Supporting Information

Evaluation of the pharmacophoric role of the O-O bond in synthetic anti-leishmanial compounds: comparison between 1,2-dioxanes and tetrahydropyrans

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**X-ray crystallography**

The X-ray intensity data were measured on a Bruker Apex II CCD diffractometer. Cell dimensions, and the orientation matrix were initially determined from a least-squares refinement on reflections measured in three sets of 20 exposures, collected in three different $\omega$ regions, and eventually refined against all data. A full sphere of reciprocal space was scanned by 0.3° $\omega$ steps. The software SMART\(^1\) was used for collecting frames of data, indexing reflections and determination of lattice parameters. The collected frames were then processed for integration by the SAINT program,\(^1\) and an empirical absorption correction was applied using SADABS.\(^2\) The structures were solved by direct methods (SIR 2014)\(^3\) and subsequent Fourier syntheses and refined by full-matrix least-squares on $F^2$ (SHELXTL),\(^4\) using anisotropic thermal parameters for all non-hydrogen atoms. The aromatic, methylene, methine and methyl hydrogen atoms were placed in calculated positions, refined with isotropic thermal parameters $U(H) = 1.2 \ U_{eq}(C)$ or $U(H) = 1.5 \ U_{eq}(C_{methyl})$ and allowed to ride on their carrier carbons. Molecular drawings were generated using Mercury.\(^5\)

![Molecular structure](image)

**Figure S1.** Determination of the relative stereochemistry of 2-methoxy tetrahydropyran 9b through X-ray crystallographic analysis (thermal ellipsoids are drawn at 30% of the probability level).
Table S1. Crystal data and structure refinement for compound 9b.

| Compound | 9b |
|----------|----|
| Formula  | C₂₁H₂₄O₄ |
| Fw       | 340.40 |
| T, K     | 296  |
| λ, Å     | 0.71073 |
| Crystal symmetry | Monoclinic |
| Space group | P2₁/c |
| a, Å     | 7.8520(7) |
| b, Å     | 19.4333(19) |
| c, Å     | 12.8156(13) |
| α        | 90 |
| β        | 106.256(3) |
| γ        | 90 |
| Cell volume, Å³ | 1877.4(3) |
| Z        | 4 |
| Dc, Mg m⁻³ | 1.204 |
| μ(Mo-Kα), mm⁻¹ | 0.082 |
| F(000)   | 728 |
| Crystal size/ mm | 0.20 x 0.15 x 0.10 |
| θ limits, ° | 1.959 - 24.999 |
| Reflections collected | 16176 |
| Unique obs. Reflections [Fo > 4σ(Fo)] | 3143 [R(int) = 0.1289] |
| Goodness-of-fit-on F² | 1.072 |
| R₁ (F)  | 0.0978 |
| wR²  | 0.2296 |
| Largest diff. peak and hole, e. Å⁻³ | 0.287 and -0.378 |

a R₁ = Σ||Fo|−|Fc||/Σ|Fo|.
b wR² = [Σw(Fo²−Fc²)²/Σw(Fo²)²]¹/² where w = 1/[σ²(Fo²) + (aP)² + bP] where P = (Fo² + Fc²)/3.
**Cis-stereopreference in tetrahydropyrans 9**

The favored *cis* relative stereochemistry established by X-ray crystallographic analysis for product 9b was extended to the other tetrahydropyrans 9a and 9c obtained using the same stereoselective synthetic approach (Scheme S1).

![Scheme S1](image)

**Scheme S1.** Reagents and conditions: Pd/C (20% w/w), H₂ (filled balloon), MeOH, rt, 12-16 h.

In Scheme S2 we present a hypothesis which could explain the *cis*-stereopreference in the construction of tetrahydropyrans 9.

![Scheme S2](image)

**Scheme S2.** Hypothesis justifying the *cis*-stereoselective synthesis of tetrahydropyran 9a.
Susceptibility test on *Leishmania* promastigotes with 10% FBS in the HOMEM assay medium

As the 20% FBS that is employed in the assay medium is a very nutrient- and antioxidant-rich environment, which could potentially prevent a stronger activation of endoperoxides 2 in comparison to the non-peroxidic analogs 3, we performed additional susceptibility tests for selected compounds by employing *L. donovani* cultures in HOMEM 10% FBS and we compared the results with those obtained by employing 20% FBS.

We observed a slight reduction of the IC$_{50}$ of all the tested compounds with 10% FBS in the assay medium as compared to 20% FBS. However, the bioactivities of endoperoxides 2 and tetrahydropyrans 3 remained comparable, suggesting a not significant pharmacophoric role of the O-O bond also under these bioassay-conditions. The antioxidant-rich environment (ie 20% FBS) does not appear to play a significant role in blocking the activation of endoperoxides. We can speculate that the increased antileishmanial activity of both peroxides and tetrahydropyrans in the presence of 10% FBS can be caused by a decreased parasite vitality with lower FBS concentration.

**Table S2.** Inhibitory activity of tetrahydropyrans 3 and endoperoxides 2 against promastigotes of *L. donovani* in the presence of 20% FBS and 10% FBS, respectively, in the medium.

| Compound | IC$_{50}$ 20% FBS | IC$_{50}$ 10% FBS |
|----------|------------------|------------------|
| 3a       | 3.4              | 1.9              |
| 2a       | 7.5              | 2.7              |
| 3b       | 5.8              | 3.2              |
| 2b       | 6.3              | 1.2              |
Cytotoxicity in THP-1 cell line

We performed additional cytotoxicity tests employing THP-1 cell line. We also calculated the corresponding selectivity indexes, as the ratio between CC$_{50}$ of THP-1 cell line and IC$_{50}$ on amastigotes of *L. donovani*.

We found that three compounds 3a, 3d and 2d showed a slightly higher cytotoxic effect on THP-1 cells than on VERO cells. On the other hand, compounds 2a, 3b and 2b showed a lower cytotoxic effect on THP-1 cells than on VERO cells; thus showing a not significant variance of selectivity index.

Table S3. Inhibitory activity of tetrahydropyrans 3 and endoperoxides 2 against amastigotes of *L. donovani*, cytotoxicity in human acute monocytic leukemia cell line (THP-1) and selectivity index (SI).

| Tetrahydropyran | IC$_{50}$ (µM)$^b$ | CC$_{50}$ (µM)$^c$ | SI$^d$ | Endoperoxide | IC$_{50}$ (µM)$^b$ | CC$_{50}$ (µM)$^c$ | SI$^d$ |
|-----------------|----------------|----------------|------|--------------|----------------|----------------|------|
| 3a              | 3.4 ±0.6       | 26.0           | 7.6  | 2a           | 12.2 ±1.2      | 86.0           | 7.0  |
| 3b              | 3.2 ±0.9       | 50.0           | 15.6 | 2b           | 5.0 ±1.0       | 52.5           | 10.5 |
| 3d              | 2.8 ±0.7       | 64.1           | 22.9 | 2d           | 16.5 ±1.5      | 142.5          | 8.6  |

$^a$ Compounds tested as racemates. $^b$ IC$_{50}$ represents the concentration of a compound that causes 50% growth inhibition. Results represent the mean (± standard deviation, SD) of three independent experiments performed in duplicate. $^c$ CC$_{50}$ represents 50% cytotoxic concentration on THP-1 cells. $^d$ Selectivity index (SI) = CC$_{50}$/IC$_{50}$. 

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Effect of iron chelator DFO on bioactivity of 2b and 3b

As for compounds 2a and 3a, we evaluated the bioactivity of tetrahydropyran 3b and endoperoxide 2b against promastigotes of *L. donovani* in the presence of the iron chelator DFO to investigate whether the IC₅₀ values of these compounds were affected by the presence of DFO. The investigation was performed by co-incubating 2b and 3b with DFO at different concentrations (100 μM, 50 μM, 25 μM, 15 μM). Table S4 shows how the different doses of DFO don’t adduct visible variation of IC₅₀ of the compounds, thus confirming a scarce influence of the iron on the compound bioactivity, confirming the same behavior observed for 2a and 3a.

**Table S4.** Inhibitory activity of tetrahydropyran 3b and endoperoxide 2b against promastigotes of *L. donovani* in the presence or absence of the iron chelator DFO.

| Compound | DFO (μM) | IC₅₀ (μM) |
|----------|----------|-----------|
| 3b       |          |           |
|          | -        | 6.6       |
|          | 100      | 4.9       |
|          | 50       | 6.6       |
|          | 25       | 7.5       |
|          | 15       | 7.5       |
| 2b       |          |           |
|          | -        | 7.5       |
|          | 100      | 5.1       |
|          | 50       | 5.5       |
|          | 25       | 5.3       |
|          | 15       | 6.0       |

a Compounds tested as racemates. b IC₅₀ represents the concentration of a compound that causes 50% growth inhibition. DFO = desferrioxamine.

The isobole technique depicts synergistic (FIC values < 0.5) or antagonistic (FIC values > 4.0) interactions between DFO and the two tested compounds. Has shown in Figure S2, combination of DFO with 2b or 3b resulted in additive effects, since no antagonism or synergism was observed. The same trend of compounds 2a and 3a was confirmed.
Figure S2. Isobolograms depicting the interaction of: A) tetrahydropyran 3b with iron chelator DFO; B) endoperoxide 2b with iron chelator DFO. Dark grey line is line of additivity. X axes depict the fractional inhibitory concentration (FIC; FIC = IC_{50} of the drug in the combination/IC_{50} of the drug when tested alone). Y axes depict the fractional of DFO. Square in the figure indicates Σ FIC values from each drug combination.
Effect of the iron chelator DFP on bioactivity of 2a and 3a

To investigate the role of iron in the activation of compounds 2a and 3a, we also tested these compounds against promastigotes of *L. donovani* in the presence of the iron chelator Deferiprone (DFP), which is a more lipophilic iron-chelator than DFO.

IC$_{50}$ (DFP) = 219 μM; CC$_{50}$ (DFP) = 143 μM.

Table S5. Inhibitory activity of tetrahydropyran 3a and endoperoxide 2a against promastigotes of *L. donovani* in the presence or absence of the iron chelator DFP.

| Compound | DFP (μM) | IC$_{50}$ (μM)$^b$ |
|----------|----------|-------------------|
| 3a       | -        | 3.4               |
|          | 200      | 4                 |
|          | 100      | 6.2               |
|          | 50       | 6.5               |
|          | 25       | 6.1               |
| 2a       | -        | 7.5               |
|          | 200      | 7.1               |
|          | 100      | 13                |
|          | 50       | 13.2              |
|          | 25       | 13                |

$^a$ Compounds tested as racemates. $^b$ IC$_{50}$ represents the concentration of a compound that causes 50% growth inhibition.

DFP = deferiprone.

Also in this case, we observed a not significant variation of the IC$_{50}$ values when the concentration of the iron-chelator was modified. At the highest concentration of the iron-chelator, the lowest IC$_{50}$ was recorded, confirming the activity of these compounds even in the absence of low molecular weight iron-species.
As shown in isobolograms depicted in Figure S3, we observed a not significant variation of the IC$_{50}$ values when the concentration of the iron-chelator increased. FIC values for both compounds are > 0.5 and < 4, therefore no antagonism or synergism was observed.

**Figure S3** Isobolograms depicting the interaction of: A) tetrahydropyran 3a with iron chelator DFP; B) endoperoxide 2a with iron chelator DFP. Light purple line is line of additivity. X axes depict the fractional inhibitory concentration (FIC; FIC = IC$_{50}$ of the drug in the combination/IC$_{50}$ of the drug when tested alone). Y axes depict the fractional of DFP. Square in the figure indicates Σ FIC values from each drug combination.
Calculated LogP

The LogP values of the compounds tested on *L. donovani* promastigotes were calculated using the software ChemDraw Professional 15.0.

**Estimated LogP**

| Compound | LogP | St. deviation | Ref. |
|----------|------|---------------|------|
| 2a       | 3.48 | 0.47          | J.Chem.Inf.Comput.Sci., 27, 21 (1987). |
| 3a       | 3.81 | 0.47          | J.Chem.Inf.Comput.Sci., 27, 21 (1987). |

**Other properties**

| Compound | tPSA | CLogP | CMR | LogS | pKa |
|----------|------|-------|-----|------|-----|
| 2a       | 55.32| 4.2224| 11.033| -4.823| 8.677|
| 3a       | 46.09| 4.5434| 11.2167| -5.023| 9.165|
Estimation of logarithm of Partition Coefficient [n-Octanol/Water] Log(p)

- **2b**
  - tPSA: 55.32
  - CLogP: 3.1264
  - CMR: 12.345
  - LogS: -5.604
  - pKa: 8.584

Log(p)........: 3.63
St.deviation.: 0.47
by Crippen's fragmentation: J.Chem.Inf.Comput.Sci.,27,21(1987).

Log(p)........: 3.73
St.deviation.: 0.49
by Viswanadhan's fragmentation: J.Chem.Inf.Comput.Sci.,29,163(1989).

- **3b**
  - tPSA: 46.09
  - CLogP: 3.4474
  - CMR: 12.5287
  - LogS: -5.546
  - pKa: 9.079

Estimation of logarithm of Partition Coefficient [n-Octanol/Water] Log(p)

Log(p)........: 3.69
St.deviation.: 0.47
by Crippen's fragmentation: J.Chem.Inf.Comput.Sci.,27,21(1987).

Log(p)........: 3.74
St.deviation.: 0.49
by Viswanadhan's fragmentation: J.Chem.Inf.Comput.Sci.,29,163(1989).
### Estimation of logarithm of Partition Coefficient [n-Octanol/Water] Log(p)

**2c**

- tPSA: 55.32
- CLogP: 1.0484
- CMR: 8.2502
- LogS: -2.624
- pKa: 8.669

Log(p).......: 0.84  
St. deviation.: 0.47  
by Crippen's fragmentation: J.Chem.Inf.Comput.Sci.,27,21(1987).

Log(p).......: 0.86  
St. deviation.: 0.49  
by Viswanadhan's fragmentation: J.Chem.Inf.Comput.Sci.,29,163(1989).

### Estimation of logarithm of Partition Coefficient [n-Octanol/Water] Log(p)

**3c**

- tPSA: 46.09
- CLogP: 1.3694
- CMR: 8.4339
- LogS: -2.521
- pKa: 9.157

Log(p).......: 1.31  
St. deviation.: 0.47  
by Crippen's fragmentation: J.Chem.Inf.Comput.Sci.,27,21(1987).

Log(p).......: 1.30  
St. deviation.: 0.49  
by Viswanadhan's fragmentation: J.Chem.Inf.Comput.Sci.,29,163(1989).
2d

\[
\begin{align*}
\text{tPSA: } & 64.88 \\
\text{CLogP: } & 8.7696 \\
\text{CMR: } & 20.95 \\
\text{LogS: } & -11.27 \\
\text{pKa: } & \text{N/A}
\end{align*}
\]

3d

\[
\begin{align*}
\text{tPSA: } & 55.65 \\
\text{CLogP: } & 9.0906 \\
\text{CMR: } & 21.1337 \\
\text{LogS: } & -11.2 \\
\text{pKa: } & \text{N/A}
\end{align*}
\]

2f

\[
\begin{align*}
\text{tPSA: } & 137.51 \\
\text{CLogP: } & 6.3691 \\
\text{CMR: } & 13.7383 \\
\text{LogS: } & -7.214 \\
\text{pKa: } & 9.039
\end{align*}
\]
Estimation of logarithm of Partition Coefficient [n-Octanol/Water] Log(p)

Log(p)........: 7.83
St.deviat.: 0.47
by Crippen's fragmentation: J.Chem.Inf.Comput.Sci.,27,21(1987).

Log(p)........: 7.68
St.deviat.: 0.49
by Viswanadhan's fragmentation: J.Chem.Inf.Comput.Sci.,29,163(1989).

Estimation of logarithm of Partition Coefficient [n-Octanol/Water] Log(p)

Log(p)........: 8.16
St.deviat.: 0.47
by Crippen's fragmentation: J.Chem.Inf.Comput.Sci.,27,21(1987).

Log(p)........: 7.97
St.deviat.: 0.49
by Viswanadhan's fragmentation: J.Chem.Inf.Comput.Sci.,29,163(1989).
NMR spectra
$^1$H NOEDIFF NMR analysis.
$^1$H NOEDIFF NMR analysis.
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HPLC analyses

Compound 3a (method B):

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 B, Sig=230,4 Ref=380,40

| Peak RetTime | Width  | Area   | Height | Area % |
|--------------|--------|--------|--------|--------|
| 1            | 1.238  | 0.1240 | 3983.99561 | 535.34558 | 100.0000 |
Compound 3b (method A):

| Peak  | RetTime | Width | Area    | Height  | Area % |
|-------|---------|-------|---------|---------|--------|
| 1     | 1.630   | 0.180 | 1303.888 | 120.570 | 0.7568 |
| 2     | 9.729   | 1.164 | 1.69999  | 1172.168 | 98.6715|
| 3     | 13.444  | 0.240 | 984.9029 | 66.1993 | 0.5717 |

Area Percent Report

Sorted By: Signal
Multiplier: 1.0000
Dilution: 1.0000
Use Multiplier & Dilution Factor with ISTDs
Compound 3c (method B):

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**Area Percent Report**

Sorted By: Signal
Multiplier: 1.0000
Dilution: 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sigs=254, A Ref=380, 40

| Peak RetTime | Type | Width | Area    | Height | Area   |
|-------------|------|-------|---------|--------|--------|
| 1           | BB   | 0.6263| 5.05425e4 | 963.09241 | 100.0000 |
Compound 3d (method B):

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=254,4 Ref=380,40

| # | Ret Time | Type | Width | Area   | Height | Area % |
|---|----------|------|-------|--------|--------|--------|
| 1 | 1.221 PB |      | 0.1721| 472.37149| 34.84564| 100.0000 |
Compound 3e (method A):

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**Area Percent Report**

**Sorted By**: Signal
**Multiplier**: 1.0000
**Dilution**: 1.0000
Use Multiplier & Dilution Factor with ISTDs

**Signal 1**: DAD1 D, Sig=280,4 Ref=380,40

| Peak | RetTime | Type | Width | Area  | Height | Area   | %     |
|------|---------|------|-------|-------|--------|--------|-------|
| 1    | 6.811   | M    | 2.8849| 5589.02539 | 32.17752 | 100.0000 |

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Compound 2e (method B):

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Area Percent Report
---

Sorted By : Signal  
Multiplier : 1.0000  
Dilution : 1.0000  
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 D, Sig=280,4 Ref=380,40

| Peak RetTime Type | Width | Area    | Height  | Area % |
|-------------------|-------|---------|---------|--------|
|                   | [min] | [min]   | [mAU*s] | [mAU]  |        |
| 1                 | 0.957 | 0.1273  | 23.51208| 3.07771| 3.6182 |
| 2                 | 1.197 | 0.1788  | 626.31610| 58.39663| 96.3818|

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Compound 2f (method B):

![Graph with peak at 5.84 min]

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 B, Sig=230.4 Ref=380.40

| Peak RetTime Type Width  | Area [mAU] | Height [mAU] | Area [%] |
|-------------------------|------------|--------------|---------|
| 1                       | 5.844 MM   | 699.20667    | 153.15910 | 100.0000 |

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References

(1) SMART & SAINT Software Reference Manuals, version 5.051 (Windows NT Version); Bruker Analytical X-ray Instruments Inc.: Madison, WI, 1998.

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