Leishmanicidal, Antiplasmodial, and Cytotoxic Activity of Novel Diterpenoid 1,2-Quinones from *Perovskia abrotanoides*: New Source of Tanshinones

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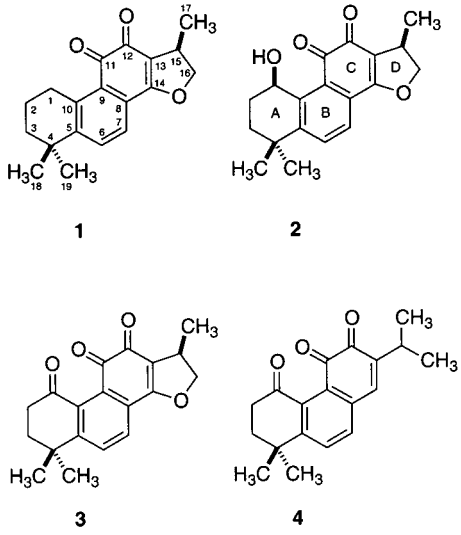
Cryptotanshinone (**1**), a quinoid diterpene with a nor-abietane skeleton, and three new natural products, 1β-hydroxycryptotanshinone (**2**), 1-oxocryptotanshinone (**3**), and 1-oxomiltirone (**4**), were isolated from roots of the Iranian medicinal plant *Perovskia abrotanoides*. Their structures were established using homo- and heteronuclear two-dimensional NMR experiments, supported by HRMS. The total amount of tanshinones isolated from dry roots of *Perovskia abrotanoides* was about 1.5%. The compounds exhibited leishmanicidal activity in vitro (IC_{50} values in the range 18−47 μM). These findings provide a rationale for traditional use of the roots in Iran as a constituent of poultices for treatment of cutaneous leishmaniasis. The isolated tanshinones also inhibited growth of cultured malaria parasites (3D7 strain of *Plasmodium falciparum*), drug-sensitive KB-3-1 human carcinoma cell line, multidrug-resistant KB-V1 cell line, and human lymphocytes activated with phytohaemagglutinin A (IC_{50} values in the range 5−45 μM). The toxicity of tanshinones toward the drug-sensitive KB-3-1 and the multidrug-resistant KB-V1 cells was the same, indicating that the compounds are not substrates for the P-glycoprotein drug efflux pump.

Introduction

Tanshinones are a group of red pigments present in danshen (Tan-Seng), a well-known drug of traditional Chinese medicine.[1,2] Danshen is a dried root of the Chinese red-rooted sage *Salvia militiorrhiza* Bunge (Lamiaceae) and is clinically useful for treatment of coronary heart and cerebrovascular diseases, viral hepatitis, and other ailments.[1−3] Tanshinones are believed to be the principal pharmacologically active constituents of danshen. They exhibit a variety of biological activities,[4] including effects on cardiac function,[5−7] antioxidant acitivty,[8,9] aldose reductase inhibitory activity,[10] interaction with the benzodiazepine receptor,[11,12] cytotoxicity,[13−16] and apoptosis induction.[17,18] Because danshen preparations constitute a basis for considerable commercial activity in China, there is a continued interest in development of biotechnology-based approaches to production of tanshinones.[19−21] Numerous methods of total synthesis of tanshinones have been developed.[12,22−30]

Chemically, tanshinones are 20-norditerpenes with an abietane-type skeleton containing a quinone moiety in the C-ring. Over 50 tanshinones have so far been identified in *S. militiorrhiza* and other *Salvia* species.[10,14,25,31−40] In the present work, we report that *Perovskia abrotanoides* Kar. (Lamiaceae) is a new, rich source of tanshinones. In addition to the well-known cryptotanshinone (**1**), three new natural products, **2**−**4**, have been isolated and identified; although **4** was previously obtained as a synthetic material, its was encountered as a natural product for the first time. Activity of the compounds against cultured *Plasmodium falciparum* parasites, *Leishmania major* promastigotes, two human cancer cell lines, and human lymphocytes is described.



P. abrotanoides is an herb used to treat leishmaniasis in Iranian folk-medicine tradition. Thus, villagers in the Isfahan province of Iran apply a poultice, made of crushed roots of the plant, water, sesame oil, and wax, on lesions caused by cutaneous leishmaniasis (own observations by the authors). The present study demonstrates that *P. abrotanoides* indeed contains copious amounts of fat-soluble leishmanicidal constituents, thus providing a rationale for the medicinal use of the plant.

Results and Discussion

Fractionation of ethyl acetate extract of roots of *P. abrotanoides* by VLC afforded several fractions, from which

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Table 1. 400 MHz ^{1}H NMR Spectral Data for Compounds **1**−**4** ($CDCl_3$)[a]

proton	**1**	**2**	**3**	**4**
H-1	3.22 (t), $J_{1,2} = 6.4$ Hz	5.04 (br, q), $J_{1,2} \approx J_{1,OH} \approx 3.5$ Hz[d]		
H-2	1.76−1.82 (m)	2.11[e] (m) and 1.88 (m)	2.93[f] (t), $J_{2,3} = 7.2$ Hz	2.90[f] (t), $J_{2,3} = 7.2$ Hz
H-3	1.65−1.67 (m)	2.16[e] (m) and 1.55 (m)	2.07[f] (t)	2.05[f] (t)
H-6[b]	7.63 (d), $J_{6,7} = 8.1$ Hz	7.72 (d), $J_{6,7} = 8.1$ Hz	7.57 (d), $J_{6,7} = 8.1$ Hz	7.53 (d), $J_{6,7} = 8.1$ Hz
H-7	7.50 (d)	7.61 (d)	7.64 (d)	7.33[g] (d)
H-15	3.60 (m)	3.61 (m)	3.61 (m)	3.02 (dsp), $J_{15,16} = J_{15,17} =$ 6.9 Hz, $J_{14,15} = 1.0$ Hz
H-16[c]	4.36 (dd, H-16β) and 4.89 (t, H-16α), $J_{16\alpha,16\beta} = J_{15,16\alpha} =$ 9.3 Hz, $^3J_{15,16\beta} = 6.1$ Hz	4.40 (dd, H-16β) and 4.92 (t, H-16α), $J_{16\alpha,16\beta} = J_{15,16\alpha} =$ 9.3 Hz, $J_{15,16\beta} = 5.5$ Hz	4.39 (dd, H-16β) and 4.92 (t, H-16α), $J_{16\alpha,16\beta} = J_{15,16\alpha} =$ 9.3 Hz, $J_{15,16\beta} = 6.1$ Hz	1.16 (d)
H-17	1.36 (d), $J_{15,17} = 6.8$ Hz	1.37 (d), $J_{15,17} = 6.8$ Hz	1.36 (d), $J_{15,17} = 6.9$ Hz	1.16 (d)
H-18, H-19	1.31 (br, s)	1.26 (s) and 1.40 (s)	1.33 (s) and 1.34 (s)	1.33 (s)
other		OH: 4.51 (br, d), $J_{1,OH} \approx 3.5$ Hz		H-14: 7.07[g] (d)

[a] Multiplicity of signals is given in parentheses: s, singlet; d, doublet; t, triplet; q quartet; sp, septet; m, multiplet; br, broad; coupling constants are specified only once at the first occurrence of the coupled proton. [b] Distinguished from H-7 on the basis of NOE to H-18/H-19. [c] H-16α and H-16β resonances in **1**−**3** were assigned from NOEs to H-15 and H-17, respectively. [d] Triplet ($J_{1,2\alpha} \approx J_{1,2\beta} \approx 3.5$ Hz) upon addition of traces of CF_3COOH. [e] Chemical shift of overlapping resonances read from a COSY spectrum. [f] H-2 and H-3 assigned on the basis of strong NOE between H-3 and H-18/H-19. [g] NOE between H-7 and H-14 observed.

four orange-red compounds were isolated after repeated chromatography. The identity of the major constituent was established by use of COSY, NOESY, and HMBC experiments as cryptotanshinone (**1**). ^{1}H and ^{13}C NMR spectra, melting point, and optical rotation data match those reported in the literature well;[10,28] however, the assignments of ^{13}C resonances of C-5 and C-10 and those of C-8 and C-9 reported by Tezuka et al.[10] have to be interchanged, as shown by HMBC spectra.

The second most abundant constituent differed from **1** by the presence of a hydroxy group in the A-ring, as evident from the presence of an oxygenated methine group in ^{1}H and ^{13}C NMR spectra (δ 5.01 and 63.38, respectively). A COSY spectrum demonstrated the presence of a $-CH(OH)-CH_2CH_2-$ spin system. That the hydroxy group was placed at C-1 and not at C-3 was apparent from a NOESY experiment, which showed NOE effects between the geminal methyl groups (C-18 and C-19) and the protons attached to C-3, whereas the other methylene group (protons attached to C-2) showed NOE to the oxygenated methine group H-1 and to the hydroxy proton. Moreover, the chemical shift of C-4 (δ 35.13), the signal of which is easily recognizable as the only aliphatic quaternary resonance present in the spectrum, is closely similar to that of C-4 in **1** (δ 34.86), which is incompatible with oxygenation of C-3. Further confirmation of the structure **2** and assignment of all carbon resonances were obtained from an HMBC spectrum. The observed change of chemical shift of the carbonyl group carbon C-11 from δ 184.28 in **1** to δ 186.31 in **2** is as expected from the presence of a hydrogen bond from the 1-hydroxy group. The 1-hydroxycryptotanshinone (**2**) was rather unstable, presumably due to reactivity of the secondary, benzylic hydroxy group.

Two minor constituents isolated from the extract were assigned structures **3** and **4**. The presence of additional carbonyl groups was apparent from ^{13}C NMR spectra (δ 198.92 for **3** and 198.84 for **4**). The chemical shift of C-4 (δ 35.23 in **3** and 34.93 in **4**) provided evidence for the site of oxygenation at C-1. Each compound exhibited a pair of triplets at about 2.9 ppm and about 2.0 ppm, which can be assigned respectively to C-2 and C-3 methylene group protons on the basis of the chemical shift values alone. In addition, NOESY spectra of **3** and **4** exhibited correlations to the geminal methyl groups from the latter but not from the former triplet. The remaining ^{1}H and ^{13}C resonances of **3** were closely similar to those of **1** and **2**, whereas **4** showed the presence of an isopropyl group. The presence of a 1,2-quinone moiety and not a 1,4-quinone moiety in **4** was apparent, inter alia, from the chemical shift[41] of H-7 and from an NOE between the peri protons H-7 and H-14. The NMR data for **1**−**4** are collected in Tables 1 and 2.

Table 2. 100 MHz ^{13}C NMR Spectral Data for Compounds **1**−**4** ($CDCl_3$)

carbon	**1**[a]	**2**[a]	**3**[b]	**4**[b]
C-1	29.68	63.38	198.92	198.84
C-2	19.09	26.91	36.22	36.15
C-3	37.83	31.92	36.49	36.58
C-4	34.86	35.13	35.23	34.93
C-5	152.39	152.11	155.70	153.78
C-6	132.58	134.09	129.67	130.94
C-7	122.52	124.54	126.60	131.87
C-8	126.30	126.93	127.29	132.82[d]
C-9	128.44	129.83	128.29	135.51[d]
C-10	143.70	143.13	138.04	138.03[d]
C-11	184.28	186.31	183.70	183.14[e]
C-12	175.73	175.37	177.39	183.76[e]
C-13	118.32	118.49	119.37	146.47
C-14	170.77	170.71	169.10	137.85
C-15	34.64	34.59	34.69	27.00
C-16	81.47	81.81	81.86	21.56
C-17	18.84	19.13	18.84	21.56
C-18	31.92	31.19[c]	28.77	28.83
C-19	31.92	31.57[c]	28.77	28.83

[a] Assignments based on one-bond ^{1}H,^{13}C chemical shift correlation and HMBC spectra. [b] Assignments based on one-bond ^{1}H,^{13}C chemical shift correlation. [c−e] These assignments may be interchanged.

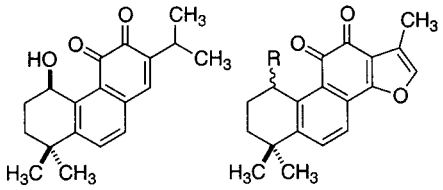
It can be assumed that **2** and **3** are biosynthetic derivatives of **1** and, hence, have the same absolute configuration at C-15, i.e., 15*R*.[42] This is supported by the optical rotation of **3** ($[\alpha]_D$ −59.3°), similar to that of **1** ($[\alpha]_D$ −81.8°). According to the classical rule of optical superposition, like functional groups in like surroundings make like contributions to the optical rotation.[43] This rule holds quite well when stereogenic centers are separated by three to five carbons.[43] Since the 1-hydroxycryptotanshinone **2** is strongly dextrorotatory ($[\alpha]_D$ +235°), its rotation appears to be dominated by the new stereogenic center at C-1. Because the two stereogenic centers in **2** are distant, the rotation of **2** may be compared with that of appropriate model compounds. Thus, the closely related dextrorotatory alcohol **5** ($[\alpha]_D$ +990°) was assigned the *R* configuration on the basis of Horeau's method.[44] On the basis of this determination, the *R*-hydroxy group gives a positive contribution to rotation of derivatives of 5-hydroxy-5,6,7,8-tetrahydrophenanthrene-3,4-dione, and thus the configuration of **2**

Table 3. Leishmanicidal, Antiplasmodial, and Cytotoxic Activity of Tanshinones **1**−**4** and Reference Compounds[a]

compound	IC_{50}, μM				
	Leishmania major	*Plasmodium falciparum*	KB-3-1 cells	KB-V1 cells	lymphocytes
1	18.4 ± 0.8	12.5 ± 0.1	6.9 ± 1.3	7.5 ± 1.2	37.4 ± 3.6
2	46.7 ± 3.1	26.9 ± 2.1	5.0 ± 1.1	5.6 ± 0.4	16.0 ± 3.3
3	25.7 ± 2.4	17.6 ± 1.5	ND	ND	12.4 ± 4.1
4	17.9 ± 0.9	13.0 ± 0.1	46.6 ± 0.4	47.8 ± 3.2	44.6 ± 6.9
amphotericin B	0.16 ± 0.03				
chloroquine		96.5 ± 2.0 nM			
rhodamine 123			1.6 ± 0.2	>500	

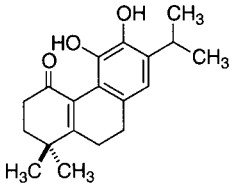
[a] Concentration necessary to inhibit growth by 50%, in μmol/L unless stated otherwise; ND: not determined.

is 1*R*. To support this assignment, the generalized Mosher's method[45,46] was applied for **2**. Thus, the epimeric esters of **2** with (*S*)- and (*R*)-methoxy(trifluoromethyl)phenylacetic acid (MTPA esters) were prepared and analyzed using ^{1}H NMR.[45] The resonances of the C-2 and C-3 methylene groups and those of the methyl groups attached to C-4 had higher δ values in the *S*-esters than in the *R*-esters ($\delta_S - \delta_R = \Delta\delta > 0$). Consistently, the signals of the other end of the molecule (ring D) showed $\Delta\delta < 0$, although the chemical shift differences were smaller because of the long distance to the MTPA esterification site. Rings A and D are thus respectively to the right and to the left of the "MTPA plane",[45,46] which corresponds to the *R* configuration of C-1, in agreement with the conclusion based on the consideration of the optical activity of **2**.



5

6 R = OH
7 R = H



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An analogue of **2** having a double bond between C-15 and C-16, i.e., compound **6**, has previously been isolated in minute amounts from danshen.[47] However, the alcohol proved to be optically inactive[47] and was considered to be an artifact originating from photooxidation of **7**,[48] which is a major constituent of danshen. By contrast, the alcohol **2** is a major constituent of *P. abrotanoides* and is optically active, and must hence be a genuine natural product.

The quinone **4** has previously been obtained by Gonzalez et al.[35] as a product of oxidation with Ag_2O of the hydroquinone **8**, isolated from *S. mellifera*. The ^{1}H NMR data reported for the oxidation product[35] match well those obtained with **4** (Table 1). The hydroquinone corresponding to **4** has been isolated from *S. argentea* by Michavila et al.[44] and named arucadiol, and later from *S. militiorrhiza* by Ginda et al.,[49] who were unaware of the former work and called the same compound miltiodiol. However, the quinone **4**, which is a 1-oxo derivative of miltirone,[12] has apparently not previously been described as a natural product.

The elemental composition of all compounds isolated from *P. abrotanoides* was confirmed by high-resolution MALDI FT MS. All compounds yielded abundant $[MNa]^+$ ions.

Apart from *S. militiorrhiza*, tanshinones have previously been isolated from several other *Salvia* species.[34,35,39,44,51−57] However, tanshinones are not universal in this genus, since the majority of *Salvia* species produce royleanone type diterpenes, in which C-20 is preserved.[58−60] Outside *Salvia*, tanshinones have been reported only from the related species *Meriandra benghalensis*[36] and *Rosmarinus officinalis*[61] and, rather unexpectedly, from a taxonomically distant species *Pseudostreblus indica*,[62] which belongs to Moraceae. In the two latter cases,[61,62] insufficient characterization of the isolated material was presented. *Perovskia*, a small genus represented in an area stretching from northeast Iran to northwest India, is closely related to *Salvia*.[63] Only essential oils[64−66] and two unusual triterpenes[67,68] have previously been reported from *Perovskia*. The total amount of isolated tanshinones **1**−**4** in *P. abrotanoides* roots was about 1.5% (dry weight), which appears to exceed what is normally found in danshen.[14,19,69,70]

Results of biological evaluation of **1**−**4** are shown in Table 3. All four compounds exhibit moderate toxicity in vitro to *Leishmania major* promastigotes, cultured *Plasmodium falciparum* parasites, and proliferating human lymphocytes. The antileishmanial activity of the tanshinones is in accord with the above-mentioned use of roots of *P. abrotanoides* in Iran as an antileishmanial herb. Moreover, **1**, **2**, and **4** were tested for cytotoxicity using two human carcinoma cell lines: the drug sensitive KB-3-1 cell line and the multidrug-resistant KB-V1 cell line. The KB-V1 cells display the complete multidrug-resistance phenotype including expression of the P-170 glycoprotein efflux pump.[71−73] The resistance index of the KB-V1 cells relative to the KB-3-1 cells is 210 for vinblastine and even higher for other cytotoxic drugs.[71,74] The tanshinones **1** and **2** exhibit practically identical toxicity, whereas the tricyclic quinone **4** is considerably less active (Table 3). No difference between the toxicity toward the drug-sensitive and drug-resistant cells was observed, whereas the resistance index for the test compound rhodamine 123 was about 500. This indicates that tanshinones are not substrates for the glycoprotein efflux pump. This is of particular interest since tanshinones were recently shown to suppress growth of hepatic tumors in vivo.[15] The presence of the D ring in tanshinones is apparently necessary for the high cytotoxicity, as illustrated by the increased IC_{50} value for **4** (Table 3). On the other hand, the toxicity of **4** toward *L. major* and *P. falciparum* is equal to or better than that of **1**−**3**. Therefore, it is conceivable that increased selectivity toward the parasites can be achieved by manipulation of the structure of tanshinones.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Perkin-Elmer 241 polarimeter. NMR spectra were recorded at 25 °C on a Bruker AMX 400 spectrometer (proton frequency 400.13 MHz) in chloroform-*d*, using TMS as internal standard. NOESY spectra were obtained with mixing times of 500−800 ms. HMBC spectra were optimized for $^{n}J_{C,H}$ = 5 and 17 Hz. $^{1}H,^{13}C$ chemical shift correlations were obtained using polarization transfer with BIRD decoupling. High-resolution mass spectra were obtained on an IonSpec Ultima 4.7 T Fourier transform mass spectrometer equipped with matrix-assisted laser desorption ionization (MALDI) source based on a 337 nm nitrogen laser, using 2,5-dihydroxybenzoic acid for matrix preparation. All spectra were peak-matched using the peak at *m/z* 273.03936 ($[2M - 2H_2O + H]^+$) as a reference peak. VLC separations were performed on Merck silica gel 60H for TLC. Column chromatography was performed on Matrex silica gel 60A, 37−70 μm (Millipore). Fractions were monitored by TLC (Merck precoated silica gel 60 F_{254} plates) eluted with toluene−EtOAc, 9:1 to 7:3. Melting points were determined in capillaries and are uncorrected.

Plant Material. Roots of *Perovskia abrotanoides* Kar. (Persian name: brazambel) were collected at an altitude of 2100 m in the area between Natanz and Kashan, Isfahan, Iran, in July 1999; the plant was authenticated by K. Bagherzadeh and M. Majid. A voucher specimen (Alt. 11015) has been deposited in the herbarium of the Isfahan Research Centre of Natural Resources and Animal Science, Isfahan, Iran.

Extraction and Isolation. Air-dried and powdered plant material (300 g) was extracted at room temperature with 2 × 3 L of EtOAc. The combined extracts were evaporated and the residue (17 g) fractionated on a VLC column (9 × 10 cm i.d.) with toluene containing increasing amounts of EtOAc, to afford eight fractions. Fraction 1 (1 g) was eluted with 1.5 L of neat toluene; fraction 2 (0.87 g) was eluted with 1 L of toluene−EtOAc (9:1); fraction 3 (2.84 g) was eluted with a further 1.5 L of the same solvent; fraction 4 (1.35 g) was eluted with 1 L of toluene−EtOAc (8:1); fraction 5 (1.1 g) was eluted with 0.5 L of toluene−EtOAc (4:1); fraction 6 (2.1 g) was eluted with a further 1.5 L of the same solvent; fraction 7 (2.2 g) was eluted with 1.5 L of EtOAc−MeOH (5:1); fraction 8 (3.2 g) was eluted with 0.5 L of neat MeOH.

Fraction 3 appeared to be homogeneous by TLC; crystallization (CH_2Cl_2−pentane) afforded 2.4 g of pure **1**. Further chromatography of fraction 1 on silica gel (57 × 3 cm column) using toluene−EtOAc (19:1) gave 5.5 mg of **4**. No well-defined material could be obtained from fraction 2. Chromatography of fraction 4 (57 × 3 cm silica column eluted with toluene−EtOAc, 8:1) afforded 30 mg of **3**. From fraction 5, 0.9 g of **2** was isolated by chromatography on a 52 × 2.5 cm silica column eluted with toluene−EtOAc, 4:1. Fraction 6 similarly afforded an additional amount (1.1 g) of crude **2**. Fractions 7 and 8 appeared to contain only polymeric material (TLC).

Cryptotanshinone [(*R*)-1,6,6-trimethyl-1,2,6,7,8,9-hexahydrophenanthro[1,2-*b*]furan-10,11-dione] (1): yield 2.4 g (0.8%); orange-red needles; mp 190−190.5 °C, lit.[28] mp 188−190 °C; $[\alpha]^{25}_D$ −81.8° (*c* 0.11, $CHCl_3$), lit.[28] $[\alpha]^{25}_D$ −84.5°; HR MALDI FT MS *m/z* 297.1485 (21%, $[MH]^+$), $[C_{19}H_{20}O_3 + H]^+$ requires 297.1485, 319.1337 (100%, $[MNa]^+$), $[C_{19}H_{20}O_3 + Na]^+$ requires 319.1305.

1β-Hydroxycryptotanshinone [(1*R*,9*R*)-9-hydroxy-1,6,6-trimethyl-1,2,6,7,8,9-hexahydrophenanthro[1,2-*b*]furan-10,11-dione] (2): yield 2.0 g (0.66%); red resin; $[\alpha]^{25}_D$ +235° (*c* 0.25, $CHCl_3$); HR MALDI FT MS *m/z* 335.1254 (100%, $[MNa]^+$), $[C_{19}H_{20}O_4 + Na]^+$ requires 335.1254, 317.1149 (69%, $[MNa - H_2O]^+$), $[C_{19}H_{20}O_4 + Na - H_2O]^+$ requires 317.1148.

1-Oxocryptotanshinone [(*R*)-1,6,6-trimethyl-1,6,7,8-tetrahydro-2*H*-phenanthro[1,2-*b*]furan-9,10,11-trione] (3): yield 30 mg (0.01%); orange-red resin; $[\alpha]^{25}_D$ −59.3° (*c* 0.07, $CHCl_3$); HR MALDI FT MS *m/z* 333.1092 (100%, $[MNa]^+$), $[C_{19}H_{18}O_4 + Na]^+$ requires 333.1097.

1-Oxomiltirone (2-isopropyl-8,8-dimethyl-7,8-dihydro-6*H*-phenanthrene-3,4,5-trione) (4): yield 5.5 mg (0.0018%); orange resin; HR MALDI FT MS *m/z* 319.1320 (100%, $[MNa]^+$), $[C_{19}H_{20}O_3 + Na]^+$ requires 319.1305.

Determination of the Absolute Configuration of 2 by Mosher's Method. Samples of **2** (50 mg, 0.16 mmol) in dry pyridine (0.2 mL) were treated with (*S*)- or (*R*)-methoxy-(trifluoromethyl)phenylacetyl chloride (50 μL, 27 mmol). After 30 min the reaction was complete according to TLC. The mixture was added to 10 mL of 0.1 M HCl and extracted with 3 × 10 mL of AcOEt, the organic phase was washed with 10 mL of 5% aqueous $NaHCO_3$ and saline, dried ($MgSO_4$), and evaporated, and the residue was analyzed by ^1H NMR in $CDCl_3$. The signals of the C-2 and C-3 protons in the *S* ester appeared at δ 2.29, 2.08, 1.94, and 1.63, and the corresponding signals in the *R* ester appeared at δ 2.25, 1.94, 1.57, and 1.41 ($\Delta\delta > 0$). The signals of the C-18 and C-19 methyl groups appeared at δ 1.40 and 1.29 in the *S* ester and at δ 1.23 and 1.26 in the *R* ester ($\Delta\delta > 0$). The C-17 methyl group appeared at δ 1.35 in the *S* ester and at δ 1.36 in the *R* ester ($\Delta\delta < 0$). For the remaining D-ring protons the chemical shift differences were less than 0.01 ppm, but still with $\delta_S - \delta_R < 0$.

Assay for Leishmanicidal Activity. Promastigotes from a WHO reference vaccine strain of *Leishmania major* were maintained at 26 °C in RPMI 1640 medium containing 25 mM HEPES, 4 mM L-glutamine, 0.02 mg/mL gentamicin, and 10% of heat-inactivated fetal calf serum. The effect of plant extract and pure compounds on the growth of promastigotes was assessed by monitoring inhibition of [^{3}H]thymidine uptake similarly to that previously described.[75] The compounds for testing were dissolved in DMSO, the stock solution was diluted appropriately with the growth medium, and aliquots were incubated in 96-well microtiter plates with promastigotes (1×10^7 per mL, 180 μL/well) for 2 h. After addition of [^{3}H]-thymidine, the plates were incubated for 18 h, the cells were harvested, and the incorporation of radioactivity was determined by liquid scintillation counting.

Assay for Antiplasmodial Activity. A modification of Desjardins' radioisotope method[76] for measuring growth of a chloroquine-sensitive strain of *Plasmodium falciparum* (3D7) was adopted, using uptake of [^{3}H]phenylalanine as an index of growth. Thus, 50 μL of the growth medium (RPMI 1640 containing 0.45% Albumax II, 11 mM D-glucose, 25 mM HEPES, 3.4 mM L-glutamine, 133 μM hypoxanthine, 44 μg/mL gentamycin sulfate, and 24 mM sodium bicarbonate) containing test substances added from a DMSO stock was mixed with 50 μL of a suspension of parasitic erythrocytes (5% hematocrit, 2−3% parasitemia) in 96-well microtiter plates. The maximal final DMSO concentration was 0.5%. Each concentration of the test substance was tested in triplicate. The plates were incubated at 37 °C for 24 h before the addition of [^{3}H]phenylalanine. After an additional 24 h incubation period, the parasites were harvested, and incorporation of radioactivity was determined by liquid scintillation counting.

Assay for Cytostatic Activity. Mycoplasma-free carcinoma cell lines KB-3-1 and KB-V1, selected for resistance with vinblastine from the KB-3-1 cells,[71] were obtained from the Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD. The cells were maintained in monolayers at 37 °C in an atmosphere containing 5% CO_2 (humidity 98%), using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, glucose (4.5 g/L), L-glutamine (0.58 g/L), sodium pyruvate (1 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). The KB-V1 cells were grown in the absence of cytostatic pressure from vinblastine, but the cells from passage numbers 5−15 preserved unchanged the degree of resistance[71] to rhodamine 123 and were used for the assay. The KB-3-1 cells and the KB-V1 cells were harvested by trypsinization at 70−80% and 60−70% confluence, respectively, applied into 96-well plates (4×10^3 of KB-3-1 cells or 7×10^3 of KB-V1 cells per well) in 75 μL of the culture medium and grown for 24 h. Test substances were

applied in 75 μL of a solution prepared by mixing 10 μL of a DMSO stock with 1990 μL of the medium and appropriately diluted to the required concentrations with the medium, and the cells were grown for an additional 48 h. No well contained more than 0.25% of DMSO, which was also present in the control wells. Six repeats of each concentration of the test substances were used, and the reported IC_{50} values are the result of at least three separate determinations with different passages of the cells. Time of incubation and the amounts of the cells used were such that the cells in the control wells reached 70% (KB-3-1) or 60−70% (KB-V1) confluence at the end of the 72 h growth period (24 h of preincubation and 48 h of incubation with test substances). After the incubation, the medium was removed and the amount of cells determined using the CellTiter 96 aqueous cell proliferation assay kit from Promega Corporation. The MTS/PMS reagent[77] was freshly prepared by mixing 2.0 mL of MTS solution (2 mg/mL) with 99.4 μL of PMS solution (0.92 mg/mL) and 10.4 mL of the growth medium (without phenol red and serum). After addition of 120 μL of the reagent mixture to each well, the plate was incubated for 50 min (or until the absorbance at 492 nm reached 0.8 for control wells) and the extent of inhibition of cell proliferation determined from comparison of the absorbance with controls.

Lymphocyte Proliferation Assay. Inhibition of proliferation of phytohaemagglutinin A-stimulated human peripheral blood mononuclear cells was determined as described by Bygbjerg et al.[78]

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