New Natural Sesquiterpenes as Modulators of Daunomycin Resistance in a Multidrug-Resistant *Leishmania tropica* Line[||,⊥]

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Received May 3, 1999

The effects produced by nine dihydro-*β*-agarofuran sesquiterpenes isolated from *Crossopetalum tonduzii* (**1**−**8**) and *Maytenus macrocarpa* (**9**) (Celastraceae) on the reversion of the resistant phenotype on a multidrug-resistant *Leishmania* line and their binding to recombinant C-terminal nucleotide-binding domain of *Leishmania* P-glycoprotein-like transporter were studied. The structures of the new compounds (**1**−**5**) were elucidated by spectroscopic methods, including $^{1}H-^{13}C$ heteronuclear correlation (HMQC), long-range correlation spectra with inversal detection (HMBC), ROESY experiments, and chemical correlations. The absolute configuration of one of them (**1**) was determined by CD studies. The structure−activity relationship is discussed.

Introduction

As part of an intensive course of research into biologically active metabolites from Celastraceae, used in folk medicine,[1] *Crossopetalum tonduzii* (Loes) Lund[2] was studied. The sesquiterpene esters, based on the dihydro-*β*-agarofuran [5,11-epoxy-5*β*,10α-eudesm-4(14)-ene] skeleton, are chemotaxonomic indicators of the family,[3] and they have shown a wide range of biological activities such as cytotoxic,[4] antitumor-promoting,[5] immunosuppressive,[6] insect antifeedant, and insecticidal properties.[7] Besides, dihydro-*β*-agarofuran sesquiterpene derivatives, isolated from the root of *Celastrus orbiculatus*, partially or completely reversed multidrug resistance (MDR) phenotype in cancer cells.[8] On the other hand, Torilin (8,11-dihydroxy-8-angeloyl-11-acetyl-4-guaien-3-one), a non-agarofuran sesquiterpene isolated from the fruits of *Torilis japonica* (Umbelliferae), reverts the MDR phenotype in cancer cells due to the increase of the intracellular accumulation of anticancer drugs.[9,10]

The MDR phenotype due to P-glycoprotein (Pgp) has been extensively documented as a very efficient mechanism to reduce intracellular drug accumulation in cancer cells[11,12] and parasitic protozoans including *Plasmodium*[13,14] and *Leishmania*.[15−17] Pgp belongs to the ATP-binding cassette (ABC) superfamily of transporter proteins and is thought to function as an ATP-dependent pump that binds and exports a broad range of drugs out of the cell, decreasing their intracellular drug concentration. Structural analysis of a Pgp sequence indicates two homologous halves, each composed of a transmembrane domain involved in drug efflux and a cytosolic nucleotide-binding domain (NBD) involved in ATP binding and hydrolysis. Numerous modulators or chemosensitizers are known to alter the ability of Pgp to maintain subtoxic intracellular drug concentrations: among others the calcium channel blockers such as verapamil and immunosuppressants such as cyclosporin A. These compounds are known to reverse MDR in cancer cells by competing with drug binding to the transmembrane domains of Pgp.[18] However, they produce undesirable side effects; thus, there is a need to develop new classes of modulators of Pgp with less toxicity for the host. Recently, it has been described that the NBDs can also be a target of Pgp inhibitors. Thus, flavonoids bind to these domains partly overlapping the ATP-binding site and a vicinal hydrophobic binding region,[19−21] inhibiting drug efflux activity and reversing the resistant phenotype of a *L. tropica* MDR line.[20]

This paper reports the effects produced by nine dihydro-*β*-agarofuran sesquiterpenes isolated from the aerial parts of *C. tonduzii* (**1**−**8**) and the roots of *M. macrocarpa* (R. and P.) Buquet (**9**)[22] (Celastraceae) on the reversion of daunomycin (DNM) resistance on a MDR *L. tropica* line and their binding to the recombinant C-terminal nucleotide-binding domain (NBD2) of *Leishmania* Pgp-like transporter, which leads to an improved understanding of the molecular site of action of these compounds on Pgps. The structures of the new compounds (**1**−**5**) were elucidated by spectroscopic methods, including $^{1}H-^{13}C$ heteronuclear correlation (HMQC), long-range correlation spectra with inverse detection (HMBC), ROESY experiments, and chemical correlations. The absolute configuration of **1** was deter-

[||] This paper is warmly dedicated to Prof. Antonio González, Universidad de La Laguna, on the occasion of his 81st birthday.

[⊥] Abbreviations: ABC, ATP-binding cassette; DNM, daunomycin; MDR, multidrug resistance; NBD, nucleotide-binding domain; NBD2, C-terminal nucleotide-binding domain; Pgp, P-glycoprotein; ONic, nicotinate; OAc, acetate; OBz, benzoate; OMeBut, 2-methylbutyrate; CD, circular dichroism; EtOAc, ethyl acetate; EtOH, ethanol; MeCN, acetonitrile.

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10.1021/jm991066b CCC: $18.00
Published on Web 09/24/1999

Table 1. ^{1}H NMR (δ, $CDCl_3$, J in Hz in parentheses) Data of Compounds **1**−**5**

compd	H-1	H-2	H-6	H-7	H-8	H-9	H-15
1	4.60 m	5.51 m	5.19 s	2.65 d (3.3)	5.71 dd (3.3, 9.8)	6.18 d (9.8)	4.62, 5.13 d_{AB} (13.2)
2	5.46 d (3.3)	5.36 m	6.47 s	2.52 d (3.2)	5.47 dd (3.2, 9.8)	6.04 d (9.8)	4.62, 4.93 d_{AB} (13.4)
3	5.48 d (3.4)	5.39 m	5.20 s	2.61 d (3.2)	5.59 dd (3.2, 10.0)	6.05 d (10.0)	4.59, 4.93 d_{AB} (13.4)
4[a]	5.99 d (3.6)	5.92 m	6.59 s	2.37 d (3.0)	5.87 d (3.0)	6.37 s	5.13, 5.34 d_{AB} (12.7)
5	5.48 dd (3.8, 11.8)	2.06 m	6.32 s	2.53 d (3.0)	5.61 d (3.0)	5.98 s	4.54, 4.81 d_{AB} (12.7)

[a] C_6D_6.

Table 2. ^{13}C NMR (δ, $CDCl_3$) Data[a] of Compounds **1**−**5**

C	**1**	**2**	**3**	**4**	**5**
C-1	72.2 d	75.6 d	76.9 d	71.0 d	72.8 d
C-2	73.2 d	67.6 d	67.3 d	68.8 d	23.6 t
C-3	41.4 t	42.0 t	41.6 t	42.1 t	37.9 t
C-4	72.0 s	69.6 s	72.1 s	69.9 s	70.5 s
C-5	91.5 s	92.1 s	91.5 s	91.6 s	91.4 s
C-6	76.9 d	75.4 d	75.3 d	71.3 d	76.2 d
C-7	53.4 d	52.1 d	53.5 d	53.3 d	53.4 d
C-8	74.0 d	73.8 d	74.2 d	77.3 d	77.4 d
C-9	76.6 d	75.3 d	75.1 d	75.8 d	71.0 d
C-10	51.1 s	51.4 s	50.6 s	54.8 s	54.2 s
C-11	84.3 s	84.3 s	84.6 s	83.2 s	83.2 s
C-12	26.2 q	25.7 q	26.2 q	25.1 q	25.6 q
C-13	30.0 q	29.7 q	30.1 q	29.1 q	29.7 q
C-14	24.4 q	24.7 q	24.1 q	24.4 q	22.8 q
C-15	61.7 t	61.7 t	61.7 t	65.1 t	63.9 t

[a] Data are based on DEPT, HMQC, and HMBC experiments.

mined by CD studies. The structure−activity relationship is also discussed.

Results and Discussion

Repeated chromatography on Sephadex LH-20 and silica gel of the ethanol extract of the aerial parts of *C. tonduzii* yielded, in addition to the known metabolites (**6**−**8**),[23] five new sesquiterpenes (**1**−**5**) (Table 5). Compound **1** had the molecular formula $C_{35}H_{43}NO_{12}$ by HREIMS; its IR spectrum showed absorption bands for hydroxy (3424 cm^{-1}) and ester (1731 cm^{-1}) groups. The mass spectrum contained fragments attributable to the presence of nicotinate (*m*/*z* 124), benzoate (*m*/*z* 105), 2-methylbutyrate (*m*/*z* 85), and acetate ($M^+ - 60$, *m*/*z* 609) groups; this was confirmed by the ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2), which included signals for nine aromatic protons between δ 7.36−9.32 for benzoate and nicotinate groups, one acetate methyl as a singlet at δ 1.91, signals for a 2-methylbutyrate group at δ 1.03 as a triplet (3H, J = 7.4 Hz) and at δ 1.34 (3H, J = 7.0 Hz) as a doublet, two methylene protons as two multiplets at δ 1.67 and 1.86, and a multiplet at δ 2.65 (1H). In its ^{1}H NMR spectrum (Table 1) also was observed signals assignable to protons on the carbons bearing three secondary ester groups at δ 6.18 d (H-9), 5.71 dd (H-8), and 5.51 m (H-2); two protons of a primary ester group at δ 4.62 and 5.13 d_{AB} (H-15); two geminal protons to secondary hydroxyl groups at δ 5.19 s (H-6) and 4.60 m (H-1); a angular methyl group at 1.75 s (Me-14) attached to a carbon at δ 72.0 bearing a hydroxyl group; and two angular methyls at δ 1.73 and 1.62 s (Me-12 and Me-13), which were confirmed by ^{13}C NMR (Table 2). All these data indicate that **1** was a polyester sesquiterpene with the dihydro-β-agarofuran skeleton. The regiosubstitution characteristics were determined by an HMBC experiment (Table 3); thus, the acetate group was sited at C-8, the benzoate at C-9, the 2-methylbutyrate at C-15, the nicotinate at C-2, and the secondary hydroxy groups at C-1 and C-6.

The relative configuration of **1** was determined on the basis of the coupling constants and the results of a ROESY experiment (Figure 1). For example, in the COSY experiment the coupling constants of H_1-H_2 and H_8-H_9 ($J_{1,2}$ = 3.1 Hz and $J_{8,9}$ = 9.8 Hz) indicated a *cis*-relationship between H-1 and H-2 and a *trans*-relationship between H-8 and H-9.[24] In its ROESY experiment significant cross-peaks were observed between H-1 and H-9 and H-2; between H-15 and H-6, H-8, and Me-14; and finally between Me-12 and H-9. Its absolute configuration was resolved by the dibenzoate chirality method, an extension of the CD exciton chirality method.[25] The dihedral angle between the two benzoate chromophores is 70.2° (calculated from J value data and by molecular mechanics calculations using the PC model[26]), and it was therefore considered suitable for CD study. The CD spectrum showed a split curve with a first positive Cotton effect at 229.1 nm ($\Delta\epsilon$ +8.5) and a second negative one at 215.1 nm ($\Delta\epsilon$ −0.4). The absolute configuration of **1** was accordingly established as (1*R*,2*S*,4*S*,5*S*,6*R*,7*R*,8*S*,9*S*,10*S*)-8-acetoxy-9-benzoyloxy-15(2)-methylbutyroyloxy-2-nicotynoyloxy-1,4,6-trihydroxy-dihydro-β-agarofuran.

The structures of compounds **2**−**4** were elucidated by spectral methods, including 2D ^{1}H−^{1}H and ^{1}H−^{13}C correlations, ROESY experiments, and chemical correlations (Tables 1−3). Thus, acetylation of **3** gave a product whose spectroscopic data were identical to those of **2**.

Compound **5**, with the molecular formula $C_{37}H_{45}NO_{12}$ (HREIMS), was shown to be a dihydro-β-agarofuran sesquiterpene with two acetate, one benzoate, one 2-methylbutyrate, one nicotinate, and one tertiary hydroxyl groups, positioned at 1α, 4β, 6β, 8α, 9β, and 15, on the basis of the study of its IR, UV, ^{1}H, and ^{13}C data (Tables 1, 2) and 2D experiments. An HMBC experiment (Table 3) allowed us to establish the substitution patterns, the new compounds having the basic polyhydroxy skeletons of 8-epi-4β-hydroxyalatol (**1**−**3**),[27] 9-epi-4β-hydroxyalatol (**4**),[27] and 9-epi-isoalatol (**5**).[28]

Agarofuran sesquiterpenes are particularly promising modulators of the MDR phenotype in eukaryotic organisms, as previously described.[8] The in vivo reversing effect of sesquiterpenes was studied on the growth of resistant parasites in the presence of DNM by comparison with wild-type parasites without drug (Table 4). After 72-h incubation of resistant parasites in medium

Table 3. Three-Bond $^1H-C^{13}$ Coupling (HMBC) in Compounds **1**−**5**

H	1	2	3	4	5
H-1	C-2*, C-9, C-10, C-15	C-9, C-10*, C-15, OAc	C-9, C-10*, C-15, OAc	C-10*, C-15, OAc	C-10*, C-15
H-2		OAc	OAc	OAc	
H-6	C-5*, C-11	C-5*, C-7*, C-8, C-11, OAc	C-5*, C-8, C-11	C-5*, C-8, C-11, OAc	C-5*, C-10, C-11, OAc
H-7	C-5, C-6*, C-9	C-5, C-8*, C-9	C-5*, C-6*, C-8	C-5, C-8*, C-9	C-5, C-8*, C-9
H-8	C-7*, C-9*, C-11, OAc	C-9*, C-11, OAc	C-9*, C-11, OAc	C-6, C-9*, ONic	C-6, C-9*, C-10, ONic
H-9	C-1, C-8*, C-10, C-15, OBz	C-1, C-8*, C-10, C-15, OBz	C-8*,C-10*, C-15, OBz	C-5, C-7, C-8*, C-15, OBz	C-5, C-8*, C-10, C-15, OBz
H-15	C-5, C-9, C-10*, OMeBut	C-1, C-5, C-10*, OMeBut	C-5, C-9, C-10*, OMeBut	C-1, C-5, OMeBut	C-5, C-9, C-10*, OMeBut

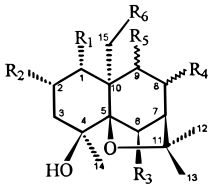
* Two-bond coupling enhancement observed.

Table 4. Effect of Sesquiterpenes on Cytotoxicity of DNM in a MDR *L. tropica* Line

	growth inhibition[a] (%)			
	15 μM		30 μM	
compd	W	DNM-R150	W	DNM-R150
1	9.3 ± 0.8	20.8 ± 7.3	8.4 ± 1.1	88.3 ± 7.9
2	4.9 ± 0.5	40.9 ± 4.9	9.5 ±1.4	86.1 ± 13.8
3	7.5 ± 1.0	26.8 ± 3.2	11.1 ± 1.8	91.7 ± 12.1
4	7.5 ± 0.9	32.3 ± 4.8	6.2 ± 0.9	82.5 ± 10.7
5	6.9 ± 0.7	92.9 ± 10.3	13.8 ± 1.7	96.1 ± 13.4
6	25.5 ± 2.8	77.8 ± 10.1	62.9 ± 8.7	95.4 ± 10.3
7	9.8 ± 1.2	90.5 ± 11.8	30.0 ± 4.2	96.0 ± 11.2
8	5.3 ± 0.6	83.1 ± 9.2	4.0 ± 0.5	92.6 ± 11.5
9	13.5 ± 1.4	16.3 ± 1.9	16.9 ± 2.5	49.8 ± 5.6

[a] Wild-type (W) and MDR (DNM-R150) parasites were treated with 15 and 30 μM of different sesquiterpenes, in the absence of presence of 150 μM DNM, respectively. The values represent the percentage of growth inhibition relative to control growth in the absence of inhibitory agent. The data shown are the average of three independent experiments ±SD.

Table 5. Affinity Binding of Sesquiterpenes **1**−**9** to *Leishmania* NBD2[a]



compd	R_1	R_2	R_3	R_4	R_5	R_6	% quenching at 50 μM[b]
1	OH	ONic	OH	β-OAc	α-OBz	OMeBut	31.1
2	OAc	OAc	OAc	β-OAc	α-OBz	OMeBut	22.8
3	OAc	OAc	OH	β-OAc	α-OBz	OMeBut	30.8
4	OAc	OAc	OAc	α-ONic	β-OBz	OMeBut	31.8
5	OAc	H	OAc	α-ONic	β-OBz	OMeBut	34.3
6	OBz	OH	OAc	β-OAc	α-OBz	OMeBut	ND
7	OBz	OH	OH	β-OAc	α-OBz	OMeBut	31.4
8	OAc	OH	OH	β-OMeBut	α-OBz	OMeBut	27.5
9	OAc	H	OAc	β-OAc	α-OBz	OAc	27.0

[a] The NBD2 domain was incubated, under conditions previously described in the Experimental Section. [b] The quenching of intrinsic fluorescence was plotted as a function of concentration, and the quenching produced at 50 μM was graphically determined. ND, not determined due to the high intrinsic fluorescence of this compound.

containing 150 μM DNM and 15 μM of sesquiterpenes (**5**−**8**), we observed a high inhibition of the growth (more than 75%), as compared to the same conditions without sesquiterpenes (Table 4). The substituent at the C-2 position seems to be important for the reversal activity since the presence of protons on C-2 produces a 3-fold higher chemosensitization with respect to the presence in the same position of an acetate group (**5** versus **4**).

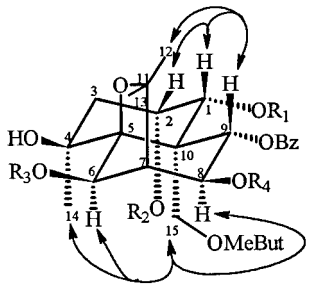


Figure 1. ROESY experiment of compound **1**.

This result suggests that the size of the substituent could strongly affect the reversal activity of the compound. Interestingly, the other three active compounds (**6**−**8**) have in common the presence of a hydroxyl group at this C-2 position with respect to an acetate group in the less active compounds (**1**−**4**), supporting the relevance of this position. Higher concentrations of all compounds (30 μM) produce more than 80% growth inhibition, with the exception of **9** (50%) which lacks the OMeBut group on C-15, clearly showing that such a group is essential for activity in this type of compounds.

The presence of a benzoate group at C-1 increases the cytotoxic effects of the compounds as observed for **6** and **7**. These compounds produce in wild-type parasites a 62.9% and 30% inhibition of growth, respectively, at 30 μM, while at 50 μM the inhibition reached 72.4% and 78.6%, respectively (data not shown). The other compounds produced limited effects on wild-type parasites (around 10% inhibition of growth at 30 μM), which could correspond to some low binding to other cellular targets. These results clearly demonstrate that sesquiterpenes **5**−**8** at 15 μM and **1**−**4** at 30 μM revert the DNM-resistant phenotype in *Leishmania*.

Sesquiterpenes maintain neither a conjugated planar ring nor a substituted tertiary amino group, as described for other MDR reversal agents;[18] however, they share a significant hydrophobicity region. Recently, the presence of a hydrophobic interaction region has been characterized near to the ATP-binding site of the NBDs of Pgps.[19−21] The binding of hydrophobic compounds such as non-Pgp-transported steroids (i.e. RU486) to this region could partly explain their reversal effects.[21] Also, addition of hydrophobic substituents to flavonoids significantly increases the affinity binding to the recombinant domain, increasing drug efflux inhibition in a drug-resistant *L. tropica* line and reversing the resistant phenotype at lower concentrations.[20] Probably, this effect could be due to strengthening of the interaction at this hydrophobic binding region. To determine whether the sesquiterpenes could produce their reversal effect, at least partly, by interacting with this hydrophobic

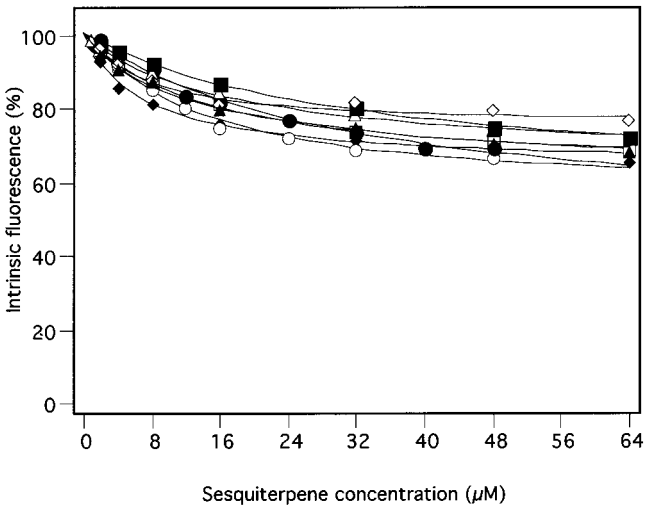


Figure 2. Interaction of recombinant *Leishmania* NBD2 with sesquiterpenes. The intrinsic fluorescence of recombinant NBD2 was measured under conditions described in the Experimental Section, in the presence of increasing concentrations of sesquiterpenes **1** (□), **2** (◇), **3** (▲), **4** (●), **5** (○), **7** (◆), **8** (△) and **9** (■) as dimethyl sulfoxide solutions.

region of the cytosolic domains of the Pgp transporter, we studied their binding to purified NBD2 of a *Leishmania* Pgp-like transporter by measuring the quenching of recombinant NBD2 intrinsic fluorescence, as previously described.[19−21] The different sesquiterpenes exhibited a low-affinity binding for the recombinant domain (Figure 2), with fluorescence quenching at 50 μM in the same range independently of the presence of different substituents at the R_1-R_6 positions (Table 5). Thus, while **4** and **5** present similar quenching at 50 μM (31.8 and 34.8, respectively), the in vivo reversal effects of both compounds show significant differences at 15 μM, **5** being 3-fold more effective as a reversal agent (Table 4).

Our results show that the in vivo ability of dihydro-β-agarofuran sesquiterpenes to inhibit growth of resistant parasites is not related to the binding to the cytosolic NBDs, suggesting the binding to the transmembrane domains of *Leishmania* Pgp-like transporter and blocking DNM efflux. Further efforts to elucidate the specific target of dihydro-β-agarofuran sesquiterpenes, in the Pgp-like transporter and to determine if these compounds are transported, are necessary for a better knowledge of their mechanism of action. Also, it is necessary to extend the assays to a higher number of compounds for a further structure−activity relationship. In vitro and in vivo experiments to answer these questions are underway in MDR cancer and parasite cells by using differently substituted sesquiterpenes and will be reported in due course.

Experimental Section

General Experimental Procedures. IR spectra were recorded in $CHCl_3$ on a Bruker IFS 55 spectrophotometer and UV spectra were collected in absolute EtOH on a Jasco V-560. ^1H and ^{13}C NMR spectra were recorded on a Bruker at 400 and 100 MHz, respectively. Specific rotations were measured on a Perkin-Elmer 241 automatic polarimeter and CD spectra on a Jasco J-600 spectropolarimeter. EIMS and HREIMS were recorded on a Micromass Autospec spectrometer. TLC 1500/LS 25 Schleicher and Schuell foils were used for thin-layer chromatography, while silica gel (0.2−0.63 mm) and Sephadex LH-20 were used for column chromatography.

Plant Material. *C. tonduzii* was collected at Boquete, Chiriquí, Panamá, in August 1991. A voucher specimen (FLORPAN 882) was deposited at the Herbarium of the University of Panamá.

Extraction and Isolation. The aerial parts (1.5 kg) of *C. tonduzii* were extracted with EtOH in a Soxhlet apparatus. Removal of the solvent under vacuum gave 190 g of residue, which was chromatographed on a silica gel column, using mixtures of *n*-hexanes−EtOAc as solvent. In this way, after several chromatographic separations on Sephadex LH-20 and silica gel, compounds **1** (9 mg), **2** (12 mg), **3** (13 mg), **4** (20 mg), and **5** (15 mg) were obtained. Compound **1** used for CD was purified by HPLC using a semipreparative μ-porasil column and eluted with a mixture of *n*-hexane−EtOAc (1:1).

(1*R*,2*S*,4*S*,5*S*,6*R*,7*R*,8*S*,9*S*,10*S*)-8-Acetoxy-9-benzoyloxy-15(2)-methylbutyroyloxy-2-nicotynoyloxy-1,4,6-trihydroxy-dihydro-β-agarofuran (1): colorless oil; $[\alpha]_D^{27}$ = +42.1 (c = 1.03, $CHCl_3$); UV λ_{max} (EtOH) 263, 252, 223, 202 nm; CD λ_{ext} (MeCN) 229.1 ($\Delta\epsilon$ = +8.5), 215.1 ($\Delta\epsilon$ = −0.4) nm; IR ν_{max} ($CHCl_3$) 3424, 3017, 2924, 2851, 1731, 1592, 1451, 1368, 849, 739, 711 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.03 (3H, t, J = 7.4 Hz), 1.34 (3H, d, J = 7.0 Hz), 1.62 (3H, s), 1.67 (1H, m), 1.73 (3H, s), 1.75 (3H, s), 1.86 (1H, m), 1.91 (3H, s), 2.12 (1H, dd, J = 3.9, 15.2 Hz), 2.22 (1H, dd, J = 3.9, 15.2 Hz), 2.68 (1H, m), 3.16 (1H, s), 7.36 (2H, m), 7.47 (1H, dd, J = 4.9, 8.0 Hz), 7.52 (1H, m), 7.86 (2H, m), 8.41 (1H, d, J = 8.0 Hz), 8.80 (1H, d, J = 4.9 Hz), 9.32 (1H, s), for other signals, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz) δ OAc [20.8 (CH_3), 169.8 ($-CO_2-$)], OBz [128.4 (2 × CH), 129.3 (C), 129.6 (2 × CH), 133.4 (CH), 167.4 ($-CO_2-$)], ONic [123.6 (CH), 125.3 (C), 137.2 (CH), 151.1 (CH), 153.8 (CH), 165.0 ($-CO_2-$)], OMeBut [11.7, 16.7 (2 × CH_3), 26.6 (CH_2), 41.6 (CH), 176.1 ($-CO_2-$)], for other signals, see Table 2; MS (EI) m/z (%) 669 (M^+, 1), 654 (1), 609 (3), 484 (3), 304 (3), 262 (4), 244 (6), 216 (10), 164 (12), 124 (25), 105 (100), 85 (14), 57 (33). HRMS (EI) m/z Calcd for $C_{35}H_{43}NO_{12}$: 669.27853. Found: 669.27936.

1α,2α,6β,8β-Tetraacetoxy-9α-benzoyloxy-15(2)-methylbutyroyloxy-4β-hydroxy-dihydro-β-agarofuran (2): colorless oil; $[\alpha]_D^{27}$ = +11.8 (c = 0.4, $CHCl_3$); UV λ_{max} (EtOH) 274.4, 261.0, 230.8, 212.8 nm; IR ν_{max} ($CHCl_3$) 3525, 2890, 1742, 1735, 1725, 1442, 1225, 1030, 750 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.03 (3H, t, J = 7.4 Hz), 1.36 (3H, d, J = 7.0 Hz), 1.49 (3H, s), 1.52 (3H, s, −OAc), 1.56 (3H, s), 1.67 (2H, m), 1.72 (3H, s), 1.88 (3H, s, −OAc), 1.95 (2H, m), 2.09 (3H, s, −OAc), 2.13 (3H, s, −OAc), 2.79 (1H, m), 7.42 (2H, m), 7.56 (1H, m), 7.91 (2H, m), for other signals, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz) δ OAc [20.5, 20.8, 21.1, 21.4 (4 × CH_3), 169.5 (2 × $-CO_2-$), 169.7 (2 × $-CO_2-$)], OBz [128.6 (2 × CH), 129.3 (C), 129.6 (2 × CH), 133.5 (CH), 165.6 ($-CO_2-$)], OMeBut [11.6, 16.3 (2 × CH_3), 26.6 (CH_2), 41.2 (CH), 176.4 ($-CO_2-$)], for other signals, see Table 2; MS (EI) m/z (%) 675 (M^+ − 15, 1), 630 (2), 588 (3), 571 (3), 570 (7), 555 (2), 528 (3), 468 (2), 408 (2), 336 (12), 202 (26), 105 (100), 85 (4), 57 (28). HRMS (EI) m/z Calcd for $C_{34}H_{43}O_{14}$: 675.2653. Found: 675.2671 [M^+ − 15].

1α,2α,8β-Triacetoxy-9α-benzoyloxy-15(2)-methylbutyroyloxy-4β,6β-dihydroxy-dihydro-β-agarofuran (3): colorless oil; $[\alpha]_D^{27}$ = + 5.4 (c 0.52, $CHCl_3$); UV λ_{max} (EtOH); 274.2, 260.8, 212.2 nm; IR ν_{max} ($CHCl_3$) 3350, 2890, 1740, 1735, 1725, 1595, 1355, 1228, 1030, 756 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.05 (3H, t, J = 7.4 Hz), 1.36 (3H, d, J = 7.0 Hz), 1.55 (3H, s, −OAc), 1.64 (3H, s), 1.67 (3H, s), 1.71 (2H, m), 1.74 (3H, s), 1.76 (3H, s, −OAc), 2.05 (2H, m), 2.11 (3H, s, −OAc), 2.63 (1H, m), 3.10 (1H, s, H−OH), 7.41 (2H, m), 7.56 (1H, m), 7.88 (2H, m), for other signals, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz) δ OAc [20.4, 20.8, 21.1 (3 × CH_3), 169.4, 169.5, 169.9 (3 × $-CO_2-$)], OBz [128.6 (2 × CH), 129.3(C), 129.5 (2 × CH), 133.5 (CH), 165.6 ($-CO_2-$)], OMeBut [11.7, 16.8 (2 × CH_3), 26.6 (CH_2), 41.2 (CH), 176.2 ($-CO_2-$)], for other signals, see Table 2; MS (EI) m/z (%) 633 (M^+ − 15, 2), 588 (4), 570 (2), 528 (1), 495 (1), 451 (1), 406 (1), 331 (1), 304 (1), 287 (2) 244 (3), 202

(11), 164 (6), 149 (15), 105 (100), 85 (26), 69 (16), 57 (38). HRMS (EI) *m/z* Calcd for $C_{32}H_{41}O_{13}$: 633.2547. Found: 633.2562 [$M^+ - 15$].

Acetylation of 3. Acetic anhydride (4 drops) was added to compound **3** (2.5 mg) dissolved in pyridine (2 drops) and the mixture left at room temperature for 16 h. EtOH (3 × 2.0 mL) was added and the mixture reduced almost to dryness in a rotavapor; this process was repeated with $CHCl_3$ (3 × 2.0 mL) to give product **2** (2 mg).

1α,2α,6β-Triacetoxy-9β-benzoyloxy-15(2)-methylbutyroyloxy-4β-hydroxy-8α-nicotynoyloxy-dihydro-β-agarofuran (4): colorless oil; $[\alpha]_D^{27} = -33.1$ ($c = 0.54$, $CHCl_3$); UV λ_{max} (EtOH) 264, 229, 210, 202 nm; IR ν_{max} ($CHCl_3$) 3525, 3410, 2890, 2810, 1741, 1728, 1715, 1580, 1360, 1255, 1220, 1100, 1020, 750, 700 cm^{-1}; ^1H NMR (C_6D_6, 400 MHz) δ 0.80 (3H, t, $J = 7.4$ Hz). 1.24 (3H, d, $J = 7.0$ Hz). 1.35 (3H, s). 1.53 (3H, s). 1.56 (6H, s). 1.67 (3H, s). 1.72 (2H, m). 1.86 (1H, dd, $J = 3.1$, 12.2 Hz). 1.91 (3H, s). 2.13 (1H, dd, $J = 3.1$, 12.2 Hz). 2.53 (1H, m), 2.60 (1H, s), 6.77 (1H, dd, $J = 4.9$, 8.0 Hz), 7.15 (3H, m), 8.27 (2H, m), 8.42 (1H, m), 8.53 (1H, d, $J = 4.9$ Hz), 9.86 (1H, s), for other signals, see Table 1; ^{13}C NMR (C_6D_6, 100 MHz) δ OAc [20.1 (CH_3), 20.5 (2x CH_3), 169.0 (2 × $-CO_2-$), 169.1 ($-CO_2-$),], OBz [128.4 (2 × CH), 129.1 (C), 130.4 (2 × CH), 133.4 (CH), 164.4 ($-CO_2-$)], ONic [123.0 (CH), 125.9 (C), 136.9 (CH), 151.6 (CH), 153.8 (CH), 163.9 ($-CO_2-$)], OMeBut [11.3, 16.5 (2 × CH_3), 26.6 (CH_2), 41.2 (CH), 175.7 ($-CO_2-$)], for other signals, see Table 2; MS (EI) *m/z* (%) 753 (M^+, 17), 738 (1), 711 (3), 694 (8), 651 (2), 630 (2), 588 (2), 553 (2), 446 (2), 349 (2), 244 (3), 216 (5), 164 (4), 124 (44), 105 (100), 85 (9), 57 (3). HRMS (EI) *m/z* Calcd for $C_{39}H_{47}NO_{14}$: 753.29966. Found: 753.30135.

1α,6β-Diacetoxy-9β-Benzoyloxy-15(2)-methylbutyroyloxy-8α-nicotynoyloxy-4β-hydroxy-dihydro-β-agarofuran (5): colorless oil, $[\alpha]_D^{27} = -31.3$ ($c = 0.84$, $CHCl_3$); UV λ_{max} (EtOH) 264, 228, 209, 202 nm; IR ν_{max} ($CHCl_3$) 3505, 3400, 2855, 2800, 1725, 1710, 1580, 1445, 1355, 1265, 1220, 1100, 1015, 955, 750, 705 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.74 (3H, t, $J = 7.4$ Hz), 1.07 (3H, d, $J = 7.0$ Hz), 1.34 (3H, s), 1.39 (2H, m), 1.51 (3H, m), 1.60 (3H, s), 1.62 (2H, m), 1.71 (3H, s), 2.11 (3H, s), 2.35 (1H, m), 2.67 (1H, s), 7.46 (3H, m), 7.60 (1H, m), 8.06 (2H, m), 8.43 (1H, d, $J = 7.9$ Hz), 8.82 (1H, d, $J = 4.7$ Hz), 8.36 (1H, s), for other signals, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz) δ OAc [20.7, 21.4 (2 × CH_3), 169.7, 169.9 (2 × $-CO_2-$)], OBz [128.5 (2 × CH), 128.6 (C), 130.1 (2 × CH), 133.8 (CH), 164.4 ($-CO_2-$)], ONic [123.3 (CH), 125.7 (C), 137.3 (CH), 151.3 (CH), 153.8 (CH), 163.9 ($-CO_2-$)], OMeBut [11.3, 16.2 (2 × CH_3), 23.6 (CH_2), 40.9 (CH), 176.1 ($-CO_2-$)], for other signals, see Table 2; MS(EI) *m/z* (%) 695 (M^+, 15), 680 (3), 653 (16), 635 (11), 574 (7), 530 (5), 474 (4), 368 (2), 246 (3), 228 (5), 206 (27), 164 (10), 124 (45), 105 (100), 85 (7), 57 (16). HRMS (EI) *m/z* Calcd for $C_{37}H_{45}NO_{12}$: 695.29418. Found: 695.29407.

Biological Assays. 1. Parasite Culture and in Vivo Experiments. The wild-type *L. tropica* LRC strain used was a clone, obtained by agar plating as described.[29] A *L. tropica* line resistant to DNM (DNM-R150) was maintained in the continuous presence of 150 μM DNM and was used as previously described.[17] This resistant line shows a MDR phenotype similar to that described in tumor cells, with a cross-resistance to different unrelated drugs and overexpression of a Pgp-like transporter involved in drug efflux.[17] Cells were grown at 28 °C in RPMI 1640-modified medium (Gibco), as detailed[30] and supplemented with 20% heat-inactivated fetal bovine serum (Gibco). The growth sensitivities of wild-type and drug-resistant parasites to sesquiterpenes were ascertained as described.[20,31]

2. Overexpression, Protein Purification, and Interaction of *Leishmania* NBD2 with Sesquiterpenes. The recombinant *Leishmania* Pgp NBD2 was overexpressed in *Escherichia coli* M15 [pREP4] cells and purified by affinity chromatography as previously described.[20] Fluorescence experiments were performed at 25.0 ± 0.1 °C using a SLM-Aminco 8000C spectrofluorometer with spectral bandwidths of 2 and 4 nm for excitation and emission, respectively. The measurements were corrected for wavelength dependence on the excitation light intensity by using rhodamine B in the reference channel. All spectra were corrected for buffer Raman effect and for dilution. NBD2 (0.2−0.5 μM final concentration) fluorescence was performed in 1 mL of diluting buffer (50 mM potassium phosphate, pH 8.5, 1 M NaCl, 20% (w/v) glycerol, 0.05% (w/v) HECAMEG, 1 mM β-mercaptoethanol, and adjusting the final imidazole concentration to 10 mM), in the presence of increasing concentrations of dimethyl sulfoxide solutions of sesquiterpenes and by scanning emission in the range 300−350 nm upon excitation at 288 nm, to minimize the interference of imidazole, as previously described.[20] The binding of the different compounds was monitored by the quenching of emission fluorescence produced by addition of increasing sesquiterpene concentrations. Corrections for inner-filter effect and dimethyl sulfoxide dilution (up to 2% final concentration) were determined under the same conditions by using a mix of *N*-acetyltryptophanamide and *N*-acetyltyrosinamide in the same ratio, 3:7, as tryptophan and tyrosine residues present in NBD2. Curve fitting of ligand binding related to fluorescence decrease was accomplished with the Grafit program (Erithacus Software) as detailed previously.[32]

Acknowledgment. We thank Prof. J. T. Vázquez for carrying out the CD experiments and Dr. Attilio Di Pietro for his help in the affinity binding assays. We also thank Pilar Navarro for help with the parasite culture and Amparo Casado for her technical assistance in the in vivo experiments. This work was supported by the DGES (Projects PB96-1039 and PB96-1033) and the Spanish Grants Ref. PM97/0139 and UE97-0019 (F.G.) and The Instituto Tecnológico de Canarias. B. Mamani would like to acknowledge the Gobierno Autónomo de Canarias for economical support. J. M. Pérez-Victoria was the recipient of a fellowship from the Junta de Andalucia, Spain.

Supporting Information Available: IR, UV, MS, HRMS, and NMR spectra for the new compounds described and the CD spectrum of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM991066B