Stereospecific Synthesis of Chiral 2,3-Dihydro-1,4-benzodithiine and Methyl-2,3-dihydro-1,4-benzodithiine Derivatives and their Toxic Effects on *Trypanosoma brucei*

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Preparation of chiral 2,3-dihydro-1,4-benzodithiine and methyl-2,3-dihydro-1,4-benzodithiine derivatives with known absolute configurations from the easily accessible chiral synthons benzyl 4-O-trifloxy-2,3-anhydro-β-L-ribopyranoside and benzyl 4-O-trifloxy-2,3-anhydro-α-D-ribopyranoside is described. These compounds showed significant in vitro toxicity of the bloodstream form of Trypanosoma brucei *with an IC_{50} of 11 μM. The parasites' energy metabolism and consumption of oxygen were found to be affected during incubation.*

Introduction

Sulfur-containing sugar derivatives are of interest from both the chemical and pharmaceutical point of view. For example, bisdithiocarbamate derivatives of carbohydrates possess antifungal activity;[1] thiodeoxy nucleoside analogues in which the ring oxygen atom is replaced by a sulfur atom exhibit antiviral, antibacterial, and antitumor activities;[2,3] 5-thio-D-glucopyranose inhibits the transport of D-glucose and release of insulin;[4] 4′-thio-2′,3′-dideoxynucleosides show in vitro activity against HIV;[5] and 2,2′-anhydro-4′-thio-1-β-D-arabinofuranosylcytosine has a comparable antitumor activity to 1-β-D-arabinofuranosylcytosine, which is used clinically against acute leukemia and lymphoma.[6] Non-carbohydrate organic compounds that contain a benzodithiine moiety have been reported to possess interesting antiviral activities.[7] However, to the best of our knowledge, no tests on the biological effects of sugar-embedded benzodithiines have been reported.

In connection with our interest of developing new synthetic methodologies for regio- and stereoselective syntheses of chiral heterocyclic systems on carbohydrate templates,[8,9] we report herein the preparation of chiral 2,3-dihydro-1,4-benzodithiine and methyl-2,3-dihydro-1,4-benzodithiine derivatives with known absolute configurations from the easily accessible chiral synthons benzyl 4-*O*-trifloxy-2,3-anhydro-β-L-ribopyranoside (**1**) and benzyl 4-*O*-trifloxy-2,3-anhydro-α-D-ribopyranoside (**2**).

African trypanosomes, causative agents of the lethal diseases sleeping sickness in man and Nagana in cattle throughout the tropical part of Africa, have been used as a possible target for these newly synthesized sugar analogues. Due to the absence of a functional respiratory chain, bloodstream forms of these parasites depend exclusively on glycolysis for their energy metabolism in the mammalian host. As in other members of the order kinetoplastida, most of the glycolytic enzymes are contained within glycosomes, a specific organelle of peroxisomal origin. Because of compartmentation of this metabolic pathway, an additional oxidase is indispensably involved in balancing the redox state of the cell.[10] This oxidase is a non-heme iron protein of the inner mitochondrial membrane that is insensitive to cyanides and has been termed trypanosomal alternative oxidase (TAO). It is indirectly responsible for the reoxidation of NADH in the glycosome, an essential reaction for the glycolytic pathway. Due to structural similarities between the sugar derivatives used in this work and the TAO inhibitor ascofuranone,[11,12] we were prompted to test the former compounds for trypanocidal activity and, in particular, inhibition of oxygen consumption.

Results and Discussion

For the preparation of the benzodithiine derivatives, the epoxy triflates **1** and **2** were allowed to react with benzene-1,2-dithiole in THF at −5 °C to room temperature to yield the chiral benzodithiine derivatives **3** and **5**, respectively, after conventional work up (Scheme 1). The preparation of chiral methyl-2,3-dihydro-1,4-benzodithiines starts with the reaction of epoxy triflates **1** and **2** with the dianion of 3,4-dimercaptotoluene at −5 °C to yield the corresponding chiral methyl-2,3-dihydro-1,4-benzodithiines **4** and **6**, respectively.

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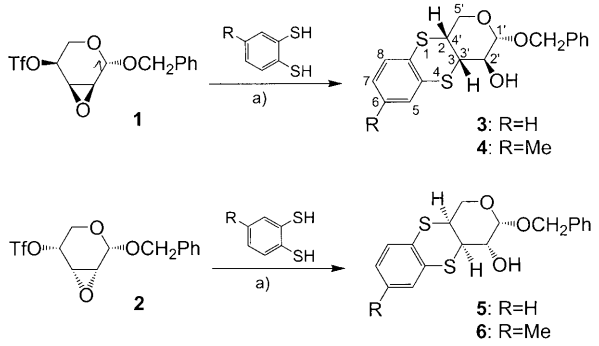

Scheme 1. Synthesis of chiral benzodithiine and methylbenzodithiine derivatives. a) THF, RT, 0 °C, 2.5 h.

The ^{13}C NMR spectra[13] of the benzodithiine derivatives **3** and **5** show two methine carbons (C-3′, C-4′) that resonate at 47.5, 44.6 and 44.3, 43.4, respectively; whereas the two methine carbons (C-3′, C-4′) of **4** and **6** arise at 47.3, 44.5 and 44.0, 44.4 ppm, respectively. The predominant conformation of these compounds is extracted from the coupling constants of the ^1H NMR spectra. For compounds **3** and **4**, the H1′/H2′ coupling constants of $J = 6.1$ Hz indicate a diaxial relationship, therefore, the predominant conformation is $^1C_4$. In the ^1H NMR spectra of compounds **5** and **6**, H1′/H2′ couplings of $J = 3.3$ Hz are observed; this indicates an equatorial orientation and thus a $^4C_1$ conformation.

Compound **4** has shown significant toxicity against the bloodstream form of *Trypanosoma brucei*. Figure 1 shows the dose–toxicity relation of the compound, clearly indicating its

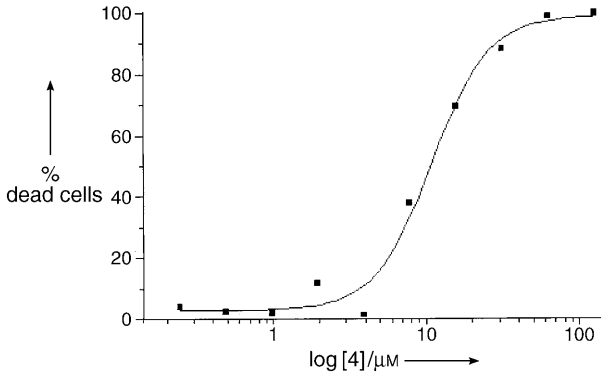

Figure 1. Dose–toxicity relation of bloodstream-form *T. brucei* treated with compound **4**. Cells were incubated for 24 h in 96-well plates under culture conditions with an initial density of 2×10^5 mL^{-1}. Control cells treated with solvent (ethanol) only did not reveal any toxic effects. The IC_{50} was determined as 11 μM.

trypanocidal effect in the low micromolar range. An IC_{50} of 11 μM was determined under the conditions described below.

For the initial experiments, 20 μM of all compounds were used. Compounds **5** and **6** showed only limited toxic activity, reducing cell numbers by half after 21 h. In the case of compound **4**, on the other hand, cells had already died after 30 minutes. Since trypanosomes treated with compound **4** exhibited a decrease in motility, as judged by light microscopy, glycolytic metabolites were determined. Bloodstream-form *T. brucei* rely exclusively on glycolysis for energy metabolism; this leads to pyruvate as the sole end product which is released into the surrounding media. After treatment of trypanosomes (2×10^7 mL^{-1}) with compound **4** (120 μM), a decrease in pyruvate production of about 30% was measured (data not shown). Moreover, measurements of the parasites' glycerol production (Figure 2) showed a significant increase in the case of compound **4**-treated cells in comparison to the control. This might suggest inhibition of TAO, since glycerol is only released by bloodstream forms that are incapable of reoxidizing NADH in the glycosome. However, inhibition of this enzyme by salicylhydroxamic acid (SHAM) was significantly stronger.

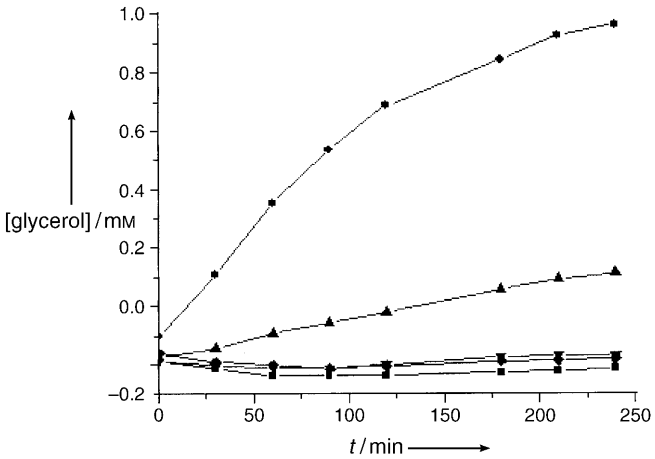

Figure 2. Glycerol production of bloodstream-form *T. brucei* during incubation with compound **4** under culture conditions. Cells were maintained at a density of 2×10^7 mL^{-1}. Solvent-treated control (■), 50 μM salicylhydroxamic acid (●), 120 μM compound **4** (▲), 60 μM compound **4** (▼), 30 μM compound **4** (◆). During incubation with 120 μM compound **4**, production of glycerol can clearly be observed.

Figure 3 shows the relative consumption of oxygen by cells treated with compound **4** in comparison to untreated control cells. Since bloodstream-form trypanosomes lack a functional respiratory chain, oxygen is only consumed by means of TAO.

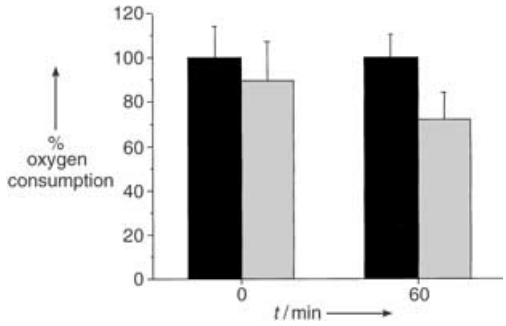

Figure 3. Relative oxygen consumption of bloodstream-form *T. brucei* treated with 120 μM compound **4**. Cells were treated for the indicated duration at a density of 2×10^7 mL^{-1}. After 60 min, a decrease in oxygen consumption of approximately 30% can be measured; this supports the data obtained by the determination of glycerol production.

Thus, the relative decrease in oxygen consumption of treated cells goes with the measured glycerol production, also pointing towards partial inhibition of TAO. Oxygen consumption was reduced by approximately 30%; this supports the value observed for pyruvate production with the same concentration of compound **4** and the same cell density. The high concentration of the compound in relation to its IC_{50} value was required due to the elevated cell density, which was 100-fold higher in the metabolic assays than it was in the trypanocidal activity test.

In conclusion, compound **4** has been shown to exhibit a significant toxic effect on *Trypanosoma brucei* with an IC_{50} of 11 μM; this makes it a good lead compound for further design of structurally related trypanocidal molecules. Moreover, the cytotoxicity assay was carried out by using an incubation period of only 24 h. For reasons of comparability with the IC_{50} of other trypanocidal compounds, it should be noted that some in vitro tests such as the Alamar Blue assay employ incubation times of 72 h and therefore might provide even lower values than 11 μM.[14] This circumstance also applies to the initial compound-screening assays with an incubation period of 21 h. With the result of reduced pyruvate production upon treatment, the parasites' energy metabolism was shown to be affected by the compound. The measured production of glycerol, along with the decrease in oxygen consumption, suggests TAO as one of the cellular targets of compound **4**. On the other hand, even in this case, the relative inhibition value of approximately 30% would not explain the toxic effect of the compound per se. Hence, the initial assumption of TAO as a target of the employed benzodithiine derivatives could only be partially confirmed. Experiments are underway to further characterize its mode of action.

Experimental Section

All chemicals and reagents were obtained from commercial suppliers and used without further purification. Solvents were dried and distilled according to standard procedures. The reactions were monitored by thin-layer chromatography, carried out on 0.25 mm silica gel plates (60 F-254, Merck, Darmstadt, Germany). Plates were visualized under UV light (where appropriate), sprayed with an orcinol/H_2SO_4/$FeCl_3$ solution and heated to develop. Column chromatography was performed on silica gel 60 (0.063–0.200 mm, Merck, Darmstadt, Germany) by using the indicated solvent system. ^1H and ^{13}C NMR spectra were obtained in $CDCl_3$ on a Bruker AC 250 (^1H NMR: 250 MHz, ^{13}C NMR: 63 MHz) or a Bruker WM 400 spectrometer (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz). The chemical shifts are reported in parts per million (ppm) on a δ scale from TMS as internal standard. The EI, FAB, and FD mass spectra were recorded on a Finnigan MAT 312 and 711A mass spectrometer connected to a PDO 11/34 (DEC) computer system. Optical rotations were obtained with an LEP AZ polarimeter (Zeiss, Jena, Germany) at 546 nm. All melting points are uncorrected.

General procedure for the preparation of chiral benzodithiines and methylbenzodithiines: Benzene-1,2-dithiol (2.5 mmol) or 3,4-dimercaptotoluene (2.5 mmol) were added to a suspension of NaH (50 mg, 2.3 mmol, 65% mineral oil) in dry THF (20 mL) at −5 °C under argon. After this mixture had been stirred at the same temperature, a solution of the triflate sugar **1** or **2** (254 mg, 1 mmol) in THF (10 mL) was added, and the resultant mixture was stirred for an additional 2 h. The temperature was allowed to rise to room temperature, and the mixture was stirred until TLC showed completion of the reaction. The mixture was quenched by the addition of saturated NH_4Cl, extracted with EtOAc (3×30 mL), dried over Na_2SO_4, and concentrated under reduced pressure. Purification of the oily residue by column chromatography with 10% petroleum ether/CH_2Cl_2 yielded the title compounds.

2,3-Dihydro(benzyl 3′,4′-dideoxy-α-D-arabinopyranoso)-[4′,3′-*b*]-1,4-benzodithiine (3): White solid, 315 mg (91% yield); m.p. 148–149 °C; $[\alpha]_D^{20} = -163.1°$ ($c = 0.08$, CH_2Cl_2); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 7.22–7.18$ (m, 5H; C_6H_5), 7.16–6.84 (m, 4H; H-7, H-8, H-9, H-10), 4.76 (d, $J = 11.5$ Hz, 1H; OC*H*HPh), 4.44 (d, $J = 11.5$ Hz, 1H; OCH*H*Ph), 4.31 (d, $J = 6.1$ Hz, 1H; H-1), 4.02 (dd, $J = 3.3$, 9.1 Hz, 1H; H-5), 3.72 (ddd, $J = 2.7$, 3.3, 10.1 1H; H-4), 3.69(dd, J=6.5, 9.7 Hz, 1H; H-2), 3.56 (dd, $J = 3.3$, 7.6 Hz, 1H; H-3), 3.27 (dd, $J = 3.6$, 8.95 Hz, 1H; H-5′), 2.48 ppm (brs, 1H; OH); ^{13}C NMR ($CDCl_3$, 63 MHz): $\delta = 125.8–129.5$, (C_6H_5, C-7–10), 102.8 (C-1), 70.6 (OCH_2Ph), 70.0 (C-2), 65.9 (C-5), 47.6 (C-3), 44.6 (C-4); HR FAB MS calcd for $C_{18}H_{18}O_3S_2$: 346.4689 $[M+H]^+$, found: 346.4725.

2,3-Dihydro(benzyl 3′,4′-dideoxy-β-L-arabinopyranoso)-[4′,3′-*b*]-1,4-benzodithiine (5): White solid, 301 mg (87% yield); m.p. 128–130 °C; $[\alpha]_D^{20} = 170.2°$ ($c = 0.10$, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 7.20–7.13$ (m, 5H; C_6H_5), 7.09–6.80 (m, 4H; H-7, H-8, H-9, H-10), 4.86 (d, $J = 3.3$ Hz, 1H; H-1), 4.63 (d, $J = 11.5$ Hz, 1H; OC*H*HPh), 4.40 (d, $J = 11.5$ Hz, 1H; OCH*H*Ph), 4.05 (dd, $J = 2.75$, 12.25 Hz, 1H; H-5), 3.99 (dd, $J = 3.3$, 8.5 Hz, 1H; H-2), 3.63–3.53 (m, 2H; H-4, H-5′), 3.45 (dd, $J = 2.4$, 6.1 Hz, 1H; H-3), 2.10 ppm (brs, 1H; OH); ^{13}C NMR ($CDCl_3$, 63 MHz): $\delta = 129.0–125.5$ (C_6H_5, C-7–10), 97.6 (C-1), 70.0 (OCH_2Ph), 68.6 (C-2), 62.2 (C-5), 44.0 (C-3), 43.9 (C-4); HR FAB MS calcd for $C_{18}H_{18}O_3S_2$: 346.4689 $[M+H]^+$, found: 346.4685.

6-Methyl-2,3-dihydro(benzyl 3′,4′-dideoxy-α-D-arabinopyranoso)-[4′,3′-*b*]-1,4-benzodithiine (4): White solid, 259 mg (72% yield); m.p. 96–97 °C; $[\alpha]_D^{20} = -47.2°$ ($c = 0.18$, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz,): $\delta = 7.87–7.83$ (m, 1H; H-10), 7.35–7.25 (m, 5H; C_6H_5), 7.17–7.08 (m, 2H; H-7, H-8), 4.88 (d, $J = 11.5$ Hz, 1H; OC*H*HPh), 4.56 (d, $J = 11.9$ Hz, 1H; OCH*H*Ph), 4.43 (d, $J = 6.6$ Hz, 1H; H-1), 4.13 (dd, $J = 2.2$, 5.7 Hz, 1H; H-5), 3.85 (dd, $J = 6.4$, 5.7 Hz, 1H; H-5′), 3.78 (dd, $J = 2.6$, 9.2 Hz, 1H; H-2), 3.67 (ddd, $J = 3.1$, 7.5, 4.0 1H; H-4), 3.36 (m, 1H; H-3), 2.24 (s, 3H; CH_3) 2.58 ppm (brs, 1H; OH); ^{13}C NMR ($CDCl_3$, 100 MHz,): $\delta = 136.9–127.8$, (C_6H_5), 127.0 (C-7–10), 126.8, 126.7, 102.8 (C-1), 70.4 (OCH_2Ph), 69.6 (C-2), 65.7 (C-5), 47.5 (C-3), 44.5 (C-4), 21.0 (CH_3); HR FAB MS calcd for $C_{19}H_{20}O_3S_2$: 360.4959 $[M+H]^+$, found: 360.4950.

6-Methyl-2,3-dihydro(benzyl 3′,4′-dideoxy-β-L-arabinopyranoso)-[4′,3′-*b*]-1,4-benzodithiine (6): White solid, 273 mg (76% yield); m.p. 140–141 °C; $[\alpha]_D^{20} = 54°$ ($c = 0.12$, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz,): $\delta = 7.37–7.28$ (m, 5H; C_6H_5), 7.13–6.82 (m, 3H; H-7, H-8, H-10), 4.79 (d, $J = 11.4$ Hz, 1H; OC*H*HPh), 4.56 (d, $J = 11.4$ Hz, 1H; OCH*H*Ph), 4.36 (d, $J = 3.3$ Hz, 1H; H-1), 4.20 (dd, $J = 2.6$, 12.3 Hz, 1H; H-5), 4.03 (dd, $J = 3.1$, 9.7 Hz, 1H; H-2), 3.85 (dd, $J = 2.2$, 5.7 Hz, 1H; H-5′), 3.77–3.58 (m, 1H; H-3,4, 5′), 3.67 (ddd, $J = 3.1$, 7.5, 4.0 Hz, 1H; H-4), 2.58 (brs, 1H; OH), 2.24 ppm (s, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 136.8–125.1$ (C-7–10, C_6H_5), 97.5 (C-1), 70.0 (OCH_2Ph), 68.2 (C-2), 62.2 (C-5), 44.4 (C-4), 44.0 (C-3), 20.7 (CH_3); HR FAB MS calcd for $C_{19}H_{20}O_3S_2$: 360.4959 $[M+H]^+$, found: 360.4942.

Parasites: *Trypanosoma brucei* MITat 1.2 (VSG-variant 221) of the monomorphic strain EATRO 427 were used throughout the experiments. Bloodstream forms were grown in axenic culture at 37 °C and 5% CO_2.[15,16] For the experiments described here, parasites

were taken from culture stabilates and seeded at a cell density of 2×10^5 cells mL^{-1}, then grown for about 18 h to a final cell density of 8×10^5 cells mL^{-1}. By adding fresh medium, the cell density was adjusted to 2×10^5 trypanosomes per mL, and the medium was divided among the desired number of individual culture flasks. For metabolite determinations, trypanosomes were grown to a density of 10^6 cells per mL in a preculture step and used at a cell density of 2×10^7 cells per mL for further studies.

Oxygen electrode: Consumption of oxygen was measured with a Clark electrode.[17] A total number of 1×10^7 cells were spun down (3000 rpm, 4 °C, 5 min) and washed twice with electrode buffer (20 mM phosphate buffer, pH 7.4, 5 mM KCl, 80 mM NaCl, 2 mM $MgCl_2$, 0.15% bovine serum albumin, 5 mM glucose). The Clark electrode was equilibrated with electrode buffer (332 μL) before cells (1×10^7 in 20 μL) were added, and O_2 consumption was detected. Inhibition was achieved by addition of SHAM (4 μL, 50 mM).

Determination of glycolytic metabolites: Glucose, pyruvate, and glycerol were determined by using standard assays from Roche. Briefly, aliquots of culture medium (60 μL) were withdrawn at denoted times and immediately precipitated by using perchloric acid (70%) to yield a final concentration of 5%. Appropriate enzymes (hexokinase/glucose 6-phosphate dehydrogenase, lactate dehydrogenase, glycerol kinase/glycerol-3-phosphate dehydrogenase) were used to determine the amounts of NADPH, NAD, or NADH, respectively. Final measurements were performed on an ELISA plate reader (MRX TC Revelation from Dynex, Dettenhofen, Germany). Chemicals were bought from Roche or from Sigma.

Cytotoxicity assay: Cytotoxicity due to compound **4** was measured by the release of acid phosphatase from lysed cells by using a modification of the assay described previously.[18] Trypanosomes were grown from culture stabilates, as described above. For the toxicity assay, they were resuspended in culture medium without phenol red at a density of 2×10^5 cells mL^{-1} and transferred to a 96-well plate in aliquots of 200 μL for each well. At this point, compound **4** (1 μL per well) was added at the denoted concentrations. After an incubation time of 36 h under culture conditions, lysis buffer (20 μL, 10 mg mL^{-1} *p*-nitrophenylphosphate, 1 M NaAc, 1% Triton® X-100 *v*/*v*) was added, and the lysate was incubated for another 6 h. Afterwards, the absorbance at $\lambda = 405$ nm was measured in an ELISA reader (MRX TC Revelation from Dynex, Dettenhofen, Germany).

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Keywords: antibiotics · benzodithiine · oxidases · sugars · *Trypanosoma brucei*

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