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Total Synthesis and Antileishmanial Activity of the Natural Occurring Acetylenic Fatty Acids 6-Heptadecynoic acid and 6-Icosynoic acid

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Abstract

The first total syntheses of the naturally occurring acetylenic fatty acids 6-heptadecynoic acid (59% overall yield) and 6-icosynoic acid (34% overall yield) was accomplished in four steps. Using the same synthetic sequence the naturally occurring fatty acids (6*Z*)-heptadecenoic acid (46% overall yield) and (6*Z*)-icosenoic acid (27% overall yield) were also synthesized. The Δ^6 acetylenic fatty acids displayed good antiprotozoal activity towards *Leishmania donovani* promastigotes (EC_{50} = 1–6 μg/mL), but the 6-icosynoic acid was the most effective in the series. In addition, the (6*Z*)-icosenoic acid was a much better antiprotozoal compound (EC_{50} = 5–6 μg/mL) than the (6*Z*)-heptadecenoic acid (EC_{50} > 25 μg/mL). The saturated fatty acids *n*-heptadecanoic acid and *n*-eicosanoic acid were not effective towards *L. donovani*, indicating that the Δ^6 unsaturation in these fatty acids is necessary for leishmanicidal activity. In addition, both the 6-icosynoic acid and the (6*Z*)-icosenoic acid were inhibitors of the leishmania DNA topoisomerase IB enzyme ($EC_{50's}$ = 36–49 μM), a possible intracellular target for these compounds. This is the first study assessing fatty acids as inhibitors of the leishmania DNA topoisomerase IB enzyme.

Keywords

Acetylenic fatty acids; Antiprotozoal activity; *Leishmania donovani*; Synthesis; Topoisomerase IB

Introduction

Many acetylenic fatty acids are natural plant constituents, but those with a C-6 triple bond, such as the 6-nonadecynoic acid, have displayed strong antifungal activity [1]. Recently, the novel 6-heptadecynoic acid (**1a**) and 6-icosynoic acid (**1b**) were isolated from the plant *Sommera sabiceoides* [1]. While **1a** is a new natural product, **1b** has been known for sometime as a trace constituent of *Alvaradoa amorphoides* seed oil [2]. Neither of these two acetylenic fatty acids **1a** and **1b** have been explored for antiprotozoal activity nor their olefinic analogs (6*Z*)-heptadecenoic acid (**6a**) and (6*Z*)-icosenoic acid (**6b**), which are also natural products. The (6*Z*)-heptadecenoic acid (**6a**) was identified in the opisthobranch *Haminaea templadoi* as well as in a marine *Micrococcus* bacterium [3,4], while **6b** is also a minor constituent of some seed oils [2].

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Some fatty acids display antiprotozoal activity, in particular against *Leishmania donovani,* the causative agent of leishmaniasis [5]. Visceral leishmaniasis is a very serious disease characterized by the invasion of spleen and liver macrophages, anaemia, fever and ultimately death if not diagnosed on time and left untreated. Visceral leishmaniasis is mostly found in India, Bangladesh, Indonesia [6] and Sudan [7] and in many cases is associated with an opportunistic infection in immunocompromised AIDS patients [8]. Pentavalent antimonials (meglumine antimoniate and sodium stibogluconate) were used as first-line drugs despite the early reported resistances [9]. An emerging alternative to these compounds are the oral chemotherapy drugs, such as the alkylphosphocholine derivatives (miltefosine and edelfosine) and the antibiotic paromomycin [10,11]. Miltefosine - originally developed as an anticancer drug - was confirmed as a major advance against visceral leishmaniasis since it was the first oral drug with high healing rates in phase IV studies, including pentavalent antimonials relapsing cases [12]. However, reproductive toxicity, its arbitrary use and its serious tendency to select resistant strains in the laboratory [13], suppose a high risk of emerging resistances during treatment with this drug, making it necessary to continue the search of new drugs for the treatment of this disease.

The toxicity of fatty acids towards *L. donovani* promastigotes is known, but it has not been thoroughly studied. Probably, the most representative work is that of G. Chaudhuri et al. where the growth inhibition of a series of C_4–C_{18} fatty acids towards *L. donovani* promastigotes was investigated [5]. It was found that the longest fatty acids (up to 18 carbon atoms) were the most inhibitory and that unsaturated fatty acids were more toxic than their corresponding saturated analogs. For example, oleic acid displayed a MIC of 0.09 μmole/mL against *L. donovani* promastigotes, while octadecanoic acid resulted in a MIC of 0.18 μmole/mL [5]. *n*-Decanoic acid was less effective against *L. donovani* with a MIC of 1.39 μmole/mL. No conclusions regarding the inhibitory mechanism of these fatty acids could be drawn, but the Chaudhuri group found that growth inhibition was not due to leakage of metabolites from the protozoan cells or from the potential ability of these molecules to affect the surface tension of the growth medium. However, it was found that the studied fatty acids inhibited the uptake of glucose and leucine by *L. donovani*. An earlier work by S. S. Kuwahara et al. reported that *n*-decanoic, *n*-dodecanoic, and *n*-hexadecanoic acids, at levels of 100 μg/mL, inhibited the motility of *L. donovani* promastigotes, but oleic acid had no effect [14].

The aim of the present study was to synthesize, for the first time, the naturally occurring 6-heptadecynoic acid (**1a**) and 6-icosynoic acid (**1b**), as well as the (6*Z*)-heptadecenoic acid (**6a**) and (6*Z*)-icosenoic acid (**6b**), and assess their antileishmanial activity against *L. donovani* promastigotes. The objective of the investigation was to determine, by keeping the chain length fixed, if a higher degree of unsaturation favors a better antileishmanial activity. In addition, we also studied the inhibition of the leishmania DNA topoisomerase IB enzyme by these fatty acids as a possible intracellular target.

Materials and Methods

General Experimental Procedures

^{1}H NMR (300 or 500 MHz) and ^{13}C NMR (75 or 125 MHz) were either recorded on a Bruker DPX-300 or a Bruker DRX-500 spectrometer. ^{1}H NMR chemical shifts are reported with respect to internal $(CH_3)_4Si$, ^{13}C NMR chemical shifts are reported in parts per million relative to $CDCl_3$ (77.0 ppm). GC/MS analyses were recorded at 70 eV using either a Hewlett Packard 5972A MS ChemStation or an Agilent 5975C MS ChemStation coupled to an Agilent 7890A GC where both instruments were equipped with a 30 m × 0.25 mm special performance capillary column (HP-5MS) of polymethyl siloxane crosslinked with 5% phenyl methylpolysiloxane. IR spectra were recorded on a Nicolet Magna 750 FT-IR spectrophotometer (Thermo-Nicolet, Madison, WI, USA). High resolution mass spectral data

was performed at the Emory University Mass Spectrometry Center on a thermo LTQ-FTMS using APCI as the probe.

5-Bromo-1-[(tetrahydropyran-2-yl)oxy]pentane (2)—To 5-bromo-1-pentanol (4.0g, 23.9 mmol) in 20 mL of $CHCl_3$ was added dropwise 2,3-dihydro-2*H*-pyran (DHP) (4.4 mL, 47.9 mmol) and catalytic amounts of *p*-toluenesulfonic acid (*p*-TSA). The reaction mixture was stirred for 90 min. at room temperature. The organic layer was washed with water (1 × 50 mL), $NaHCO_3$ (1 × 50 mL), water (1 × 75 mL) and dried over Na_2SO_4. The crude product was purified using silica gel column chromatography eluting with hexane:ether (9:1) affording 5.93 g of **2** as a colorless oil for a 99% yield.

General Procedure for the Acetylide Coupling Reaction

Into a 100 mL round-bottomed flask at 0 °C containing dry THF (5.0 – 7.0 mL) was added the terminal alkyne (6.0 – 7.2 mmol) followed by the dropwise addition of 2.5M *n*-BuLi (12.0 – 14.4 mmol). The mixture was stirred at 0 °C for 80 min. The temperature was then lowered to −60 °C and HMPA (10.0 – 14.0 mL) was added followed by the addition of **2** (6.0 – 7.2 mmol). The mixture was stirred for 24 h and then washed with brine (2 × 15 mL), extracted with hexane (2 × 15 mL) and the organic phase dried over Na_2SO_4 and evaporated *in vacuo* affording **3a** (1.47 g, 54% yield) or **3b** (1.60 g, 79% yield) after purification by Kugelrohr distillation at 170 °C/3 mm Hg.

2-(Heptadec-6-ynyloxy)-tetrahydro-2H-pyran (3a)—Was obtained as a pale yellow oil in a 79% yield from the reaction of 1-dodecyne (1.3 mL, 6.0 mmol) with *n*-BuLi in dry THF (5.0 mL) and **2** (1.510 g, 6.0 mmol) according to the general procedure described above; IR (neat) ν_{max}: 2923, 2853, 1455, 1440, 1351, 1136, 1120, 1077 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.56 (1H, m), 3.85-3.73 (2H, m), 3.48-3.37 (2H, m, H-1), 2.12 (4H, m), 1.69-1.35 (6H, m), 1.26 (22H, brs, -CH_2-), 0.87 (3H, t, J = 6.8 Hz, -CH_3); ^{13}C NMR ($CDCl_3$, 75MHz) δ 98.8, 80.4 (C-7), 79.9 (C-6), 67.5, 62.3, 31.9, 30.7, 29.6, 29.5, 29.3, 29.3, 29.2, 29.0, 28.9, 26.9, 25.5, 22.7, 19.6, 18.7, 18.7, 14.1; GC-MS (70eV) *m/z* (relative intensity) 336 (M^+, 4), 265 (3), 264 (3), 263 (12), 252 (2), 251 (2), 195 (5), 181 (6), 167 (4), 135 (2), 125 (2), 95 (17), 93 (11), 86 (6), 85 (100), 81 (17), 79 (19), 69 (9), 67 (25), 57 (14), 55 (25); HRMS calcd for $C_{22}H_{40}O$ $[M+H]^+$ 337.3101, found 337.3102.

2-(Icos-6-ynyloxy)-tetrahydro-2H-pyran (3b)—Was obtained as a pale yellow oil in a 54% yield from the reaction of 1-pentadecyne (1.9 mL, 7.2 mmol) with *n*-BuLi in dry THF (7.0 mL) and **2** (1.82 g, 7.2 mmol) according to the general procedure described above; IR (neat) ν_{max}: 2922, 2853, 1464, 1351, 1322, 1136, 1120, 1077 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.57 (1H, m), 3.91-3.68 (2H, m), 3.55-3.34 (2H, m, H-1), 2.14 (4H, m), 1.90-1.40 (6H, m), 1.25 (28H, brs, -CH_2-), 0.87 (3H, t, J = 6.8 Hz, -CH_3); ^{13}C NMR ($CDCl_3$, 75MHz) δ 98.8, 80.4 (C-7), 79.9 (C-6), 67.5, 62.3, 31.9, 30.7, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.00, 28.9, 26.9, 25.5, 22.7, 19.7, 18.7, 18.7, 14.1; GC-MS (70eV) *m/z* (relative intensity) 378 (M^+, 1), 305 (4), 293 (2), 195 (6), 125 (2), 101 (10), 97 (4), 95 (11), 93 (6), 86 (5), 85 (100), 83 (7), 67 (19), 57 (12), 55 (18); HRMS calcd for $C_{25}H_{46}O_2$ $[M+H]^+$ 379.3571, found 379.3573.

General Procedure for the Tetrahydropyranyl Deprotection

To a mixture of methanol (10 mL) and either **3a** or **3b** (0.91–1.46 g, 2.7–3.9 mmol) was added catalytic amounts of *p*-toluenesulfonic acid (*p*-TSA) and the reaction mixture was stirred at 60 °C for 24 h. After this time the solvent was evaporated and then the organic extract was washed with a saturated solution of sodium bicarbonate (3 × 25 mL), dried over Na_2SO_4, filtered, and evaporated *in vacuo*, affording 0.68–0.95 g (83–99% yield) of the alkynols **4a** and **4b**. The

products were deemed sufficiently pure so as to be used as such for the next steps without further purification.

6-Heptadecynol (4a)—Was obtained as a pale yellow oil in a 99% yield from the reaction of **3a** (0.91 g, 2.7 mmol) with catalytic amounts of *p*-TSA in methanol (10 mL) according to the general procedure described above; IR (neat) ν_{max}: 3355, 2923, 2853, 1460, 1377, 1333, 1119, 1047, 1010, 724 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 3.63 (2H, t, *J* = 6.5 Hz, H-1), 2.13 (4H, m), 1.40–1.61 (6H, m), 1.24 (16H, brs, -CH_2-), 0.87 (3H, t, *J* = 6.8 Hz, -CH_3); ^{13}C NMR ($CDCl_3$, 75MHz) δ 80.5 (C-7), 79.8 (C-6), 62.9 (C-1), 32.3, 31.9, 29.7, 29.6, 29.6, 29.3, 29.2, 29.1, 28.9, 24.9, 22.7, 18.7, 18.7, 14.1; GC-MS (70eV) *m/z* (relative intensity) 252 (M^+,1), 152 (2), 135 (5), 124 (6), 108 (30), 98 (45), 97 (32), 95 (37), 93 (62), 91 (22), 84 (22), 83 (37), 82 (100), 81 (62), 80 (62), 79 (85), 70 (36), 69 (38), 67 (91), 65 (13), 57 (31), 55 (86); HRMS calcd for $C_{17}H_{32}O$ $[M+H]^+$ 253.2526, found 253.2527.

6-Icosynol (4b)—Was obtained as a semi solid in a 83% yield from the reaction of **3b** (1.46 g, 3.9 mmol) with catalytic amounts of *p*-TSA in methanol (10 mL) according to the general procedure described above; IR (neat) ν_{max}: 3321, 2922, 2852, 1461, 1376, 1350, 1120, 1055, 721 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 3.63 (2H, t, *J* = 6.5 Hz, H-1), 2.12 (4H, m), 1.51 (4H, m), 1.24 (24H, brs, -CH_2-), 0.86 (3H, t, *J* = 7.0 Hz, -CH_3); ^{13}C NMR ($CDCl_3$, 75MHz) δ 80.5 (C-7), 79.8 (C-6), 62.9 (C-1), 32.3, 31.9, 29.6, 29.6, 29.4, 29.2, 28.9, 24.9, 22.7, 19.7, 18.7, 14.1; GC-MS (70eV) *m/z* (relative intensity) 292 (M^+, 1), 194 (2), 135 (9), 126 (5), 111 (26), 108 (47), 98 (60), 97 (39), 95 (43), 93 (74), 91 (27), 82 (100), 81 (67), 80 (69), 79 (95), 69 (37), 68 (33), 67 (96), 57 (43), 55 (98); HRMS calcd for $C_{20}H_{38}O$ $[M+H]^+$ 295.2995, found 295.2998.

General Procedure for the Oxidation of the Alcohols

To a solution of pyridinium dichromate (PDC) (5.6–11.9 mmol) and 2 mL of dimethylformamide (DMF) was added under argon a solution of the alcohol (1.11–2.38 mmol) in 6.6 mL of DMF and the reaction mixture was left stirring at room temperature for 48 h. The mixture was washed with water (3 × 25 mL), extracted with hexane (1 × 25 mL), the organic phase was dried over Na_2SO_4, and the solvent removed *in vacuo* affording the corresponding carboxylic acids **1a** (0.26 g) and **1b** (0.48 g) in 76% yields.

6-Heptadecynoic acid (1a)—Was obtained as a white solid (mp 38–40 °C, lit[1] mp 39 °C) in a 76% yield from the reaction of **4a** (0.6 g, 2.38 mmol) and PDC (4.5 g, 11.9 mmol) in DMF according to the general procedure described above. Compound **1a** presented spectral data similar to the one reported in the literature [1].

6-Icosynoic acid (1b)—Was obtained as a white solid (mp 52–54°C, lit[1] mp 53 °C) in a 76% yield from the reaction of **4b** (0.3 g, 1.11 mmol) and PDC (2.1 g, 5.6 mmol) in DMF according to the general procedure described above. Compound **1b** also presented spectral data similar to the one reported in the literature [1].

General Procedure for the Catalytic Hydrogenation of the Alkynes

Into a 25 mL two-neck round-bottom flask were placed dry hexane, the alkynes **4a** (0.056 g) or **4b** (0.295 g), quinoline (0.18 – 0.8 mL), and 10% palladium on activated carbon (Pd/C). One of the necks was capped with a rubber septum and the other was connected via tygon tubing to a 25 mL graduated pipet ending in a 150 mL beaker with distilled water. While stirring at room temperature a 20 mL syringe with needle was used to withdraw air from the system and to draw water up into the graduated pipet to the 0.0 mL mark. Hydrogen was then introduced into the system using a balloon filled with hydrogen attached to a hose barb-to-luer-lock adapter with a stopcock and a needle. The reaction mixture consumed 5.4–24.6 mL of

hydrogen during 1h. The mixture was filtered and the solvent removed *in vacuo* obtaining the desired alkenols **5a** (0.046 g, 70% yield) and **5b** 0.208 g (82 % yield) after purification by Kugelrohr distillation at 110 °C/3 mm Hg.

(6Z)-Heptadecenol (5a)—Was obtained as a colorless oil (0.046 g, 0.18 mmol) in a 82% yield from the catalytic hydrogenation described in the general procedure above; IR (neat) ν_{max}: 3328, 3004, 2922, 2853, 1658, 1461, 1377, 1074, 1053, 720 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.32 (2H, m, H-6, H-7), 3.61 (2H, t, J = 6.6 Hz, H-1), 2.00 (4H, m), 1.55 (3H, m, $-CH_2-$, -OH), 1.24 (20H, brs, $-CH_2-$), 0.87 (3H, t, J = 6.8 Hz, $-CH_3$); ^{13}C NMR ($CDCl_3$, 75MHz) δ 130.2 (C-7), 129.5 (C-6), 63.0 (C-1), 32.7, 31.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 27.2, 27.1, 25.4, 22.7, 14.1; GC-MS (70eV) *m/z* (relative intensity) 254 (M^+,1), 236 (6), 208 (2), 180 (2), 123 (13), 97 (25), 96 (65), 95 (61), 82 (100), 81 (64), 69 (40), 68 (54), 67 (92), 57 (32), 55 (69); HRMS calcd for $C_{17}H_{34}O$ $[M+H]^+$ 255.2682, found 255.2684.

(6Z)-Icosenol (5b)—Was obtained as a semi solid (0.21 g, 0.71 mmol) in a 70% yield from the catalytic hydrogenation described in the general procedure above; IR (neat) ν_{max}: 3346, 3002, 2919, 2850, 1460, 1363, 1062, 724 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.35 (2H, m, H-6, H-7), 3.63 (2H, m, J = 6.6 Hz, H-1), 2.02 (4H, m), 1.57 (2H, m), 1.25 (26H, brs, $-CH_2-$), 0.88 (3H, t, J = 6.5 Hz, $-CH_3$); ^{13}C NMR ($CDCl_3$, 125MHz) δ 130.2 (C-7), 129.5 (C-6), 63.0 (C-1), 32.7, 31.9, 29.8, 29.70, 29.67, 29.6, 29.5, 29.4, 29.3, 27.3, 27.2, 25.4, 22.7, 14.1; GC-MS (70eV) *m/z* (relative intensity) 296 (M^+,1), 279 (2), 278 (9), 250 (2), 222 (2), 138 (6), 124 (10), 123 (11), 97 (26), 96 (61), 95 (52), 82 (100), 81 (59), 69 (41), 68 (55), 67 (89), 57 (41), 55 (77); HRMS calcd for $C_{20}H_{40}O$ $[M+H]^+$ 297.3152, found 297.3154.

General Procedure for the Oxidation of the Alkenols

To a solution of pyridinium dichromate (PDC) (1.27–1.77 mmol) and 2 mL of dimethylformamide (DMF) was added under argon a solution of the alcohol (0.25–0.35 mmol) in 5.0 mL of DMF and the reaction mixture was left stirring at room temperature for 48 h. The mixture was washed with water (3 × 25 mL), extracted with hexane (1 × 25 mL), the organic phase was dried over Na_2SO_4, and the solvent was removed *in vacuo* affording the carboxylic acids **6a** (0.067g, 73% yield) and **6b** (0.069 g, 86% yield).

(6Z)-Heptadecenoic acid (6a)—Was obtained as a viscous oil in a 73% yield from the reaction of **5a** (0.09 g, 0.35 mmol) and PDC (0.69 g, 1.77 mmol) in DMF according to the general procedure described above; IR (neat) ν_{max} 3264, 3005, 2922, 2853, 1708 (C=O), 1459, 1412, 1378, 1342, 1201, 1119, 935, 720 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.37 (2H, m, H-6, H-7), 2.37 (2H, t, J = 7.6 Hz, H-2), 2.14 (4H, m), 1.67 (2H, m), 1.27 (18H, brs, $-CH_2-$), 0.89 (3H, t, J = 7.0 Hz, $-CH_3$); ^{13}C NMR ($CDCl_3$, 125MHz) δ 179.6 (C-1), 130.5 (C-7), 128.9 (C-6), 33.9, 31.9, 29.7, 29.6, 29.5, 29.32, 29.29, 29.2, 29.1, 27.2, 26.7, 24.3, 22.7, 14.1; GC-MS (70eV) *m/z* (relative intensity) 268 (M^+, 4), 250 (15), 208 (7), 206 (6), 166 (6), 165 (3), 152 (5), 151 (5), 138 (6), 137 (7), 123 (11), 111 (17), 110 (15), 97 (35), 96 (27), 95 (29), 81 (47), 69 (45), 67 (59), 57 (36), 55 (100); HRMS calcd for $C_{17}H_{32}O_2$ $[M-H]^-$ 267.2330, found 267.2334.

(6Z)-Icosenoic acid (6b)—Was obtained as a white solid in a 86% yield from the reaction of **5b** (0.075g, 0.25 mmol) and PDC (0.48 g, 1.27 mmol) in DMF according to the general procedure described above; mp 31–34 °C; IR (neat) ν_{max} 3200–3095, 3002, 2922, 2852, 1709 (C=O), 1462, 1411, 1377, 1348, 1120, 1075, 936, 797, 719 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.33 (2H, m, H-6, H-7), 2.34 (2H, m, J = 7.2 Hz, H-2), 2.10 (4H, m), 1.63 (2H, m), 1.38 (4H, m), 1.24 (20H, brs, $-CH_2-$), 0.86 (3H, t, J = 7.1 Hz, $-CH_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 179.5 (C-1), 130.8 (C-7), 129.2 (C-6), 34.0, 32.2, 29.94, 29.91, 29.88, 29.8, 29.6, 29.5, 29.4, 29.3, 29.2, 27.5, 27.0, 24.5, 22.9, 14.3; GC-MS (70eV) *m/z* (relative intensity) 310 (M^+, 4),

292 (25), 211 (7), 208 (4), 250 (5), 249 (4), 248 (4), 194 (3), 165 (4), 164 (4), 152 (4), 151 (5), 123 (11), 122 (8), 111 (18), 110 (14), 109 (12), 97 (34), 95 (27), 85 (17), 81 (46), 67 (51), 55 (100); HRMS calcd for $C_{20}H_{36}O_2$ $[M-H]^-$ 309.2799, found 309.2802.

Cell Cultures

L. donovani (MHOM/ET67/L82 strain) promastigotes were propagated in a completely defined medium 199, supplemented with 10% heat inactivated fetal calf serum (FCS) and penicillin/streptomycin cocktail (containing 50 U/ml penicillin, 50 μg/ml streptomycin). The IC_{50} values were determined after 48 h incubation with different concentrations of the fatty acids dissolved in DMSO (ranging from 0.6 to 25 μg/ml) added to cultures and cell population assessed by Coulter.

Murine macrophages (RAW 264.7) were grown at 37 oC in RPMI 1640 medium, supplemented with 10% FCS antibiotics (containing 50 U/ml penicillin, 50μg/ml streptomycin). Toxicity IC_{50} values were determined after 48 h incubation with different concentrations of the compounds dissolved in DMSO (0.3 to 100 μg/ml) using vital alamarBlue® dye (Invitrogen, Carlsbad Ca. USA), according manufacturer recommendations.

Purification of Recombinant Leishmanial TopIB

Expression of a recombinant topoisomerase IB from *Leishmania donovani* (LdTopIB) in a topoisomerase IB-deficient *Saccharomyces cerevisiae* strain has been described elsewhere [15]. Purification of recombinant LdTopIB was done according to Diaz-Gonzalez and coworkers [16]. Briefly, LdTopIB overexpressing yeasts were disrupted with one freeze/thaw cycle at −80 °C, with the purpose of weakening the yeast wall; after lysis with 425–600 μm acid-washed glass beads, the extracts were cleared by centrifugation at 15000 × *g* for 30 min at 4 °C. The protein suspension was loaded onto a phosphocellulose (P-11) column, previously equilibrated as manufacturer indications. LdTopIB was eluted at 4 °C with a discontinuous gradient of KCl (0.2, 0.4, 0.6, 0.8 and 1 M) in TEEG buffer, supplemented with 0.1 mg/ml sodium bisulphite, 0.8 mg/ml NaF and the protease inhibitors cocktail. Active fractions were further loaded onto a phenyl-sepharose column (Sigma-Aldrich, St Louis, US), eluted with a discontinuous inverse gradient of ammonium sulphate (1, 0.8, 0.6, 0.4 and 0.2 M) and then concentrated by Microcon YM-30 (Millipore) before use.

DNA Relaxation Assays

DNA topoisomerase I activity was assayed by the relaxation of negatively supercoiled plasmid DNA. The reaction mixture in a total volume of 20 μl contained 0.2 μg of supercoiled pHOT plasmid, 10 mM Tris-HCl buffer pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/ml bovine serum albumin, 50 mM KCl and various extracts containing altered proteins or wild type enzyme, starting with 1 unit LdTopIB. The reaction mixtures were incubated for 30 min at 37 °C. The enzyme reactions were stopped by the addition of up to 1% SDS- final concentration- and digested by 2 mg/mL proteinase K with 1 h incubation to remove protein bonded to the DNA fragment. The extent of plasmid DNA relaxation was assessed by electrophoresis in a 1% agarose gel in 0.1 M Tris acetate EDTA (TAE) buffer pH 8.0 at 2 V/cm for 14 h. The gels were visualized under UV illumination after being stained with ethidium bromide (0.5 mg/ml) and a posterior electrophoresis in the presence of 0.1 mg/ml ethidium bromide, in order to separate the nicked DNA from the relaxed topoisomers. One unit of LdTopIB is defined as the amount of purified protein able to relax 0.2 μg of pHOT supercoiled DNA per 30 min at 37 °C.

Results and Discussion

Our approach towards the total synthesis of either 6-heptadecynoic acid (**1a**) or 6-icosynoic acid (**1b**) started with commercially available 1-dodecyne or 1-pentadecyne which were

successfully alkylated with 2-(5-bromopentyloxy) tetrahydropyran (**2**) in the presence of *n*-BuLi in THF-HMPA at 0 °C affording the tetrahydropyranyl protected alkynes **3a** (79% yield) and **3b** (54% yield) (Scheme 1). Deprotection of either **3a** or **3b** was achieved with *p*-toluenesulfonic acid (PTSA) in methanol at 60 °C, which yielded the desired 6-heptadecynol (**4a**) (99% yield) or 6-icosynol (**4b**) (83% yield). Final oxidation of either **4a** or **4b** with pyridinium dichromate (PDC) in DMF afforded the desired 6-heptadecynoic acid (**1a**) or 6-icosynoic acid (**1b**) in 76% yields. The overall yields for the last three steps of the syntheses of **1a** (59% yield) and **1b** (34% yield) favored the shorter chain acid **1a**. The syntheses shown in Scheme 1 represent the first synthesis for both **1a** and **1b**.

Taking advantage of the same synthetic methodology (Scheme 1) we synthesized the naturally occurring fatty acids (6*Z*)-heptadecenoic acid (**6a**) and (6*Z*)-icosenoic acid (**6b**). For example, diverting the alkynols **4a** or **4b** to a catalytic hydrogenation under Lindlar's conditions resulted in the (6*Z*)-heptadecenol (**5a**) (82% yield) or the (6*Z*)-icosenol (**5b**) (70% yield) with a 100% *cis* stereochemistry for the double bonds. Oxidation of either **5a** or **5b** with pyridinium dichromate (PDC) in DMF afforded the desired (6*Z*)-heptadecenoic acid (**6a**) (73% isolated yield) and (6*Z*)-icosenoic acid (**6b**) (86% isolated yield). The overall yields for the four-step synthesis of **6a** (36%) and **6b** (21%) from **2** again favored the shorter chain acid **6a**. Surprisingly, this is also the first reported synthesis for either the alkenoic acids **6a** or **6b**.

Aimed at exploring the antiprotozoal activity of the acetylenic fatty acids **1a** and **1b** as well as that of the olefinic fatty acids **6a** and **6b** we studied their toxicity towards *L. donovani* promastigotes. The results of our investigation are shown in Table 1. As the table shows the acetylenic fatty acids **1a** and **1b** as well as the olefinic fatty acid **6b** displayed good antileishmanial activities towards *L. donovani* promastigotes (EC_{50} = 1–7 μg/mL). Surprisingly, the (6*Z*)-heptadecenoic acid (**6a**) was not effective against *L. donovani* (EC_{50} > 25 μg/mL). The saturated fatty acids *n*-heptadecanoic acid and *n*-icosanoic acid were tested as a control and they were also not effective towards *L. donovani* promastigotes (EC_{50} > 25 μg/mL), thus corroborating the previous literature report that unsaturation is an important factor for the antileishmanial activity of fatty acids [5]. From the results with the *L. donovani* promastigotes we can conclude that the C_{20} fatty acids were the more effective antileishmanial compounds since both acids **1b** and **6b** displayed a better therapeutic index ($IC_{50}/EC_{50} \approx 5$) over either **1a** or **6a**. Among the C_{17} fatty acids the acetylenic acid **1a** was the most efficient antiprotozoal compound, thus corroborating the importance of the C-6 triple bond in the antileishmanial activity of these compounds. Therefore, these results tend to indicate that naturally occurring Δ^6 acetylenic fatty acids could be valuable fatty acids that can be optimized for developing more potent antileishmanial agents and thus merit further scrutiny in the search for better analogs.

As a possible mechanism of toxicity we also studied the inhibition of these Δ^6 fatty acids towards the leishmania DNA topoisomerase IB (LdTOPIB) enzyme since nothing is known in the literature as to the interaction of fatty acids with this particular enzyme. One aim here was to determine a possible selectivity of the studied fatty acids towards the LdTOPIB enzyme as a function of the level of unsaturation. The LdTOPIB enzyme is substantially different from the human topoisomerase I enzyme since the LdTOPIB enzyme is a heterodimer resulting from two separate genes encoding for the core and catalytic domains of the enzyme [9]. Our results with the acetylenic acids **1a** and **1b** are shown in Fig. 1 and with the alkenoic acids **6a** and **6b** in Fig. 2, while the corresponding inhibition plots are shown in Fig. 3. As can be seen from Fig. 1 and Fig. 3 the 6-icosynoic acid (**1b**) was more efficient (EC_{50} = 49.04 ± 4.82 μM) than the 6-heptadecynoic acid (**1a**) (EC_{50} = 71.72 ± 3.84 μM) in inhibiting the LdTOPIB enzyme. On the other hand, the (6*Z*)-icosenoic acid (**6b**) also displayed reasonably good inhibition of the enzyme (EC_{50} = 36.67 ± 1.51 μM) (Fig. 2 and Fig. 3), but the (6*Z*)-heptadecenoic acid (**6a**) displayed a weak inhibition of the enzyme (EC_{50} = 80.43 ± 9.29 μM), which seems to

explain its lack of toxicity towards the *L. donovani* promastigotes. The saturated fatty acids *n*-heptadecanoic acid and *n*-icosanoic acid did not inhibit the enzyme, even at concentrations as high as 1mM (gel not shown). Therefore, the 6-icosynoic acid (**1b**) and the (6*Z*)-icosenoic acid (**6b**) were among the best inhibitors in the series of the LdTOPIB enzyme indicating that both unsaturation and chain length are important factors when considering inhibition of the LdTOPIB enzyme by fatty acids. Therefore, the LdTOPIB enzyme seems to be a likely intracellular target for these C_{20} unsaturated fatty acids. We believe that our results should pave the way for the development of more potent antileishmanial fatty acids towards *L. donovani* and similar protozoa such as the trypanosomes, who share a similar topoisomerase IB enzyme.

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Abbreviations

DNA	Deoxyribonucleic acid
EC_{50}	Effective concentration 50%
GC-MS	Gas chromatography-mass spectrometry
HRMS	High resolution mass spectrometry
IC_{50}	Inhibitory concentration 50%
IR	Infrared spectroscopy
LdTOPIB	Leishmania DNA topoisomerase IB
MIC	Minimum Inhibitory concentration
NMR	Nuclear Magnetic Resonance
PDC	Pyridinium dichromate

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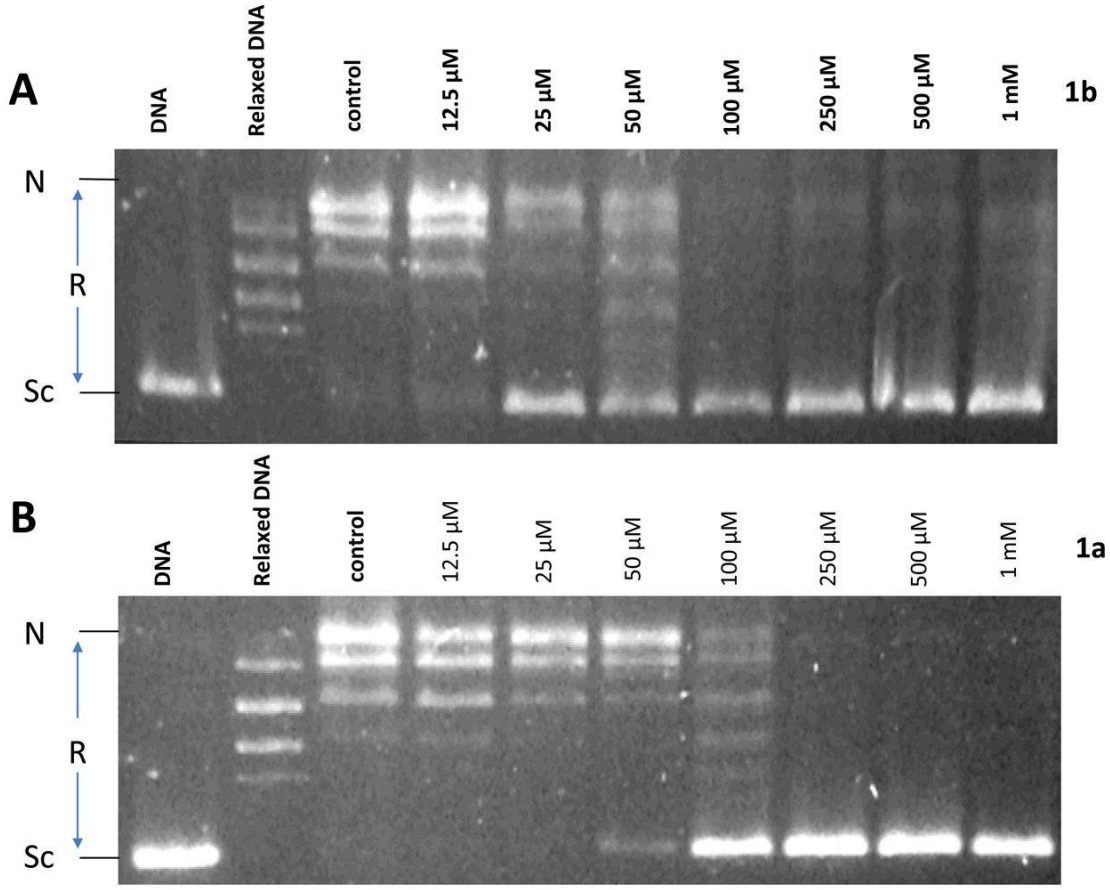


Fig. 1.

Inhibition of the relaxation activity of recombinant LdTopIB by the 6-icosynoic acid (**1b**) (top) and the 6-heptadecynoic acid (**1a**) (bottom). One unit of recombinant LdTop1 was assayed in a plasmid DNA relaxation assay for 30 min at 37 °C (as described under "Materials and Methods") in the presence of 12.5 to 1000 µM compounds **1a** and **1b**. Reaction products were resolved in agarose gel and subsequently visualized by ethidium bromide staining. The relative position of the negatively supercoiled DNA substrate is indicated by Sc, N is the nicked DNA, whereas the ladder of relaxed DNA topoisomer bands is labeled R. Reactions were stopped with a mixture of 1 % SDS and 6.1 µg of proteinase K. Lane 1 contains 0.2 µg of pHOT plasmid DNA and lane 2 is a relaxed marker.

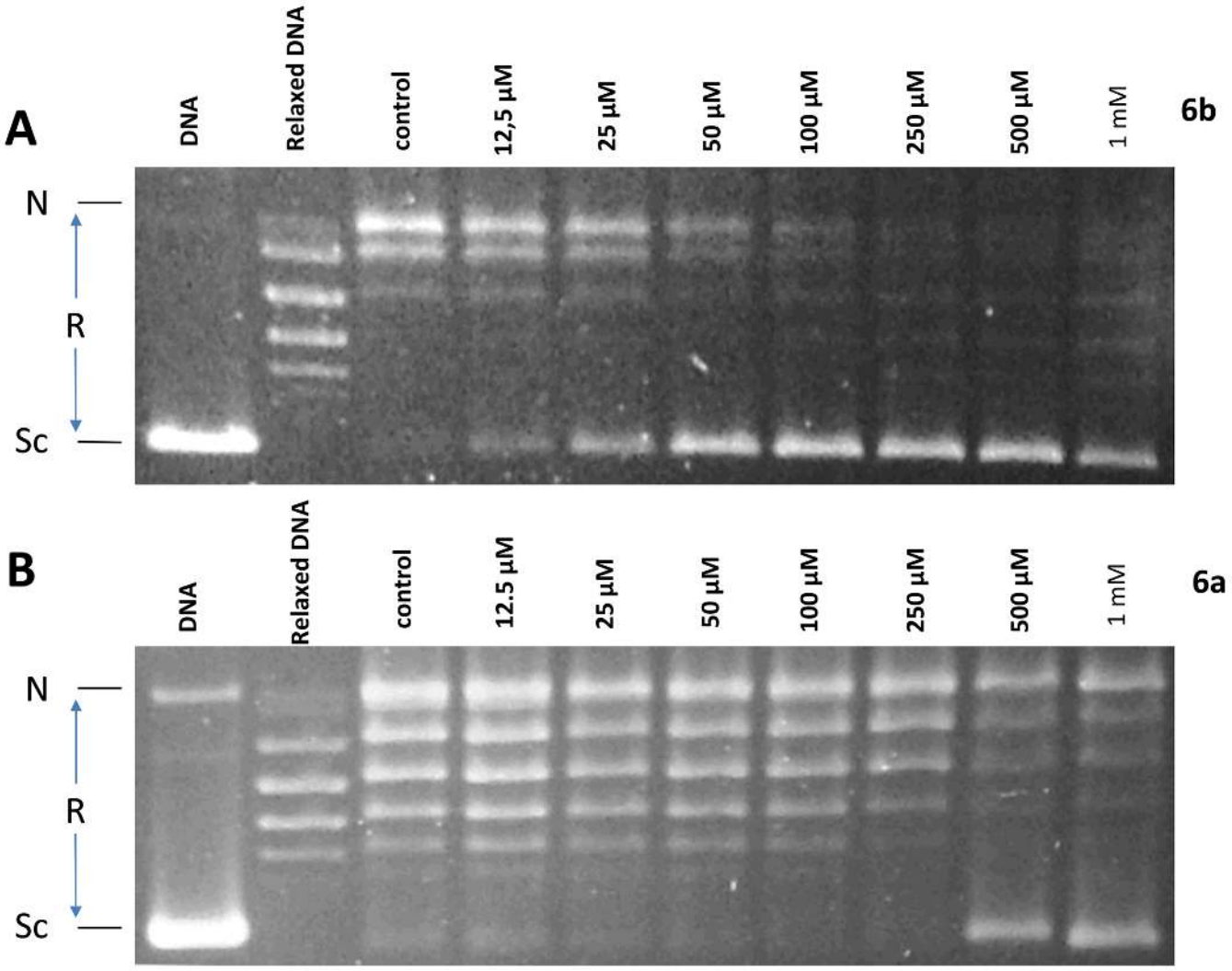


Fig. 2.

Inhibition of the relaxation activity of recombinant LdTopIB by the (6Z)-icosenoic acid (**6b**) (top) and the (6Z)-heptadecenoic acid (**6a**) (bottom). One unit of recombinant LdTop1 was assayed in a plasmid DNA relaxation assay for 30 min at 37°C (as described under "Materials and Methods") in the presence of 12.5 to 1000 μM compounds **6a** and **6b**. Reaction products were resolved in agarose gel and subsequently visualized by ethidium bromide staining. The relative position of the negatively supercoiled DNA substrate is indicated by Sc, N is the nicked DNA, whereas the ladder of relaxed DNA topoisomer bands is labeled R. Reactions were stopped with a mixture of 1% SDS and 6.1 μg of proteinase K. Lane 1 contains 0.2 μg of pHOT plasmid DNA and lane 2 is a relaxed marker.

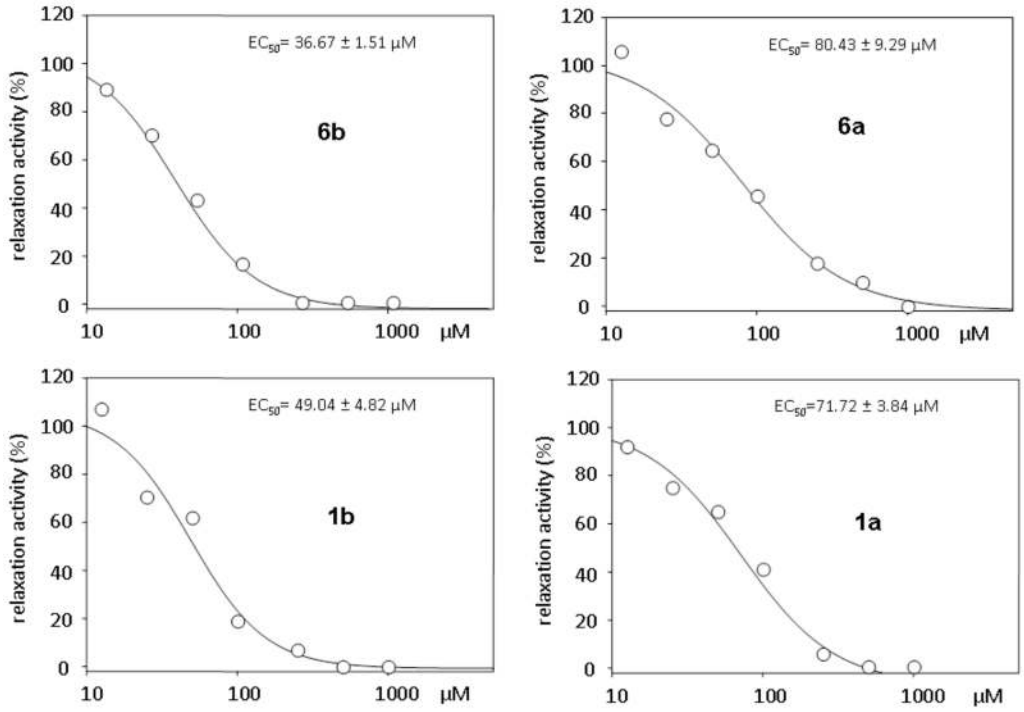
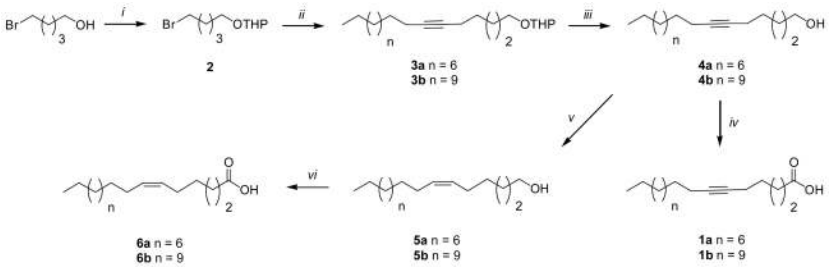


Fig. 3.

Plots of relaxation activities (%) of the LdTOPIB enzyme vs. concentration (µM) of the acetylenic fatty acids **1a** and **1b**, and the olefinic fatty acids **6a** and **6b**. The EC_{50} values are shown at the top of each graph.



i) DHP, p-TSA, $CHCl_3$, rt, 90 min; *ii*) 1-dodecyne or 1-pentadecyne, *n*-BuLi, THF-HMPA, 0 °C, 80 min; *iii*) p-TSA, MeOH, 60 °C, 24h; *iv*) PDC, DMF, 48 h; *v*) H_2, Pd/C (10%), quinoline, hexane; *vi*) PDC, DMF, 48 h.

Scheme 1.
Synthesis of the acetylenic acids **1a** and **1b** and the olefinic acids **6a** and **6b**.

Table 1

Antileishmanial Activities (in μg/mL) of the Studied Fatty Acids[a]

Fatty Acids	*L. donovani* promastigotes EC_{50}	Murine macrophages IC_{50}	Therapeutic index IC_{50}/EC_{50}
6-Heptadecynoic (**1a**)	6.67 ± 1.42	5.66 ± 0.92	0.85
(6*Z*)-Heptadecenoic (**6a**)	> 25	11.86 ± 1.63	n/a
Heptadecanoic (17:0)	> 25	14.18 ± 7.48	n/a
6-Icosynoic (**1b**)	1.11 ± 0.28	5.35 ± 2.14	4.82
(6Z)-Icosenoic (**6b**)	5.67 ± 1.07	28.20 ± 7.54	4.97
Icosanoic (20:0)	> 25	21.93 ± 2.61	n/a

[a] As a positive control we determined that miltefosine displays an IC_{50} of 0.79 ± 0.14 μM against *L. donovani* and an EC_{50} of 1.95 ± 0.37 μM against murine macrophages.

n/a: not assessed