Antiprotozoal Compounds from *Asparagus africanus*

H. A. Oketch-Rabah and S. F. Dossaji

Phytochemistry Department, National Museums of Kenya, P. O. Box 40658, Nairobi, Kenya

S. Brøgger Christensen, Karla Frydenvang, and Else Lemmich*

Department of Medicinal Chemistry, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark

Claus Cornett

Department of Analytical and Pharmaceutical Chemistry, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark

Carl E. Olsen

Chemistry Department, Royal Veterinary and Agricultural University, Thorvaldsensvej, DK-1871 Frederiksberg, Denmark

Ming Chen† and Arsalan Kharazmi

Department of Clinical Microbiology and Center for Medical Parasitology, University Hospital 7806, Tagensvej 20, DK-2200 Copenhagen N, Denmark

Thor Theander

Center for Medical Parasitology, Institute of Medicinal Microbiology and Immunology, Copenhagen University, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark

Received April 25, 1997[⊗]

Two antiprotozoal compounds have been isolated from the roots of *Asparagus africanus* Lam. (Liliaceae), a new sapogenin, 2β,12α-dihydroxy-(25*R*)-spirosta-4,7-dien-3-one (**1**), which was named muzanzagenin, and the lignan (+)-nyasol (**2**), (*Z*)-(+)-4,4′-(3-ethenyl-1-propene-1,3-diyl)-bisphenol. The structure of the sapogenin was elucidated by MS and by 1D and 2D NMR methods and established by a single crystal X-ray analysis. (+)-Nyasol potently inhibits the growth of *Leishmania major* promastigotes, the IC_{50} being 12 μM, and moderately inhibits *Plasmodium falciparum* schizonts with the IC_{50} 49 μM. These concentrations only moderately affect the proliferation of human lymphocytes. Muzanzagenin showed a moderate in vitro activity in all three tests, the IC_{50} against leishmania promastigotes was 70 μM, and against four different malaria schizont strains the IC_{50} values were 16, 163, 23, and 16 μM, respectively.

Leishmaniases and malaria are protozoal diseases that are responsible for widespread morbidity and mortality. Protozoa of the genus *Leishmania* cause a complex of diseases ranging from self-healing cutaneous to fatal visceral leishmaniases in many parts of Africa, Asia, and Latin America.[1] It is estimated that more than 12 million people are infected with these parasites, and approximately 350 million people are at risk of being infected.[2] The development of resistance to the first choice drugs, pentavalent antimonials, has created a desperate need for new drugs for treatment of leishmaniases.[3,4]

Malaria is one of the worlds most serious health problems, endemic in parts of Africa, Asia, and Latin America.[5] An estimated 300−500 million people are infected. In Africa alone, the disease is estimated to be responsible for the death of about 1 million children yearly.[6] The spread of drug resistant strains of *Plasmodium falciparum*, which causes the most serious form of malaria, steadily increases the problem of controlling the disease.[6,7] Thus, there is an urgent need for new drugs.

Asparagus africanus Lam. (Liliaceae), a scrambling, woody shrub found in many districts of Kenya[8,9] has been used for centuries as a traditional drug.[10] The Akamba tribe in Kenya uses the roots of this plant to treat splenomegaly, which is one of the clinical manifestations of leishmaniasis infections,[11] or repeated malaria infections. Because both diseases are endemic in this region, this use of *A. africanus* roots encouraged us to submit the root extracts to an in vitro screening for activity against promastigotes of *Leishmania major*. Previous phytochemical investigations have led to isolation of steroidal sapogenins from the genus *Asparagus*,[12] and recently the lignan (+)-nyasol has been isolated from *A. cochinchinensis*.[13] *A. africanus*, however, has not previously been investigated. A bioactivity-guided fractionation of the extract led to the isolation of two compounds, a new steroid sapogenin (**1**), which was named muzanzagenin, and (+)-nyasol (**2**). This paper describes the isolation of the two compounds and the characterization of compound **1** by ^{1}H- and ^{13}C-NMR

* To whom correspondence should be addressed. Phone: +45 35 37 08 50. FAX: +45 35 37 22 09. E-mail: el@charon.dfh.dk.
† In addition, attached to the State Serum Institute, Copenhagen, Denmark.
⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

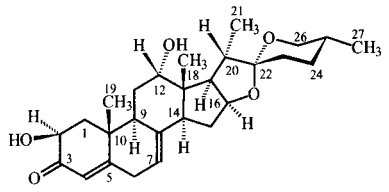
S0163-3864(97)00217-6 CCC: $14.00

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for Compound **1**

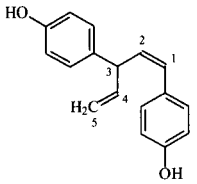
C[a]	δ (ppm)	H[b]	δ (ppm)	J (Hz)
C-1	39.1	H-1α	1.6[c]	
		H-1β	2.42 dd	J_{gem} 13.6; $J_{1\alpha,2\alpha}$ 5.6
C-2	69.1	H-2	4.24 dd	$J_{1\beta,2\alpha}$ 13.8
C-3	199.8			
C-4	118.9	H-4	5.84 d	$J_{4,6\beta}$ 1.6
C-5	170.6			
C-6	33.0	H-6α	2.7 br[d]	
		H-6β	3.20 d, br	J_{gem} 17.8
C-7	116.4	H-7	5.20 dd	$J_{6\alpha,7}$ 5.3; $J_{6\beta,7}$ 2.0
C-8	139.4			
C-9	39.4	H-9	2.86 m	
C-10	46.0[e]			
C-11	30.1	H-11α	1.9 m	
		H-11β	1.9 m	
C-12	71.3	H-12	3.83 m	
C-13	39.4[e]			
C-14	46.5	H-14	2.6 m[d]	
C-15	30.8	H-15α	2.05 ddd	J_{gem} 12.8; J_{vic} 7.4 and 4.6
		H-15β	1.6[c]	
C-16	79.9	H-16	4.46 m	
C-17	53.4	H-17	2.52 dd	$J_{16,17}$ 6.8; $J_{17,20}$ 8.8
C-18	17.1	H-18	0.72 s	
C-19	23.7	H-19	1.17 s	
C-20	42.2	H-20	1.82 m	
C-21	14.4	H-21	1.00 d	$J_{20,21}$ 7.0
C-22	109.3			
C-23	31.4[f]	H-23	1.5−1.8[c]	
C-24	28.8[f]	H-24	1.6[c]	
C-25	30.3	H-25	1.6[c]	
C-26	66.9	H-26eq	3.49 ddd	J_{gem} 10.9; $J_{25ax,26eq}$ 2; $J_{24eq,26eq}$ 1
		H-26ax	3.38 t	J_{gem}= $J_{25ax,26ax}$ 10.9
C-27	17.1	H-27	0.79 d	$J_{25,27}$ 6.4

[a] 100 MHz. [b] 400 MHz. [c,d] Overlapping signals; br = broad signal. [e,f] Values in the same column are interchangeable.

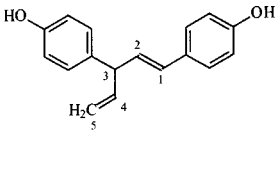
spectral assignments and by an X-ray diffraction study. In addition, the in vitro antiprotozoal properties are reported.



1



2



3

Results and Discussion

An EtOAc extract of the roots of *A. africanus* showed a marked inhibition of the growth of promastigotes of *L. major*. Fractionation of this extract using the in vitro antileishmanial activity as a guide led to the isolation of a new steroidal sapogenin (**1**) as a crystalline compound and of (+)-nyasol (**2**) as a colorless oil, which did not crystallize.

The EIMS of compound **1** showed a base peak at 328 *m/z* and the molecular ion at *m/z* 442. The ^{13}C-NMR signals (Table 1), in combination with the information from a DEPT spectrum, indicated the presence of 27 carbon atoms, including a carbonyl carbon at δ 199.8, two quaternary vinylic carbons at δ 170.6 and δ 139.4, and two tertiary vinylic groups at δ 118.9 and δ 116.4. A signal at δ 109.3 indicated a possibility of an acetalic quaternary carbon. ^{13}C-NMR signals at δ 79.9, 71.3, and 69.1, corresponding to methine carbons and a methylene carbon at δ 66.9, indicated the presence of a number of oxygenated carbons in the structure. Combination of these data with the interpretation of the remaining ^{13}C-NMR signals and with the MS data allowed deduction of the empirical formula $C_{27}H_{38}O_5$.

The total number of carbon atoms and the presence of an acetalic carbon indicated the possibility of a spirostane structure. This was supported by the fragmentation pattern in the EIMS spectrum in which a major peak at *m/z* 139, and others at *m/z* 370, 328, 299, 126, and 115 all are indicative of a spiroketal moiety.[14] In addition, the base peak *m/z* 328, the prominent peak at *m/z* 139, and the peak at *m/z* 126 indicate the absence of other substituents on ring F than the methyl group.[15]

Signals analogous to the ^{13}C-NMR signals originating from C-20 to C-27 of diosgenin were found in the spectrum of **1** and were assigned accordingly.[16,17] Some of the attached protons could be assigned from the ^{1}H−^{13}C HETCOR spectrum, but the signals from the H-23−H-25 protons were overlapping mutually and with other signals (Table 1). The orientation of the C-27 methyl group in compound **1** must be equatorial, since the vicinal coupling $J_{26\alpha,25\beta}$ = 10.9 Hz verified an axial−axial coupling. The ^{1}H-COSY spectrum enabled assignments of H-14 to H-17, using H-20 as an anchoring point. From the positions of the protons, the ^{1}H−^{13}C HETCOR spectrum made possible the assignments of signals for C-14 to C-17 and C-20. The α- and β-protons at C-15 were assigned by means of the NOESY spectrum, as an interaction was seen between H-14 (δ 2.60) and H-15α (δ 2.05) and between the protons attached to C-18 (δ 0.72) and H-15β (δ 1.6). In the ^{1}H-NMR spectrum three separate coupling systems remained. The first of these consisted of the H-1 protons and H-2, which formed an ABX pattern, while the second one consisted of H-4, the two H-6 protons, and H-7. In the NOESY spectrum an interaction was seen between the H-19 (δ 1.17) and the signal at δ 3.20, confirming that this signal originates in H-6β. The third coupling system was formed by H-9, the two H-11, and H-12. The two H-11 protons could not be distinguished as the chemical shift of the two protons are almost identical, resulting in a complex pattern. The coupling constants between H-12 (triplet-like pattern, *J* = 1−2 Hz) in β-position and the two protons at C-11 are very small, in accordance with the influence of an axial OH group at C-12.[18] In the ^{1}H−^{1}H COSY a cross peak between δ 2.86 and δ 5.20 (*J* = 2.0 Hz) and between δ 2.86 and δ 3.20 indicated allylic coupling between H-9 and H-7 and homoallylic coupling between H-9 and H-6β. The corresponding carbons were assigned from the HETCOR spectrum. The protons at the methyl groups C-18 and C-19 were assigned δ 0.79 and δ 1.17, respectively, after comparing the data with other similar compounds[16,17] and using the information from the NOESY spectrum.

An X-ray analysis was performed in order to establish the relative configuration of the isolated compound **1**. The crystal structure contained two independent molecules in the asymmetric unit (labeled A and B). The

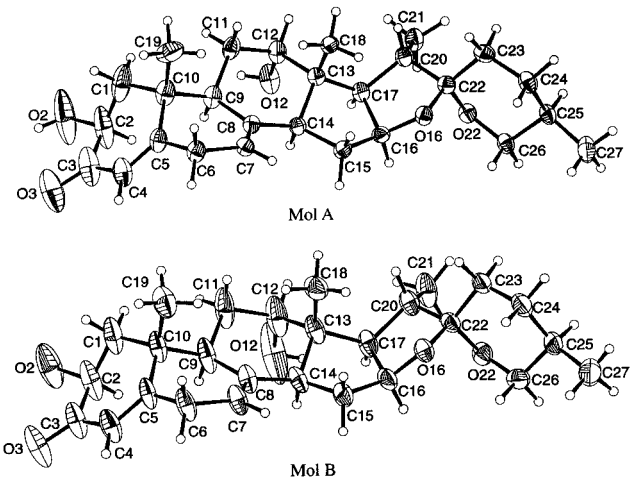


Figure 1. Perspective drawings (ORTEPII[19]) of the two molecules in the asymmetric unit of muzanzagenin (**1**). Displacement ellipsoids of the non-hydrogen atoms are shown at the 50% probability level. Hydrogen atoms are represented by spheres of arbitrary size.

molecular structures of the two molecules are shown in Figure 1. Differences in the conformations of molecules A and B are located in the A, B, and C rings of the steroid skeleton. The torsion angles differ by up to 10°. Some disorder is observed for these rings and especially for the hydroxyl groups, O2 and O12. The disorder is illustrated by the displacement ellipsoids in Figure 1. In the crystal several hydrogen bonds are observed, between hydroxyl groups ($O2_A \cdots O12_B$, $O2_B \cdots O2_A$) and between hydroxyl and carbonyl groups ($O12_A \cdots O3_B$). No hydrogen bond is observed involving the carbonyl group $O3_A$. Residual densities (up to 1.75 eÅ^{-3}) might indicate the presence of solvent molecules located in special positions. The residual densities are near the hydroxyl groups, $O2_B$ and $O12_B$ (ca. 3 Å). The structure of compound **1** was thus established as 2β,12α-dihydroxy-(25*R*)-spirosta-4,7-dien-3-one.

In a recent paper[13] on the isolation of (+)-nyasol, Tsui and Brown concluded, based on comparison with previously published data, that the two naturally occuring lignans hinokiresinol (**3**), obtained from *Chamaecyparis obtusa*,[20] and (−)-nyasol, obtained by hydrolysis of the glycoside nyasoside isolated from *Hypoxis nyasica*,[21] both possess the *Z*-stereochemistry and both should be named nyasol. On the basis of literature studies[22−24] and our own observation, however, we conclude that the two isomers can be distinguished by the following data. The *cis* isomer nyasol has a coupling constant of $J_{1,2}$ of 11−12 Hz, in contrast to the *trans* isomer, hinokiresinol (**3**), in which the coupling constant is 16 Hz;[25] the chemical shift of C-3 in nyasol is δ 46−48[12,25] but δ 51 in hinokiresinol,[24] and the λ_{max} for nyasol is 258 nm[26] and 264 nm for hinokiresinol.[20] Finally, a band in the IR spectrum at 965 cm^{-1} is present only in hinokiresinol (**3**), the *trans* isomer.[20] In conclusion we believe that both isomers are naturally occurring.

Compound **1** was tested for in vitro antileishmanial and antiplasmodial activity as well as for the toxicity to human lymphocytes, using an in vitro test on the inhibition of the phytohemaglutinin A (PHA)-induced proliferation of human lymphocytes. The results are presented in Table 2. Compound **1** showed the dose-dependent growth inhibitory activity against various strains of *P. falciparum* and against *L. major* promastigotes. In antileishmanial tests an IC_{50} of 31 ± 4 μg/mL (70 ± 9 μM) was obtained comparable to that of pentostam, a conventional antileishmanial drug that gave an IC_{50} of 53 ± 11 μg/mL. In the antiplasmodial tests IC_{50} values of 61 and 23 μM were obtained for the chloroquine-susceptible strains K39 and 3D7, respectively, while for the chloroquine-resistant strains, V1/S and Dd2, the IC_{50} values were determined to be 163 and 16 μM, respectively.

Table 2. In Vitro Activity of Muzanzagenin (**1**) and (+)-Nyasol (**2**) against *Leishmania major* Promastigotes and *Plasmodium falciparum* and Inhibition of PHA-Induced Proliferation of Human Lymphocytes

	muzanzagenin		(+)-nyasol		chloroquine
	IC_{50} (μM)[a]	SI[b]	IC_{50} (μM)[a]	SI[b]	IC_{50} (μM)[a]
L. major	70 ± 9 (7)	1.8	49 ± 5 (8)	4.1	
P. falciparum strains:					
K39[c]	61 ± 9 (3)	2.1			0.009
V1/Sd[d]	163 ± 23 (3)	0.8			0.14
3D7[c]	23 ± 2 (7)	5.6	12 ± 3 (8)	16.7	0.025
Dd2[d]	16 ± 2 (11)	8.0	12 ± 1 (9)	16.7	0.28
lymphocytes	129 ± 2 (7)		200 ± 6 (6)		

[a] Concentration of test compound that inhibits by 50% the growth of the parasites, presented as mean ± SEM with the number of replicates in parenthesis. [b] Selectivity index (SI) is defined as the IC_{50} for lymphocyte proliferation assay test/IC_{50} for parasite growth inhibition assay. [c] Chloroquine-susceptible strains. [d] Chloroquine-resistant strains.

Experimental selectivity index (SI) can be used as a measure of selectivity for crude extracts and biologically active compounds. This ratio was calculated using the formula: SI = IC_{50} of drug for inhibition of PHA-induced lymphocyte proliferation test/IC_{50} of inhibition of parasite growth. The higher the value, the more selective the drug is, and a ratio of 1.0 indicates lack of selectivity. In the antileishmanial tests the SI was less than 1.8, while in the antiplasmodial tests a SI value of 5.6 was obtained for a chloroquine-sensitive strain 3D7 and 0.8 for V1/S, a multidrug-resistant strain (Table 2).

Nyasol (**2**) like other phenolic compounds undergoes chemical changes on exposure to air, turning from colorless to red. A comparison of the activity of freshly prepared samples and a sample that had changed to red over a week indicated no significant difference in the inhibition of *P. falciparum* growth between the two samples (data not shown).

Comparison of the IC_{50} values of chloroquine and **2** indicates that chloroquine is 500 times more active against 3D7 and only 50 times more active against Dd2. There appears to be no cross resistance between chloroquine and **2** as **2** equally inhibits the growth of chloroquine-susceptible and chloroquine-resistant strains (Table 2).

The high SI values indicate a high degree of selectivity for **2** especially against *Leishmania* protozoa. Although in vitro tests do not always reflect the in vivo situation, the antiplasmodial and antileishmanial activity elicited by **2** coupled with the low toxicity to lymphocytes demonstrated in this study indicate that this compound could be of future interest as a template for developing antiprotozoal compounds.

Experimental Section

General Experimental Procedures. NMR spectra were recorded in $CDCl_3$ at 200 MHz or 400 MHz (Bruker) for ^{1}H spectra and at 50 MHz or 100 MHz for

^{13}C spectra with TMS as internal standard. UV spectra were recorded on a UV/vis Perkin-Elmer spectrophotometer 265 and IR on an IR Perkin-Elmer spectrophotometer 781, specific rotation on a Perkin-Elmer 241 polarimeter. Mass spectrometry was performed on a JEOL AX505W mass spectrometer. X-ray analysis was carried out on an Enraf-Nonius CAD-4 diffractometer with $\omega/2\theta$ scan mode, λ(Cu $K\alpha$) = 1.5418 Å. The crystal was cooled with an Enraf−Nonius low-temperature device. Liquid chromatography was performed on Si gel 60 (0.0063−0.200 mm) and low pressure liquid chromatography on Si gel (0.040−0.063mm) or on LiChroprep RP-18 (40−60 μm). TLC was performed on TLC Si gel 60 F_{254} or on TLC RP-18 or RP-8. Zones on TLC plates were visualized under UV light (254/366 nm) and sprayed with phosphomolybdic acid (5% in EtOH). Analytical HPLC over RP-18 (Spherisorb ODS-2, 5 μm) on Waters liquid chromatograph equipped with a model 501 pump, a model 484 UV variable detector, or on a Waters 6000A pump with a Shimadzu SPD-6 A UV-vis detector. Low-pressure liquid chromatography was performed using a glass column fitted with stainless steel and teflon tubings (Separo, Sweden) and FMI lab pump model QD. Melting points were determined on a Reichert melting point apparatus and are uncorrected.

Plant Material. The roots of *Asparagus africanus* Lam. (Liliaceae) were collected from Kithembe Hill in Machakos District, Kenya, in July, 1993. A voucher specimen has been deposited at the Herbarium of National Museums of Kenya where identification of the plant was confirmed as *A. africanus* by the taxonomist Mr. Onesmus Mwangangi.

Extraction of Plant Material. Root material (500 g) was defatted with petroleum ether 40−60° and successively extracted with CH_2Cl_2, EtOAc, and MeOH at room temperature. The residues, 3.8, 4.6, and 10 g, respectively, were tested in vitro for antileishmanial activity, as described in the section on biological activity testing. The EtOAc fraction was selected for the bioactivity-guided fractionation and was chromatographed on Si gel (450 g) eluted with CH_2Cl_2 containing increasing amounts of EtOAc. Fractions were monitored by TLC, Si gel (CH_2Cl_2−EtOAc, 9.5:0.5 and 9:1). Fractions with similar profiles were combined into 12 fractions of which fraction 6−12 showed activity in the antileishmanial activity testing.

Isolation of Muzanzagenin (1). Fraction 10 (730 mg) had a high antileishmanial activity and was rechromatographed by low-pressure liquid chromatography on Si gel (100 g) eluting with $CHCl_3$−EtOAc−MeOH (20:1:0.5). Fractions were monitored by TLC using the same eluting solvent system. Further purification by repeated low-pressure liquid chromatography (RP-18, 40−60 μm; 25 g) using MeOH−H_2O (75:25) yielded 120 mg of **1**. Crystallization from MeOH−H_2O (80:20) gave pale yellow needles of **1** (32 mg). HPLC analysis on RP-18 (LiChrosorb RP-18, 5 μm, mobile phase MeOH−H_2O (80:20); detection at 220 nm) only revealed one peak. The total amount of **1** in the plant material is estimated 0.13%.

Muzanzagenin (1) (2*β*,12α-dihydroxy-(25*R*)-spirosta-4,7-dien-3-one): obtained as pale yellow needles (MeOH−H_2O); mp 230 °C; $[\alpha]^{21}_{589}$ −99.9, $[\alpha]^{21}_{578}$ −103.9, $[\alpha]^{21}_{546}$ −119.8 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ϵ) 237 (4.18) nm; IR (KBr) ν_{max}, 3460 (OH), 1675 (C=O, α,β-unsaturated ketone), 1630 (isolated C=C), 976, 919, 902, 862 (spiroketal moiety) cm^{-1}; EIMS (70 eV) *m/z*, $[M]^+$ 442 (20), 424 (65) $[M - H_2O]^+$, 370 (15), 328 (100), 310 (60), 299 (35), 281 (30), 139 (60); NMR data see Table 1.

Single-Crystal X-ray Crystallography of 1. Suitable single crystals were obtained from a solution in DMSO. The crystal (0.23 × 0.07 × 0.06 mm) belongs to the monoclinic system, space group C2 with a = 32.15(1), b = 6.528(2), c = 28.445(6) Å, β = 122.08(2)°, V = 5058(3) Å^3, Z = 8, D_{calc} = 1.162 g/cm^3, T = 122 K, and μ(Cu $K\alpha$) = 0.63 mm^{-1}; θ_{max} = 75°, $-40 \le h \le 40$, $-8 \le k \le 8$, $-35 \le l \le 35$. Of the 11 445 reflections collected, 10 024 reflections were unique (R_{int} = 0.0115). Data reduction was performed using DREADD.[27] Structure was solved using SHELXS86[28] and refined by full-matrix least-squares procedure based on F^2 (SHELXL93[29]) to a final $R(F)$ = 0.094 and w$R(F^2)$ = 0.245 for 7590 observed reflections [$I \ge 2\sigma(I)$] and 589 variables; $w = 1/[\sigma^2(F_o2) + (0.1665P)^2 + 9.73P]$, $P = (F_o2 + 2F_c2)/3$; GooF = S = 1.073. The hydrogen atoms could not be localized in the difference electron density maps, and they have been included in calculated positions. Hydrogen atoms of the hydroxyl groups have been calculated and included if they were part of a hydrogen bond (H atom of $O2_B$ excluded). During refinements the hydrogen atoms have been kept riding on the atoms to which they are bonded. Residual electron densities remain to be explained.

The relative configuration (in correspondence to natural steroid configuration) of compound **1** was determined by X-ray analysis.[30] Due to the fact that the compound consists only of C, H, and O atoms with two independent molecules in the asymmetric unit, the absolute configuration cannot be determined reliably.

The following tables have been deposited: (a) fractional atomic coordinates and equivalent displacement parameters for non-hydrogen atoms; (b) fractional atomic coordinates for calculated hydrogen atoms and isotropic displacement parameters; (c) anisotropic displacement parameters for non-hydrogen atoms; (d) bond lengths, bond angles, torsion angles, and hydrogen bond dimensions; and (e) observed and calculated structure factors.[31]

Isolation of (+)-Nyasol (2). Fraction 6 (380 mg) had a high antileishmanial activity and showed a major compound on TLC. This fraction was further purified by low pressure liquid chromatography on RP-18 (MeOH−H_2O, 80:20) to yield nyasol (**2**) (130 mg) as a colorless oily substance that appeared as one major peak on TLC (CH_2Cl_2−EtOAc, 9.5:0.5). Repeated low-pressure liquid chromatography on Si gel with EtOAc−$CHCl_3$ (98:2 to 90:10) yielded a colorless oily compound **2** (35 mg) that did not crystallize. HPLC analysis on RP-18 (MeOH−H_2O, 80:20, detection at 220 nm) showed the compound to be of high purity. NMR data and optical rotation confirmed that the compound was identical to nyasol recently obtained from *Asparagus cochinchinensis*.[13] The total amount of **2** in the plant material is estimated at 0.02%.

Antileishmanial Activity. A WHO reference vaccine strain of *L. major*, originally isolated from a patient in Iran and kindly provided by R. Behin, WHO Immunology Research and Training Center, Lausanne, Switzerland, was used in the tests. The promastigotes

were cultured in medium 199 containing 0.02 mg/mL gentamycin, 25 mM *N*-(2-hydroxyphenyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 4 mM L-glutamine, and 10% heat-inactivated (56 °C, 30 min) fetal calf serum (HFCS). The parasites were incubated at 26 °C. The effects of different plant extracts and pure compounds on the growth of promastigotes was assessed by monitoring the inhibition of ^{3}[H]thymidine uptake as previously described.[32] A parasite concentration of $1-3 \times 10^6$/mL from a 4-day-old culture was used in the tests. Parasites (180 μL) were incubated in the presence of different concentrations of the extracts, compounds, or the medium alone. Stock solution of 1 mg/50 μL in DMSO or EtOH 70% was diluted with medium 199 containing 2% heated fetal calf serum to achieve final well concentrations of 100, 50, 10, and 5 μg/mL for the crude extracts and in the range of 100−1.5 μg/mL for the isolated compound. A final concentration of DMSO below 0.5% in the test solution was shown to have no effect on the parasite growth by including solvent controls alongside the tests. Each concentration was tested in triplicate. The plates were incubated at 26 °C for 2 h in 5%−9% CO_2 after which, 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added to each well. The promastigotes were further incubated under the same conditions and harvested 18 h later on filter paper using a cell harvester (Skatron, Lierdyen, Norway) and counted in scintillation counter (Minaxi Ti-Carb 4000, United Technologies, Packard, Downers Grove, IL).

Antiplasmodial Activity. Two chloroquine-susceptible strains of *P. falciparum* (K39 and 3D7) and two chloroquine-resistant strains (V1/S and Dd2) were used in the tests. The parasites were all laboratory-maintained cultures. K39 and V1/S were maintained in O positive red blood cells (RBC) as previously described,[33] while 3D7 and Dd2 were maintained in O positive RBC in standard RPMI medium supplemented with 5% normal rhesus-positive human serum, and 50 mg/100 mL glucose, according to a described method.[34]

Modifications of Desjardin's radioisotopic method[35] for measuring parasite growth were adopted for the in vitro antiplasmodial tests of crude extracts and compounds. For the strains K39 and V1/S the tests were performed as described by Watkins *et al*.,[33] whereby parasites were incubated for 48 h before the addition of ^{3}H-hypoxanthine and harvested 24 h later. For 3D7 and Dd2, the radiolabel was added after 24 h and harvested 24 h later.[36] After the second incubation period, the parasites were harvested on filter papers, dried in the oven at 60 °C for 30 min, and the [^{3}H]-hypoxanthine incorporation was measured by liquid scintillation counting. The test compounds were prepared as described in the section for antileishmanial testing, the only difference being the media used for dilution from the stock solution.

Lymphocyte Proliferation Assay. The effects of the crude extracts and the isolated compound on the proliferation of phytohemaglutinin-stimulated human peripheral blood mononuclear cells was assessed by monitoring the uptake of radiolabeled thymidine as previously described.[37] The drugs were tested in concentrations ranging from 200.0 to 3.0 μg/mL.

Data Analysis. Percentage inhibition was determined using the following formulae: inhibition = [(cpm control − cpm drug)/(cpm control − cpm background)] × 100; where cpm drug = cpm for drug; cpm control = cpm for the non-treated parasite/lymphocytes in the experiment, and cpm background = cpm for wells with medium alone. The percentage inhibition data was used to derive the drug concentration causing 50% inhibition of [^{3}H]hypoxanthine incorporation into nucleic acids (IC_{50}) using GRAFIT 3.0,[38] whereby the data are fitted to the equation below. The concentration−response curve is plotted with the drug concentration displayed logarithmically on the *x*-axis and the percentage inhibition on the *y*-axis. Radioimmunoassay equation for the concentration−response curve expressed in terms of IC_{50} value was, thus, $y = [a/[(1 + 1/IC_{50}c)\, x^c]] + d$, where a is the maximum y range, d is the background y value, and c is a slope factor. The statistical differences between mean IC_{50} values were examined by the Student's *t*-test.

Acknowledgment. H.A.O. is grateful to DANIDA for a fellowship and to the National Museums of Kenya for personal support. The NMR spectrometers were purchased through grants from the Alfred Benzon Foundation, Ib Henriksen Foundation, and Thorkild Steenbeck Foundation. K.F. is thankful to Alfred Benzon Foundation for an Investigator Fellowship and to Mr. Flemming Hansen for collecting X-ray diffraction data.

References and Notes

(1) WHO *Tropical Disease Research, Progress 1991*−92. Eleventh Programme Report of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). WHO: Geneva, 1993; pp 15−29.
(2) Berenguer, J.; Moreno, S.; Cercenado, E.; Bernaldo de Quiros, J. C.; Garcia de la Fuente, A.; Bouza, E. *Ann. Intern. Med.* **1989**, *111*, 129−132.
(3) WHO *UNDP/WORLD BANK/WHO. The Leishmaniases. WHO Special Programme for Research and Training in Tropical Diseases. Progress in international research. 1987−1988.* WHO: Geneva 1989; pp 10−30.
(4) *UNDP/WORLD BANK/WHO. Special Programme for Research and Training in Tropical Diseases. TDR News* **1990**, *34*, 1 and 7.
(5) WHO. *Weekly Epidemiol. Rec.* **1993**, *34*, 245−252.
(6) Schuster, B. G.; Milhous, W. K. *Parasit. Today* **1993**, *9*, 167−168.
(7) Panisko, D. M.; Keystone, J. S. *Drugs* **1990**, *39*, 160−189.
(8) Beentje, H. *Kenya Trees, Shrubs and Lianas*; National Museums of Kenya: Nairobi, 1994.
(9) Agnew, A. D. Q.; Agnew, S. *Upland Kenya Wild Flowers. A flora of the ferns and herbaceous flowering plants of Upland Kenya*; East African Natural History Society: Nairobi, 1994.
(10) Watt, J. M.; Breyer-Brandwijk, M. G. *Medicinal and Poisonous Plants of Southern and Eastern Africa*; E. S. Livingstone: Edinburgh, 1962.
(11) Chang, K. P.; Fong, D.; Bray, R. S. *Leishmaniases.* Chang, K. P., Bray, R. S. Eds.; Elsevier: Oxford, 1985; pp 1−30.
(12) Sharma, S. C.; Chand, R.; Sati, O. P. *Pharmazie* **1980**, *33*, 711−712.
(13) Tsui, W.-T.; Brown, G. D. *Phytochemistry* **1996**, 1413−1415.
(14) Budzikiewicz, H. C.; Djerassi, C.; Williams, D. H. *Structure Elucidation of Natural Products by Mass Spectrometry. Volume II: Steroids, terpenoids, sugars, and miscellanous classes.* Holden-Day: San Fransisco, 1964; pp 110−120.
(15) Faul, W. H.; Djerrasi, C. *Org. Mass Spectrom.* **1970**, *3*, 1187−1189.
(16) Agrawal, P. K.; Jain, D. C.; Pathak, A. K. *Magn. Reson. Chem.* **1995**, *33*, 923−953.
(17) Agrawal, P. K.; Jain, D. C.; Gupta, R. K.; Thakur, R. S. *Phytochemistry* **1985**, *24*, 2479−2496.
(18) Williams, D. H.; Flemming, I. *Spectroscopic Methods in Organic Chemistry*; McGraw-Hill Book, Limited: Berkshire, 1989; pp 92−101.
(19) Johnson, C. K. ORTEPII. *Report ORNL-5138.* Oak Ridge National Laboratory: Oak Ridge, TN, 1976.
(20) Hirose, Y.; Oishi, N.; Nagaki, H.; Nakatsuka, T. *Tetrahedron Lett.* **1965**, 3665−3668.
(21) Sibanda, S.; Ntabeni, O.; Nicoletti, M.; Galeffi, C. *Biochem. Sys. Ecol.* **1990**, 481−483.

(22) Beracierta, A. P.; Whiting, D. A. *Tetrahedron Lett.* **1976**, *27*, 2367−2370.
(23) Beracierta, A. P.; Whiting, D. A. *J. Chem. Soc., Perkin Trans. 1* **1978**, 1257−1262.
(24) Enzell, C. R.; Hirose, Y.; Thomas, B. R. *Tetrahedron Lett.* **1967**, 793−798.
(25) Ameer, F.; Drewes, E.; Drewes, M. W.; Roos, P. H. P.; Watson, M. C. *J. Chem. Soc., Perkin Trans. 1* **1988**, 1425−1430.
(26) Marini-Bettolo, G. B.; Nicoletti, M.; Messana, I. *Tetrahedron* **1985**, 665−670.
(27) Blessing, R. H. *Crystallogr. Rev.* **1987**, *1*, 3−58.
(28) Sheldrick, G. M. *Acta Crystallogr.* **1990**, *A46*, 467−473.
(29) Sheldrick, G. M. *SHELXL93. Program for the Refinement of Crystal Structures.* University of Göttingen, Germany, 1993.
(30) Flack, H. D. *Acta Crystallogr.* **1983**, *A39*, 876−881.
(31) Atomic coordinates for this structure have been deposited with the CCDC and are available, free of charge, on request to the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, England.
(32) Pearson, R. D.; Souza, A. Q. *Principles and Practice of Infectious Diseases*; Mandell, G. L., Douglas, R. G., Bennet, J. E., Eds.; Churchhill Livingstone: New York, 1990; pp 2066−2077.
(33) Watkins, W. M.; Chulay, J. D.; Sixsmith, D. G.; Spencer, H. C.; Howells, R. E. *J. Pharm. Pharmacol.* **1987**, *39*, 261−265.
(34) Trager, W.; Jensen, J. B. *Science* **1976**, *193*, 673−675.
(35) Desjardins, R. E.; Canfield, R. E.; Hayness, C. J.; Chulay, J. D. *Antimicrob. Agents Chemother.* **1979**, *16*, 710−718.
(36) Chen, M.; Theander, T. G.; Christensen, S. B.; Hviid, L.; Zhai, L.; Kharazmi, A. *Antimicrob. Agents Chemother.* **1994**, *38*, 1470−1475.
(37) Bygbjerg, I. C.; Theander, T. G.; Andersen, B. J.; Flachs, H.; Jepsen, S.; Larsen, P. B. *Trop. Med. Parasitol.* **1986**, *37*, 245−247.
(38) Leatherbarrow, R. J. *GraFit*; Erithacus Software Ltd. Staines, UK, 1990.

NP970217F