

## Five New Cassane Diterpenes from *Myrospermum frutescens* with Activity against *Trypanosoma cruzi*

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Received January 10, 2003

Five novel cassane diterpenes (**1–5**) with activity against *Trypanosoma cruzi* were isolated from leaves of *Myrospermum frutescens*. The structures were determined as 18-hydroxycassan-13,15-diene (**1**), 6 $\beta$ ,18-dihydroxycassan-13,15-diene (**2**), 6 $\beta$ -hydroxy-18-acetoxycassan-13,15-diene (**3**), 18-acetoxy-13,15-diene-19-cassanoic acid (**4**), and 6 $\beta$ ,13 $\beta$ -dihydroxy-18-acetoxycassan-14(17),15-diene (**5**). Structures were elucidated by spectroscopic analysis (NMR and HRCIMS) and by the synthesis of derivatives **2a** and **2b**. Compounds **3** and **5** were more active against the extracellular form of the parasite (11 and 16  $\mu$ M, respectively) than the intracellular forms, while compounds **1** and **2** were more active against the more clinically relevant intracellular forms of the parasite (17  $\mu$ M). Compounds **1** and **2** were approximately 9-fold more toxic toward *T. cruzi* than toward human fibroblasts, the cell type that serves as the parasite's mammalian host cell.

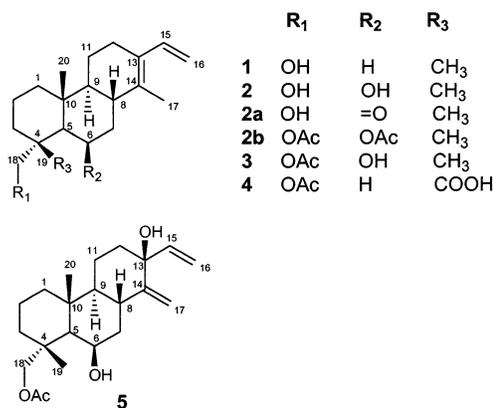
As part of the activities of the Panama International Cooperative Biodiversity Groups Program,<sup>1</sup> a program to find treatments for parasitic diseases, cancer, and HIV, we report the isolation of five novel cassane diterpenes (**1–5**) from the leaves of *Myrospermum frutescens* Jacq. (Fabaceae, subfamily Papilionaceae). Compounds **1–5** demonstrate activity against *Trypanosoma cruzi*, the causative agent of Chagas' disease, a parasitic disease that outranks the combined burden of malaria, schistosomiasis, and leishmaniasis in terms of its economic impact in Latin America.<sup>2</sup> Current estimates from the World Health Organization indicate that 16–18 million people are infected, with an additional 100 million at risk.<sup>3</sup> There is an urgent need for novel treatments for Chagas' disease, as there are no treatments currently available for use on a large-scale nor have any vaccines been developed.

There are no ethnopharmacologic uses for this plant reported in the literature. Previous chemical investigations of *M. frutescens* were limited to the isolation of oils from the seeds.<sup>4,5</sup> There are reports of diterpenes with activity against *T. cruzi*, the majority of which possess kaurane skeletons.<sup>6–8</sup> Compounds with the cassane skeletons, such as those described herein, have been isolated from the genus *Caesalpinia* (Fabaceae, subfamily Caesalpinaceae), the majority of which are furanoditerpenoids.<sup>9–11</sup>

The structures of the cassane diterpenes **1–5** were determined primarily by NMR techniques, in particular 1D <sup>1</sup>H and <sup>13</sup>C NMR (broad band and DEPT) and 2D NMR (COSY-90, NOESY, HSQC, and HMBC), irradiation, and NOE differential experiments. Herein we describe the isolation, structural characterization, and anti-trypanosomal activity of these diterpenes.

### Results and Discussion

An MeOH/EtOAc extract of leaves of *M. frutescens* showed activity against the extracellular form of the *T.*



*cruzi* parasite. The extract was suspended in H<sub>2</sub>O and extracted successively with CH<sub>2</sub>Cl<sub>2</sub> and EtOAc. The residue obtained from the CH<sub>2</sub>Cl<sub>2</sub> layer was then partitioned between hexane and 10% aqueous MeOH. Activity against *T. cruzi* was found in the hexane, EtOAc, and MeOH fractions. The MeOH fraction was selected for bioassay-guided fractionation due to the enhanced likelihood of finding compounds of medium polarity and measurable anti-trypanosomal activities, yielding the novel cassane diterpenes **1–5**.

Compound **1** was obtained as an amorphous solid. The molecular formula was assigned as C<sub>20</sub>H<sub>32</sub>O on the basis of the [M + H]<sup>+</sup> ion that appeared at *m/z* 289.2537 in HRCIMS experiments. The IR spectrum showed an absorbance at 3425 cm<sup>-1</sup>, consistent with a hydroxyl group. The <sup>1</sup>H NMR spectrum showed three methyl singlets at  $\delta$  0.80, 0.89, and 1.73, which were assigned to positions C-19, C-20, and C-17, respectively. Two doublets at  $\delta$  3.12 (1H, *J* = 11 Hz) and 3.39 (1H, *J* = 11 Hz) indicated the presence of two diastereotopic protons on a hydroxyl-bearing carbon. <sup>1</sup>H NMR spectra also showed the presence of olefinic protons at  $\delta$  4.95 (1H, d, *J* = 11 Hz), 5.03 (1H, d, *J* = 17 Hz), and 6.80 (1H, dd, *J* = 11, 17 Hz), consistent with a terminal double bond. The <sup>13</sup>C NMR experiments showed signals for 20 carbon atoms, four of which were olefinic

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carbons at  $\delta$  111.0, 128.9, 135.9, and 137.2 and one signal for a hydroxyl-bearing carbon at  $\delta$  72.6. Carbon-bound protons were assigned from HSQC 2D NMR spectral data. Using HMBC 2D NMR data, the methyl group at  $\delta$  1.73 was assigned to C-17, on the basis of the correlation of its protons to C-8, C-13, and C-14. The hydroxyl-bearing methylene group was assigned to C-18 on the basis of the correlation of the carbon-bound protons to C-3, C-4, and C-5. The NOESY spectrum of **1** showed a correlation between H-18a ( $\delta$  3.12) and H-18b ( $\delta$  3.39) with H-5 ( $\delta$  1.20) and H-19 ( $\delta$  0.80). Correlations between H-15 ( $\delta$  6.80) and H-16 ( $\delta$  4.95 and 5.03) and H-17 ( $\delta$  1.73) were observed, in addition to correlations between the protons of H-16 and H-12 ( $\delta$  2.02 and 2.31). These data correspond to a diterpene with a cassane skeleton with no additional substituents on the A, B, or C rings.<sup>12</sup> The absence of any NOE correlation between H-20 and the protons on C-18 was consistent with the placement of the hydroxymethylene group at C-4 in the  $\alpha$  position, an observation consistent with the NOEs between the H-18 protons and H-5.

Compound **2** was also obtained as an amorphous solid. The molecular formula was assigned as  $C_{20}H_{32}O_2$  on the basis of the  $[M + H]^+$  ion that appeared at  $m/z$  305.2470 in the HRCIMS, 16 amu greater than **1**. The IR spectrum showed a broad band at  $3433\text{ cm}^{-1}$  consistent with a hydroxyl moiety. The  $^1\text{H}$  NMR spectrum of **2** was very similar to that of compound **1**, the major difference being a resonance for a single proton at  $\delta$  4.38 in the spectrum of compound **2**, consistent with its attachment to a hydroxyl-bearing carbon. The  $^{13}\text{C}$  NMR spectrum showed signals for 20 carbon atoms, including one resonance at  $\delta$  68.1 suggesting a hydroxyl-bound carbon. The HSQC spectrum revealed that H-6 ( $\delta$  4.38) was attached to C-6 ( $\delta$  68.1), an observation consistent with the HMBC correlations of  $\delta$  4.38 to C-4 and C-5. The NOESY spectrum of **2** showed correlations between H-6 and H-7a, H-18a, and H-18b. Irradiation of the signal at  $\delta$  4.38 in a differential NOE experiment showed correlations with protons at H-5, H-7a, and H-18a. The absence of any NOE correlation between protons at H-6, H-19, and H-20 suggests that the C-6 bound hydroxyl group is in the  $\beta$  position, consistent with the NOEs observed between the H-6 and H-18 protons.

To confirm the structure of **2**, two derivatives were synthesized. Oxidation of **2** with Jones reagent yielded a mixture of products from which ketone **2a** was isolated in 14% yield. The IR spectrum of derivative **2a** showed the presence of hydroxyl and carbonyl groups. The  $^1\text{H}$  NMR signal at  $\delta$  4.38 in the  $^1\text{H}$  NMR of **2**, assigned to position 6, was no longer evident in **2a**. The  $^{13}\text{C}$  NMR spectrum of **2a** showed 20 carbon signals with one signal at  $\delta$  211.8, consistent with the carbonyl carbon of a cyclohexanone. The assignment of the carbonyl group to position C-6 was based upon correlations to protons H-5 ( $\delta$  2.40), H-7a ( $\delta$  2.67), and H-8 ( $\delta$  2.44) observed in HMBC experiments. The NOESY spectrum showed correlation between H-18a and H-18b with H-19 ( $\delta$  1.17). NOE correlations between protons H-18 with H-20 were not observed. Irradiation of the H-18 protons in a differential NOE experiment showed a correlation only with H-19, providing additional support for the proposed relative configuration at C-4. Irradiation of H-20 ( $\delta$  0.84) showed correlations with C-19 methyl protons ( $\delta$  1.17) and H-8 ( $\delta$  2.44).

Treatment of **2** with pyridine and acetic anhydride yielded compound **3** (61%) and the diacetate **2b** (11%). The IR spectrum of **2b** showed a broad band at  $1730\text{ cm}^{-1}$ , consistent with the carbonyl groups of the two acetyl

residues. The  $^1\text{H}$  NMR spectrum indicated the presence of two methyls, also assigned to the acetyl moieties. The  $^1\text{H}$  NMR spectrum revealed a doublet at  $\delta$  5.39 ( $J = 2.4\text{ Hz}$ ), which was assigned to the proton bound to C-6. Irradiation at H-6 in a differential NOE experiment showed correlations with H-18, H-7a, and H-5, while irradiation of the H-18 proton showed correlations with the H-6, H-5, H-19, and H-18 protons. These results confirmed the relative configurations at C-4 and C-6. The  $^{13}\text{C}$  NMR indicated the presence of 24 carbons, including resonances for carbonyl carbons and methyl groups associated with the acetate moieties.

Compound **3** (first isolated as a natural product and then as a product of acetylation from **2**) was isolated as an amorphous solid. The molecular formula was assigned as  $C_{22}H_{34}O_3$  on the basis of HRCIMS data. The IR,  $^1\text{H}$  NMR, and  $^{13}\text{C}$  NMR were consistent with the presence of an acetyl moiety. The HMBC experiment showed correlations of the diastereotopic protons attached to C-18 with the carbonyl carbon of the acetyl moiety and with C-19. The protons attached to C-19 also showed correlations with the C-18 protons and to the quaternary carbon at C-4. These data are consistent with the attachment of the acetyl group to C-18.

Compound **4** showed an apparent molecular ion of  $m/z$  360.2300, consistent with the formula  $C_{22}H_{32}O_4$ . The IR spectrum showed absorbances consistent with a carboxylic acid moiety. The  $^1\text{H}$  NMR spectrum showed the presence of two methyl groups. The  $^{13}\text{C}$  NMR spectrum showed two signals for carbonyl carbons, one associated with a carboxylic acid moiety at  $\delta$  180.5 and the other assigned to an acetyl group at  $\delta$  171.5. HMBC data showed correlations between the carbonyl of the carboxylic acid group with H-18a ( $\delta$  4.45), H-18b ( $\delta$  3.92), H-5 ( $\delta$  1.25), and H-3a ( $\delta$  1.01), consistent with the carboxyl group at C-4. The absence of any detectable NOE between H-18 and H-20 was consistent with the relative configuration shown.

Compound **5** was assigned a molecular formula of  $C_{22}H_{34}O_4$  on the basis of HRCIMS data. The IR spectrum showed absorbances consistent with a hydroxyl group and the carbonyl carbon of an acetyl moiety. The  $^1\text{H}$  NMR spectrum showed evidence for two terminal double bonds. One terminal double bond showed an ABX system where the X part of the system was at  $\delta$  6.09 (1H, dd,  $J = 11$  and  $15\text{ Hz}$ ) and the AB part of the system was at  $\delta$  5.37 (1H, dd,  $J = 1.3$  and  $11\text{ Hz}$ ) and 5.38 (1H, dd,  $J = 1.3$  and  $15\text{ Hz}$ ). The other terminal double bond gave an AB system at  $\delta$  4.92 (1H, d,  $J = 1.7\text{ Hz}$ ) and 5.08 (1H, d,  $J = 1.7\text{ Hz}$ ). The  $^1\text{H}$  NMR spectrum showed signals for three methyl groups. The  $^{13}\text{C}$  NMR showed 22 signals including four olefinic carbons, comprising one quaternary carbon, two exomethylene carbons, and one  $\text{R}=\text{C}(\text{H})\text{R}'$  moiety. Compound **5** also revealed one oxygen-bound quaternary carbon at  $\delta$  86.0, subsequently assigned to C-13. The spectral data were consistent with two double bonds, one between C-15 and C-16 as seen in compounds **1–4**, and another between C-14 and C-17. Correlations observed in HSQC and HMBC experiments also supported the proposed structure for compound **5**. NOESY experiments showed correlations between the H-17 proton at  $\delta$  4.92 and both C-7 protons ( $\delta$  1.51, 1.90, dt,  $J = 3$  and  $13\text{ Hz}$ ), as well as correlations between H-15 with H-12 ( $\delta$  1.58), H-16 ( $\delta$  5.37), and the H-17 proton at  $\delta$  5.08. These data are consistent with a hydroxyl group on C-13 in the  $\beta$  position.

The anti-trypansomal activities of cassane diterpenes **1–5** are presented in Table 3. Bioassay-guided fractionation of *M. frutescens* employed the extracellular (epimas-

**Table 1.** <sup>1</sup>H NMR Data for Compounds **1–5**<sup>a</sup>

proton	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>
1	0.90 br 1.74 br	0.89 br 1.71 br	0.90 br 1.75 br	0.91 br 1.79 br	0.90 br 1.75 br
2	1.52 br 1.65 br	1.52 br 1.69 br	1.51 br 1.68 br	1.17 br 1.50 br	1.62 br
3	1.28 br	1.17 br 1.36 br	1.33 br 1.41 br	1.01 br 2.23 br	1.41 br 1.52 br
5	1.20 br	1.20 br	1.18 br	1.25 br	1.22 br
6	1.30 br 1.55 br	4.38 d (2,3)	4.27 d (1,7)	1.78 br	4.36 d (1,7)
7	0.89 br 2.13 br	1.19 br 2.13 dt (3,13)	1.19 br 2.13 dt (3,13)	2.16 br	1.51 br 1.90 dt (3,13)
8	2.02 br	2.40 br	2.42 br	1.93 br	2.71 br
9	0.96 br	0.92 br	0.92 br	0.84 br	0.92 br
11	0.96 br 1.70 br	1.70 br 2.02 br	1.18 br 1.85 br	1.78 br	1.52 br 1.69 br
12	2.02 br 2.31 br	1.99 br 2.31 br	2.03 br 2.34 br	2.25 br	1.58 br 2.22 dt (3,13)
15	6.80 dd (11,17)	6.78 dd (11,17)	6.77 dd (11,17)	6.79 dd (11,17)	6.09 dd (11,15)
16	4.95 d (11) 5.03 d (17)	4.93 d (11) 5.08 d (17)	4.94 d (11) 5.10 d (17)	4.95 d (11) 5.09 d (17)	5.37 dd (1,3,11) 5.38 dd (1,3,15)
17	1.73 s	1.72 s	1.73 s	1.66 s	4.92 d (1,7) 5.08 d (1,7)
18	3.12 d (11) 3.39 d (11)	3.19 d (11) 3.51 d (11)	3.69 d (11) 4.02 d (11)	3.92 d (10) 4.45 d (10)	3.73 d (11) 4.04 d (11)
19	0.80 s	1.16 s	1.25 s		1.30 s
20	0.89 s	1.15 s	1.19 s	0.72 s	1.25 s
CH <sub>3</sub> CO-18			2.02 s	1.97 s	2.06 s

<sup>a</sup> All spectra were recorded in CDCl<sub>3</sub>, 300 MHz at 27 °C using TMS as internal reference (δ in ppm, *J* are given in Hz).

**Table 2.** <sup>13</sup>C NMR Data for Compounds **1–5**<sup>a</sup>

carbon	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>
1	38.6	40.4	41.2	38.8	41.1
2	18.7	18.3	18.7	19.2	19.3
3	35.5	38.4	38.3	33.1	37.6
4	38.0	36.0	37.6	48.2	37.8
5	48.9	51.2	51.6	51.7	50.4
6	22.5	68.1	68.2	24.5	67.2
7	31.8	40.4	40.5	32.0	39.9
8	41.7	37.6	36.4	41.6	32.0
9	54.1	54.4	55.0	53.8	56.6
10	36.9	36.9	37.6	37.4	37.3
11	21.6	21.1	21.6	27.0	18.3
12	26.9	26.3	26.8	26.9	33.1
13	128.9	129.1	129.7	129.0	86.0
14	137.2	136.2	136.6	136.8	151.7
15	135.9	135.4	135.8	135.8	139.4
16	111.0	110.5	111.2	111.3	111.5
17	16.1	15.8	16.3	16.1	115.7
18	72.6	72.3	73.2	72.7	73.9
19	17.7	19.7	20.1	180.5	19.9
20	15.0	17.0	17.5	13.0	18.0
Ac-18			171.6 21.3	171.5 21.2	171.8 21.0

<sup>a</sup> δ in ppm, 75 MHz in CDCl<sub>3</sub>.

tigote) stage of *T. cruzi*. However, *T. cruzi* are obligatory intracellular organisms and require a host cell in order to survive and multiply in the mammalian host, and the intracellular forms (trypomastigote and amastigote) are those that are responsible for disease manifestations in humans.<sup>13</sup> Compounds **3** and **5** were more active against the extracellular form of the parasite (11 and 16 μM, respectively) than the intracellular form, while compounds **1** and **2** were more active against the more clinically relevant intracellular form of the parasite (17 and 16 μM, respectively). Compound **4** showed no significant activity against the extracellular form of the parasite (IC<sub>50</sub> = 104 μM) and was not isolated in sufficient quantity for testing against the intracellular forms of the parasite.

To accurately interpret the results of the intracellular assay, it was necessary to determine whether the measured toxicity of a compound resulted from the killing of the

**Table 3.** Activities of Compounds **1–5** against *Trypanosoma cruzi*<sup>a</sup>

compound	extracellular	intracellular	cytotoxicity <sup>b</sup>
	IC <sub>50</sub> (μM)	IC <sub>50</sub> (μM)	IC <sub>50</sub> (μM)
<b>1</b>	48.6 ± 15.8	17.4 ± 4.1	149
<b>2</b>	56 ± 3.8	16.6 ± 1.5	148
<b>3</b>	11.5 ± 8.4	25.9 ± 1.7	118
<b>4</b>	104 ± 0.74	ND <sup>c</sup>	ND
<b>5</b>	16.5 ± 1.2	35.8 ± 0.4	124
amphotericin B	1 ± 0.12	ND	ND
nifurtimox	ND	11 ± 1.92	ND

<sup>a</sup> Results show the IC<sub>50</sub> value ± the SD (*n* = 3). Compounds **2a** and **2b** were assayed in the extracellular *T. cruzi* assay and showed IC<sub>50</sub> values of 36 and 59 μM, respectively. <sup>b</sup> Experiments performed with human foreskin fibroblasts. <sup>c</sup> ND = not determined.

parasite or from simply killing the host cells. Accordingly, we determined the cytotoxicity of these compounds against mammalian cell lines using primary human foreskin fibroblasts (HFF), the cell line routinely employed to culture the intracellular forms of *T. cruzi*.<sup>14</sup> HFF cells were grown in 96-well culture plates and incubated in the presence and absence of the pure compounds for 6 days, followed by the addition of MTT, a reagent that is actively metabolized by living cells and routinely used to determine cell viability.<sup>15</sup> In the case of compounds **1** and **2**, the intracellular form of the parasite was approximately 9-fold more sensitive to the compounds than were the HFF cell lines (Table 3). The enhanced toxicity of compounds **1** and **2** toward the intracellular form of the parasite as compared to the HFF cell line is particularly relevant to the development of new drugs for treating Chagas' disease, as it involves the more clinically and biologically relevant form of the parasite for which there are currently no effective treatments. Compounds **3** and **5** showed approximately 8- and 10-fold greater effect, respectively, on the extracellular form of the parasite than on the HFF cells. Efforts are currently underway in our laboratories to better understand how substituents on the cassane skeleton affect the potency and specificity of their anti-trypanosomal activity.

## Experimental Section

**General Experimental Procedures.** Melting points were determined using an Electrothermal 9100 apparatus and are uncorrected. Optical rotations were measured on either JASCO DIP-370 or Perkin-Elmer 141 polarimeters. IR spectra were recorded on a Shimadzu FTIR-8300 spectrophotometer. The NMR spectra were recorded on a Bruker Avance 300 spectrometer (300 MHz for protons and 75 MHz for carbon) with TMS as an internal standard. Irradiation experiments were recorded on Bruker AMX-500 and Bruker Avance 300 instruments. HRCIMS were recorded on UG-Autospec or Kratos MS50TC instruments.

**Plant Material.** Mature leaves of *M. frutescens* were collected from Barro Colorado Nature Monument in Gatún Lake in the Republic of Panama in January 2001. The material was identified by Professor Mireya Correa of the University of Panama and the Smithsonian Tropical Research Institute. Vouchers of the plants have been deposited in the herbarium of University of Panama (PMA 50913 and PMA 48733).

**Extraction and Isolation.** Upon collection, mature leaves were transferred to sealed plastic bags, kept on ice, and processed within 6 h. After removal of the stems, 0.3 kg of fresh leaves was homogenized in 30 g aliquots with 240 mL of cold MeOH for 30 s in a Waring blender followed by treatment with a Polytron homogenizer (Brinkmann Instruments) for at least 2 min or until the suspension of leaf material was homogeneous. The mixture was filtered under vacuum through Whatman # 4 filter paper, and the marc was then washed with 150 mL of EtOAc. The MeOH and EtOAc fractions were combined and filtered through Whatman # 1 filter paper. The extract was concentrated by rotary evaporation, yielding a total of 116 g of crude extract, which was stored at  $-80^{\circ}\text{C}$  until further use. In 15 g aliquots, the crude extract was dissolved in 500 mL of  $\text{H}_2\text{O}$  and extracted with  $\text{CH}_2\text{Cl}_2$  ( $4 \times 250$  mL). The  $\text{CH}_2\text{Cl}_2$  was removed under reduced pressure, and the extract was dissolved in 250 mL of 1:9  $\text{H}_2\text{O}/\text{MeOH}$ , which was then extracted with hexane ( $4 \times 250$  mL). Removal of MeOH under reduced pressure yielded a total of 21 g of extract, which was chromatographed in 5 g aliquots on a column of silica gel 60 (37–75  $\mu\text{m}$ , Geduran) eluting sequentially with 500 mL of 60:40  $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ , 200 mL of EtOAc, 300 mL of 1:1 EtOAc/MeOH, and 250 mL of MeOH. The fractions were combined according to composition by TLC into four main fractions (fractions 1–4). Fraction 1 (1.5 g) was subjected to column chromatography with silica gel (7GF, VWR Scientific) and eluted with 200 mL of  $\text{CH}_2\text{Cl}_2$  and 200 mL of  $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ , 4:1, to yield compounds **1** (6.0 mg), **3** (292.0 mg), and **5** (9.0 mg). Fraction 2 (5.6 g) was chromatographed on a column of silica gel 60 (63–200  $\mu\text{m}$ ) and eluted sequentially with hexane/EtOAc in the following proportions: 90:10 (200 mL), 85:15 (500 mL), 80:20 (400 mL), 70:30 (500 mL), 60:40 (300 mL), and 40:60 (300 mL), followed by 300 mL of EtOAc, 300 mL of 1:1 EtOAc/MeOH, and 300 mL of MeOH, which were pooled into six fractions (fractions 1a–6a). Fraction 3a (2.04 g) was subjected to silica gel column chromatography (7GF) and eluted with 250 mL of 60:20:20 hexane/toluene/acetone to yield compound **2** (392.0 mg). Fraction 3a was also subjected to preparative TLC (Whatman PK5F silica gel 150 A plates) followed by elution with a 40:40:20 mixture of hexane/ $\text{CHCl}_3$ /THF, yielding compounds **2** (14.0 mg) and **4** (17.0 mg).

**18-Hydroxycassan-13,15-diene (1):** amorphous powder;  $[\alpha]_D^{25} -49^{\circ}$  ( $c$  0.0015, MeOH); IR ( $\text{CHCl}_3$ )  $\nu_{\text{max}}$  3425, 2928, 2866, 1631, 1385  $\text{cm}^{-1}$ ; for  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR data, see Tables 1 and 2; HRCIMS ( $\text{CH}_4$ )  $m/z$  289.2537  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{20}\text{H}_{33}\text{O}$ , 289.2531) 288  $[\text{M}^+]$  (45), 271 (100), 259 (22), 203 (20), 163 (47), 107 (73).

**6 $\beta$ ,18-Dihydroxycassan-13,15-diene (2):** white solid; mp  $128-130^{\circ}\text{C}$ ;  $[\alpha]_D^{25} -131^{\circ}$  ( $c$  0.0037, MeOH); IR ( $\text{CHCl}_3$ )  $\nu_{\text{max}}$  3433, 2928, 1645, 1254, 1042  $\text{cm}^{-1}$ ; for  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR data, see Tables 1 and 2; HRCIMS ( $\text{CH}_4$ )  $m/z$  305.2470  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{20}\text{H}_{33}\text{O}_2$ , 305.2480), 305 (3), 287 (25), 269 (18), 257 (11), 231 (6), 116 (30), 78 (16), 60 (42), 28 (100).

**Oxidation of 2.** Compound **2** (176 mg) was dissolved in acetone (previously distilled over  $\text{KMnO}_4$ ), and Jones reagent

was added dropwise and stirred at room temperature until color persisted, at which time the reaction was quenched with MeOH, extracted with  $\text{CH}_2\text{Cl}_2$ , and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The products were purified by column chromatography on silica gel 60 (37–75  $\mu\text{m}$ ) and eluted with  $\text{CHCl}_3$ , yielding ketone **2a** (26 mg, 14%): amorphous solid, mp  $96-98^{\circ}\text{C}$ ;  $[\alpha]_D^{25} -22.8$  ( $c$  0.86,  $\text{CHCl}_3$ ); IR ( $\text{CHCl}_3$ )  $\nu_{\text{max}}$  3433, 2930, 2868, 1705, 1231, 1041  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  6.77 (1H, dd,  $J = 11, 17$  Hz, H-15), 5.15 (1H, d,  $J = 17$  Hz, H-16a), 5.01 (1H, d,  $J = 11$  Hz, H-16b), 3.51 (1H, d,  $J = 11$  Hz, H-18a), 3.17 (1H, d,  $J = 11$  Hz, H-18b), 2.67 (1H, d,  $J = 4.2$  Hz, H-7a), 2.40 (1H, br, H-5), 2.12 (1H, br, H-7b), 1.69 (3H, s, H-17), 1.45 (1H, br, H-9);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz)  $\delta$  211.8 (C=O, C-6), 134.0 (CH, C-15), 133.1 (C, C-14), 128.4 (C, C-13), 110.9 ( $\text{CH}_2$ , C-16), 71.4 ( $\text{CH}_2$ , C-18), 60.5 (CH, C-5), 52.3 (CH, C-9), 46.8 ( $\text{CH}_2$ , C-7), 41.6 (CH, C-8), 40.3 (C, C-10), 37.2 ( $\text{CH}_2$ , C-1), 36.0 (C, C-4), 35.3 ( $\text{CH}_2$ , C-3), 25.4 ( $\text{CH}_2$ , C-12), 20.5 ( $\text{CH}_2$ , C-11), 16.9 ( $\text{CH}_2$ , C-2), 16.7 ( $\text{CH}_3$ , C-19), 15.0 ( $\text{CH}_3$ , C-20), 14.4 ( $\text{CH}_3$ , C-17); HRCIMS ( $\text{CH}_4$ )  $m/z$  302.2241  $[\text{M}^+]$  (calcd for  $\text{C}_{20}\text{H}_{30}\text{O}_2$ , 302.2245), 302  $[\text{M}^+]$  (91), 284 (64), 269 (94), 257 (28), 229 (32), 203 (18), 167 (39), 149 (34), 137 (44), 123 (100), 109 (83), 91 (81).

**Acetylation of 2.** To 30 mg of **2** were added 2 mL of acetic anhydride and 2 mL of pyridine, which was stirred overnight at room temperature. Ice was added to the mixture, which was then extracted with ether. The ether layer was washed successively with 2 N HCl, 6%  $\text{Na}_2\text{CO}_3$ , and water and then dried over anhydrous  $\text{Na}_2\text{SO}_4$ . After removal of solvent by rotary evaporation the residue (27 mg) was purified by column chromatography on silica gel (7GF) which was eluted with  $\text{CHCl}_3$  to yield the diacetate **2b** (4 mg, 13%) and the monoacetate **3** (22 mg, 73%). Diacetate **2b**:  $[\alpha]_D^{24} -24.1$  ( $c$  0.59,  $\text{CHCl}_3$ ); IR ( $\text{CHCl}_3$ )  $\nu_{\text{max}}$  3020, 2928, 2858, 1730, 1246, 1217, 1035  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$  6.81 (1H, dd,  $J = 11, 17$  Hz, H-15), 5.39 (1H, d,  $J = 2.4$  Hz, H-6), 5.14 (1H, d,  $J = 17$  Hz, H-16a), 4.99 (1H, d,  $J = 11$  Hz, H-16b), 3.86 (1H, d,  $J = 11$  Hz, H-18a), 3.76 (1H, d,  $J = 11$  Hz, H-18b), 2.29 (1H, br, H-7a), 1.72 (3H, s, H-17), 1.35 (1H, br, H-5), 1.15 (1H, br, H-7b), 1.01 (1H, br, H-9);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz)  $\delta$  171.4 (MeCO-), 170.6 (MeCO-6), 135.9 (C, C-14), 135.7 (CH, C-15), 129.9 (C, C-13), 111.5 ( $\text{CH}_2$ , C-16), 73.2 ( $\text{CH}_2$ , C-18), 70.8 (CH, C-6), 54.6 (CH, C-9), 51.4 (CH, C-5), 40.7 ( $\text{CH}_2$ , C-1), 38.4 ( $\text{CH}_2$ , C-3), 37.8 (C, C-4), 37.5 (C, C-10), 37.0 (CH, C-8), 36.9 ( $\text{CH}_2$ , C-7), 26.8 ( $\text{CH}_2$ , C-12), 22.2 ( $\text{CH}_3\text{CO}$ -6), 21.6 ( $\text{CH}_2$ , C-11), 21.3 ( $\text{CH}_3\text{CO}$ -18), 19.4 ( $\text{CH}_3$ , C-19), 18.5 ( $\text{CH}_2$ , C-2), 17.1 (CH, C-20), 16.3 ( $\text{CH}_3$ , C-17); HRCIMS ( $\text{CH}_4$ )  $m/z$  387.2520  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{24}\text{H}_{35}\text{O}_4$ , 387.2535), 386  $[\text{M}^+]$  (15), 362 (9), 344 (20), 328 (100), 301 (10), 270 (30), 253 (40), 229 (16), 185 (24), 159 (39), 145 (39), 135 (61), 107 (55), 95 (59).

**6 $\beta$ -Hydroxy-18-acetoxycassan-13,15-diene (3):** white solid, mp  $127-130^{\circ}\text{C}$ ;  $[\alpha]_D^{25} -75$  ( $c$  0.00325, MeOH); IR ( $\text{CHCl}_3$ )  $\nu_{\text{max}}$  3516, 2860, 1724, 1628, 1038  $\text{cm}^{-1}$ ; for  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR data, see Tables 1 and 2; HRCIMS ( $\text{CH}_4$ )  $m/z$  347.2583  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{22}\text{H}_{35}\text{O}_3$ , 347.2586), 347  $[\text{M} + \text{H}]^+$  (2), 329 (64), 287 (32), 269 (100), 146 (11), 107 (17), 61 (78), 45 (52), 29 (57), 19 (84).

**18-Acetoxy-13,15-diene-19-cassanoic acid (4):** white solid;  $85-88^{\circ}\text{C}$ ;  $[\alpha]_D^{24} -11.1$  ( $\text{CHCl}_3$ ,  $c$  0.45); IR ( $\text{CHCl}_3$ )  $\nu_{\text{max}}$  3680, 2937, 1730, 1244, 1037  $\text{cm}^{-1}$ ; for  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR, see Tables 1 and 2; HRCIMS ( $\text{CH}_4$ )  $m/z$  360.2300  $[\text{M}^+]$  (calcd for  $\text{C}_{22}\text{H}_{32}\text{O}_4$ , 360.2300) 360  $[\text{M}^+]$  (89), 346 (13), 300 (89), 255 (35), 243 (87), 227 (28), 187 (38), 159 (49), 135 (79), 105 (98), 91 (100).

**6 $\beta$ ,13 $\beta$ -Dihydroxy-18-acetoxycassan-14(17),15-diene (5):** amorphous powder;  $[\alpha]_D^{25} 19^{\circ}$  (MeOH,  $c$  0.000733); IR ( $\text{CHCl}_3$ )  $\nu_{\text{max}}$  3423, 2868, 1720, 1645, 1036  $\text{cm}^{-1}$ ; for  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR, see Tables 1 and 2; HRCIMS ( $\text{CH}_4$ )  $m/z$  363.2507  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{22}\text{H}_{35}\text{O}_4$ , 363.2490), 363  $[\text{M} + \text{H}]^+$  (42), 301 (41), 284 (49), 259 (72), 241 (70), 229 (18), 146 (65), 129 (100), 113 (32), 83 (24), 71 (35).

**Intracellular *T. cruzi* Bioassay.** The recombinant *T. cruzi* clone C4 of *T. cruzi* that expresses  $\beta$ -galactosidase ( $\beta\text{Gal}$ ) as a reporter enzyme was used in the assay.<sup>12</sup> Trypomastigotes were grown on monolayers of human foreskin fibroblasts (HFF) as previously described.<sup>13</sup> Both the parasite

and the HFF were grown in RPMI-1640 medium without phenol red (Gibco BRL) plus 10% fetal calf serum. Briefly, 96-well tissue culture plates (Costar) were seeded with HFF at  $1 \times 10^3$  per well in 100  $\mu\text{L}$  volumes and incubated overnight.  $\beta\text{Gal}$ -expressing trypomastigotes were then added at  $1 \times 10^3$  per well in 50  $\mu\text{L}$  volumes. After incubation overnight, the drug or test compounds were added in serial dilutions in 50  $\mu\text{L}$  volumes. The final concentrations of the test samples after parasite addition are 50, 10, and 2  $\mu\text{g}/\text{mL}$  in 200  $\mu\text{L}$  of media. At 7 days after infection, the assays were developed by addition of the chlorophenolred- $\beta$ -D-galactopyranoside (CPRG) to a final concentration of 100  $\mu\text{M}$  and Nonidet P-40 to a final concentration of 0.1%. Plates were incubated for 4–6 h at 37 °C, and the colorimetric reaction was quantified with a Benchmark microplate reader (BIORAD) at an optical density of 570 nm. Negative controls were identical to the conditions described above but with DMSO alone at final concentrations identical to those employed with test substances. The final DMSO concentration did not exceed 0.1%, which has no measurable effect on parasite growth (data not shown). The absorbance observed at 570 nm from the negative control was subtracted from the  $\text{IC}_{50}$  value determined in the presence of test substance. Each test substance as well as control experiment was tested in duplicate, and the results represent a minimum of three separate experiments which were used to calculate the standard deviation (SD). Results are expressed as  $\text{IC}_{50}$ , the concentration of compound that inhibited growth of the parasites by 50%  $\pm$  SD. Nifurtimox was chosen as a positive control for the intracellular form of *T. cruzi*, as it is the drug routinely used for the treatment of acute Chagas' disease in humans.<sup>13</sup>

**Extracellular *T. cruzi* Bioassay.** Recombinant epimastigotes were grown in liver infusion tryptone (LIT) medium supplemented with 10% calf serum at 28 °C as previously described.<sup>13</sup> Cultures were initiated with a cell density of  $1 \times 10^5$  epimastigotes per mL and were incubated in the presence of test compounds. Three days after seeding of the culture with parasite, the epimastigotes were treated with CPRG and Nonidet P-40 as described above for the intracellular *T. cruzi* bioassay, followed by quantification by measuring the optical density at 570 nm. Amphotericin B was employed as a positive control for the extracellular form of *T. cruzi*.<sup>13</sup> Inhibition of the parasites by test compounds or controls is presented as  $\text{IC}_{50}$  as described above for the intracellular assay.

**Cytotoxicity Tests with Human Foreskin Fibroblasts.** Primary human foreskin fibroblasts (HFF) adhered to 96-well plates were used to evaluate the toxicity of the compounds purified from *M. frutescens* on the basis of the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide<sup>14</sup> (MTT, Sigma). After treatment with the test compounds, cell viability was evaluated after a 3 day incubation at 37 °C in  $\text{CO}_2$ . Cytotoxicity to the cell lines is presented as  $\text{IC}_{50}$  as described above for the intracellular *T. cruzi* assay.

**Acknowledgment.** The authors extend special thanks to Drs. Phyllis D. Coley and Thomas A. Kursar, who were instrumental in establishing the present bioprospecting program in Panama. Funding comes from the International Cooperative Biodiversity Groups Program (grant number #IUOI TWO1021-01 to PC) from the NIH, National Science Foundation, and U.S. Department of Agriculture. We thank F. Buckner from the University of Washington, Seattle, for the recombinant Tulahuen clone C4 of *T. cruzi*. We gratefully acknowledge the Smithsonian Tropical Research Institute Equipment Fund for partial funding for the Bruker Avance NMR. We thank Dr. Kerry McPhail from the Department of Pharmacy of Oregon State University for expert assistance with MS and NMR experiments. We also thank Dr. Ricardo Riguera from the Department of Organic Chemistry of the Universidad de Santiago de Compostela for assistance with NMR experiments.

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NP0300100