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Original article

Synthesis of some 1-(dihydroxypropyl)pyrazolo[3,4-d]-pyrimidines and *in vivo* evaluation of their antileishmanial and antitrypanosomal activity

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Summary — A number of 1-(2,3-dihydroxypropyl)- and 1-[2-(1,3-dihydroxypropyl)]pyrazolo[3,4-d]pyrimidines were synthesized. Some of these compounds were evaluated for their activity against *Leishmania infantum* and *Trypanosoma brucei brucei* in mice. The highest degree of antileishmanial activity was displayed by the 4-amino-1-(RS)-(2,3-dihydroxypropylpyrazolo[3,4-d]pyrimidine **11a**, which yielded a 87% and 96% parasite inhibition in a standard 5-day test and in a long-term (14 days) treatment, respectively, though full cures of animals were not achieved. On the contrary, this derivative did not significantly prolong the survival of *T b brucei* infected mice.

Résumé — On a synthétisé quelques 1-(2,3-dihydroxypropyl et 1-[2-(1,3-dihydroxypropyl)]pyrazolo[3,4-d]pyrimidines. *On a testé l'activité contre la* Leishmania infantum *et le* Trypanosoma brucei brucei *chez la souris pour une partie de ces composés: l'activité la plus forte a été développée par le 4-amino-1-(2,3-dihydroxypropyl)pyrazolo[3,4-d]pyrimidine* ***11a****, qui a provoqué une inhibition du parasite de 87% dans un test standard de 5 jours et de 96% par un traitement à long terme (14 jours), mais n'a pas donné une guérison complète des animaux. Au contraire, ce dérivé ne prolonge pas significativement la survivance des souris infestées par* T b brucei.

5-Aminopyrazoles / pyrazolo[3,4-d]pyrimidines / antileishmanial activity / antitrypanosomal activity

Introduction

Allopurinol ribonucleoside **1** has been shown to be 300 times more active than aglycon allopurinol against *Leishmania donovani in vitro* [1]. Recently, both the 4-aminopyrazolo[3,4-d]pyrimidine (4-APP) and its ribonucleoside **2** were found to be several times more active than **1** against promastigotes of *L braziliensis* and *L mexicana* [2].

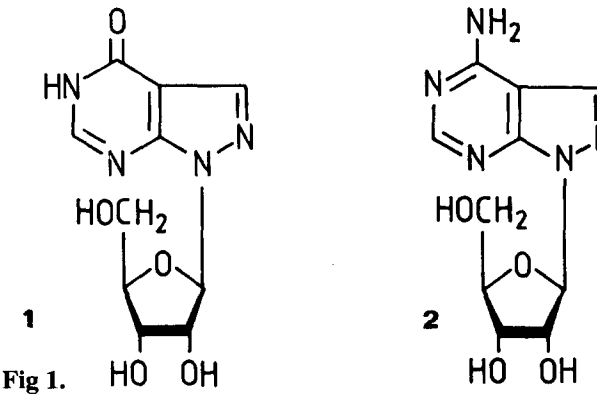


Fig 1.

The antiparasitic properties of allopurinol, 4-APP and their corresponding nucleosides, and the observation that certain analogues of the normal nucleosides, in which the ribose unit was replaced by a truncated acyclic residue, all displayed the same potent biological activity as antiviral agents [3], suggested that it could be of interest to prepare some hitherto unreported 1-(2,3-dihydroxypropyl)- and 1-[2-(1,3-dihydroxypropyl)]pyrazolo[3,4-d]-pyrimidines **3** and **4**, with the aim of studying their effect against *L infantum* and *T brucei brucei in vitro*.

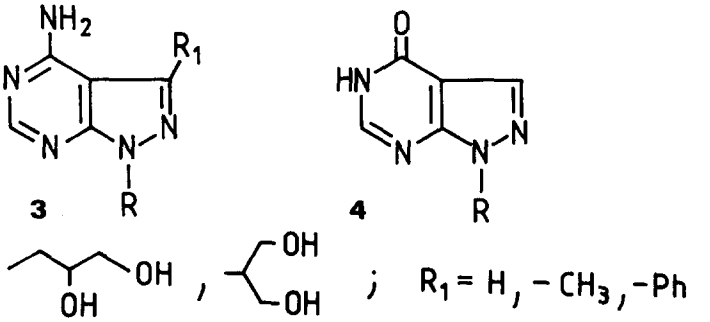
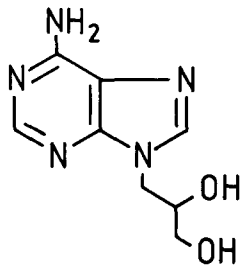


Fig 2.

*Correspondence and reprints

The 1-(2,3-dihydroxypropyl)pyrazolo[3,4-d]pyrimidines contain the acyclic chain of the antiviral (S)-9-(2,3-dihydroxypropyl)adenine (S)-DHPA:



(S)-DHPA

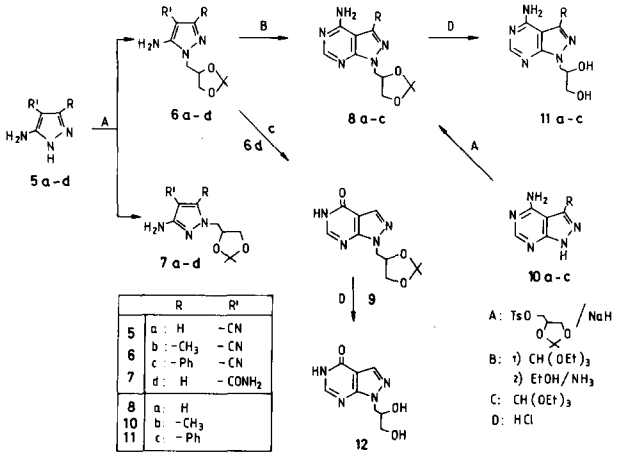
Fig 3.

Since it was found that the activity of the pure (S) enantiomer is comparable with that of the racemic derivative (RS)-DHPA (4), the compounds described in this paper were prepared as the better accessible racemates.

Chemistry

1-(2,3-Dihydroxypropyl)pyrazolo[3,4-d]pyrimidines

5-Aminopyrazoles 5, prepared according to methods in the literature (see *Experimental protocols*), were the starting material for the synthesis outlined in



Scheme 1.

scheme 1. Reaction of sodium salts of **5**, prepared *in situ* by treatment with sodium hydride in anhydrous dimethylformamide, with 1-*O*-*p*-toluensulfonyl-2,3-*O*-isopropylidene-glycerol [5] provided a mixture of the isomers **6** and **7** in comparable yields, except **7c** obtained in very poor yield (6%) because of steric hindrance. The isomers were easily separated by chromatography on silica gel.

The structure assignments of both isomers were substantiated by ^{1}H NMR spectroscopy. Compound **7a** showed in $CDCl_3$ the H-3 proton at δ 7.60 ppm and the $-NH_2$ signal at 4.20 ppm (partially overlapped with the peaks of the side chain protons), shifted respectively downfield and upfield from the signals observed at δ 7.46 and 4.95 in the other isomer **6a**. Also the isomers **6d–7d** and **6b–7b** revealed the same difference in their NMR spectral data: in fact the proton resonance value of H-3 in **6d** (δ 7.62 ppm) and the methyl group signal in **6b** (δ 2.03) were lower than the corresponding signals in **7d** (δ 7.88) and **7b** (δ 2.14), while the $-NH_2$ groups of **6d** (δ 6.06) and **6b** (δ 6.40) appeared shifted downfield from the same signals of **7d** (δ 5.38) and **7b** (δ 5.36). This finding is in full agreement with a published ^{1}H NMR study on isomeric glycosides of five-membered nitrogen heterocycles [6].

In the spectrum of **7c** the phenyl ring was represented by a sharp singlet at 7.47 ppm, while the corresponding phenyl ring of compound **6c** gave 2 main peaks at δ 7.80 and 7.32 ppm, thus revealing its conjugation with the carbon-nitrogen double bond (the 'ortho' hydrogens were shifted downfield).

By reaction with triethylorthoformate followed by ring closure with ethanolic ammonia compounds **6a–c** were converted to 4-amino-1-(2,2-dimethyl-1,3-dioxolan-4-yl)pyrazolo[3,4-d]pyrimidines **8** in yields ranging from 55 to 65%. Compound **6d** instead, simply by refluxing in triethylorthoformate, gave an 82% yield of the allopurinol derivative **9**.

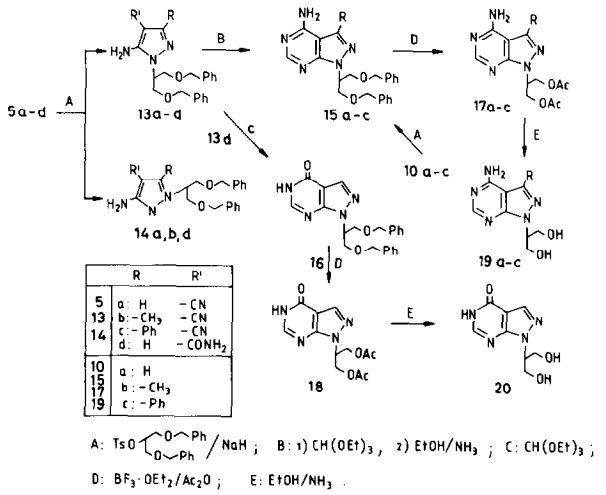
An alternative synthetic route to compound **8**, though only in about 35% yield, involved the direct condensation of 4-aminopyrazolo[3,4-d]pyrimidines **10** with sodium hydride/1-*O*-*p*-toluensulfonyl-2,3-*O*-isopropylidene-glycerol in dimethylformamide, under the same conditions previously described for the preparation of the isomeric pyrazoles **6** and **7** and of the analogues triazolopyrimidines [7].

Treatment of compounds **8** and **9** with diluted hydrochloric acid at 60°C readily removed the isopropylidene group to provide essentially quantitative yields of the desired 1-(2,3-dihydroxypropyl) derivatives **11** and **12**.

1-[2-(1,3-Dihydroxypropyl)]pyrazolo[3,4-d]pyrimidines

By condensing sodium salts of **5** with (1,3-di-*O*-benzyl-2-*O*-*p*-toluensulfonyl)glycerol [8], following the same procedure described for **6** and **7**, a mixture of the isomers **13** and **14** was obtained (scheme 2). As expected, compound **5c**, because of steric hindrance, yielded compound **13c** as the sole product.

Structural assignments, as shown before for the corresponding isomers **6** and **7**, were made by ^{1}H NMR spectroscopy.



Scheme 2.

The syntheses of the pyrazolo[3,4-*d*]pyrimidines **15** and **16** were obtained by the same routes outlined in scheme 1 for compounds **8** and **9**.

Cleavage of the benzyl ether functionalities of **15** and **16** was first attempted by hydrogenolysis. Standard catalytic reduction with 10% Pd/C at 3 atm was unsuccessful. The benzyl groups were cleaved by acetolysis reaction with acetic anhydride and boron trifluoride ethyl etherate at room temperature to give diacetylderivatives **17** and **18** in good yields. Ammonolysis of the latter compounds by treatment with ethanolic ammonia at room temperature gave a 70–80% yield of the expected 1[2-(1,3-dihydroxypropyl)]pyrazolo[3,4-*d*]pyrimidines **19** and **20**.

Pharmacology

In vivo activity on *Leishmania infantum* was evaluated in mice, using a quantitative method for parasite load determination previously described [9]. Three groups of experiments were carried out: a rapid standard test, in which all compounds were tested at the single daily dose of 100 mg/kg for 5 consecutive days; a long-term treatment, in which a daily dosage of 100 mg/kg was administered for a maximum of 42 consecutive days; a dose-response test, carried out by treating groups of mice with 5 different doses for 5 consecutive days. The last 2 groups of experiments were carried out on the compound which showed the highest activity on *Leishmania* at the rapid standard test. All experiments were performed in triplicate, using *N*-methylglucamine antimonate (Glucantime) as reference drug.

In vivo activity on *Trypanosoma brucei brucei* was also tested by treating infected mice according to different schedules and by considering the survival time as index. In this case, pentamidine (Lomidine) was used as a reference drug.

Acute toxicity tests were also carried out in mice.

Results and Discussion

The results on the *in vivo* activity on *Leishmania infantum* of all compounds examined though the standard 5-day test are reported in table I. The highest activity on *Leishmania* was displayed by the 4-aminopyrazolo-[3,4-d]pyrimidines. The compound **11a** showed the strongest inhibitory effect, which was highly reproducible in the replicates of the experiments (SE ± 0.4). In 1 of the experiments, the activity was even higher than that of reference compound, Glucantime.

Table I. Activity of some 1-(dihydroxypropyl)pyrazolo-[3,4-d]pyrimidines on *Leishmania infantum* in Balb/c mice. Drugs were administered sc at the dose of 100 mg/kg for 5 consecutive d. Glucantime was used as reference compound.

Compound	*% Parasite inhibition (± SE)*
11a	87.0 (± 0.4)
19a	86.6 (± 1.2)
20	83.9 (±13.5)
12	78.1 (± 0.7)
Glucantime	90.0 (± 3.1)

11a was selected for the long-term treatment of *Leishmania infantum* infected mice. Results are shown in fig 4. Even at the daily dosage of 100 mg/kg for 42 consecutive d, this compound failed to cure the animals. However, a constant parasite inhibition of about 96% was reached after 14 d of treatment. On the contrary, Glucantime, administered according to the same schedule, cured the mice between d 14 and 28. The dose-response curve of **11a** (fig 5) revealed a high activity at very low doses, ED_{50} being 0.27 mg/kg x 5 d (95% CL: 0.17–0.43). On the other hand, to obtain an inhibitory effect of 90 and 95%, high doses were necessary, *ie* 131 (95% CL: 62–279) and 749 (95% CL: 292–1, 920) mg/kg x 5 d respectively.

11a Activity was also tested on *T brucei brucei*. Different doses and administration routes were employed. No significant differences were observed when surviving curves of treated and control animals were compared according to Mantel Haenszel test (10), as shown in table II.

Toxicity tests were carried out on **11a**. Even at the dose of 3 000 mg/kg this compound showed no gene-

ral toxic effects on mice treated, except for a slight tissue necrosis at the site of the injection when administered sc.

Allopurinol alone or in combination with sodium stibogluconate has been successfully used for the treatment of some cases of visceral leishmaniasis by *L donovani* unresponsive to antimonial drugs [11–13]. Whether or not unresponsiveness to antimonials is actually due to an increasing resistance of *Leishmania* strains to these compounds, it appears that this phenomenon is now also spreading to areas where human visceral leishmaniasis is caused by *L infantum* [14]. The use of pyrazolopyrimidine derivatives more active than allopurinol would be desirable, taking into account, however, that these compounds are probably leishmanistatic rather than leishmanicidal [15]. Therefore, pyrazolopyrimidines should be used in combination with other leishmanicidal agents. The present study on the activity on *L infantum* of some 1-(dihydroxypropyl)pyrazolo[3,4-d]pyrimidines has confirmed that this class of compounds does not free infected mice from parasites even when used at very high doses. However, at least **11a** produces a strong inhibitory effect on the leishmanial parasite load (up to 96%) when used in long-term treatment, without having toxic effect. A possible role of these compounds in the chemotherapy of visceral leishmaniasis might be considered.

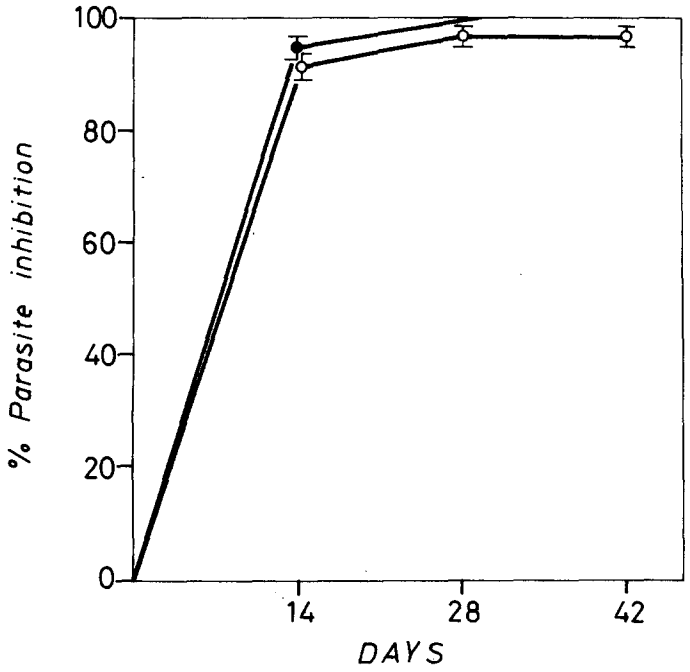


Fig 4. Effect of long-term treatment with **11a** on *Leishmania infantum* in Balb/c mice. Glucantime was used as reference compound. Drugs were administered sc at the daily dose of 100 mg/kg. (○) **11a**, (●) Glucantime.

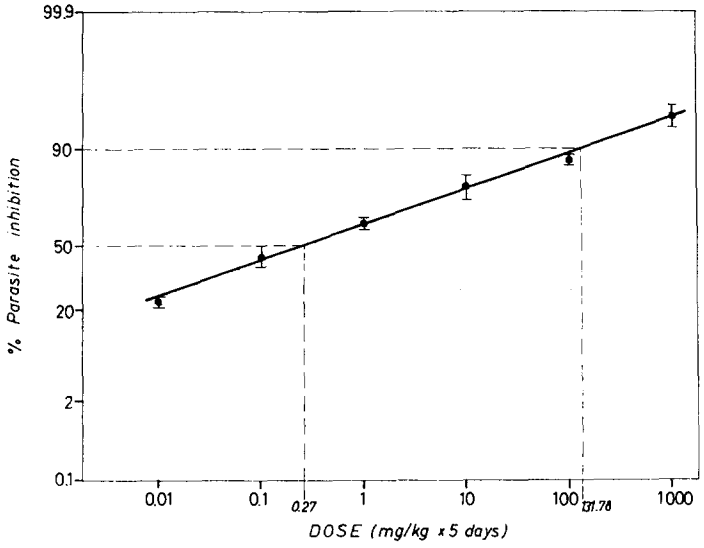


Fig 5. Dose-response curve **11a** on *Leishmania infantum* in Balb/c mice. Drug was administered sc for 5 consecutive d.

Table II. Activity of compound **11a** on *Trypanosoma brucei brucei* in CD and Balb/c mice. Lomidine was used as reference compound. [a]Increased Life Span as compared to controls. [b]Not significant.

Compound	*Animal*	*Dose (mg/kg)*	*Route and no of administrations*	*% ILS* [a]
11a	CD	500	po x 1	0
id	id	100	po x 3	+ 6 (ns)[b]
id	id	100	sc x 3	+29 (ns)
id	Balb/c	250	sc x 5	+16 (ns)
Lominide	CD	5	iv x 1	cured
id	Balb/c	4	iv x 2	cured

Experimental protocols

Chemistry

Melting points were determined on a Köfler hot stage apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian T-60 spectrometer with TMS as internal standard. Column chromatographic separations were obtained on Merck silica gel (70-230 mesh). Purity of compounds were checked by TLC on silica gel 60_{F254} plates, and components were visualized by a UV fluorescent lamp. Solutions in organic solvents were dried over anhydrous sodium sulfate before concentration *in vacuo* (rotary evaporator). Microanalyses were performed by the Microanalytical Section of our Institute and the analytical results were within ± 0.3% of the theroretical values for C, H, N.

The preparation of **5a** (16), **5b** (17), **5c** (18), **5d** (16), **10a** (16), **10b** (17) and **10c** (19) has been reported elsewhere.

*5-Amino-4-cyano- and 3-amino-4-cyano-1-(RS)-(2,2-dimethyl-1,3-dioxolan-4-yl)methyl-pyrazoles 6 and **7a–d**. 5-Amino-4-cyano- and 3-amino-4-cyano-1-[2-(1,3-benzyloxypropyl)]pyrazoles **13a–d** and **14a,b,d**.*

Each compound **5** (0.1 mol) was dissolved in dry DMF (80 ml) and added dropwise over 30 min to a cooled and stirred suspension of NaH (5.3 g, 50% oil dispersion, 0.11 mol) in DMF (60 ml). The mixture was allowed to warm to room temperature and was stirred for 1 h. It was then cooled in an ice/water bath and 1-*O*-*p*-toluensulfonyl-2,3-*O*-isopropylideneglycerol (5) (31.4 g, 0.11 mol) for compounds **6** and **7**, or (1,3-di-*O*-benzyl-2-*O*-*p*-toluensulfonyl)glycerol [8] (46.7 g, 0.11 mol) for compounds **13** and **14**, was added in 15 min. The mixture was heated with stirring at 90–100°C for 2 h. After cooling, the reaction mixture was carefully diluted with water and extracted with chloroform. The solvent was evaporated and the residue chromatographed on a silica gel column by eluting with ethyl acetate (**6, 7d** and **13, 14d**) or an ethyl acetate/n-hexane 2:1 (**6, 7a–c**) or 1:2 (**13a–c, 14a, b**) mixture. Compounds **6** and **13** were eluted first, followed by **7** and **14** respectively.

6a: mp 108–110°C (AcOEt/hexane), 35%. Anal $C_{10}H_{14}N_4O_2$.
6b: mp 178–180°C (AcOEt/hexane), 32%. Anal $C_{11}H_{16}N_4O_2$.
6c: mp 165–167°C (Ethanol), 51%. Anal $C_{16}H_{18}N_4O_2$.
6d: mp 153–155°C (AcOEt), 37%. Anal $C_{10}H_{16}N_4O_3$.
7a: mp 97– 99°C (AcOEt/hexane), 45%. Anal $C_{10}H_{14}N_4O_2$.
7b: mp 169–171°C (AcOEt), 30%. Anal $C_{11}H_{16}N_4O_2$.
7c: mp 163–165°C (Ethanol), 6%. Anal $C_{16}H_{18}N_4O_2$.
7d: mp 172–174°C (AcOEt/), 28%. Anal $C_{10}H_{16}N_4O_3$.
13a: mp 86– 88°C (AcOEt/hexane), 34%. Anal $C_{21}H_{22}N_4O_2$.
13b: amorphous, 29%. Anal $C_{22}H_{24}N_4O_2$.
13c: mp 113–115°C (AcOEt/hexane), 50%. Anal $C_{27}H_{26}N_4O_2$.
13d: mp 104–106°C (AcOEt/hexane), 26%. Anal $C_{21}H_{24}N_4O_3$.
14a: mp 111–113°C (AcOEt), 36%. Anal $C_{21}H_{22}N_4O_2$.
14b: mp 88– 90°C (AcOEt/hexane), 27%. Anal $C_{22}H_{24}N_4O_2$.
14d: mp 101–103°C (benzene/hexane), 31%. Anal $C_{21}H_{24}N_4O_3$.

*4-Amino-1-(RS)-(2,2-dimethyl-1,3-dioxolan-4-yl)methyl-pyrazolo[3,4-d]pyrimidines **8a–c***
*4-Amino-1-[2-(1,3-benzyloxypropyl)]pyrazolo[3,4-d]pyrimidines **15a–c***

Method A. Compounds **8** and **15** were prepared following the procedure described for **6, 7** and **13, 14,** by reaction of the sodium salts of **10** with the same p-toluensulfonyl-glycerols. The crude reaction products were purified by chromatography over silica gel by eluting with ethyl acetate.

Method B. A solution of **6** or **13** (2 g) in triethylorthoformate (20 ml) was refluxed for 8 h. Excess orthoformate was removed *in vacuo* and the crude ethoxymethyleneimino derivative which formed was treated with ethanol saturated with ammonia (20 ml). The reaction mixture was stirred overnight at room temperature, then evaporated to dryness. The residue was chromatographed on silica gel and eluted with ethyl acetate to yield compounds **8** or **15**.

8a: mp 132–134°C (AcOEt), 38% A, 55% B. Anal $C_{11}H_{15}N_5O_2$.
8b: mp 141–143°C (AcOEt), 34% A, 62% B. Anal $C_{12}H_{17}N_5O_2$.
8c: mp 152–154°C (Ethanol), 37% A, 64% B. Anal $C_{17}H_{19}N_5O_2$.
15a: amorphous, 33% A, 65% B. Anal $C_{22}H_{23}N_5O_2$.
15b: mp 118–120°C (AcOEt), 35% A, 73% B. Anal $C_{23}H_{25}N_5O_2$.
15c: amorphous, 30% A, 53% B. Anal $C_{28}H_{27}N_5O_2$.

*1-(RS)-(2,2-dimethyl-1,3-dioxolan-4-yl)methyl-5H-pyrazolo[3,4-d]pyrimidin-4-one **9***
*1-[2-(1,3-benzyloxypropyl)]-5H-pyrazolo[3,4-d]pyrimidin-4-one **16***

A solution of **6d** or **13d** (2 g) in triethylorthoformate (20 ml) was refluxed for 12 h. The solvent was evaporated and the residue crystallized.

9: mp 203–205°C (Ethanol), 82%. Anal $C_{11}H_{14}N_4O_3$.
16: mp 106–108°C (AcOEt), 74%. Anal $C_{22}H_{22}N_4O_3$.

*4-Amino-1-(RS)-(2,3-dihydroxypropyl)pyrazolo[3,4-d]pyrimidines **11a–c***
*1-(RS)-(2,3-dihydroxypropyl)-5H-pyrazolo[3,4-d]pyrimidin-4-one **12***

A mixture of each compound **8** or **9** (1 g) and HCl N (20 ml) was warmed at 60°C for 2 h, then evaporated to dryness. The residue was directly crystallized. Compounds **11** were isolated as hydrochlorides.

11a·HCl: mp 210–213°C (Methanol), 90%. Anal $C_8H_{12}N_5O_2Cl$.
11b·HCl: mp 196–198°C (Methanol/ether), 88%. Anal $C_9H_{14}N_5O_2Cl$.
11c·HCl: mp 247–250°C (Methanol), 77%. Anal $C_{13}H_{15}N_5O_2Cl$.
12: mp 247–250°C (Methanol), 86%. Anal $C_8H_{10}N_4O_3$.

*4-Amino-1-[2-(1,3-diacetoxypropyl)]pyrazolo[3,4-d]pyrimidines **17a–c***
*1-[2-(1,3-diacetoxypropyl)]-5H-pyrazolo[3,4-d]pyrimidin-4-one **18***

Boron trifluoride etherate (1 ml) was added to a stirred and cooled solution of **15** or **16** (2 g) in acetic anhydride (30 ml). The solution was kept overnight at room temperature, then evaporated to dryness. The residue was chromatographed over silica gel by eluting with ethyl acetate, then crystallized to give pure samples.

17a: mp 142–144°C (AcOEt/hexane), 46%. Anal $C_{12}H_{15}N_5O_4$.
17b: mp 120–122°C (AcOEt/hexane), 50%. Anal $C_{13}H_{17}N_5O_4$.
17c: mp 163–165°C (AcOEt),62%. Anal $C_{18}H_{19}N_5O_4$.
18: mp 132–134°C (AcOEt), 39%. Anal $C_{12}H_{14}N_4O_5$.

*4-Amino-1-[2-(1,3-dihydroxypropyl)]pyrazolo[3,4-d]pyrimidines **19a–c***
*1-[2-(1,3-dihydroxypropyl)]-5H-pyrazolo[3,4-d]pyrimidin-4-one **20***

A solution of compounds **17** or **18** (2 g) in ethanolic ammonia (20 ml) was stirred in a stoppered flask at room temperature for 20 h, then evaporated to dryness. The residue obtained after solvent evaporation was crystallized.

19a: mp 203–205°C (Methanol), 78%. Anal $C_8H_{11}N_5O_2 \cdot H_2O$.
19b: mp 208–210°C (Methanol), 86%. Anal $C_9H_{13}N_5O_2$.
19c: mp 194–196°C (Methanol), 68%. Anal $C_{13}H_{15}N_5O_2$.
20: mp 192–194°C (Ethanol), 80%. Anal $C_8H_{10}N_4O_3$.

Pharmacology

For the *in vivo* test on *Leishmania*, the strain MCAN/IT/82/-ISS29, isolated from a dog in Italy and cryopreserved as amastigote form obtained from infected hamster spleen, was used. This strain was isoenzymatically typed and found indistinguishable from the WHO reference strain for *L infantum*. A semi-purified suspension containing 6 x 10^6 amastigotes was inoculated *via* the tail vein in groups of Balb/c mice weighing 18–20 g. On d 7 after infection the increase in parasite load was monitored by killing one animal from the control group. Liver was weighed, from which imprints were made and amastigotes counted according to the Stauber's method [20]. Amastigotes were counted against 1000 liver cell nuclei, and their numbers were expressed as 'LD units' *ie* the number of parasites per liver cell nucleus multiplied by the weight of the organ in mg. The compounds to be tested were dissolved in phosphate buffered saline and administered sc

daily from d 10 after injection. In the rapid standard test the compounds were tested at the daily dose of 100 mg/kg for 5 consecutive d and mice were killed on d 15. In long-term treatment, a dosage of 100 mg/kg was administered for 42 consecutive d. In this case, groups of both treated and untreated mice were killed 14, 28 and 42 d after the onset of treatment. In the dose-response test, groups of mice were treated at daily doses of 0.01, 0.1, 1, 10, 100 and 1000 mg/kg for 5 consecutive d. The parasite load of killed mice was assessed as described above, and the mean parasite count of each treated group was expressed as a percentage of the mean parasite count of control group. *N*-Methylglucamine antimonate (Glucantime) was used as a reference drug, administered sc at the daily dose of 100 mg/kg.

In vivo activity on *Trypanosoma brucei brucei* was tested on a strain kindly provided by AH Fairlamb, Rockefeller University, NY. 0.1 ml of infected rat blood containing 5×10^5 parasites was injected ip in groups of CD and Balb/c mice. The drug was administered sc or *po* according to different schedules and starting from d 0. The *Trypanosoma* strain employed rapidly kills mice, therefore, the pharmacological effect of the drug was evaluated on the basis of increased life span (ILS) of treated animals compared to controls.

Acute toxicity tests were carried out on CD1 mice by treating a group of mice with increasing doses of drug administered sc and *po*.

Acknowledgments

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