Identification of Isoflavone Derivatives as Effective Anticryptosporidial Agents in Vitro and in Vivo

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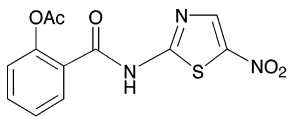
We report the preparation and antiparasitic activity in vitro and in vivo of a series of isoflavone derivatives related to genistein. These analogues retain the 5,7-dihydroxyisoflavone core of genistein: direct genistein analogues (2-H isoflavones), 2-carboethoxy isoflavones, and the precursor deoxybenzoins were all evaluated. Excellent in vitro activity against *Cryptosporidium parvum* was observed for both classes of isoflavones in cell cultures, and the lead compound **19**, RM6427, shows high in vivo efficacy against an experimental infection.

Introduction

Although a number of new antiparasitic drugs[1] have become available over the past decade, with specifically mefloquine,[2] albendazole,[3] ivermectin,[4] atovaquone−proguanil,[5] and nitazoxanide[6] having been approved in the United States, there is still a perceived need for effective new agents. Without question, intestinal parasitic infections remain a leading cause of mortality, especially in developing countries, and emerging and resistant intestinal protozoa pose a wider threat. In the case of *Cryptosporidium parvum*, especially, significant outbreaks occur in the Western world and there is a lack of effective therapies, as discussed in a recent article.[7]

The nitrothiazole derivative nitazoxanide **1**,[8] now approved for both human and veterinary use in the United States and already extensively used in Latin America, has excellent broad-spectrum activity against a wide range of intestinal protozoa and helminths.[9] It therefore represents a clear benchmark for any new antiparasitic agent: in addition it possesses highly significant antibacterial[10] and antiviral[11] activity. There could be advantages, nevertheless, in a very active but highly specific antiparasitic agent. Absence of other activities, notably antibacterial, could avoid the development of further resistant bacterial strains—a problem familiar from the overuse of antibacterial agents, especially penicillins.



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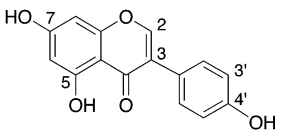

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We now report the synthesis and biological evaluation of a selection from a series of isoflavone derivatives having excellent antiparasitic activity in vitro but lacking antibacterial activity. Our starting point was the naturally occurring isoflavone genistein **2**,[12] which has been extensively studied as a potential therapeutic agent. In particular, genistein is known to be an inhibitor of protein Tyr kinases[12] found in epidermal growth factor receptors (EGRFs). Since the EGRF peptides can mediate host−parasite infections, they appear to be potential targets in antiparasitic chemotherapy, and genistein therefore became a logical starting point.

Chemistry

Condensation of phloroglucinol **3** with commercial arylacetonitriles **4**−**7**, catalyzed by $ZnCl_2$ and gaseous HCl (Hoesch reaction),[13] afforded the open-chain ketones ("deoxybenzoins") **8**−**11** (Scheme 1). It proved more reliable to use a preformed solution of $ZnCl_2$ in Et_2O. The first products were imines, which without isolation were hydrolyzed to ketones **8**−**11** with aq HCl using a cosolvent (ethanol, THF) for solubility if required. This step was often slow: careful TLC monitoring was important. We found later that addition of EDTA often accelerated this step, presumably by complexation of remaining traces of Zn^{2+}.

An alternative was the direct condensation of arylacetic acids with phloroglucinol **3** catalyzed by $BF_3 \cdot Et_2O$,[14] affording ketones akin to **8**−**11** directly. We found this procedure worked very well with aryl groups containing electron-donating substituents, e.g., 4-methoxyphenylacetic acid, but most of the substituents of interest to us were electron-withdrawing, and the corresponding acids did not react satisfactorily.

C-formylation of ketones **8**−**11** followed by closure to 2-H isoflavones **12**−**15** could be achieved in one pot using Vilsmeier conditions,[15] by the use of acetic formic anhydride[16] or using DMF dimethyl acetal[17] (Scheme 1).The Vilsmeier conditions employing methanesulfonyl chloride ($MeSO_2Cl$) activation reported by Bass[18] worked very well provided *freshly distilled* $MeSO_2Cl$ was used. In cases where a 2′-substituent was present it was sometimes difficult to remove traces of solvent DMF from the products, but there were no such difficulties with compounds **12**−**15**.

Alternatively, condensation of ketones **8**−**11** with ethyl oxalyl chloride using K_2CO_3 as base with a phase-transfer catalyst[19,20] afforded the 2-carboethoxyisoflavones **16**−**19** (Scheme 1). We found later that the use of a phase-transfer catalyst was unnecessary, and yields, though variable, approached 80% in favorable cases. Here again it was important to use *freshly distilled* ethyl oxalyl chloride for good yields. In some cases a

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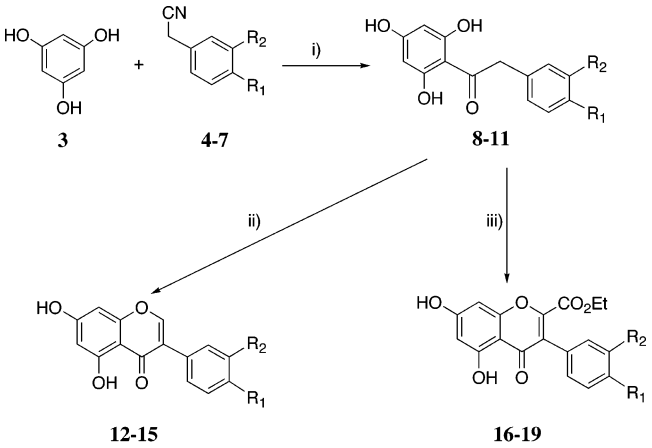
Scheme 1[a]

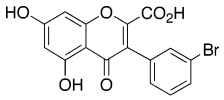


4, 8,12, 16: $R_1 = NO_2$, $R_2 = H$
5, 9, 13, 17: $R_1 = H$, $R_2 = NO_2$
6, 10, 14, 18: $R_1 = Br$, $R_2 = H$
7, 11, 15, 19: $R_1 = H$, $R_2 = Br$

[a] Reagents, conditions: (i) anhydrous HCl, $ZnCl_2$–Et_2O, then aq HCl, heat; (ii) $MeSO_2Cl$, Me_2N•CHO, aq workup; (iii) ClCO•CO_2Et, $^nBu_4N^+Br^-$, acetone, K_2CO_3, heat.

little overreaction occurred by acylation of the phenols giving carbonates, separable by chromatography.

Esters **16**−**19** are not simply prodrugs for the free carboxylic acids. Thus, we prepared the free acid form **20** of compound **19** by hydrolysis of **19** with aq NaOH followed by acidification with an acidic ion-exchange resin, then evaporation and recrystallization of the residue. The activity of **20** was significantly less than that of **19**: see below.



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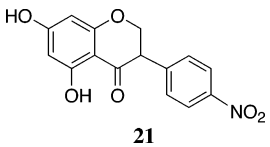
We also evaluated a partially reduced analogue. Thus, reduction of the 2,3-double bond in **12** without reduction of the aryl nitro group was achieved using $NaBH_4$ in 2-propanol: the saturated ketone **21** resulted in good yield as evinced by a characteristic A_2X pattern in the ^1H NMR spectrum. Here again, **21** was less active than **12** against *Cryptosporidium parvum*: see below.



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Spectroscopic analysis of these derivatives was generally straightforward. In particular, the open-chain ketones **8**−**11** showed characteristic two-proton singlets at around δ 3.0 for the ArCH_2CO protons, while the 3- and 5-protons of the phloroglucinol moiety resonated as a singlet at around δ 6.5. After cyclization, the 3- and 5-protons resonated as two narrow one-proton doublets in both the 2-H and 2-CO_2Et isoflavone classes. The 2-H proton in isoflavones **12**−**15** resonated as a sharp singlet around δ 8.0.

Biology: In Vitro Activity

The activity of compounds **8**−**19**, **20**, and **21** against the coccidian parasite *Cryptosporidium parvum* grown in cell cultures is shown in Table 1. A fuller account of the biological activity of these and related isoflavones has been published elsewhere.[21] The data are presented to show mean IC_{50} values (micromolar) for each compound as measured in a specified number of determinations. Nitazoxanide **1** has been added to the Table as a reference compound effective against *C. parvum*.

Table 1. Antiparasitic Activities of Compounds **8**−**19**, **20**, and **21** Expressed as IC_{50} Values (μM), Number of Observations in Brackets[a]

compound	*Cryptosporidium parvum*
8	70.8 (2)
9	464.5 (2)
10	15.9 (2)
11	17.2 (2)
12	1.8 (2)
13	0.3 (2)
14	1.3 (2)
15	0.4 (3)
16	64.1 (2)
17	15.8 (2)
18	0.6 (3)
19	1.6 (3)
20	20.1 (3)
21	11.7 (2)
nitazoxanide **1**	3.25 ± 0.85

[a] The strain of parasite used is given in the Experimental Section data.

It will be seen that in general the open-chain ketones **8**−**11** were significantly less active in this assay. Also 2′-substituted analogues, not shown, were less active. From the isoflavones, the 2-H, 3′- and 4′-NO_2 derivative **12** and **13** and the 4′-Br and 3′-Br compounds in both 2-H and 2-CO_2Et series, viz., **14**, **15**, **18**, and **19**, exhibited excellent activity. Furthermore, when checked for regrowth of the parasite after up to 7 days these assays still proved negative. We have selected compounds **18** and **19** as promising candidates for further evaluation in comparison with nitazoxanide.

The specificity of activity against *C. parvum* is striking. Compounds **18** and **19** have also shown useful activity against the microsporidian species *Vittaforma corneae* and *Encephalitozoon intestinalis* with IC_{50} of ca. 10 μg/mL (E. Didier, personal communication). However, when we assayed compounds **8**−**19**, **20**, and **21** against *Trypanosoma brucei*, *Trichomonas vaginalis*, and *Giardia lamblia*, very little activity was seen. Compounds **12** and **14** showed slight activity against *T. brucei* (IC_{50}s from 2 to 20 μM) and similar figures against *G. lamblia*; also **12** showed weak activity against *T. vaginalis*, $IC_{50} = 8$ μM. The remaining compounds were inactive. Against a chloroquine-sensitive strain of the malaria parasite *Plasmodium falciparum*, compound **12** showed weak activity ($IC_{50} = 20$ μM); the other compounds were inactive.

In Vivo Activity

The activity of the leading compounds **18** and **19** was studied in vivo against immunosuppressed gerbils, *Meriones unguiculatus*, infected with *Cryptosporidium parvum*. Immunosuppression was achieved by injection with dexamethasone over a 10 day period, and *C. parvum* was administered by oral inoculation (see the Experimental Section). The drugs were administered orally twice daily: compound **19** was administered for 8 days consecutively, **1** and paromomycin for 12 days (see the Experimental Section). In addition dexamethasone was administered until the end of the experiment (12 day point). Compound **19**, nitazoxanide **1**, and paromomycin were used in direct comparison, and statistical significance was demonstrated ($P < 0.05$ considered significant).

The results (Table 2) show essentially the same effectiveness for **19** at doses of either 200 or 400 mg/kg/day; **1** was tested at

Table 2. In Vivo Activity of Compounds **18** and **19** versus Reference Compounds against *C. Parvum* Infection

compound	dose mg/kg/day (no. of days)	animals deparasitized at day 8	mean oocyst reduction in stool at day 8 (%)	*P* value (vs control)
19	200 (8)	6/10 (60%)	90.5	<0.0001
19	400 (8)	6/10 (60%)	92	<0.0001
1	200 (12)	2/14 (14%)	55	<0.0008
Paromomycin	100 (12)	2/14 (14%)	17.6	NS
18	400 (12)	2/15 (13%)[a]	53.5[b]	0.006[c]
	controls	0/10	0	

[a] At day 12. [b] 67% at day 12. [c] $P = 0.018$ at day 12.

200 mg/kg/day and paromomycin at 100 mg/kg/day. Both **19** (at either dose) and nitazoxanide **1** showed statistically significant reduction of oocyst shedding with $P < 0.0001$ and $P < 0.0008$, respectively. Under these conditions paromomycin did not show significant inhibition compared to that of controls. Most significantly, **19** totally inhibited oocyst shedding in 6/10 gerbils compared to 2/14 for **1** and paromomycin. In summary, after 8 days, **19** was shown to be clearly superior to the other agents (Table 2), with 60% of animals fully deparasitized and a mean oocyst reduction in stools of over 90%. In comparison, compound **18** was less effective than **19** in vivo, despite its superior MIC value in vitro. Finally we note that these compounds have been shown not to be cytotoxic at the dose used.[21]

Experimental Section

Chemistry. Organic extracts were washed finally with saturated aq NaCl and dried (over anhydrous Na_2SO_4 unless otherwise stated) prior to rotary evaporation at <30 °C. Analytical thin-layer chromatography was performed using Merck Kieselgel 60 F 254 silica plates. Preparative column chromatography was performed on Merck 938S silica gel. Infra-red spectra were recorded for Nujol mulls on a Perkin Elmer RX1 FT1R instrument. Unless otherwise stated, ^{1}H and ^{13}C NMR spectra were recorded on $CDCl_3$ solutions using either Bruker 250 or 400 MHz instruments with tetramethylsilane as internal standard. Mass spectra in the chemical ionization (CI) mode were obtained using a VG7070E mass spectrometer. Both low- and high-resolution electrospray mode (ES) mass spectra were obtained using a Micromass LCT mass spectrometer operating in the +ve or −ve ion mode as indicated.

Typical Procedure for Open-Chain Ketones: 4′-Nitro-2,4,6-trihydroxydeoxybenzoin (8). Zinc chloride (3.56 g, 26.2 mmol) was added to an ice-cold solution of phloroglucinol **3** (3.00 g, 23.8 mmol) and 4-nitrophenylacetonitrile **4** (2.62 g, 16.2 mmol) in ether (100 mL) at 0 °C, then dry HCl gas was passed through the solution for 4 h with continuous stirring. Passage of the gas was discontinued, and the mixture was stirred for a further 16 h at 20 °C, then the mixture was filtered and the solid dissolved in 1 M HCl (2 equiv) and heated at reflux for 3 h. The solution was cooled and filtered, then the filtrate was warmed with a solution of EDTA, sodium salt (4.43 g, 11.9 mmol) in water for 3 h. The solid was filtered off while hot and washed with warm water, then heated at reflux in 1 M HCl for 5 h. The resulting solid was filtered off, washed with water, and dried overnight to give the product **8**[22] (2.39 g, 51%); mp 212−214 °C (lit.[23] mp 250−252 °C). Anal. ($C_{14}H_{11}NO_6$) C, H, N. ^{1}H NMR (250 MHz, $CDCl_3$): 4.64 (2 H, s, Ar CH_2), 6.03 (2 H, s, ArH), 7.49 and 8.20 (4 H, dd, ArH). ^{13}C NMR (100 MHz, $(CD_3)_2SO$): 49.2, 95.1(×2), 104.1(×2), 123.4-(×2), 144.7, 146.6, 164.6(×3), 165.5 and 201.3. *m*/*z* (ES −ve mode): 289 (M^+, 10%), 288 (100%), and 244 (10%). High-resolution mass (ES) calculated for $C_{14}H_{10}NO_6$ $[M - H]^+$: 288.0502. Found: 288.0508.

Similarly Prepared Were Compounds 9, 10, and 11 (See the Supporting Information). Typical Procedure for 2-H Isoflavones: 5,7-Dihydroxy-3-(3-Nitrophenyl)-chromen-4-one (13). $BF_3{\cdot}Et_2O$ (0.175 mL, 0.196 g, 1.38 mmol, 4 equiv) was added dropwise to a solution of deoxybenzoin **8** (0.100 g, 0.346 mmol, 1 equiv) in anhydrous DMF (1 mL). A solution of methanesulfonyl chloride (0.085 mL, 0.126 g, 1.104 mmol, 3 equiv) in anhydrous DMF (0.3 mL) was added to the mixture, which was heated on a steam bath for 1.5 h, cooled, and poured into water. The crude product was filtered off and recrystallized by adding as a hot THF solution to water and cooling. Filtration afforded pure **13** (0.077 g, 74%); mp 266−268 °C (THF aq). Anal. ($C_{15}H_9NO_6$) C, H, N. ^{1}H NMR (400 MHz, CD_3OD): 6.21 (1 H, s, ArH), 6.34 (1 H, s, ArH), 7.64 (1 H, dd, $J = 8.3$ and 5.1 Hz, ArH), 7.93 (1 H, dd, $J = 9.3$ and 5.1 Hz, ArH), 8.20 (1 H, dd, $J = 9.3$ and 8.3 Hz, ArH), 8.25 (1 H, s, ArH) and 8.45 (1 H, s, 2-H). *m*/*z* (CI, NH_3): 300 (MH^+, 100%) and 270 (50%). High-resolution mass (ES) calculated for $C_{15}H_{10}NO_6$ (MH^+): 300.0508. Found: 300.0516.

Similarly Prepared Were Compounds 12, 14, and 15 (See the Supporting Information) (Refs 24, 25). Typical Procedure for 2-Carboethoxyisoflavones: 3-(3-Bromophenyl)-5,7-dihydroxy-4-oxo-4H-chromene-2-carboxylic Acid, Ethyl Ester (19). Freshly distilled ethyl chlorooxoacetate (1.14 mL, 10.2 mmol, 4 equiv) was added dropwise to a stirred mixture of **11** (0.824 g, 2.55 mmol, 1 equiv) and K_2CO_3 (2.11 g, 15.3 mmol, 6 equiv) in anhydrous acetone (50 mL) under N_2. The reaction mixture was stirred at room temperature for 2 h and then heated to 50 °C for a further 16 h. The solvent was removed, then the residue was extracted into ethyl acetate and the base neutralized with aqueous citric acid (20%). The organic layer was separated, washed with water and brine, and dried over $MgSO_4$ before removal of the solvent under reduced pressure to afford a brown oil, which was purified by column chromatography eluting with ethyl acetate−hexane (3:7). Recrystallization from ethyl acetate−hexane yielded **19** as bright yellow crystals (0.538 g, 52%), mp 202−203 °C. Anal. ($C_{18}H_{13}BrO_6$) C, H. ν_{max} cm^{-1} 1738 and 1710. ^{1}H NMR (400 MHz, CD_3OD): 1.05 (3 H, t, $J = 7.1$ Hz, CH_3CH_2O), 4.16 (2 H, q, $J = 7.1$ Hz, CH_3CH_2O), 6.28 (1 H, d, $J = 1.7$ Hz) and 6.42 (1 H, d, $J = 1.7$ Hz, 6-H and 8-H), 7.26 (1 H, d, $J = 7.6$ Hz, 6′-H), 7.35 (1 H, t, $J = 7.6$ Hz, 5′-H), 7.48 (1 H, t, $J = 1.6$ Hz, 2′-H) and 7.59 (1 H, d, $J = 7.6$ Hz, 4′-H).^{13}C NMR (100 MHz, $(CD_3)_2SO$): 13.6, 62.6, 94.4, 99.9, 104.6, 121.2, 123.8, 129.4, 130.3, 131.2, 132.8, 133.5, 150.2, 157.1, 160.3, 161.9, 165.8, and 180.7. *m*/*z* (CI, NH_3) 405 (100%, MH^+). High-resolution mass (CI) calculated for $C_{18}H_{14}{}^{79}BrO_6$ (MH^+): 404.99738. Found: 404.99786.

Similarly Prepared Were Compounds 16, 17, and 18 (See the Supporting Information) (Refs 19, 25). 3-(3-Bromophenyl)-5,7-dihydroxy-4-oxo-4H-chromene-2-carboxylic Acid (20). 1 M NaOH (8.2 mL, 2.6 equiv) was added in one portion to a stirred solution of ester **19** (1.90 g, 4.7 mmol) in acetone (70 mL) at 20 °C. After 1 h Amberlite IR-120 (H^+: 4.3 g, 8.2 mmol) was added, and after stirring for 15 min, the resin was filtered off and the filtrate was evaporated to dryness. The residual oil was partitioned between EtOAc and 5% aq citric acid, then the organic layer was separated, washed with water and brine, dried ($MgSO_4$), and evaporated to give a yellow oil which on recrystallization from EtOAc−hexane afforded the title compound **20** (1.18 g, 68%), mp 223−224 °C. Anal. ($C_{16}H_9BrO_6$) C, H. ^{1}H NMR ($(CD_3)_2SO$, 400 MHz): 3.83, 3.92 (2 H, 2 s, D_2O exch., 2×OH), 6.56, 6.75 (2 H, 2 d, $J = 2.2$ Hz, 6-H and 8-H), 7.22 (1 H, d, $J = 7.7$ Hz, 6′-H), 7.34 (1 H, t, $J = 7.8$ Hz, 5′-H), 7.43 (1 H, t, $J = 1.7$ Hz, 2′-H) and 7.55 (1 H, approximately dd, $J = 7.8$ and 1.9 Hz, 4′-H). ^{13}C NMR (100 MHz, CD_3OD): 94.1, 99.6, 104.8, 123.2, 129.0, 129.6, 131.1, 132.9, 133.4, 152.2, 157.6, 162.2, 162.4, 165.9, and 181.2. *m*/*z* (CI, NH_3) 377 (100%, MH^+). High-resolution mass (CI) calculated for $C_{16}H_{10}BrO_6$ (MH^+): 376.96606. Found: 376.96630.

5,7-Dihydroxy-3-(4-nitrophenyl)-chroman-4-one (21). Sodium borohydride (0.014 g, 0.36 mmol) was added to an ice-cold solution of **12** (0.100 g, 0.33 mmol) in THF (6 mL) and 2-propanol (4 mL). After 1 h the solution was quenched by addition of 20% aq citric acid (final pH 6−7), then the solution was evaporated and the residue was dissolved in ethyl acetate, washed with water and brine, dried ($MgSO_4$), and evaporated to give an orange solid. Chromatography, eluting with EtOAc−hexane, 3:7, afforded on pooling and evaporation of appropriate fractions the title compound **21**

(0.097 g, 98%), mp 194−196 °C. Anal. ($C_{15}H_{11}NO_6$) C, H, N. υ_{max} cm^{-1} 1644 and 1525 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): 4.20 (1 H, t, 3-H), 4.63 (2 H, d, 2 × 2-H), 5.93 (2 H, d, ArH), 7.57 and 8.21 (4 H, dd, ArH). ^{13}C NMR (100 MHz, CD_3OD): 52.4, 72.2, 96.6, 97.9, 103.7, 125.1, 131.4, 145.0, 149.3, 165.0, 166.4, 169.2, and 196.7. *m*/*z* (CI, NH_3): 302 (MH^+, 12%), 289 (12%), and 272 (100%). High-resolution mass (CI) calculated for $C_{15}H_{12}NO_6$ (MH^+): 302.06647. Found: 302.06649.

Biological Activity. In Vitro Antiparasitic Assays. Anticryptosporidial Screen (Professor L. Favennec and Colleagues). Human epithelial cells HCT-8 were obtained from the ATCC collection and seeded in 16-well tissue culture chambers. Sporozoites of *C. parvum* were obtained from oocysts of experimentally infected calves and used to infect the above HCT-8 cells by addition of 50 mL of freshly excysted sporozoite medium (inoculum 2.5 × 10^5 mL^{-1}) to each well. After incubation for 2 h at 37 °C, medium containing nonpenetrated sporozoites was removed and replaced with either 200 μL of medium containing test compound at a defined concentration or medium only. Developing parasites were detected by immunofluorescence using a reported procedure.[26] Following addition of each synthetic compound **8**−**19**, **20**, or **21**, the effect of treatment was defined by the percent reduction in development (treated values vs control).

Other Parasite Screens. The incorporation of tritiated thymidine or hypoxanthine as a measure of parasite viability, reported by Adagu et al.,[27] was employed in the assessment of the biological activity of the compounds against *T. brucei* (strain 427), *T. vaginalis* (strain UCH-1), *G. lamblia* (strain JKH-1), and *P. falciparum* (clone T9-96). To convert the anticryptosporidial data from percent growth inhibition to IC_{50} values, as in Table 1, the "logit" transformation was used.[28]

In Vivo Testing. Male and female gerbils (*Meriones unguiculatus*) weighing 22−27 g were immunosuppressed by injection with dexamethasone at 0.8 mg/kg every 2 days for 10 days before oocyst injection. Prior to the start of treatment, each gerbil was inoculated orally by gavage with 10^5 *C. parvum* oocysts. Compound **19**, nitazoxanide **1**, and paromomycin were suspended in 5% carboxymethylcellulose and administered twice daily orally, under a constant volume of 20 mL/kg, commencing 4 h after infection: **19** for 8 consecutive days, **1** and paromomycin for 12 days.

Cryptosporidium infection was assessed by measuring oocyst shedding in feces collected for 24 h from each animal on days 4, 8, and 12 post infection. Feces were suspended in 10% (w/v) formalin solution and homogenized, and oocysts were counted by phase contrast microscopy examination of smears prepared by mixing fecal suspensions with a carbol fuchsine solution. Oocyst numbers were expressed per microscopic field at a magnification of 400.

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Supporting Information Available: Spectroscopic data for compounds **9**−**12** and **14**−**18**; analytical data for target compounds **8**, **13**, and **19**−**21**. This material is available free of charge via the Internet at http://pubs.acs.org.

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