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First Total Synthesis of ω -Phenyl 6 Fatty Acids and their Leishmanicidal and Anticancer Properties

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Abstract

The first total synthesis of ω -phenyl 6 fatty acids (FA) and their cytotoxicity (A549) and leishmanicidal (*L. infantum*) activities are described. The novel 16-phenyl-6-hexadecynoic acid (**1**) and the known 16-phenylhexadecanoic acid (**2**) were synthesized in 7-8 steps with overall yields of 46 % and 41 %, respectively. The syntheses of the unprecedented 10-phenyl-6-decynoic acid (**3**), 10-cyclohexyl-6-decynoic acid (**4**) and 10-(4-methoxyphenyl)-6-decynoic acid (**5**) was also performed in 3 steps with 73-76 % overall yields. The use of lithium acetylide coupling enabled the 4-step synthesis of 10-phenyl-6-*Z*-decenoic acid (**6**) with a 100 % cis-stereochemistry. The cytotoxicity of these novel FA was determined against A549 cells and *L. infantum* promastigotes and amastigotes. Among the ω -phenylated FA, the best cytotoxicity towards A549 was displayed by **1**, with an IC₅₀ of 18 ± 1 μ M. On the other hand, among the C₁₀ acids, the ω -cyclohexyl acid **4** presented the best cytotoxicity (IC₅₀ = 40 ± 2 μ M) towards A549. Based on caspase-3/7 studies neither of the FA induced apoptosis in A549, thus implying other mechanisms of cell death. The antileishmanial studies were performed with the top *Leishmania donovani* topoisomerase IB (*LdTopIB*) inhibitors, namely **1** and **2** (EC₅₀ between 14 and 36 μ M, respectively), acids that did not stabilize the cleavage complexes between *LdTopIB* and DNA. Acids **1** and **2** displayed cytotoxicity towards *L. infantum* amastigotes (IC₅₀ = 3-6 μ M) and *L. infantum* promastigotes (IC₅₀ = 60-70 μ M), but low toxicity towards murine splenocytes. Our results identified **1** as the optimum ω -phenylated acid of the series.

Keywords

Acetylenic Fatty Acids; Cancer; Leishmaniasis; *Leishmania infantum*; Synthesis; Topoisomerase IB

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CONFLICT OF INTEREST

The authors confirm that this article has no conflict of interest.

INTRODUCTION

Fatty acids (FA) are important biomolecules, and at the physiological level they are used to produce energy (β oxidation), serve as messengers (hormones), and are present in phospholipids, structural components of cell membranes [1-3]. For decades, unusual FA have been isolated from natural sources with biomedical applications; previous studies have shown the potential of FA against bacteria, parasites, viruses and cancer cells [4-7]. The study of the relevance of this type of lipids is limited because most of the efforts have focused on their antibacterial and antifungal properties [8]. Recently, it has been demonstrated that saturated and unsaturated FA have inhibitory properties against validated therapeutic targets for cancer and leishmaniasis, such as the human topoisomerase IB (hTopIB) and the *Leishmania* topoisomerase IB (*L*TopIB) enzymes, respectively [9-11].

Cancer and leishmaniasis are diseases that are difficult to treat; both are afflictive and fatal without appropriate treatments [12]. During 2017, approximately 1.7 million new cancer cases and 0.6 million cancer related deaths were estimated in the United States alone [13]. Prostate and lung cancer are among the most common cancers worldwide. Unfortunately, lung cancer is developing chemotherapy resistance, limiting the availability of effective treatments [14]. The non-small cell lung cancer (NSCLC) is the most common lung cancer representing 85 % of all reported lung cancer cases [15].

Palmitic acid (PA) inhibits hTopI and displays cytotoxicity against the A549 cell line, a NSCLC *in vitro* cell model, with a half maximal inhibitory concentration (IC_{50}) of 150 μ M [16]. It was proposed that PA exerts its toxicity via inhibition of the DNA topoisomerase I (topo I) as well as by the induction of autophagy in A549 as determined by the accumulation of LC3 [16]. Interestingly, PA did not induce apoptosis based on caspase assays and DNA fragmentation studies. Based on these results, it is a worthwhile proposition to synthesize other FA analogs that might effectively induce the same cellular responses and be more effective against A549 or other cancer cell lines.

Leishmaniasis is a neglected tropical disease (NTD) caused by kinetoplastids, which in its most severe form, visceral leishmaniasis (VL), has a high mortality rate [17]. Worldwide, this disease ranks second after malaria as the parasitic disease with the highest mortality. Annually more than 300,000 new cases of infection are reported, but the actual treatments are toxic (nephrotoxic and hepatotoxic), expensive, and not very effective due to the development of drug resistance and geographical variability of the outcomes [18]. Recently, we studied the antileishmanial properties of the naturally occurring 6-heptadecynoic acid (6a -17:1), 6Z-heptadecenoic acid (6 -17:1), and *n*-heptadecanoic acid (*n*-17:0), and the two former acids inhibit *L*TopIB with EC_{50} values between 72 ± 4 μ M and 80 ± 9 μ M, respectively [19]. Our results demonstrated that the 6-alkynoic FA are better inhibitors of *L*TopIB than other olefinic and saturated analogs of comparable chain length. Therefore, our research team has been actively studying FA as antiparasitic and anticancer agents to determine the structural characteristics that impart toxicity [19].

Recent studies with other FA structural motifs revealed that introduction of a phenyl group into a 5,9 fatty acid increases its topo I inhibitory potential. For example, D'yakonov and collaborators found that the (5*Z*,9*Z*)-11-phenylundeca-5,9-dienoic acid displays a high inhibitory activity towards topoisomerases I and II [20]. This enhanced activity was rationalized in terms of involvement of the phenyl group in π - π stacking interactions with the aromatic structures of the nucleotides, mainly with adenine [20]. The phenyl containing acid was observed to interact more tightly with the DNA as compared to the (5*Z*,9*Z*)-eicosadienoic acid, which does not have a phenyl substituent.

Based on the data presently available in the literature it became of interest to synthesize ω -phenyl analogs of the 6 alkynoic acids, since these FA have displayed good TopIB inhibition and antileishmanial activities, the best example being the 6-icosynoic acid [21]. In this work, we present the first total syntheses of a series of C₁₀ and C₁₆ ω -phenyl 6 FA and describe their anticancer (A549) and leishmanicidal (*L. infantum*) properties.

MATERIALS AND METHODS

The analysis of all the compounds was performed by ¹H NMR (300 MHz) and ¹³C NMR (75 MHz) using a Bruker DPX-300 spectrometer. The samples were diluted in 99.8% chloroform-*d* (CDCl₃), the solvent signals at 7.26 (¹H) and 77.0 (¹³C) ppm were used as internal standards for hydrogen and carbon, respectively. Mass spectral data was acquired on a single quadrupole GC-MS (Agilent 7820/Agilent 5977E) equipped with a 30 m $\times$ 0.32 mm (film 0.25 μ m) capillary column (DB-5MS) of phenyl arylene polymer which is virtually equivalent to a (5%-phenyl)-methylpolysiloxane. The IR spectra were measured neat on a Bruker Tensor 27 FT-IR spectrometer. High-resolution mass spectral data were acquired using a quadrupole time-of-flight mass spectrometer (Q-TOF, Synapt, G2-S, Waters) with electrospray ionization in negative mode.

1. Representative procedure for the synthesis of the ω -phenylated fatty acids. Synthesis of the 16-phenyl-6-hexadecynoic acid (**1**).

2-((16-Phenylhexadec-6-yn-1-yl)oxy)tetrahydro-2H-pyran (7**).**—Into a 50-mL round-bottomed flask, was added 0.5 g (2.5 mmol) of 2-(6-heptyn-1-yloxy)tetrahydro-2*H*-pyran, 4 mL of dry THF, and the mixture was cooled until 0 °C. To the cooled solution was added 1.5 mL (3.8 mmol) of *n*-BuLi (2.5 M in hexanes) which stirred for 15 min. To the stirring solution was added dropwise 1.5 mL of 1,3-dimethyl-2-imidazolidinone (DMI) and 1.0 g (2.5 mmol) of 1-bromo-9-phenylnonane. After 20 min the cold bath was removed and the reaction was left stirring for 24 h. After the reaction time, the reaction crude was washed with a brine solution (2 $\times$ 10 mL), extracted with diethyl ether (4 $\times$ 15 mL), dried over MgSO₄ and filtered. The solvent of the filtered solution was removed *in vacuo* and the obtained orange liquid was purified using silica gel column chromatography eluting **7** with hexane/ether (9:1). Pyran **7** (0.88 g, 2.2 mmol) was obtained as a colorless oil in an 88% yield.

16-Phenyl-6-hexadecynol (8**).**—Into a 50-mL round-bottomed flask, was added **7** (0.7 g, 1.8 mmol), catalytic amounts of *p*-toluenesulfonic acid (PTSA) and 20 mL of MeOH. The stirring solution was refluxed at 65 °C for 12 h. After the reaction time, the solvent was

removed *in vacuo*, the reaction crude was purified using silica gel column chromatography eluting **8** with hexane/ether (8:2). Alkynol **8** (0.5 g, 1.6 mmol) was obtained as a colorless oil in an 88 % yield.

16-Phenyl-6-hexadecynoic acid (1).—Into a 50-mL round-bottomed flask under argon were placed 2.9 g (7.7 mmol) of pyridinium dichromate (PDC) and 20 mL of dry dimethylformamide (DMF). To the stirring solution was added 0.4 g (1.3 mmol) of **8** and the reaction was left stirring for 48 h. After the reaction time, the crude was washed with a 1M HCl solution (2 × 10 mL), extracted with diethyl ether (4 × 15 mL), dried over MgSO₄ and filtered. The solvent of the filtered solution was removed *in vacuo* and the obtained yellowish liquid was purified using silica gel column chromatography eluting **1** with hexane/ether (7:3). Acid **1** (0.4 g, 1.2 mmol) was obtained as a white solid for a 92 % yield.

2. *Leishmania infantum* studies

2.1 *L. infantum* iRFP strain—All the biological procedures involving living animals were performed according to Spanish Act (RD 53/2013) and EU legislation (2010/63/EU). Protocols were approved by the Animal Care Committee of the University of León (Spain) Project license number PI12/00104. The generation of the infrared-fluorescent strains has been described previously by the authors [22]. *L. infantum* BCN-150 was genetically modified in the laboratory with the pLEXSY-hyg2 vector (Jena Bioscience, Germany), fused to the IRFP protein and the 3' UTR region of histone Hsp70 from *Leishmania*, which increases the expression of the proteins located upstream thereof. *L. infantum* iRFP strain was subcultured at a density of 10⁶ cells/mL in Medium 199 supplemented with 25 mM HEPES (pH 6.9), 10 mM glutamine, 7.6 mM hemin, 0.1 mM adenosine, 0.01 mM folic acid, 1 × RPMI 1640 vitamin mix (Sigma) and 10% (v/v) heat-inactivated fetal calf-serum (FCS) supplemented with antibiotic cocktail containing 50 U/mL penicillin and 50 µg/mL streptomycin). These cultures were placed in 96-well black bottom microplates (Nunc, Fisher Sci), aliquoted in 200 µL volumes per well, and challenged at different concentrations of test compounds incubated at 26 °C. The infrared signal emitted at 708 nm by the cultures was recorded after 52 h using an ODYSSEY® (Li-Cor) imaging equipment. The data obtained were processed by a non-linear curve fitting with the SigmaPlot 10.0® program to obtain IC₅₀ values.

Primary splenic cell cultures were obtained from 8 to 10 week BALB/c mice inoculated i.p. with 10⁸ metacyclic *L. infantum* iRFP promastigotes. Five-weeks post-infection, mice were euthanized and spleens were aseptically removed. To obtain a suspension of individual cells, spleens were disrupted in small pieces, incubated in collagenase D (2 mg/mL) prepared in buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂ and 1.8 mM CaCl₂) for 20 min at 37 °C. This suspension was gently passed through a 100 µm cell strainer aided by freshly prepared PBS. Splenocytes were washed twice by centrifugation at 500 g for 7 min at 4 °C and resuspended in RPMI (Gibco) supplemented with 10% (v/v) FCS, 1 mM sodium pyruvate (Gibco), 1 × RPMI vitamins and antibiotics. After cell counting in Neubauer chamber, splenocytes were seeded in 96-well microplates at a density of 5 × 10⁵ cells per well and incubated at 37 °C in an atmosphere of 5% CO₂. Macrophages were incubated for 24 h to allow their perfect adhesion to the plaque [23]. After this time, cells

were challenged at different concentrations of each compound over a period of 72 h. Amastigote viability within the macrophages was assessed by the infrared signal emitted at 708 nm by the cultures using an ODYSSEY® (Li-Cor) imaging equipment. The data obtained were processed by a non-linear curve fitting with the SigmaPlot 10.0® program.

2.2 Expression and purification of recombinant *Leishmania* and human proteins—Cloning, expression and purification of recombinant *L*TopIB and hTopIB proteins were performed using *S. cerevisiae* EKY3 TopIB-deficient strain [MAT a ura3-52 his3 200leu2 1 trp1 63 top1 :TRP1] platform and conventional low-pressure chromatographic methods reported elsewhere [24]. Protein extracts were precipitated with solid (NH₄)₂SO₄ to a saturation 35% (w/v), collected by centrifugation at 10,000 rpm and resuspended in 20 mL of TEEG buffer (1x), loaded onto a column packed with phosphocellulose resin P-11 (Whatman®) eluted with 0.8 M KCl and further purified by hydrophobic interaction chromatography (PSP phenyl-sepharose-4B). All the stages were aided with a FPLC device (Akta, GE Healthcare) at 4 °C in presence of a commercial proteinase inhibitors cocktail (Roche Farma SA, Spain).

2.3 TopIB relaxation of supercoiled activity—The relaxation of supercoiled DNA by human or leishmania recombinant TopIB was assayed by measuring the intensity of different topoisomers with distinct electrophoretic mobility in agarose gel. Briefly, the reaction mixture contained in a final volume of 20 µL, 2 µL of TOP buffer (10x; 10 mM Tris HCl buffer pH 7.5, 5 mM MgCl₂, 0.1 mM EDTA, 15 µg/mL bovine serum albumin and 150 mM KCl) and 0.5 µg of purified human or leishmania TopIB. The compounds were preincubated at 4 °C 15 min before with the assay mixture lacking DNA. Reactions were started by adding the substrate; 0.5 µg of supercoiled circular pSK DNA. Incubations were carried out in eppendorf tubes for 30 min in a water bath at 37 °C. Reactions were stopped by the addition of 1% SDS (w/v) and digested with 1mg/mL proteinase K at 37 °C to remove contaminating protein linked to DNA. Topoisomers were resolved by agarose gel (1% w/v) electrophoresis in TBE (Tris-borate buffer, pH 8.0) at 2 V/cm for 16 h, and stained with BrEt solution (0.5 µg/100 mL) to visualize under UV lamp to obtain digital images using a G-Box (Syngene UK) gel-doc.

2.4 Competition assays with gimitecan (GMT)—To assess if the test compounds establish covalent linkages to DNA, an agarose cleavage assay was performed. For this purpose, the reaction mixture (20 µL) contained 2 µL of TOP buffer (10x), 0.5 µg pSK DNA, but 75 to 100 U of *L*TopIB or hTopIB was added and 1 µL of the different concentrations of the drug to be studied (dose dependent assay). For competition assays a fixed concentration of 100 µM of GMT was used [25]. The mixtures were incubated at 25 °C. After stopping the reactions with 1% (w / v) SDS, they were digested with 2 µg proteinase K for 1 h at 37 °C. The samples were then cleaned by a 1:1 (v/v) extraction with phenol-chloroform. Finally, the extracted samples were resolved on 1% (w/v) agarose gels in TBE (1x) supplemented with EtBr at a final concentration of 40 pg/µL. Electrophoresis was performed under the same conditions as in the previous sections and the bands were quantified with the ImageJ (National Institutes of Health software) gel analysis software. The amount of nicked DNA was calculated as the percentage of signal obtained for the indentation band divided by the

total signal of the lane. The results were plotted with SigmaPlot™ 11.3 statistical software (Systat Software Inc, UK).

2.5 Cytotoxicity on uninfected murine splenocytes and determination of the Selectivity Index (SI)—To obtain uninfected murine splenocytes, BALB/c mice were euthanatized to obtain the spleen under sterile conditions. Splenocytes were obtained as described previously, seeded in 96-well plates at a cell density of 2×10^5 cells/well and exposed to different concentrations of the tested compounds for 72 h at 37 °C in an atmosphere of 5% CO₂. The viability of splenocytes was determined using the Blue Alamar dye (Invitrogen) micromethod by recording the fluorescence emitted at 595 nm by a multimodal Synergy HT (BioTek) microplate reader. SI was determined as the ratio between the IC₅₀ values obtained for non-infected mouse splenocytes and the EC₅₀ values for amastigotes measured in infected splenocytes.

3. Cytotoxic studies with A549

3.1 Cell viability—A549 cells were seeded at 5×10^4 cells/mL and 100 µL per well were added into a 96-well plate. Then, cells were incubated at FA concentrations of 5-400 µM for 24 h. Next, cell viability was measured using the *CellTiter 96 aqueous non-radioactive cell proliferation assay kit* (Promega). After the incubation time, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and phenazine methosulfate (PMS) solution was added to each well (333 µg/ml MTS + 25 µM PMS) and incubated for 1h at 37 °C under a 5% CO₂ atmosphere. The absorbance of each well was measured at 492 nm in a microplate reader spectrophotometer.

3.2 Caspase-3/7 activity assay—A549 cells were grown to 80% confluency. Then, the cells were seeded (1×10^5 cells/mL) into black 96-well plates (flat clear bottom) with 100 µL of Dulbecco's Modified Eagle's Medium (DMEM) per well. After 24 h, cells were incubated with a final concentration of 50 µM of the FA for 24 h. Next, caspase-3 activity was measured using the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega). After completion of the incubation time of 24 h, the medium was removed followed by the addition of 50 µL of fresh DMEM (without phenol red to diminish background interference) and 50 µL of Apo-ONE® Caspase-3/7 Reagent (1:100 substrate diluted in lysis buffer) to each well. The plate was gently mixed using a plate shaker at 150 rpm for 1h at room temperature. After 1h, the fluorescence of each well was measured (Excitation λ = 485 nm; Emission λ = 527 nm) in the Tecan infinite M200PRO microplate reader (Tecan Trading AG, Switzerland).

EXPERIMENTAL

16-Phenyl-6-hexadecynoic acid (1)

Synthesized as a white solid in a 92 % yield. mp. 33-36 °C; IR (neat) ν_{max} : 3400-2500 (–CO₂H), 3084 (=C-H), 3063 (=C-H), 3028 (=C-H), 2927, 2915, 2848, 1694 (C=O), 1604, 1495, 1462, 1454, 1433, 1412, 1320, 1264, 1207, 1198, 1026, 930, 904, 745, 727, 697 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 7.32-7.14 (5H, m, -Ph), 2.63-2.57 (2H, t, J = 7.4 Hz, H-16), 2.40-2.35 (2H, t, J = 7.3 Hz, H-2), 2.21-2.08 (4H, m, –CH₂–C≡C–CH₂–), 1.81-1.20 (19H, m,

–CH₂–); ¹³C NMR (75 MHz, CDCl₃) δ 179.3 (s, C-1), 143.1 (d), 128.5 (d), 128.4 (d), 125.7 (d), 81.0 (s, C-7), 79.4 (s, C-6), 36.1 (t, C-16), 33.6 (t, C-2), 31.7 (t), 29.62 (t), 29.60 (t), 29.5 (t), 29.3 (t), 29.2 (t), 29.0 (t), 28.5 (t), 24.0 (t), 18.9 (t), 18.6 (t). GC/MS (70 eV) *m/z* (relative intensity): 328 (M⁺, 5), 313 (0.01), 297 (2), 281 (1), 268 (0.01), 254 (1), 243 (1), 227 (1), 187 (1), 171 (1), 157 (2), 140 (53), 117 (17), 104 (29), 91 (100), 80 (39), 67 (14), 55 (12). UPLC-HRMS (negative ion mode) Calcd for C₂₂H₃₁O₂ [M-H]⁺ 327.2324, found 327.2325.

Methyl 16-phenyl-6-hexadecynoate.—GC/MS (70 eV) *m/z* (relative intensity): 342 (M⁺, 3), 327 (0.01), 311 (2), 293 (0.01), 268 (3), 242 (0.01), 227 (2), 211 (0.01), 195 (1), 171 (2), 154 (42), 131 (16), 117 (17), 104 (33), 91 (100), 79 (28), 74 (24), 67 (18), 55 (12).

10-Phenyl-6-decynoic acid (3)

Synthesized as a colorless oil in an 89 % yield. IR (neat) $\nu_{\max}$: 3026-2665 (–CO₂H), 3084 (=C-H), 3060 (=C-H), 3026 (=C-H), 2930, 2860, 1704 (C=O), 1496, 1431, 1233, 933, 744, 698 cm^{–1}; ¹H NMR (300 MHz, CDCl₃) δ 7.34-7.17 (5H, m, -Ph), 2.73-2.68 (2H, t, *J* = 7.4 Hz, H-10), 2.43-2.37 (2H, t, *J* = 7.3 Hz, H-2), 2.26-2.15 (4H, m, –CH₂–C≡C–CH₂–), 1.87-1.51 (7H, m, –CH₂–); ¹³C NMR (75 MHz, CDCl₃) δ 178.6 (s, C-1), 142.0 (s), 128.7 (d), 128.5 (d), 126.0 (d), 80.5 (s, C-1), 80.1 (s), 35.0 (t, C-10), 33.5 (t), 30.8 (t), 28.6 (t), 24.0 (t), 18.6 (t), 18.4 (t). UPLC-HRMS (negative ion mode) Calcd for C₁₆H₁₉O₂ [M-H]⁺ 243.1385, found 243.1386.

Methyl 10-phenyl-6-decynoate.—GC/MS (70 eV) *m/z* (relative intensity): 258 (M⁺, 1), 226 (2), 209 (4), 198 (3), 184 (10), 169 (5), 154 (19), 143 (57), 129 (47), 115 (14), 104 (100), 91 (73), 77 (22), 74 (6), 5 (14), 55 (5).

10-Phenyl-6Z-decenoic acid (6)

Synthesized as a colorless oil in an 88 % yield. IR (neat) $\nu_{\max}$: 3400-2500 (–CO₂H), 3084 (=C-H), 3062 (=C-H), 3026 (=C-H), 3004, 2928, 2855, 1704 (C=O), 1603, 1584, 1496, 1404, 1074, 1052, 1030, 743, 697 cm^{–1}; ¹H NMR (300 MHz, CDCl₃) δ 7.31-7.16 (5H, m, -Ph), 5.47-5.34 (2H, m, CH=CH), 2.65-2.60 (2H, t, *J* = 7.6 Hz, H-10), 2.38-2.33 (2H, t, *J* = 7.3 Hz, H-2), 2.11-2.01 (4H, m, –CH₂–C=C–CH₂–), 1.74-1.60 (4H, m, –CH₂–), 1.46-1.36 (3H, m, –CH₂–); ¹³C NMR (75 MHz, CDCl₃) δ 179.7 (s, C-1), 142.6 (s), 130.1 (d), 129.7 (d), 128.6 (d), 128.4 (d), 125.8 (d), 35.6 (t), 34.0 (t), 31.5 (t), 29.2 (t), 26.98 (t), 26.96 (t), 24.5 (t). UPLC-HRMS (negative ion mode) Calcd for C₁₆H₂₁O₂ [M-H]⁺ 245.1542, found 245.1543.

Methyl 10-phenyl-6Z-decenoate.—GC/MS (70 eV) *m/z* (relative intensity): 260 (M⁺, 9), 242 (0.01), 228 (8), 200 (1), 186 (2), 169 (1), 145 (4), 131 (24), 117 (20), 104 (100), 91 (71), 79 (10), 74 (7), 67 (10), 55 (8).

10-Cyclohexyl-6-decynoic acid (4)

Synthesized as a white solid in a 92 % yield. mp. 32-36 °C; IR (neat) $\nu_{\max}$: 3500-2361 (–CO₂H), 1712 (C=O), 1460, 1449, 1251, 1199, 929, 885, 731, 673 cm^{–1}; ¹H NMR (300 MHz, CDCl₃) δ 2.40-2.35 (2H, t, *J* = 7.3 Hz, H-2), 2.21-2.08 (4H, m, –CH₂–C≡C–CH₂–),

1.80-1.43 (12H, m, $-\text{CH}_2-$), 1.28-0.82 (8H, m, $-\text{CH}_2-$); ^{13}C NMR (75 MHz, CDCl_3) δ 179.7 (s, C-1), 81.1 (s, C-7), 79.4 (s, C-6), 37.4 (t), 36.9 (t), 33.7 (t), 33.5 (d), 28.5 (t), 26.9 (t), 26.7 (t), 26.5 (t), 24.0 (t), 19.2 (t), 18.6 (t). UPLC-HRMS (negative ion mode) Calcd for $\text{C}_{16}\text{H}_{25}\text{O}_2$ $[\text{M}-\text{H}]^+$ 249.1855, found 249.1855.

Methyl 10-cyclohexyl-6-decynoate.—GC/MS (70 eV) m/z (relative intensity): 264 (M^+ , 0.01), 249 (0.01), 232 (1), 215 (2), 193 (0.01), 181 (3), 168 (5), 163 (9), 154 (54), 149 (27), 135 (38), 122 (27), 109 (45), 94 (100), 80 (77), 74 (18), 67 (79), 55 (65).

10-(4-Methoxyphenyl)-6-decynoic acid (5)

Synthesized as a colorless oil in a 91 % yield. IR (neat) ν_{max} : 3286 ($-\text{CO}_2\text{H}$), 3053 ($=\text{C}-\text{H}$), 3031 ($=\text{C}-\text{H}$), 3004 ($=\text{C}-\text{H}$), 2995, 2860, 2669, 1705 ($\text{C}=\text{O}$), 1512, 1432, 1243, 1176, 1035, 933, 831, 633, 516 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ 7.12-7.09 (2H, dt, $J = 2.6$ Hz, $J = 8.5$ Hz, -Ph), 6.85-6.81 (2H, dt, $J = 3.2$ Hz, $J = 8.6$ Hz, -Ph), 3.79 (3H, s, $-\text{OCH}_3$), 2.67-2.62 (2H, t, $J = 7.5$ Hz, H-10), 2.41-2.36 (2H, t, $J = 7.3$ Hz, H-2), 2.16-1.51 (11H, m, $-\text{CH}_2-$); ^{13}C NMR (75 MHz, CDCl_3) δ 179.1 (s, C-1), 158.0 (s), 134.1 (s), 129.5 (d), 113.4 (d), 80.5 (s, C-7), 80.0 (s, C-6), 55.4 (q, $-\text{OCH}_3$), 34.1 (t, C-10), 33.6 (t), 31.0 (t), 28.6 (t), 24.0 (t), 18.6 (t), 18.3 (t). UPLC-HRMS (negative ion mode) Calcd for $\text{C}_{17}\text{H}_{21}\text{O}_3$ $[\text{M}-\text{H}]^+$ 273.1491, found 273.1490.

Methyl 10-(4-methoxyphenyl)-6-decynoate.—GC/MS (70 eV) m/z (relative intensity): 288 (M^+ , 26), 257 (1), 239 (4), 227 (1), 214 (2), 199 (3), 187 (48), 173 (54), 159 (23), 146 (6), 134 (86), 121 (100), 106 (5), 91 (21), 77 (17), 74 (2), 65 (7).

RESULTS AND DISCUSSION

The synthesis of 16-phenyl-6-hexadecynoic acid (**1**) and 16-phenylhexadecanoic acid (**2**) started from commercially available 2-(hex-5-yn-1-yloxy)tetrahydro-2H-pyran (Scheme 1). The protected alkynol was successfully coupled with commercially available 3-phenyl-1-bromopropane in the presence of *n*-BuLi in a tetrahydrofuran/1,3-dimethyl-2-imidazolidinone (THF-DMI) solution at 0 °C, obtaining an 84 % yield of 2-((9-phenyl-5-nonyl-1-yl)oxy)tetrahydro-2H-pyran (**9**). The resulting tetrahydropyranyl ether was deprotected via acidic methanolysis by refluxing **9** in methanol at 60 °C with catalytic amounts of PTSA thus obtaining the 9-phenyl-5-nonyl-1-ol (**10**) in a 90 % yield. The alkynol **10** was hydrogenated using 5-10 mol % of Pd-C in hexane, obtaining 9-phenyl-1-nonanol (**11**) in a 92 % yield. Subsequently, alcohol **11** was brominated under Appel reaction conditions, with triphenylphosphine and carbontetrabromide in dichloromethane (DCM) affording the known 1-bromo-9-phenyl-1-nonanol (**12**) in a 92 % yield. The protected 6-heptynol was successfully coupled with **12** in the presence of *n*-BuLi in a THF-DMI solution at 0 °C, obtaining an 88 % yield of 2-((16-phenylhexadec-6-yn-1-yl)oxy)tetrahydro-2H-pyran (**7**). The resulting tetrahydropyranyl ether **7** was deprotected via acidic methanolysis by refluxing **7** in methanol at 60 °C with catalytic amounts of PTSA, thus obtaining the 16-phenyl-6-hexadecynol (**8**) in an 88 % yield. Subsequently, alcohol **8** was oxidized applying the Corey & Schmidt method, PDC in DMF, affording **1** in a 92 % yield. The synthesis of **1** was achieved in 7 steps with an overall yield of 46 %. Taking advantage of this route, the

known **2** [26] was also obtained by hydrogenating **1** with H₂ in 5-10 mol % Pd-C thus obtaining the saturated acid in a 90 % yield. The synthesis of **2** was achieved in 8 steps and in an overall yield of 41 %.

The synthesis of 10-phenyl-6-decynoic acid (**3**) started from commercially available 2-(6-heptyn-1-yloxy)tetrahydro-2*H*-pyran (Scheme 2). The protected alkynol was successfully coupled with commercially available 3-phenyl-1-bromopropane in the presence of *n*-BuLi in a THF-DMI solution at 0 °C, obtaining a 92 % yield of 2-((10-phenyl-6-decyn-1-yl)oxy)tetrahydro-2*H*-pyran (**13**). The resulting tetrahydropyranyl ether was deprotected via acidic methanolysis by refluxing **13** in methanol at 60 °C with catalytic amounts of PTSA, thus obtaining the 10-phenyl-6-decynol (**14**) in a 90 % yield. Subsequently, **14** was oxidized applying the Corey & Schmidt method affording 10-phenyl-6-decynoic acid (**3**) in an 89 % yield. This synthesis was achieved in 3 steps with an overall yield of 74 %. For the synthesis of the olefinic analog **6** alcohol **14** was hydrogenated under Lindlar's conditions, obtaining the 10-phenyl-6*Z*-decenol (**15**) in a 90 % yield. The 100% cis-alkenol was oxidized with PDC in DMF obtaining the corresponding 10-phenyl-6*Z*-decenoic acid (**6**) in an 88 % yield. The synthesis of **6** was achieved in 4 steps with an overall yield of 66 %. Taking advantage of this route we also synthesized the known 10-phenyldecanoic acid (**16**) [27] by hydrogenating alkynol **14**. The hydrogenation was performed using H₂ in 5-10 mol % Pd-C, obtaining the 10-phenyldecanol (**17**) in an 89 % yield. Alcohol **17** was oxidized with PDF/DMF obtaining **16** as a white solid in an 89 % yield. The synthesis of **16** was achieved in 4 steps and in an overall yield of 66 %.

The synthesis of the ω -cyclohexyl fatty acid **4** (Scheme 3) was possible by starting with 2-(6-heptyn-1-yloxy)tetrahydro-2*H*-pyran, which was successfully coupled with 1-bromo-3-cyclohexylpropane (**18**) in the presence of *n*-BuLi in a THF-DMI solution at 0 °C, obtaining an 89 % yield of 2-((10-cyclohexyl-6-decyn-1-yl)oxy)tetrahydro-2*H*-pyran (**19**). The resulting tetrahydropyranyl ether was deprotected via acidic methanolysis refluxing **19** in methanol at 60 °C with catalytic amounts of PTSA, thus obtaining the 10-cyclohexyl-6-decynol (**20**) in a 93 % yield. Subsequently, alcohol **20** was oxidized applying the Corey & Schmidt conditions affording the 10-cyclohexyl-6-decynoic acid (**4**) in a 92 % yield. The synthesis of **4** was achieved in 4 steps and in an overall yield of 76 %.

The synthesis of the 10-(4-methoxyphenyl)-6-decynoic acid (**5**) was accomplished using 2-(6-heptyn-1-yloxy)tetrahydro-2*H*-pyran (Scheme 3), which was successfully coupled with commercially available 1-bromo-3-(4-methoxyphenyl)propane in the presence of *n*-BuLi in a THF-DMI solution at 0 °C, obtaining an 89 % yield of 2-(10-(4-methoxyphenyl)dec-6-ynyloxy)-tetrahydro-2*H*-pyran (**21**). The tetrahydropyranyl ether **21** was deprotected via acidic methanolysis refluxing the protected alcohol in methanol at 60 °C with catalytic amounts of PTSA, thus obtaining the 10-(4-methoxyphenyl)-6-decynol (**22**) in a 90% yield. Subsequently, alcohol **22** was oxidized applying the Corey & Schmidt conditions affording **5** in a 91 % yield. The synthesis of **5** was achieved in 3 steps and in an overall yield of 74 %.

As discussed before, FA exert their antileishmanial and anticancer activities by a variety of mechanisms and some of these mechanisms might involve the inhibition of topo I thus promoting the activation of apoptosis and/or autophagy pathways [16]. Recently, Ok Joon

Kim and collaborators determined that PA inhibits topo I and increases the autophagic flux in A549 cells in a time-dependent manner due to an increase in the conversion of LC3 I to LC3 II [16]. Our group has also demonstrated that the 6-alkynoic acids are better inhibitors of topo I when compared to 6-alkenoic and alkanolic acids of comparable chain lengths [21]. This inhibitory activity could translate into better anticancer and/or antileishmanial activities since topo I is a validated target for both diseases. Therefore, we decided to first study the topo I inhibitory activities of the novel FA **1-6** and **16**. As a starting point for this study we determined the hTopIB and LTopIB inhibitory properties of the naturally occurring 6-hexadecynoic acid (6-HDA), which displayed a half maximal effective concentration (EC_{50}) of $127 \pm 2 \mu\text{M}$ and $61 \pm 3 \mu\text{M}$ towards hTopIB and LTopIB, respectively (Table 1). Based on these results it became clear the better affinity of 6-HDA towards LTopIB over hTopIB. When a ω -phenyl substituent was added to either 6-HDA or PA the inhibitory potential of the acid increased towards both enzymes. Presumably, the phenyl group is engaging in additional π - π stacking interactions with nucleotides of the DNA or aromatic amino acids in topo I. In the case of the human enzyme acids **1** and **2** inhibited hTopIB with EC_{50} 's of $25.0 \pm 0.1 \mu\text{M}$ and $31.2 \pm 0.3 \mu\text{M}$, respectively (Table 1). With LTopIB a higher inhibition was again observed for **1**, which displayed an EC_{50} of $14 \pm 1 \mu\text{M}$, followed by **2** with an EC_{50} of $36 \pm 6 \mu\text{M}$, both exhibiting better inhibitory activity than the natural occurring 6-HDA (EC_{50} of $61 \pm 3 \mu\text{M}$). Taken together, our data indicates that the addition of an ω -phenyl group increased the inhibitory capability of 6-HDA towards both topoisomerases. While in most cases the acids were more inhibitory towards LTopIB than towards hTopIB, an exception was observed for **2**, since the saturated acid displayed a slight preference for hTopIB over LTopIB. It should be mentioned that LTopIB differs significantly from hTopIB since it is phylogenetically unique and has an anomalous dimeric structure [19].

Reducing the carbon chain length of the acids from C_{16} to C_{10} was detrimental for the topo I inhibitory activities (Table 1). For example, acid **3**, the C_{10} analog of **1**, was not effective towards the topo I enzymes (EC_{50} 's $> 200 \mu\text{M}$). Changing the ω -phenyl group for a ω -cyclohexyl substituent, like in **4**, or even a p-methoxylated phenyl analog like in **5**, proved of no value in increasing the inhibitory potential of the short-chain analogs towards topo I. The only C_{10} acid in the series with some sort of inhibitory activity towards topo I was the known 10-phenyldecanoic acid (**16**), which displayed an EC_{50} of $200 \mu\text{M}$. These results indicated that chain length is a critical factor for the ω -phenylated FA to be effective topo I inhibitors. This could be rationalized in terms of less Van Der Waals interactions of the shorter-chain acids as compared to the longer-chain acids within the active site of the enzyme.

To assess if **1** and **2** inhibit leishmanial and human topo I enzymes or stabilize cleavage complexes with DNA, an agarose assay in the presence of ethidium bromide (EtBr) was performed. In this assay, the cleavage complexes stabilized by the drug, topo I, and DNA, were digested with proteinase K and the nicked DNA was resolved in the gel in a band appearing at the top of the lane (Fig. 1). Neither **1** or **2** produced nicked DNA, but the topo I poison GMT, a more stable camptothecin (CPT) analogue used as a positive control, did. In a second experiment both **1** and **2** were incubated with $100 \mu\text{M}$ GMT to check whether these compounds compete with GMT for the same topo I active site. The representative agarose

gel of Fig. 1 shows that the band corresponding to nicked DNA was effectively reduced by both acids, as well as the band corresponding to relaxed DNA. These results indicated that **1** and **2** share the same binding site as GMT, but they did not stabilize the cleavage complexes between topo I and DNA.

Acids **1** and **2** stood out as the best candidates of the series for further cell and/or parasitic studies. The cytotoxic properties of PA against the A549 cell line was initially determined, as our baseline data, and an IC_{50} of $145 \pm 2 \mu M$ was observed, which correlated quite well with the IC_{50} value reported in the literature ($IC_{50} = 150 \mu M$) [16]. When a C-6 unsaturation was introduced in the alkyl chain, as in 6-HDA, the cytotoxicity of the acid increased 9-fold since an IC_{50} of $16 \pm 2 \mu M$ was determined (Table 2). Addition of an ω -phenyl group, such as in **1**, did not change much the cytotoxicity of 6-HDA since the observed IC_{50} was $18 \pm 1 \mu M$ (Fig. 2). Moreover, when the ω -phenyl group was introduced into palmitic acid, such as in **2**, the cytotoxicity was comparable to the one observed for PA ($IC_{50} = 122 \mu M$ for **2** vs. $145 \mu M$ for PA). These results indicated that phenyl substitution does not help in increasing the cytotoxicity of the C_{16} acids towards A549. However, the addition of an ω -phenyl substituent was beneficial to the cytotoxicity displayed by some of the C_{10} acids, such as the 10-phenyl-6Z-decenoic acid (**6**) and the 10-phenyldecanoic acid (**16**), but detrimental to the cytotoxicity when a C-6 triple bond was present. For example, while acids **16** and **6** displayed IC_{50} 's between 56 and 61 μM , acids **3** and **5** displayed IC_{50} 's around 300 μM . Changing the ω -phenyl group in **3** for an ω -cyclohexyl group, as in **4**, restored the cytotoxicity ($IC_{50} = 40 \pm 2 \mu M$). These results demonstrate that carbon chain length and the 6 unsaturation were more important for the cytotoxicity displayed by the FA on A549 than the ω -phenyl substitution. In any instance, among the studied FA, the naturally occurring 6-HDA displayed the best cytotoxicity against A549 with an IC_{50} of $16 \pm 2 \mu M$ followed closely by acid **1** ($IC_{50} = 18 \pm 1 \mu M$).

To better understand the mechanism of cytotoxicity of these compounds we performed caspase-3/7 (CASP-3/7) activity assays and determined that cell death does not go via activation of CASP-3/7, implying that the main mechanism of toxicity does not involve apoptosis (Fig. 2). If we disregard apoptosis, it is very likely for our new FA to behave as PA and exert their toxicity via inhibition of the DNA topo I as well as by the induction of autophagy [19]. Another possibility is for the acids to destabilize the integrity and function of the A549 cell membranes due to their amphipathic character. These FA can integrate into cell membranes creating instability of the lipid bilayer thus making the lipid membranes more permeable [28].

The best candidates identified in the *L*TopIB enzymatic studies were also selected for further antiprotozoal evaluation towards *L. infantum* promastigotes and amastigotes. The selected candidates were **1** and **2** since they were again the most inhibitory towards *L*TopIB (Table 1). Among the studied ω -phenylated acids, **1** was the most toxic towards *L. infantum* promastigotes with an IC_{50} of $61 \pm 7 \mu M$ (Table 3). On the other hand, acid **2** presented the best toxicity towards *L. infantum* amastigotes with an IC_{50} of $3 \pm 1 \mu M$. Both FA displayed a higher toxicity towards amastigotes than towards promastigotes. These findings confirm that FA can distinguish between the different stages of the leishmanial parasite (amastigotes vs. promastigotes) and display differential toxicities towards each stage. This correlates with

previous studies on *L. mexicana* where amastigotes had an increased uptake rate (10-fold higher) of non-esterified fatty acids compared to promastigotes [29-30]. The results also revealed that acids **1** and **2** displayed a similar toxicity profile towards both stages of *L. infantum*. This implies that the ω unsaturation was not critical for the observed activities even though it made a difference for the *L*TopIB inhibition. It is important to mention here that 6-HDA displays toxicity towards *L. infantum* promastigotes with an $EC_{50} = 102 \mu M$ and PA is not effective at all. Therefore, the ω -phenyl substitution makes a difference in the toxicity of the FA towards *L. infantum*.

CONCLUSION

The work presented herein identified **1** as the best overall candidate against both the A549 cell line and *L. infantum* amastigotes. Acid **1** was also the best inhibitor among the studied FA against both *h*TopIB and *L*TopIB by a mechanism different from the classical poisons, such as GMT or CPT, that stabilize the ternary complex between the enzyme, DNA, and the drug. While ω -phenyl substitution was important for topo I inhibition, it was not as important for the anticancer activity displayed by the FA towards A549 since an acid devoid of the phenyl substitution, such as 6-HDA, was as good an anticancer agent as **1**. The exact mechanism by which acids such as **1** exert their toxicity (cancer or leishmania) has yet to be exactly elucidated, but it became clear that apoptosis was not the preferred mode of action based on the caspase 3/7 assays. Taking into consideration previous research with PA it is not farfetched to propose that **1** exerts its toxicity towards A549 by means of inhibition of topo I followed by autophagy [16]. This will be a topic for future research.

These observations can also be extended to the leishmanicidal action of **1** and **2**, since apoptosis is also not the preferred death mechanism by which these FA kill the parasites [5]. It might be worthwhile to investigate autophagy as the preferred death mechanism for *L. infantum* as well. Interesting was the distinction made by acids **1** and **2** between *L. infantum* promastigotes and amastigotes, displaying a 10-fold preference in toxicity for the latter. This preference makes sense given the fact that in Leishmania, amastigotes tend to internalize FA faster than promastigotes, probably due to the substrates available in their intracellular habitat [29-30].

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Biography

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Fig. (1).

FA inhibit LTopIB and hTopIB by a GMT-independent mechanism. Purified LTopIB (lanes 2 to 7) was assayed in the presence of DMSO (lane 2), 100 μ M GMT (lane 3), 100 μ M **2** (lane 4), and 100 μ M **1** (lane 5). To assess the potential competition with GMT, 100 μ M of either **1** or **2** were added simultaneously to 100 μ M GMT (lanes 6 and 7, respectively). Similarly, purified hTopIB (lanes 8 to 13) was assayed in the presence of DMSO (lane 8), 100 μ M GMT (lane 9), 100 μ M **2** (lane 10), and 100 μ M **1** (lane 12). Potential competition with GMT was assayed by adding simultaneously 100 μ M GMT to 100 μ M **2** (lane 11), and 100 μ M **1** (lane 13). Supercoiled, relaxed and nicked DNA were separated resolved in 1% agarose gel containing ethidium bromide to a final concentration of 40 μ g/mL. (N = nicked DNA; Sc = supercoiled DNA; R = relaxed DNA).

Fig. (2).

Caspase-3/7 Assay. The positive control is the chemotherapeutic agent Cisplatin (CisPt) with an $IC_{70} = 50 \mu M$. The mechanism of FA toxicity was not predominantly apoptosis. At a concentration of the FA of $50 \mu M$ the caspase-3/7 activity of the cells with the studied acids was like the untreated A549 cells. The caspase-3/7 activity units are arbitrary units (a.u.).

Scheme 1.

Synthesis of 16-phenyl-6-hexadecynoic acid (**1**) and 16-phenylhexadecanoic acid (**2**). (i) *n*-BuLi, THF-DMI, 1-bromo-3-phenylpropane, 0 °C, 24 h; (ii) MeOH, PTSA, 65 °C, 12 h; (iii) H₂ Pd-C, hexane; (iv) CBr₄, PPh₃, DCM; (v) *n*-BuLi, THF-DMI, **12**, 0 °C, 24 h; (vi) MeOH, PTSA, 65 °C, 12 h; (vii) PDC, DMF, 48 h; (viii) H₂ Pd-C, hexane.

Scheme 2.

Synthesis of 10-phenyl-6-decynoic acid (**3**), 10-phenyl-6*Z*-decenoic acid (**6**), and 10-phenyldecanoic acid (**16**). (i) *n*-BuLi, THF-DMI, 1-bromo-3-phenylpropane, 0 °C, 24 h; (ii) MeOH, PTSA, 65 °C, 12 h; (iii) PDC, DMF, 48 h; (iv) 5-10 % Lindlar catalyst, quinoline, hexane, H₂, 12 h; (v) 5-10 % Pd-C catalyst, hexane, H₂, 12 h.

Scheme 3.

Synthesis of 10-cyclohexyl-6-decynoic acid (**4**) and 10-(4-methoxyphenyl)-6-decynoic acid (**5**). (i) *n*-BuLi, THF-DMI, 1-bromo-3-cyclohexylpropane or 1-bromo-3-(4-methoxyphenyl)propane, 0 °C, 24 h; (ii) MeOH, PTSA, 65 °C, 12 h; (iii) PDC, DMF, 48 h.

Table 1.

Inhibition of hTopIB and LTopIB by HDA and the synthetic FA.

Fatty Acid	hTopIB EC ₅₀ (μM)	LTopIB EC ₅₀ (μM)
6-HDA	127 ± 2	61 ± 3
1	25.0 ± 0.1	14 ± 1
2	31.2 ± 0.3	36 ± 6
3	> 200	> 200
6	> 200	> 200
16	200	200
4	> 200	> 200
5	> 200	> 200

Table 2.

Cytotoxicity of the studied FA towards the A549 cell line, NSCLC *in vitro* cell model.

Fatty Acid	IC ₅₀ (μM) ^a
PA	145 ± 2
6-HDA	16 ± 2
1	18 ± 1
2	122 ± 2
3	306 ± 6
6	61 ± 1
16	56 ± 1
4	40 ± 2
5	> 300

^aResults are the average of three runs (n = 3).

Table 3.

Toxicity of 1 and 2 against *L. infantum* promastigotes and amastigotes.

FA	Promastigotes IC ₅₀ (μM)	Amastigotes IC ₅₀ (μM)	Cytotoxicity ^a EC ₅₀ (μM)	SI promastigotes	SI amastigotes
1	61.4 ± 6.7	6.0 ± 2.2	> 250	> 4.1	> 42.0
2	71.2 ± 6.4	2.8 ± 1.0	> 250	> 3.5	> 89.0

SI = Selectivity Index.

^aUninfected Splenocytes