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ACYCLIC PYRAZOLO[3,4-d]PYRIMIDINE NUCLEOSIDE AS POTENTIAL LEISHMANIOSTATIC AGENT*

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□ *A new synthesis of 6-amino-1-hydroxyethoxymethyl-4 (5H)-oxopyrazolo[3,4-d]pyrimidine (**4**) has been mentioned. Compound **4** exhibited inhibition of amastigotes of* Leishmania donovani *to the extent of 89% at 30 μg/mL, whereas* iso-*guanine analogue **5** had the inhibition only to the extent of 52.8% at 100 μg/mL* in vitro. *In hamster model the maximum inhibitory response for compound **4** against amastigotes multiplication was observed to be 94% at 50 mg/kg single dose for 5 consecutive days.*

Keywords *Leishmania donovani*; Amastigotes; Promastigotes; Hamster; Leishmaniostatic; Pyrazolo[3,4-d]pyrimidine

INTRODUCTION

The hemoflagellates of the genera leishmania infect nearly 40 million people every year in tropical and subtropical parts of the world due to lack of satisfactory curative and preventive chemotherapeutic agents.[1] The biochemical targets being pursued for design of suitable chemotherapeutic agents include inhibitors of purine salvage pathways,[2] DNA-gyrase,[3] tubulin binding,[4] hydroperoxide metabolism,[5] and trypanothione reductase.[6] The parasite has evolved a unique mechanism for scavenging purines and their nucleotides from the host in absence of its *de-novo* synthesis. In the process, nucleotides are dephosphorylated to nucleosides prior to the cell entry. Two scavenging channels have

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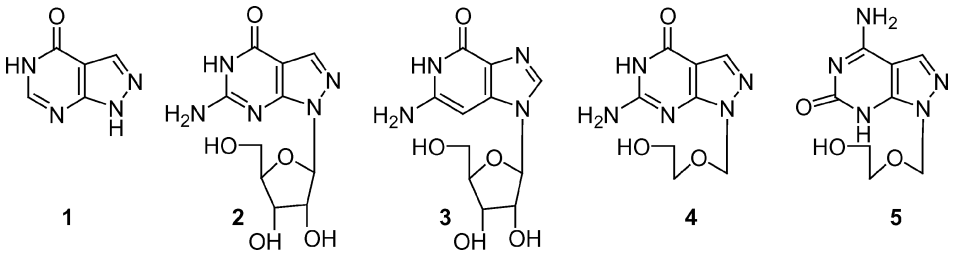
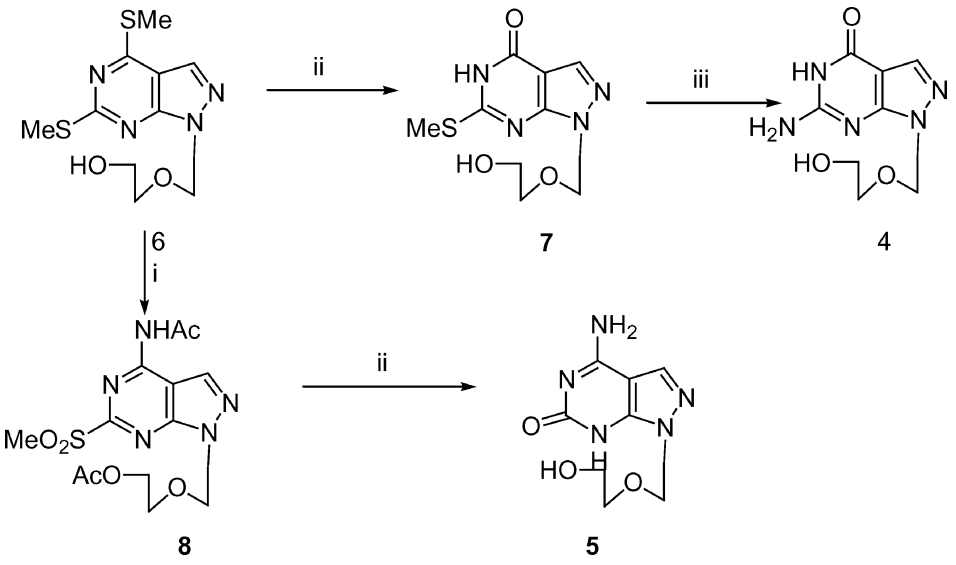
been identified on parasite cell surface.[7] One transports inosine, guanosine, and their analogues while the second carries adenosine and thymidine analogues. The promastigotes utilizing first channel concentrate on 4-hydroxypyrazolo[3,4-d]pyrimidine (HPP, **1**) assuming to be hypoxanthine from culture medium and process it to 4-amino-pyrazolo[3,4-d]pyrimidine-l-ribofuranosyl-5′-monophosphate (4-APRP-MP), which ultimately becomes lethal to the parasite. The HPP, however, is not metabolized to the corresponding guanosine stage (**2**) and, in fact, direct administration of 6-amino-4-hydroxy-pyrazolo[3,4-d]pyrimidine-riboside (**2**) did not inhibit the parasite proliferation,[8] whereas 3-deaza guanosine[9] (**3**), which is 20 times more active than 4-hydroxy-pyrazolo[3,4-d]pyrimidine (**1**, allopurinol), did. The difference in activity profile of guanosine analogues **2** and **3** suggests the strict stereospecificity requirement of salvage enzyme. The synthesis of **3** is, however, cumbersome, which prompted us to search for simpler analogues. Acyclic sugars are known to mimic the ribose portion of nucleosides,[10] which led us to prepare the analogue **4** for antileishmanial activity. The *iso*-guanine analogue **5** was also prepared for structure activity relationship study.



SCHEME 1

Compound **4** has been previously synthesized[11] by Beauchamp et al. for antiviral screening by condensation of polyacetylated 6-amino-4-hydroxy-pyrazolo[3,4-d]pyrimidine base with suitably protected glycone. Here we report another synthesis by simple condensation of 4,6-dimethylthiopyrazolo[3,4-d]pyrimidine with l-chloromethyl-2-benzoyloxyethane in a regiospecific manner to provide 4,6-dimethylthio-l-(benzoyloxyethoxymethyl)-pyrazolo[3,4-d]pyrimidine, which on treatment with methanolic ammonia at ambient temperature afforded 4,6-dimethylthio-l-hydroxyethoxymethyl-pyrazolo[3,4-d]pyrimidine (**6**).[12]

Compound **6** on heating with sodium hydroxide in dioxan yielded 6-methylthio-4(5H)-oxo-l-hydroxyethoxymethyl-pyrazolo[3,4-d]pyrimidine



SCHEME 2 Reagents and conditions: (i) Aq. NaOH, Dioxan (ii) Ethanolic NH3.

(**7**) after selective substitution of 4-methylthio group. The substitution of 6-methylthio group with ethanolic ammonia at 160°C gave 6-amino-4(5H)-oxo-l-hydroxyethoxymethyl-pyrazolo[3,4-d]pyrimidine (**4**) in good yield. For the synthesis of *iso*-guanine analogue **5**, compound **6** was converted to 4-acetylamino-6-methylsulphonyl-1-acetoxymethyl-pyrazolo[3,4-d]pyrimidine (**8**) according to our earlier procedure,[12] which on treatment with 2N sodium hydroxide solution afforded the required product 4-amino-6(7H)-oxo-1-hydroxyethoxymethyl-pyrazolo[3,4-d]pyrimidine (**5**).

BIOLOGICAL SCREENING

The method used for assessing *in-vitro* anti-leishmanial activity was similar to that used by Bhatnagar et al.,[13] except that in this case peritoneal macrophages of BALB/C mice infected with *L. donovani* (Dd8 strain) amastigotes were used. However, the rest of the procedures were similar. The percentage inhibition of amastigotes is calculated by using average number of amastigotes per macrophage in treated (PT) and in control (PC) with formula [% inhibition = 100 − (PT × 100)/PC]. Anti-leishmanial activity *in vivo* against amastigotes of *L. donovani* was determined in hamsters with Dd8 strain. Male hamsters weighing 35–40 grams were infected with 1×10^7 amastigotes, and 4 weeks later the intensity of infection was assessed by spleen biopsy. Animals with 2^+ infection having a count of 11–50 amastigotes per

100 spleen cell nuclei were chosen for screening compounds. Usually 2–3 animals were used for each dose schedule of the test compound as well as standard anti-leishmanial drug, i.e., sodium stibogluconate (SSG), while 2–3 untreated animals were kept as controls. The test animals were treated with a single daily intraperitonial injection of the compound and SSG for 5 days. The drug suspension employed for the test was prepared by grinding accurately weighed drug in distilled water (2–5 mL) with 1–2 drops of tween-80. This stock solution was suitably diluted for use. The post treatment biopsy was conducted one week after the last day of the drug administration. Intensity of infection in treated animals was compared with that of the control animals and percentage inhibition was calculated using a formula, i.e., % inhibition = 100 − (actual no. of amastigotes in treatment × 100)/(initial no. of amastigotes in treatment × increase in untreated control). A dose–response test was carried out by treating groups of infected hamsters with different doses for 5 consecutive days.

RESULTS AND DISCUSSION

Compound **4** showed marked inhibitory activity of amastigotes *in vitro* to the extent of 89% at 30 [μ]g/mL, whereas **5** had the inhibition only to 52.8% at 100 [μ]g/mL. Because of the high order of activity of compound **4**, it was tested *in vivo* in hamster against amastigotes of *L. donovani*. It showed a strong inhibitory effect, which was reproducible as indicated in replicate experiments, and the results are given in Table 1. The maximum response was obtained at day 7 post-treatment when treated at 25 mg/kg dose administered daily for five consecutive days; however, there was no complete inhibition even after post-treatment day 28 and also after increasing the dose level. The above study suggests that the compound does not free infected hamsters from parasites even after treatment at high doses. The compound only reduces the parasite load up to 94%, which therefore suggests that compound **4** has a leishmaniostatic effect and can be more effective only if used in combination with some other drugs. However, the activity of compound **4**

TABLE 1 *In vivo* Anti-Leishmanial Activity of Compound **4** against *L. donovani* Amastigotes in Hamster

S. No	Dose (mg/kg*5 days)	% Inhibition on 7th day
1	5	64.2 ± 5
2	10	84 ± 6.25
3	25	92.5 ± 12.5
4	25	74.3 ± 6.5 (on 28th day)
5	50	94 ± 16.4
6	100	Death
Sodium stibogluconate		
7	25	86.8 ± 2.4
8	50	90.4 ± 2.8

is comparable with the standard drug, sodium stibogluconate. This study at least finds a simple guanosine analogue of pyrazolo[3,4-d]pyrimidine series to have strong leishmaniostatic activity.

EXPERIMENTAL PROCEDURE

Melting points were taken with Buchi capillary apparatus (silicon bath) and are uncorrected. UV spectra were recorded on a Perkin-Elmer-202 spectrophotometer (v max in nm), IR spectra on a Perkin-Elmer-157 grating infracord (v max in cm^{-1}), and PMR spectra on a Perkin-Elmer-360L, 60 MHz (chemical shift in δ scale). The mass spectra were recorded on a JEOL-D-300 spectrophotometer. The compounds were routinely checked for purity on silica gel plates and spots were located under UV lamp/spraying with 100% sulphuric acid in ethanol followed by heating at 100°C. Evaporations were carried out at <30°C under reduced pressure.

1-(Hydroxyethylmethyl)-6-methylthio-4(5H)-oxopyrazolo[3,4-d]pyrimidine (7). A mixture of **6** (1.3 g, 4.5 mmol), dioxan (35 mL), and 2N sodium hydroxide was refluxed for 6 h. The resulting mixture was cooled, neutralized with 2N hydrochloric acid, and concentrated under reduced pressure. The residue was chromatographed on silica gel column. Elution with chloroform methanol (3%) afforded **7** (1 g, 85%); m.p: 172°C; MS (m/z): 256 (m^+); PMR ($CDCl_3$ + DMSO-d_6): 7.80 (s, 1H, H-3), 5.53 (s, 2H, H-1′), 3.55 (bs, 4H, H-3′ & H-4′), 2.5 (s, 3H, SCH_3). Anal. Calcd. For $C_9H_{12}N_4O_3S$ (C, 42.2 H, 4.7; N, 21.9. Found: C, 42.6; H, 4.9; N, 22.2%.

6-Amino-1-hydroxyethoxymethyl-4(5H)-oxopyrazolo[3,4-d]pyrimidine (4). A mixture of **7** (0.5 g, 2 mmol) and ethanolic ammonia (60 mL ethanol, saturated with ammonia at 0°C) was heated in a steel bomb at 160°C for 3 days. Ethanol and excess of ammonia were removed under reduced pressure. The product was crystallized from ethanol to give **4** (0.23 g, 57%); mp: 248–250°C (de) [Lit.[11] mp 250–253°C]; IR (KBr): 1675 (CO); 5.40 (s, 2H, H-1′), 5.1–4.5 (m, 1H, NH) 3.50 (bs, 4H, H-3′ & H-4′). Anal.Calcd for $C_8H_{11}N_5O_3.H_2O$: C, 39.5; H, 5.4; N, 28.8 Found: C, 39.9; H, 5.3; N, 28.9%.

4-Amino-6(7H)-oxo-1-hydroxyethoxymethyl-pyrazolo[3,4-d]pyrimidine (5). A mixture of **8** (2.0 g, 5 mmol) and 2N sodium hydroxide solution (40 mL) was stirred at 65°C for 3 h. The resulting mixture was extracted with ethyl acetate. The organic layer was separated, washed with water, dried (Na_2SO_4), and concentrated in *vacuo*. The crude product thus obtained was chromatographed over silica gel column. Elution of the column with $CHCl_3$: MeOH: CH_3COCH_3 (88:10:2, V/V) gave **5** (0.5 g, 45%); mp: 234–235°C; MS (m/z): 226 (m^{+1}); PMR (DMSO-d_6): 8.4 (s, 1H, H-3), 7.4 (bs, 3H, NH

and NH_2), 5.9 (s, 2H, H-1), 4.8–4.5 (m, 4H, H-2 & H-3). Anal. Calcd. for $C_8H_{11}N_5O_3$: (C, 42.7; H, 4.8; N, 31.1. Found: C, 42.8; H, 4.8; N, 31.0%.

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