30 mM dithiothreitol, 1 mM bacitracin, 50 μM CDP, and 0.25 μCi of [^{3}H]CDP. After 30 min of incubation at 37 °C, the reaction was stopped by immersing the tube in boiling water for 4 min, and the precipitate was removed by centrifugation. Nucleotides in the supernatant were converted to nucleosides by enzymatic hydrolysis. The deoxyribonucleosides were subsequently separated from the ribonucleosides by ascending polyethyleneimine-cellulose chromatography.

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Methylating Agents as Trypanocides

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Methylating agents, such as streptozotocin, procarbazine, *N*-methyl-*N*-nitrosourea, dimethyl sulfate, 1,2-dimethylhydrazine, and a series of 1,2-bis(sulfonyl)-1-methylhydrazines synthesized in this laboratory, were evaluated and shown to be therapeutically active against murine models of African trypanosomiasis. At high dose levels, methylating agents halted trypanosome proliferation and transformed cells into bizarre forms containing multiple nuclei and kinetoplasts. These cells disappeared from the bloodstream of mice bearing these organisms in 48–72 h. When administered at repetitive low doses, methylating agents induced the entire population of trypanosomes to differentiate into biochemically distinct short-stumpy forms in a synchronous manner. These results suggest that methylating agents may be used as biochemical tools in the study of trypanosome differentiation.

Trypanosomes of the brucei group are flagellated protozoa which produce lethal infections in humans and domestic mammals throughout much of sub-Saharan Africa. With the exception of α-(difluoromethyl)ornithine (DFMO), the trypanocidal drugs currently in use have been available for 25–80 years. Current treatment of early-stage infections consists of suramin for *Trypanosoma rhodesiense* and pentamidine for *Trypanosoma gambiense*.[1-3] These therapies require approximately 6 weeks of hospitalization due to drug toxicity.[1,2] The only drug available for late-stage sleeping sickness is melarsoprol.[1-4] This drug has serious side effects and up to 5% of patients die due to drug toxicity. Suramin, pentamidine, and melarsoprol are all administered by intravenous injection. Recently, DFMO has been shown to be effective against early-stage sleeping sickness in man and animals. However, there are doubts as to its efficacy in late-stage disease unless it is used in combination with other less desirable agents such as bleomycin.[5,6] Therefore, better drugs are needed to treat trypanosomiasis.

In the present study, we report the evaluation of a number of methylating agents, including several methylhydrazine derivatives synthesized in our laboratory[7], as antitrypanosomal agents against murine models of trypanosomiasis.

Biological Results and Discussion

The antitrypanosomal properties of compounds **1–16** were determined by measuring their effects on the survival time of mice bearing *T. rhodesiense*. The results are summarized in Table I. A representative compound, 1,2-bis(methylsulfonyl)-1-methylhydrazine (**5**), was also tested against a number of other trypanosome species, such as *T. gambiense*, which like *T. rhodesiense* causes a fatal disease in man,[1,2,4,8] and *Trypanosoma evansi* and *Trypanosoma equiperdum*, which are of veterinary importance[2,8] (Table II).

All of the methylating agents tested displayed significant trypanocidal activity. In general, compounds lacking a reactive methyl group, but structurally identical with the corresponding *N*-methyl analogues in all other respects (e.g., compounds **1** and **9**), or containing the methyl group, but lacking good leaving groups (compound **8**),[9] were inactive as trypanocides. Methanol was produced by these agents in aqueous solutions free from strong competing nucleophiles. Formation of this alcohol was used as a

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(1) World Health Organization "Sixth Programme Report of the UNDP/World Bank/W.H.O.," Special Programme for Research and Training in Tropical Diseases; World Health Organization: Geneva, May 1983; Publication No. TDR/PR-6/83(5)-Try.
(2) Manson-Bahr, P. E. C.; Apted, F. I. C. *Manson's Tropical Diseases, 18th Ed.*; Bailliere Tindall: Eastbourne, 1983; pp 72–92.
(3) Gutteridge, W. E.; Coombs, G. H. *The Biochemistry of Parasitic Protozoa*; Macmillan: London, 1977; pp 1–25.
(4) World Health Organization *WHO Chron.* **1985**, *39*, 176–181.
(5) McCann, P. P.; Bacchi, C. J.; Clarkson, A. B., Jr.; Seed, J. R.; Nathan, H. C.; Amole, B. O.; Hutner, S. H.; Sjoerdsma, A. *Med. Biol.* **1983**, *59*, 434–440.
(6) Clarkson, A. B., Jr.; Bacchi, C. J.; Mellow, G. H.; Nathan, H. C.; McCann, P. P.; Sjoerdsma, A. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5729–5733.
(7) Shyam, K.; Hrubiec, R. T.; Furubayashi, R.; Cosby, L. A.; Sartorelli, A. C. *J. Med. Chem.* **1987**, *30*, 2157–2161.
(8) Hoare, C. A. *Adv. Parasitol.* **1967**, *5*, 47–91.
(9) Shyam, K.; Cosby, L. A.; Sartorelli, A. C. *J. Med. Chem.* **1985**, *28*, 525–527.

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Table I. Effects of Methylating and Ethylating Agents on the Survival Time of Mice Bearing *T. rhodesiense*[a]

no.	compound	dose, mmol/kg	mean life extension[c]	RTECS designation[b]
	vehicle only (50 μL DMSO or 0.5 mL PBSG)	-	0	
1	$H_2NNH_2 \cdot 2HCl$	0.5	0	C
2	$CH_3NHNH_2 \cdot 2HCl$	0.5	1 (0.8)	C
3	$CH_3NHNHCH_3 \cdot 2HCl$	0.2	4 (1.7)	C
4	$C_2H_5NHNHC_2H_5 \cdot 2HCl$	0.2	0	C
5	$CH_3SO_2N(CH_3)NHSO_2CH_3$	0.2	12 (2.5)	N
6	$CH_3SO_2N(CH_3)NHSO_2C_6H_4$-*p*-$OCH_3$	0.2	4 (1.0)	N
7	$C_6H_5SO_2N(CH_3)NHSO_2C_6H_5$	0.2	5 (2.2)	N
8	$C_6H_5CON(CH_3)NHCOC_6H_5$	0.2	0	N
9	$C_6H_5SO_2NHNHSO_2C_6H_5$	0.2	0	N
10	$(CH_3)_2SO_4$	0.2	3 (1.3)	C
11	$(C_2H_5)_2SO_4$	0.2	0	C
12	$CH_3SO_2OCH_3$	0.2	1 (1)	C
13	*N*-methyl-*N*-nitrosourea	0.2	8 (1.7)	C
		0.4	>30 (cure)	
14	procarbazine	0.2	5 (1.3)	C
		0.9	>30 (cure)	
15	5-(3,3-dimethyl-1-triazenyl)-1*H*-imidazole-4-carboxamide	0.2	6 (1.3)	C
		0.5	>30 (cure)	
16	streptozotocin	0.2	4 (0.8)	C

[a] Mice were infected ip with approximately 10^6 cells/mouse in phosphate-buffered saline containing glucose; this results in death in 4 days postinfection. These mice were then treated ip with a single dose of drug 3 days after infection. The number of days the mice survived beyond the untreated controls was used as a measure of trypanocidal activity. The level of parasitemia in treated mice was measured at regular intervals to distinguish between parasite-related and drug-toxicity-related deaths. No toxic deaths were observed in the studies reported here. [b] Registry of toxic effects of chemical substances,[13] C = carcinogen, N = no current listing. Compounds 1–4 were obtained from Aldrich Chemical Co. Compounds 10–13 and 16 were obtained from Sigma Chemicals. Compounds 5–9 were synthesized in this laboratory as previously described.[7] Compounds 14 and 15 were obtained from the National Cancer Institute. [c] All figures for life extension represent the mean value for four animals given to the nearest whole day. The standard error is given in parentheses where appropriate.

Table II. Activity of 1,2-Bis(methylsulfonyl)-1-methylhydrazine against Several *Trypanosoma* Species[a]

species	dose, mmol/kg	mean life extension[b]
T. brucei rhodesiense (YTAT 1.1)	0.2	12 (2.5)
T. brucei gambiense (TxTAT 1.0)	0.2	13 (2.2)
T. brucei brucei (M110)	0.2	11 (2.6)
T. evansi (SN)	0.2	12 (1.4)
T. evansi (SAK)	0.2	>30 days (cure)
T. equiperdum (1559)	0.2	12 (2.4)

[a] Mice were infected with approximately 10^6 cells/mouse by ip injection in phosphate-buffered saline containing glucose. All of the above trypanosome species produce acutely lethal infections in mice. Thus, if untreated, mice die in 3–5 days depending upon the trypanosome species employed. The parasitemia levels were regularly monitored and when the parasitemia reached approximately 10^8 cells/mL animals were given a single dose of 0.2 mmol/kg of 1,2-bis(methylsulfonyl)-1-methylhydrazine. The number of days the mice survived beyond the control animals, which received $(CH_3)_2SO$ only, was used as a measure of trypanocidal activity. The precise details of this assay procedure are given in the Experimental Section. [b] All figures for life extension represent the mean value for 4 animals given to the nearest whole day. The standard error is given in parentheses.

measure of the rate of spontaneous breakdown of these compounds to generate reactive methyl groups. When aqueous, buffered (pH 7.6) solutions of a representative compound (**5**) were assayed over time for the formation of methanol, no further alcohol was generated after 15 min, indicating that decomposition was complete within this time period. This result correlated with the loss of biological activity upon aging of equivalent solutions, where essentially all antiparasitic activity was lost after aging for 15 min (i.e., 0, 21, 73, and 97% of the antitrypanosomal activity was lost after 0, 1, 5, and 15 min of aging, respectively). These findings provide strong evidence that methylation is essential for the observed biological activity of these compounds. In support of this hypothesis, a number of structurally unrelated methylating agents, but not ethylating agents (e.g., compounds **4** and **11**), were found to have significant biological activity (Table I).

Methylating agents appear to have two major effects on trypanosomes in vivo, depending upon the dose level. At high levels (0.2 mmol/kg for compound **5**), cytokinesis appears to be inhibited almost immediately and the cells are transformed into abnormally large forms which are 2–3 times the volume of normal parasites, containing multiple nuclei and kinetoplasts, within 24 h of drug administration to the host. These cells disappear from the bloodstream in 48–72 h. When administered at repetitive low doses (0.01 to 0.03 mmol/kg every 8 h for 48–64 h for compound **5**), methylating agents induce the entire population to differentiate into short-stumpy forms, as judged by morphology, NADH diaphorase positivity, and other biochemical and physiological criteria, measured as described in the literature.[10] Short-stumpy forms are terminally differentiated and are infective to the tsetse fly vector but not infective to the mammalian host. The latter property may make these agents useful biochemical tools in the study of differentiation in trypanosomes, since, with these compounds, it is possible to induce the entire population of trypanosomes to differentiate in a moderately synchronous manner and through this approach early events in the differentiation process can be studied. Both single, high-dose regimens and repetitive low doses can result in cures with a number of the agents described in this paper. When administered as 7–10 small 8-h doses, the total dose administered may be reduced to 70–80% of that required to give the same result as a single large dose.

DFMO has also been shown to induce differentiation in *Trypanosoma brucei*.[10] This effect is generally attributed to the depletion of polyamines. DFMO, however, also causes a 1000-fold increase in decarboxylated *S*-adenosylmethionine (DSAM) and *S*-adenosylmethionine (SAM).[11] These latter metabolites are weak chemical methylating agents and, therefore, may be in part re-

(10) Giffin, B. F.; McCann, P. P.; Bitonti, A. J.; Bacchi, C. J. *J. Protozool.* **1986**, *33*, 238–243.
(11) Fairlamb, A. H.; Henderson, G. B.; Bacchi, C. J.; Cerami, A. *Mol. Biochem. Parasitol.* **1983**, *7*, 209–225.

sponsible for the differentiating action of DFMO. The depletion of polyamines and trypanothione as a result of the DFMO treatment may potentiate the actions of SAM and DSAM as methylating agents by decreasing the levels of competing nucleophiles. Depletion of polyamines may also make the nucleic acids more susceptible to methylation.[12] SAM is also the methyl donor used by many methylases; therefore, enzymatically mediated methylation reactions may also be affected. We are currently investigating the role of methylation in the action of DFMO against trypanosomes.

Preliminary results indicate that compound **5** has almost identical activity with that reported in Table I when administered orally in slightly acidified aqueous solutions (8 μL/g of a 25 mM solution in 5 mM HCl). Orally active trypanocidal agents are desirable, since in areas where trypanosomiasis is endemic, other routes of drug administration frequently present problems. Although methylating agents in general are carcinogenic and mutagenic, in cases of multidrug resistant trypanosomiasis which have failed to respond to existing therapies, these compounds merit consideration as clinical agents.

Experimental Section

Trypanosome cultures were maintained by mixing equal volumes of infected mouse blood containing 10^8–10^9 parasites/mL with buffer (pH 7.4) of the following composition: 80 mM NaCl, 16.2 mM Na_2HPO_4, 3.8 mM NaH_2PO_4, 5 mM KCl, 2 mM $MgCl_2$, 20 mM glucose, 10 mM sodium citrate, and 1.5 g/L of bovine serum albumin containing 20% glycerol by volume. This mixture was stored frozen in liquid nitrogen until required. Individual mice were inoculated with 100 μL of the above mixture. The parasitemia in the infected mice was monitored daily, and when the parasitemia reached 10^8–10^9 cells/mL, blood was collected by cardiac puncture into a citrated syringe. This blood was diluted to 10^7 cells/mL with phosphate-buffered saline containing glucose (PBSG; 80 mM NaCl, 16.2 mM Na_2HPO_4, 3.8 mM NaH_2PO_4, 50 mM glucose), and 100 μL of this mixture was used to infect mice for evaluation of antitrypanosomal activity of methylating agents. Freshly isolated trypanosomes from mice were used because these gave reproducible infections.

Trypanosome concentrations were determined by diluting 5 μL of blood or cell suspension (200-fold) with PBSG and counting the trypanosomes directly in a Neubauer hemocytometer under phase contrast at a magnification of 200X.

Antitrypanosomal activity of various agents was determined in female CD-1 mice of 6–8 weeks of age and of 25–35 g body weight. Mice were injected ip with 10^6 trypanosomes prepared as described above. If untreated, mice die in 3–5 days after infection, depending upon the trypanosome species employed, when the parasitemia reaches 1–2 × 10^9 cells/mL. Infected mice were treated with either a single dose of test agent or multiple doses given every 8 h for 48–64 h, beginning when the parasitemia reached approximately 10^8 cells/mL. The number of days the mice survived beyond that of the untreated controls receiving vehicle only was used as a measure of trypanocidal activity. The level of parasitemia in treated mice was measured at regular intervals as described above to distinguish between parasite-related and test agent toxicity related deaths. Mice surviving 30 days or more were invariably cured and did not subsequently relapse. Compounds **5**, **13**, and **16** were administered by ip injection dissolved in 50 μL of $(CH_3)_2SO$ and compounds 1–4, 14, and 15 in 0.5 mL of PBSG. In the latter case, the solution was adjusted to pH 7.4 prior to dosing. In multidose studies, proportionately smaller volumes were administered. Oral administration of test agents was essentially in aqueous solutions prepared by adding 0.1 mL of 0.5 M test agent in $(CH_3)_2SO$ to 1.9 mL of 5 mM HCl (acidic solutions of 1,2-bis(sulfonyl)-1-methylhydrazines are stable). Eight microliters of this solution were administered orally per g of mouse body weight. This dose level is equivalent to 0.2 mmol/kg. The loss of biological activity of compound **5** upon aging of aqueous solutions was measured by adding 100 μL of a 1 M solution in $(CH_3)_2SO$ to 9.9 mL of 200 mM potassium phosphate buffer (pH 7.6) at 37 °C. Aliquots were removed at various time points and 20 μL/g of body weight of this mixture was injected ip into mice infected with *T. rhodesiense* (10^8 cells/mL of blood) to give an effective dose of test agent of 0.2 mmol/kg. The number of days mice survived beyond the mice treated with phosphate buffer containing 1% $(CH_3)_2SO$ was used as a measure of biological activity. Methanol generation was assayed by placing 2.2-mL aliquots of a 1:200 dilution of aged solutions of the composition described above into a Gilson oxygraph. Twenty microliters of *Pichia pastoris* alcohol oxidase (666 units/mL) was then added and the resultant O_2 consumption was used as a measure of methanol content.

The relative changes in the cellular volume of test agent treated and normal trypanosomes were measured with a Coulter particle counter by analyzing parasites purified at various times after dosing as described by Lanham and Godfrey.[14]

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(12) Wurdeman, R. L.; Gold, B. *Chem. Res. Toxicol.* **1988**, *1*, 146–147.

(13) Sweet, D. V. *Registry of Toxic Effects of Chemical Substances* (RTECS); U.S. Department of Health and Human Services. U.S. Government Printing Office: Washington, DC, 1985–1986.

(14) Lanham, S. M.; Godfrey, D. C. *Exp. Parasitol.* **1970**, *28*, 521–534.