infected mice. The mice were killed 6 hr post-dose (250 mg/kg). Six hundred pairs of worms were then collected and suspended in the above described survival medium. It is worth mentioning that the worms collected from untreated mice would have a survival time of about 10 days. In contrast, when they are collected 6 hr post-dose, the survival time decreases to 3 days.*

Under the actual conditions, the unpaired worms were recorded from the survival medium 2 days post introduction, washed in physiological serum, suspended in 0.25 M sucrose and disrupted at 4° using a Thomas–Potter homogenizer. The homogenate was centrifuged.

Test No. 4. Six hundred pairs of schistosomes were collected, washed and disrupted according to the above-mentioned method. The centrifuged homogenate was supplemented with oltipraz (1 mg/5 mL) and allowed to react for 24 hr.

Isolation and characterization of monosulphoxide and disulphoxide metabolites. The monosulphoxide and disulphoxide metabolites of oltipraz and compound **3** (see Eqn 6) were produced by the perfused liver material used by Rhone-Poulenc Sante.

2-Methyl-1-methylsulfinyl-3-methylthioindolizine had: ^{1}H NMR (270 MHz, $CDCl_3$); δ 2.20 [s, 3H, CH_3(2) or SCH_3(3)], 2.60 [s, 3H, CH_3(2) or SCH_3(3)], 3.00 [s, 3H, SCH_3(1)], 6.80 [dd, 1H, H(6), J = 7 Hz, J = 6 Hz], 7.05 [dd, 1H, H(7), J = 7 Hz, J = 9 Hz], 7.90 [d, 1H, H(8), J = 9 Hz], 8.50 [d, 1H, H(5), J = 6 Hz]. Mass spectrum (D.C.I.): m/z = 240 (MH^+); (E.I.): m/z = 239 ($M^{+\cdot}$), m/z = 224 (M-CH_3, 100%), m/z = 209 [M-$(CH_3)_2$].

1,3-Dimethylsulfinyl-2-methylindolizine had: ^{1}H NMR (270 MHz, $CDCl_3$): δ 2.50 [s, 3H, CH_3(2)], 3.00 [s, 3H, SCH_3(1) or SCH_3(3)], 3.05 [s, 3H, SCH_3(1) or SCH_3(3)], 6.90 [dd, 1H, H(6), J = 7 Hz], 7.20 [dd, 1H, H(7), J = 7 Hz, J = 9 Hz], 8.10 [d, 1H, H(8), J = 9 Hz], 9.00 [d, 1H, H(5), J = 7 Hz]. Mass spectrum (D.C.I.): m/z = 256 (MH^+); (E.I.): m/z = 255 ($M^{+\cdot}$), m/z = 240 (M-CH_3, 100%), m/z = 225 [M-$(CH_3)_2$].

* J.-P. Leroy, private communication.

RESULTS

Reductive metabolism pathway

As mentioned in the introduction, oltipraz, **1**, is metabolized *in vivo* to pyrrolo[1,2-*a*]pyrazine derivatives and the same pyrrolo pyrazine species can be obtained both by the use of nucleophiles RS^- (R = ethyl, cysteinyl, glutathionyl) [14] and electrochemical reduction [16].

Attack by EtS^- occurs at the S-2 position and is followed by $Z \rightleftharpoons E$ isomerism reaction. Intramolecular ring closure of the *E* isomer yields, after elimination of molecular sulphur, the unsymmetrical pyrrolo[1,2-*a*]pyrazine disulphide **5** as the major product (Scheme 1).

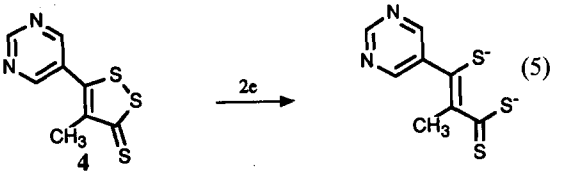
Analogues **2** and **3** behave similarly giving indolizine species **7–10** (Table 1).

The electrochemical reduction of 1,2-dithiole-3-thiones affords a convenient verification of the above suggested mechanism, making use of electrode as an electron-donor instead of a nucleophilic reagent.

Oltipraz **1**, and analogues **2** and **3**, undergo a 1-electron reversible addition (Eqn 1). The resulting thiyl anion radicals are subsequently converted into pyrrolo[1,2-*a*]pyrazine (Eqn 3), or indolizine, radicals which dimerize (Eqn 4 Scheme 2). Thus, controlled potential electrolysis affords a convenient route to symmetrical disulphides **6**, **8** and **10**.

The standard potentials E° of the redox couples thiyl anion radical/parent dithiole-thione, the ring-closure rate constants and the half-life times of the thiyl anion radicals have been deduced from voltammetric and chronoamperometric measurements [16] and are gathered in Table 2.

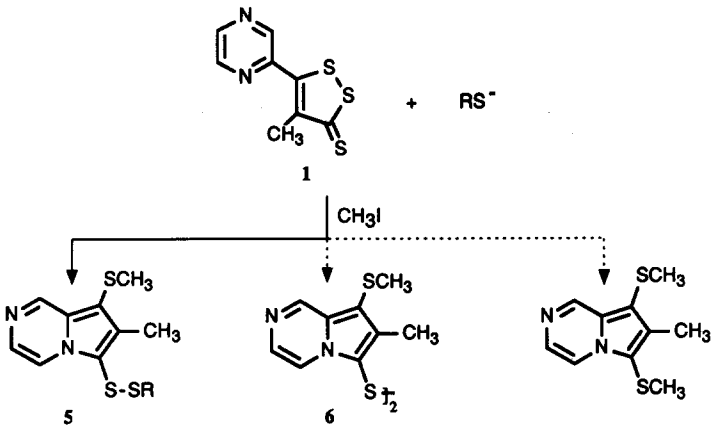
A different kind of electrochemical behaviour is related to compound **4** which does not undergo ring-closure reaction yielding pyrrolo[1,2-*a*]pyrazine or indolizine systems. In this case, only an overall irreversible 2-electron process is observed according to Eqn 5:



(5)

In vitro, enzymatic and in vivo *assays*

Under the above-mentioned experimental conditions (see test No. 1), the unsymmetrical and symmetrical disulphides **5** and **6** exhibited decreased activity (×0.3) when compared with that of oltipraz **1**, whilst **2** and its symmetrical disulphide **8** were 10-fold less active than **1**. In addition, the unsymmetrical disulphide **7** was two-fold less active than **1**, i.e. five-fold more active than the parent dithiole-thione **2**. Lastly, it is worth mentioning that inactive compound **3** yields inactive disulphides **9** and **10** (Table 1).



Scheme 1. Reactivity of oltipraz with thiolate ions.

Regarding enzymatic tests, studies were carried out in our laboratory dealing with glutamylcysteine (GC), GSH synthetases [19] and GSSG reductase [18]. In summary, the presence in *S. mansoni* of GC and GSH synthetases has been shown, but it appears that depletion of GSH level in the worm after oltipraz administration is likely not due to a direct inhibition of GSH biosynthesis. With regard to the GSSG reductase, several points should be underlined:

1. The presence of GSSG reductase in *S. mansoni* has been evidenced. Neither oltipraz nor active analogues **2** and **4** are GSSG reductase inhibitors, whereas they are active *in vivo* on the worms. In contrast, pyrrolo pyrazine disulphides **5** and **6** (or indolizines **7** and **8**) prepared from oltipraz (or active analogue **2**) showed inhibitory properties *in vitro*, whilst indolizine disulphides **9** and **10** obtained from inactive analogue **3** were devoid of effect on GSSG reductase;

2. Contrary to GSH synthetase, GSSG reductase activity, estimated from the worms after oltipraz treatment, exhibited about two-fold lower activity than that showed by worms of untreated mice;

3. Chromatographic studies showed an irreversible loss (50%) of GSSG reductase activity after incubation of the enzyme with active disulphides. Equilibrium dialysis assays confirm the binding of disulphide to GSSG reductase [18].

From *in vivo* assays it appears that:

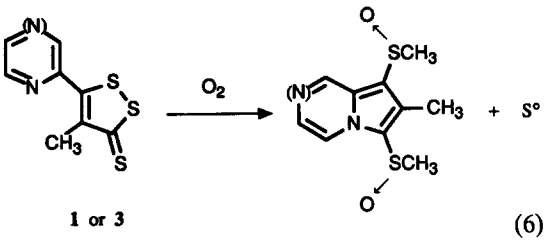
1. Unchanged oltipraz and pyrrolo[1,2-*a*]pyrazine metabolites (sulphoxides) were characterized by thin layer chromatography (TLC) in the plasma of uninfected mice after administration of oltipraz (see test No. 2). Note that monosulphoxide and disulphoxide metabolites of oltipraz, used as reference compounds, were produced by the perfused liver material. Their structural data were in agreement with those previously reported in the literature when these compounds were extracted from treated mice urine [10];

2. Neither unchanged oltipraz nor sulphoxide metabolites could be evidenced by TLC in the supernatant of schistosome's homogenate (see test No. 3 and test No. 4). In contrast, oltipraz would be quantitatively characterized after extraction in a blank i.e. in the survival medium devoid of worm's homogenate.

DISCUSSION

From the above-mentioned results, it can be deduced that: (a) oltipraz is a very slow acting drug [9]; (b) oltipraz lowers the GSH level in the parasite (in a roughly stoichiometric proportion within 24 hr when compared with the oltipraz equivalent concentration); (c) symmetrical and unsymmetrical disulphides **5–8** (Table 1) are active on worm GSSG reductase and on the worms *in vitro* provided that these disulphides derive from active parent 1,2-dithiole-3-thiones. These findings strongly suggest that oltipraz behaves as a prodrug.

According to a *chemical approach*, it was firstly suggested that molecular sulphur accompanying the ring-closure step (Eqn 3) may account for the observed antibilharzial activity. This hypothesis is not further supported by the following results: using the perfused liver material, we have found that oltipraz and analogue **3** were similarly metabolized (see Materials and Methods) as shown in Eqn 6:

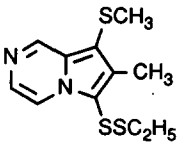
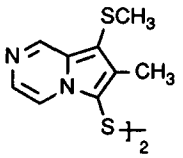
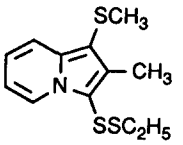
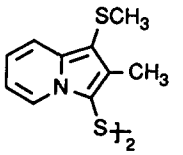
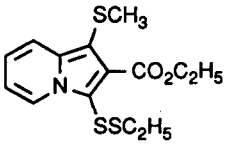
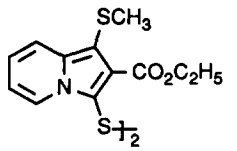


(6)

As **3** does not exhibit antischistosomal activity, it appears safe to conclude that the ability to produce molecular sulphur according to Eqn 6 does not result in pharmacological action.

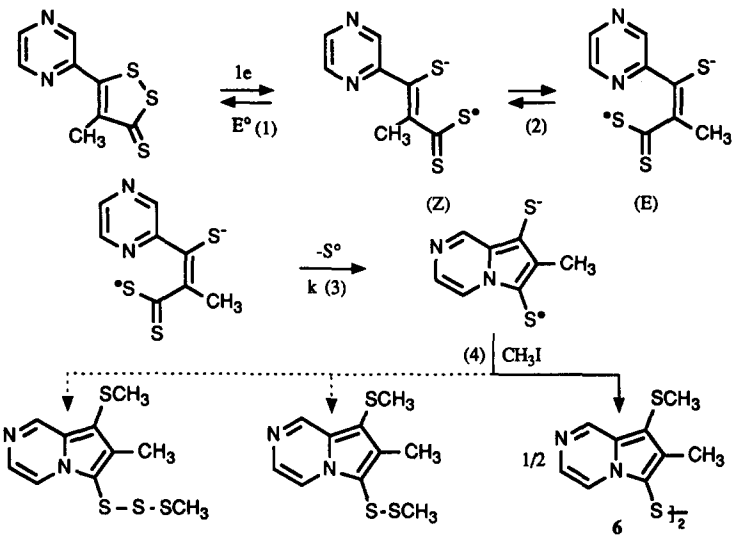
An *electrochemical interpretation* was secondly proposed: the antibilharzial activity can be critically dependent on formation of thiyl radicals [20]. This hypothesis could be further substantiated by the above mentioned electrochemical studies which bring along quantitative informations:

Table 1. Inhibition of GSSG reductase activity and schistosomicidal activity *in vitro* of symmetrical, unsymmetrical disulphides and parent dithiole-thiones

Compounds		Inhibition of *S. mansoni* GSSG reductase activity (%)*	Schistosomicidal activity *in vitro*†
Oltipraz	**1**	0	1
	**5**	50	0.3
	**6**	100	0.3
36598 RP	**3**	0	0
	**9**	0	0
	**10**	0	0
35911 RP	**2**	0	0.1
	**7**	50	0.5
	**8**	50	0.1

* The concentration of compounds was 50 μM. Assays were carried out as described in Ref. 18.

† The schistosomicidal activity *in vitro* was measured by using concentrations up to 100 μg/mL (see Materials and Methods). Activities of the studied compounds were referred to oltipraz activity.



Scheme 2. Electrochemical reduction of oltipraz: mechanistic pathway.

Table 2. Standard redox potentials E° and ring-closure constants k

Anion radical/dithiole-thione	E° mV s.c.e.	k (sec^{-1}) at 25°	$t_{1/2}$ (sec)
1'/1	−910 ± 5	0.10 ± 0.02*	7.0
2'/2	−880 ± 5	50 ± 10†	0.014
3'/3	−1050 ± 5	0.50 ± 0.1*	1.4

* Measured by potential step chronoamperometry.
† Measured by cyclic voltammetry.
For more detailed approach, see Ref. 16.

Comparison of the E° values gathered in Table 2 indicates that considering that there is a correlation between these data and schistosomicidal activity would be irrelevant as oltipraz **1** and analogue **2**, which is 10 times less active, exhibit very close values of E°;

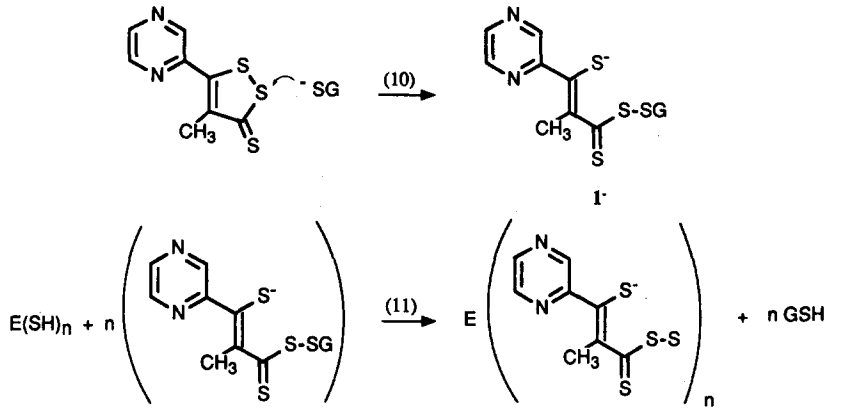
The second feature is that there is no obvious correlation between schistosomicidal effect and the respective life-times of the thiyl anion radicals formed upon addition of 1-electron (step 1 in Scheme 2). Indeed, although the respective life-times found for the thiyl anion radicals **1'** and **3'** are of the same order of magnitude (>1 sec), **1** behaves as a very potent schistosomicidal drug whereas analogue **3** is devoided of such an activity. In addition, analogue **4**, which exhibits pharmacological activity (and toxicity*) higher than that showed by **1**, yields upon addition of one electron a thiyl anion radical which instantaneously disproportionates yielding the dithiocarboxylate species (Eqn 5).

From these findings, it can be deduced that the formation of a long life-time thiyl radical anion is not prerequisite for pharmacological activity to occur.

* J.-P. Leroy, private communication.

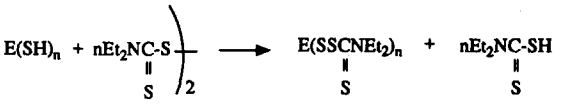
However, there seems little doubt that oltipraz requires metabolite activation to display schistosomicidal activity which would arise from thiol conjugates. Accordingly, it was previously underlined that oltipraz lowers GSH level in the parasite [9]. The high ratio (about 100:1) of GSH to GSSG is intracellularly maintained by the activity of reduced NADPH dependent GSSG reductase. The GSH level of *S. mansoni* appears specifically sensitive to low doses of oltipraz, in contrast to GSH level in the host tissues. This may result from differential sensitivity of host and parasite: many host cells have a considerable excess of GSH, whilst *S. mansoni* may have GSH concentration that is close to the minimum required for cell survival. Depletion of GSH would therefore be expected to be more damaging to the parasite than to the host.

In agreement with this reasoning, *in vitro*, enzymatic and *in vivo* assays led us to consider a third *enzymatic hypothesis*: unsymmetrical and symmetrical disulphides **5–8** (Table 1) would participate in nucleophilic heterolytic cleavage to yield mixed disulphides, as previously mentioned in the case of tetraethylthiuram disulphide (disulfiram) [21–24]. Stromme [25] suggests that sulphydryl groups of acetaldehyde dehydrogenase itself yields *in vivo* a bound material largely identified as



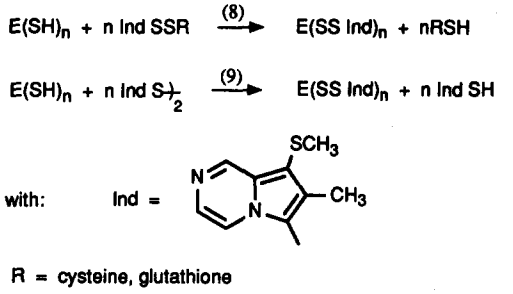
Scheme 4. Inhibition reaction of the opened anionic species 1^- on worm GSSG reductase.

diethylthiocarbamate, according to Eqn 7:



(7)

In the present case, Scheme 3 can be proposed for the inhibition reaction:



Scheme 3. Inhibition reaction pathway.

It was considered that it would be interesting to get an insight into the metabolic pathway followed by oltipraz in the schistosome to see, whether or not, the worm is able to synthesize the pyrrolo[1,2-*a*]pyrazine metabolites.

According to our results (test No. 4), it was found that neither oltipraz nor its pyrrolo pyrazine metabolites could be evidenced in the worms homogenate. The sulphoxide metabolites found in the plasma 6 hr post dose (test No. 2) were synthesized by the infected host, but they did not undergo uptake into the schistosomes. In contrast, the death of the worms 3 days after their introduction in the survival medium (test No. 3) ascertains the uptake of oltipraz into the worms at the 6 hr delay time. These findings highlight an interesting observation from a pharmacological standpoint: the sulphoxide metabolites biosynthesis occurs in the infected host solely, so that the ring-fused reaction yielding the pyrrolo pyrazine system can be regarded as a detoxifying reaction. Accordingly, analogue **4** which cannot undergo this ring-fused reaction exhibited highly toxic properties.*

Finally, remembering that the isolated symmetrical and unsymmetrical disulphides are inactive *in vivo*, it would be safe to state that the metabolite responsible for the inhibitory action on worm GSSG reductase likely is the opened anionic species 1^-, common to all the active analogues **1**, **2** and **4** (Scheme 4).

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* J.-P. Leroy, private communication.

treatment of *Schistosoma mansoni* and *Schistosoma heamatobium* infections with oltipraz (35972 RP). *Curr Chemother Inf Dis* **2**: 1109–1114, 1980.
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