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ORIGINAL ARTICLE

# Novel Very Long-Chain α-Methoxylated Δ5,9 Fatty Acids from the Sponge *Asteropus niger* Are Effective Inhibitors of Topoisomerases IB

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**Abstract** The novel fatty acids (2*R*,5*Z*,9*Z*)-2-methoxy-25-methyl-5,9-hexacosadienoic acid (**1a**) and (2*R*,5*Z*,9*Z*)-2-methoxy-24-methyl-5,9-hexacosadienoic acid (**1b**) were isolated in 80 % purity from the Caribbean sponge *Asteropus niger* by chloroform/methanol extraction followed by solvent partitioning and silica gel column chromatography. The compounds were characterized by utilizing a combination of gas chromatography-mass spectrometry, nuclear magnetic resonance, and circular dichroism. Acids **1a** and **1b** were not detected in the phospholipids (PtdCho and PtdIns) of the sponge, but rather as free FA and possibly in glycosylceramides. The mixtures of **1a** and **1b** displayed cytotoxicity towards THP-1 and HepG2 cells with $EC_{50's}$ between 41 and 35 μg/mL. Apoptosis was not the preferred mode of cell death induced by **1a**–**1b** in the THP-1 cells. This implies other types of cytotoxicity mechanisms, such as membrane disruption and/or the inhibition ($EC_{50}$ = 1.8 μg/mL) of the human topoisomerase IB enzyme (*h*TopIB), with a mechanism of inhibition different from the one displayed by camptothecin (CPT). In a separate experiment, the mixture of **1a** and **1b** also displayed cytotoxicity towards *ex vivo* mouse splenocytes infected with *Leishmania infantum* amastigotes ($IC_{50}$ = 0.17 mg/mL) and free living promastigotes ($IC_{50}$ = 0.34 mg/mL). It was also found that the FA were inhibitory of the *Leishmania* topoisomerase IB (*L*TopIB) with an $EC_{50}$ = 5.1 μg/mL. Taken together, **1a** and **1b** represent a new class of FA with potential as TopIB inhibitors that preferentially inhibit *h*TopIB over *L*TopIB.

**Keywords** *Asteropus niger* · Cancer · *Leishmania infantum* · Leishmaniasis · Methoxylated fatty acids · Sponges · Topoisomerase IB

**Abbreviations**

| | |
|---|---|
| FA | Fatty acids |
| CD | Circular dichroism |
| CPT | Camptothecin |
| ECL | Equivalent chain length |
| $EC_{50}$ | Half maximal effective concentration |
| $ED_{50}$ | Median effective dose |
| GC–MS | Gas chromatography-mass spectrometry |
| HepG2 | Human hepatocellular liver carcinoma cell line |
| *h*TopIB | Human topoisomerase IB |
| $IC_{50}$ | Half maximal inhibitory concentration |
| *L*TopIB | *Leishmania* topoisomerase IB |
| PtdCho | Phosphatidylcholine |
| PtdIns | Phosphatidylinositol |
| PtdSer | Phosphatidylserine |
| iRFP | Infrared fluorescent protein |
| THP-1 | Human monocytic leukemia cell line |
| UPLC-MS | Ultra high performance liquid chromatography—mass spectrometry |

## Introduction

Sponges are the source of novel phospholipid FA, especially the unusual long-chain Δ5,9 FA (C23–C30) with no

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counterpart in the terrestrial world [1]. Contrary to conventional animal phospholipids, which typically carry a saturated acyl group at the *sn*-1 position of the glycerol backbone and an unsaturated FA at the *sn*-2 position, the Δ5,9 FA show no preference for either position [1, 2]. One of the most characteristic FA is the (5*Z*,9*Z*)-5,9-hexacosadienoic acid (5,9-26:2), since it is found in the phospholipids of most sponges, such as in *Erylus goffrilleri*, among many other sponges [1, 3].

A considerable number of methyl-branched *iso*/*anteiso* Δ5,9 FA have also been isolated from sponges. For example, the 22-Me-5,9-24:2 and the unusual 23-Me-5,9-24:2 were identified in the lipid extract of the sponge *Geodinella robusta* [4], the 23-Me-5,9-25:2 was initially identified in the sponge *Cribrochalina vasculum* [5], while the 25-Me-5,9-26:2 and the 24-Me-5,9-26:2 were first identified in the sponge *Petrosia ficiformis* [6] and most recently in the Caribbean sponge *Pseudospongosorites suberitoides* [7]. All of these *iso*/*anteiso* Δ5,9 FA have, as a common biosynthetic precursor, either *iso*-15:0 and/or *anteiso*-15:0 that are also typically found in sponges but that definitively originate from bacterial symbionts within the sponge [8].

Another interesting group of Δ5,9 diunsaturated FA are the 2-methoxylated Δ5,9 FA, which were identified in the Caribbean sponge *Topsentia roquensis* [9] and subsequently in the sponge *E. goffrilleri* [3]. The first identified 2-methoxylated Δ5,9 FA was the long-chain 2-OMe-5,9-26:2, which occurred in very low abundance in *T. roquensis* and it was basically characterized from its mass spectral data. In recent isolation studies with *E. goffrilleri*, five 2-methoxylated Δ5,9 FA were also identified in less than 1.5 % relative abundance of the total FA composition and their characterization mainly relied on GC-MS of the different derivatives [3]. Four of the five 2-methoxylated Δ5,9 FA were a series of $C_{16}$-$C_{20}$ normal chain FA, identified as the 2-OMe-5,9-16:2, 2-OMe-5,9-18:2, 2-OMe-5,9-19:2, and the 2-OMe-5,9-20:2 acids. Also identified was the (5*Z*,9*Z*)-2-methoxy-15-methyl-5,9-hexadecadienoic acid, a short chain *iso* methyl branched 2-methoxylated Δ5,9 FA [3]. However, due to their low natural abundance in the sponge it was difficult to study the biophysical and biological properties of these intriguing methoxylated compounds.

The Δ5,9 FA have displayed biological activities as well, including the inhibition of the human topoisomerase I (*h*TopI) and cytotoxicity towards some cancer cell lines. For example, it was reported that 5,9-27:2 effectively inhibits *h*TopI with an $IC_{50}$ of 0.86 μM [10]. Other equally cytotoxic compounds include the 5,9-20:2 ($IC_{50} > 0.1$ μM), 5,9-28:2 ($IC_{50} = 1.3$ μM), the methyl branched 23-Me-5,9-26:2 ($IC_{50} = 1.1$ μM) and the fully synthetic (5*Z*,9*Z*)-11-phenyl-5,9-undecadienoic acid ($IC_{50} = 0.7$ μM) [10–12]. All of these FA, which have shown inhibitory activity against *h*TopI, do not have any structural characteristics similar to the well-known DNA *h*TopI inhibitors, such as camptothecin (CPT). The mechanism of *h*TopI inhibition can be associated with the direct interaction of these FA with the *h*TopI enzyme by binding in close proximity of the active site, thus inhibiting the DNA cleavage by a tyrosine, while still allowing the enzyme–DNA interaction [12]. It is of interest to mention that the combination of both 22-Me-5,9-24:2 and 23-Me-5,9-24:2 displays cytotoxicity against mouse Ehrlich carcinoma cells ($ED_{50} = 1.9$ μg/mL) and shows hemolytic effect on mouse erythrocytes [4]. Taken together, all of these findings tend to indicate that the Δ5,9 FA should display anticancer activities by possibly inhibiting *h*TopI, among other possible mechanisms of action.

The *h*TopI inhibitory activities of optically active very long chain ($C_{26}$–$C_{29}$) 2-methoxylated Δ5,9 FA, as well as their anticancer potential or other sort of bioactivity, have not been studied. This is due in part to the paucity of material presently available from natural sources and the troublesome synthetic procedures needed to make the optically active very long-chain acids, particularly the analogs with *iso*/*anteiso* methyl-branching. The 2-methoxylated Δ5,9 FA have the potential of being better *h*TopI inhibitors than their corresponding Δ5,9 FA. For example, it can be speculated that the α-methoxy functionality in the FA will make additional favorable binding interactions between the FA and the *h*TopI enzyme as the acidity of the FA is expected to increase (pKa 3.5 *vs* 4.8) and the extra oxygen of the methoxy group can provide an additional hydrogen bond acceptor (HBA) in the molecule. In fact, we have previously shown that the (±)-2-methoxy-6-heptadecynoic acid is a better inhibitor ($EC_{50} = 16.6$ μM) of the *Leishmania donovani* DNA topoisomerase IB enzyme (*L*TopIB) than 6-heptadecynoic acid ($EC_{50} = 71.7$ μM) [13]. Interestingly, the latter two acids were not as effective towards the *h*TopIB enzyme [13]. In fact, all the short-chain FA that we have tested so far have been more effective towards *L*TopIB than *h*TopIB [13].

The structural differences between *L*TopIB and *h*TopIB make the former an attractive target for chemotherapeutic intervention since *L*TopIB is a heterodimer arising from two separate genes encoding for the core and catalytic domains of the enzyme [14]. *Leishmaniasis* is a complex disease caused by different species of protozoan parasites belonging to the genus *Leishmania* [14]. The disease is transmitted by the bite of female Phlebotominae sand flies causing cutaneous, monocutaneous, and visceral leishmaniasis (Kala Azar) in humans [14].

In the present work we report the isolation and characterization of the novel (2*R*,5*Z*,9*Z*)-2-methoxy-25-methyl-5,9-hexacosadienoic acid (**1a**) and (2*R*,5*Z*,9*Z*)-2-methoxy-24-methyl-5,9-hexacosadienoic acid (**1b**), as well as other 2-OMe-Δ5,9 $C_{26}$-$C_{29}$ analogs, from the Caribbean sponge *Asteropus niger*. The amount of material isolated permitted

their further scrutiny as TopIB inhibitors, as well as the assessment of their cytotoxic and antileishmanial activities.

## Materials and Methods

### Instrumentation

All compounds were analyzed by $^{1}H$ NMR (500 MHz) and $^{13}C$ NMR (125 MHz) using a Bruker Avance DRX-500 spectrometer. The samples were diluted in 99.8 % chloroform-d ($CDCl_3$) and the solvent signals at 7.26 ($^{1}H$) and 77.0 ($^{13}C$) ppm were used as internal standards for hydrogen and carbon, respectively. Mass spectral data were acquired on a GC–MS (Hewlett-Packard 5972A MS ChemStation or Agilent 5975C MS Chemstation) instrument at 70 eV, equipped with a 30 × 0.25 mm (film 0.25 μm) special performance capillary column (HP-5MS) of polymethylsiloxane cross-linked with 5 % phenyl methylpolysiloxane. CD spectra were recorded on a Jasco Asia Portal J-1500 Circular dichroism spectrometer.

### Sponge Collection

*Asteropus niger* was collected during a June 2006 underwater expedition to Monito Island, Puerto Rico, and identified according to Hajdu and van Soest [15]. The sponge was shade dried and transported to the laboratory, washed in tap water to remove sand and other debris, stored at −20 °C, and then freeze-dried. A voucher specimen is stored at the Department of Chemistry, University of Puerto Rico, Rio Piedras campus.

### Extraction and Isolation of 1a–1b

The sponge (362 g dry weight) was carefully cut into small chunks and blended using a mixture of $CHCl_3$/MeOH (1:1 v/v) (4 × 1L). After filtration, the crude extract was concentrated *in vacuo* to yield a brown thick paste (25.9 g) that was suspended in $H_2O$ (1L) and extracted with *n*-hexane (3 × 500 mL). The resulting hexane crude extract was concentrated *in vacuo* to yield a brown paste (7.4 g) that was resuspended in *n*-hexane (750 mL) and extracted with methanol (2 × 250 mL). The methanol extract obtained was concentrated *in vacuo* to yield a brown paste (2 g) that was partitioned by silica gel (70 g) column chromatography using a gradient of increasing polarity with $CHCl_3$/MeOH (100:0-7:3) as mobile phase to obtain six fractions.

Fraction 2 (575.5 mg) was dissolved in THF (5.3 mL) and added to freshly prepared diazomethane in diethyl ether (30 mL). The reaction mixture was stirred at room temperature for 3 h and concentrated *in vacuo*. The brown oily paste obtained was purified by silica gel (18 g) column chromatography using *n*-hexane/EtOAc (98:2) as mobile phase and separated into seven fractions. Fraction 2.4 consisted of a colorless oil (15.6 mg) that was subsequently identified as a 1:2 mixture of the α-methoxy methyl esters of **1a** and **1b** by GC–MS and NMR. Saponification of the methyl esters with 2 M NaOH in methanol at 60 °C for 3 h yielded the free FA.

*Methyl*
*(2R,5Z,9Z)-2-Methoxy-25-methyl-5,9-hexacosadienoate*

ECL = 27.31, GC–MS *m/z* (*relative intensity*, %) $M^+$ 450 (1), 418 (4), 391 (9), 359 (8), 221 (1), 193 (3), 171 (5), 165 (10), 139 (37), 134 (12), 112 (6), 111 (31), 107 (17), 104 (100), 81 (43), 79 (41), 69 (21), 67 (28), 57 (21), 55 (29).

*Methyl*
*(2R,5Z,9Z)-2-Methoxy-24-methyl-5,9-hexacosadienoate*

ECL = 27.43, GC–MS *m/z* (*relative intensity,* %) $M^+$ 450 (2), 418 (5), 391 (10), 359 (10), 221 (1), 193 (3), 171 (5), 165 (11), 139 (39), 134 (13), 112 (6), 111 (31), 107 (18), 104 (100), 81 (45), 79 (43), 69 (20), 67 (29), 57 (30), 55 (30).

### Extraction and Isolation of Phospholipids

The sponge (5.8 g) was extracted with 2 × 200 mL of $CHCl_3$/MeOH (1:1 v/v) yielding 1.56 g of total lipids. The neutral lipids, glycolipids, and phospholipids (0.86 g) were separated by column chromatography on silica gel (60–200 mesh) using the methodology of Privett *et al.* [16]. The phospholipid classes were identified by $R_f$ values using thin-layer chromatography (silica gel H plates) and $CHCl_3$/MeOH/$NH_4OH$ (65:30:5) as the developing solvent. The main phospholipids identified were phosphatidylcholine (PtdCho) and phosphatidylinositol (PtdIns) as determined by comparison of their $R_f$ values with commercial standards.

### Preparation of Fatty Acid Methyl Esters

The fatty acyl components of the phospholipids were obtained as their methyl esters by the reaction of the phospholipid mixture with methanolic HCl followed by column chromatography on silica gel and eluting with *n*-hexane/diethyl ether (9:1).

### UPLC-MS Analysis of Fatty Acids

*A. niger* phospholipid fractions were re-dissolved in 30 μl of acetonitrile/2-propanol/water (1:1.28:1.28 by volume). The LC system consisted of a Waters ACQUITY UPLC

pump with a well-plate autosampler (Waters, Milford, MA) equipped with an ACQUITY UPLC HSS T3 column (1.8 μM, 100 A pore diameter, 2.1 × 150 mm, Waters) and an ACQUITY UPLC HSS T3 Vanguard precolumn (1.8 μM, 100 A pore diameter 2.1 × 5 mm, Waters). The column temperature was 55 °C and the autosampler temperature was 8 °C. The flow rate was 0.3 mL/min. Solvent A consisted of acetonitrile/water (40:60) with 10 μM ammonium acetate and 0.025 % acetic acid. Solvent B was acetonitrile/2-propanol (10:90) containing 10 μM ammonium acetate and 0.02 % acetic acid. Solvent B was initially held at 40 % for 0.1 min and was then increased to 99 % over 10 min using a linear gradient. Solvent B was held at 99 % for 8 min before returning to initial conditions over 0.5 min. The column was equilibrated for 2.5 min between injections.

FA were analyzed using a quadrupole time-of-flight mass spectrometer (Q-TOF, Synapt G2-S, Waters) with electrospray ionization in negative ion mode. The cone voltage was 20 V and the capillary voltage was 1.51 kV. The source and desolvation temperatures were 110 and 350 °C, respectively. The analyzer was operated with extended dynamic range at 10,000 resolution (fwhm at *m/z* 554) with an acquisition time of 0.1 s. $MS^E$ mode was used to collect data with the T-wave element alternated between a low energy of 2 V and high energy states where the transfer T-wave element voltage was from 10 to 25 [17]. The cone gas flow rate was 10 L/h and the desolvation gas flow was 1000 L/h. Leucine enkephalin (400 pg/μl, acetonitrile/water (50:50) by volume) was infused at a rate of 10 μl/min for mass correction. MassLynx V4.1 software (Waters) was used for instrument control, acquisition, and sample analysis.

## Cell lines and Culture Conditions

The *Leishmania infantum* iFRP.70 strain was used in these experiments. This strain expresses the iFRP infrared protein (λexc. 684 nm;λem. 708 nm) that allows the detection of viable cells in an Odyssey infrared system (LI-COR, USA) facility [18]. *L. infantum* IFRP.70 promastigotes were routinely cultured at 26 °C in M199 medium 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), 10 % (v/v) heat-inactivated foetal calf serum (FCS) and antibiotic cocktail (50 U/ml penicillin, 50 μg/ml streptomycin). To assess the effect of **1a**–**1b** on *L. infantum* amastigotes, an *ex vivo* culture of infected splenocytes with *L. infantum* iFRP.70 was obtained from a 5-weeks infection of BALB/c mice [18]. Briefly, susceptible BALB/c mice were intraperitoneally infected with *L. infantum* iFRP.70 metacyclic promastigotes and when the infection was set, animals were slaughtered and the spleen was aseptically recovered, and carefully minced in order to obtain a single cell suspension of splenocytes. This cellular suspension was cultured in RPMI medium, 20 % FCS, 1 mM sodium pyruvate, 1× RPMI vitamins, 10 mM HEPES and 100 U/mL penicillin and 100 μg/mL streptomycin.

Mammalian cell cultures were used to determine the toxic effects of the compound mixture. For this purpose two established human cell lines were used. The human monocytic cell line (THP1) (ATCC TIB-202) derived from an acute monocytic leukemia is easily differentiated to macrophages (target cells of *Leishmania* amastigotes) with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA). THP1 was cultured in RPMI medium supplemented with 10 % heat-inactivated FCS and 1 % streptomycin/penicillin at 37 °C and 5 % $CO_2$. By its part, the human liver carcinoma HepG2 (ATCC HB-8065) cell line was used to analyze the liver damage. HepG2 was cultured in MEM medium supplemented with 10 % FCS, 1 mM sodium pyruvate, 1 g/L glucose, 100 U/mL penicillin and 100 μg/mL streptomycin.

All experimental animal procedures herein described were carried out in strict accordance with the Spanish (Ley 32/2007) and European Union Legislation (2010/63/UE). The protocols used were approved by the Animal Care Committee of the University of León (Spain).

## Leishmanicidal Activity and Cytotoxic Assessment

To test the leishmanicidal effect of **1a**–**1b** on the *L. infantum* iFRP.70 strain we monitored the infrared emission of the viable promastigotes and infected splenocytes in an Odyssey infrared system (LI-COR, USA) facility. Briefly, for free living promastigotes, $10^6$ cells/mL were cultured in the presence of 200-0.1 μM of **1a**–**1b** at 26 °C for 48 h. For amastigote-infected splenocytes, cell suspensions were seeded into black (clear-bottom) 96-well plates to a density of 200.000 counts of infrared fluorescence. The FA were then added in serial concentrations of 200-0.1 μM or DMSO as the control. After 48 h of incubation at 37 °C and 5 % $CO_2$, the viability of the cells was assessed using the infrared emission of viable cells.

To test the toxicity of **1a**–**1b** on THP-1 and HepG2 the Alamar Blue staining method was used according to manufacturer's recommendations (Invitrogen).

## Recombinant *L. infantum* (*L*TopIB) and Human TopIB (*h*TopIB)

Cloning of *L*TopIB and *h*TopIB ORF, expression and purification of the enzymes were carried out as previously described using a TopIB-defective *Saccharomyces cerevisiae* expression platform [19].

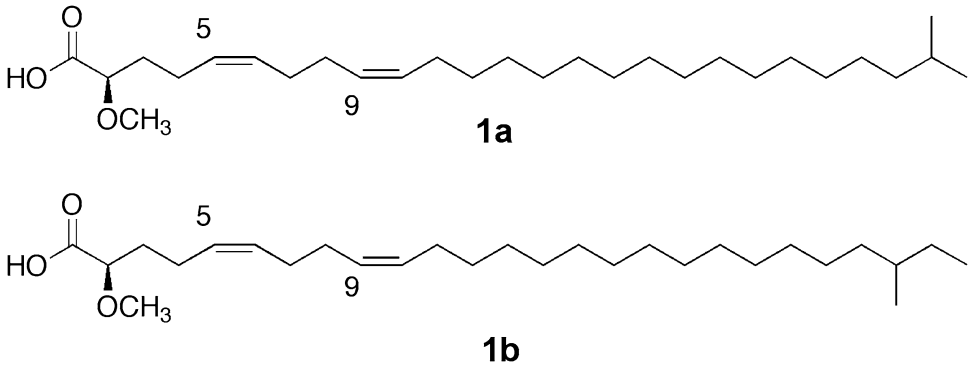


**Fig. 1** Structures of (2*R*,5*Z*,9*Z*)-2-Methoxy-25-methyl-5,9-hexacosadienoic acid (**1a**) and (2*R*,5*Z*,9*Z*)-2-Methoxy-24-methyl-5,9-hexacosadienoic acid (**1b**)

### DNA Relaxation Assays

TopIB activity was assayed by the relaxation of negatively supercoiled plasmid DNA. One unit of recombinant *L*TopIB or *h*TopIB was incubated with the mixture of FA for 15 min at 4 °C. Then, the reaction mixture, containing 0.5 μg of supercoiled pSK DNA, 10 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/ml bovine serum albumin, and 50 mM KCl, in a total volume of 20 μL, was added. Reaction mixtures were incubated for 4 min at 25 °C. Enzyme reactions were stopped by the addition of up to 1 % (wt/vol) SDS (final concentration) and digested with 1 mg/ml proteinase K at 37 °C during one extra hour to remove the protein that remained linked to DNA fragments. The extent of plasmid DNA relaxation was assessed in 1 % agarose gels by electrophoresis in 0.1 M Tris borate EDTA buffer (pH 8.0) at 2 V/cm for 16 h. Gels were visualized with UV illumination after ethidium bromide (0.5 μg/ml) staining. A further electrophoresis was run in the presence of 0.1 μg/ml ethidium bromide in order to separate nicked DNA from relaxed topoisomers [20].

### Determination of Apoptosis

FA-induced apoptosis was detected in THP-1 cells measuring the translocation of phosphatidylserine (PtdSer) to the cell surface with the Annexin V-FITC reagent (BD Pharmigen). Fraction of Annexin V-positive cells was measured with CellQuest software (BD Biosciences, San Jose, CA).

## Results

### Isolation and Characterization

*Asteropus niger*, a Caribbean sponge collected from the West Coast of Puerto Rico, was analyzed in this work [15]. Extraction of the sponge yielded a 1:2 mixture of the unprecedented (2*R*,5*Z*,9*Z*)-2-methoxy-25-methyl-5,9-hexacosadienoic acid (**1a**) and (2*R*,5*Z*,9*Z*)-2-methoxy-24-methyl-5,9-hexacosadienoic acid (**1b**) in 80 % purity (Fig. 1), which were purified as methyl esters to facilitate their isolation and their initial characterization, and subsequently saponified to yield the free FA (see "Materials and Methods").

The characterization of **1a**–**1b** was initially accomplished using gas chromatography-mass spectrometry (GC–MS) of the methyl esters. The methyl esters of **1a** and **1b** presented in their mass spectra a series of peaks that allowed their full characterization based on previous work from our laboratory on other similar structures [3]. All of the methyl esters presented a base peak at *m/z* 104 (100 %) resulting from a McLafferty rearrangement, a strong $M^+$-32 peak due to the loss of methanol, and a prominent $M^+$-59 fragment due to α-cleavage to the carbonyl ($-CO_2CH_3$). The presence of the Δ5,9 diunsaturation was revealed by the fragment at *m/z* 171 (due to allylic cleavage between carbons 7 and 8) which readily losses methanol to yield a more abundant and characteristic fragment at *m/z* 139 [3].

The $^1$H NMR of the mixture of acids **1a** and **1b** confirmed the presence of the 2-methoxy functionalities in the FA (Table 1). The α-hydrogen, which integrated for one hydrogen, resonated as a doublet of doublets at δ 3.80 and the methoxy group as a singlet of 3 hydrogens at δ 3.44. The presence of the *iso* and *anteiso* ramifications in **1a** and **1b** was confirmed by the differences in the resonance signals of the hydrogens and carbons between C-24 and C-27 (Table 1). For example, in **1a** the signals in the $^1$H-NMR spectrum at δ 1.51 (1H, *m*), 1.83 (2H, *m*) and 0.86 (6H, *d*), as well as the signals in the $^{13}$C-NMR spectrum at δ 39.1 (*t*, C-24), 27.9 (*d*, C-25), and 22.7 (*q*, C-26, C-27) confirmed the *iso* ramification in the fatty acid [4]. For the acid **1b** the *anteiso* ramification was confirmed by the signals in the $^1$H-NMR spectrum at δ 1.14 (1H, *m*), 1.24 (2H, *m*), 0.83 (3H, *d*), and 0.85 (3H, *t*) as well as the signals in the $^{13}$C-NMR spectrum at δ 34.4 (*d*, C-24), 36.7 (*t*, C-25), 11.4 (*q*, C-26), and 19.2 (*q*, C-27).

**Table 1** Nuclear magnetic resonance characterization for **1a** and **1b**

| Position | 1a (*iso*) | | 1b (*anteiso*) | |
|---|---|---|---|---|
| | $^{1}$H NMR $\delta_H$, mult. (*J* Hz) | $^{13}$C NMR (ppm) | $^{1}$H NMR $\delta_H$, mult. (*J* Hz) | $^{13}$C NMR (ppm) |
| 1 | | 175.2 *s* | | 175.2 *s* |
| 2 | 3.80 *dd* (8.8, 8.8) | 79.5 *d* | 3.80 *dd* (8.8, 8.8) | 79.5 *d* |
| 2′ (–$OCH_3$) | 3.44 *s* | 58.4 *q* | 3.44 *s* | 58.4 *q* |
| 3 | 1.84 *m* | 24.7 *t* | 1.84 *m* | 24.7 *t* |
| 4 | 2.20–2.14 *m* | 27.1 *t* | 2.20–2.14 *m* | 27.1 *t* |
| 5 | 5.30–5.40 *m* | 128.1 *d* | 5.30–5.40 *m* | 128.1 *d* |
| 6 | 5.30–5.40 *m* | 130.6 *d* | 5.30–5.40 *m* | 130.6 *d* |
| 7 | 2.09 *m* | 27.2 *t* | 2.09 *m* | 27.2 *t* |
| 8 | 2.09 *m* | 27.2 *t* | 2.09 *m* | 27.2 *t* |
| 9 | 5.30–5.40 *m* | 128.9 *d* | 5.30–5.40 *m* | 128.9 *d* |
| 10 | 5.30–5.40 *m* | 130.9 *d* | 5.30–5.40 *m* | 128.9 *d* |
| 11 | 2.02 *br q* | 27.3 *t* | 2.02 *br q* | 27.3 *t* |
| 12–23 | 1.17–1.60 *m* | 29.3–30.1 *t* | 1.17–1.60 *m* | 29.3-30.1 *t* |
| 24 | 1.83 *m* | 39.1 *t* | 1.14 *m* | 34.4 *d* |
| 25 | 1.51 *m* | 27.9 *d* | 1.24 *m* | 36.7 *t* |
| 26 | 0.86 *d* (6.7) | 22.7 *q* | 0.85 *t* (7.2) | 11.4 *q* |
| 27 | 0.86 *d* (6.7) | 22.7 *q* | 0.83 *d* (6.3) | 19.2 *q* |

The $^{1}$ H-NMR (500 MHz) and $^{13}$C-NMR (125 MHz) spectra were obtained in $CDCl_3$ as the reference solvent

The double bond geometries in **1a**–**1b** were elucidated by means of $^{13}$C-NMR spectroscopy. It is well established that carbons adjacent to *trans* double bonds tend to resonate between 29 and 38 ppm, whereas those adjacent to *cis* double bonds are normally encountered between 26 and 28 ppm [21]. In our case, the C-4 and C-7 carbons resonated at 27.1–27.2 ppm, while the C-8 and C-11 carbons resonated at 27.2–27.3 ppm. This confirmed that both double bonds in **1a**–**1b** have the *cis* configuration [21].

Circular dichroism (CD) of the **1a**–**1b** mixture (in ethanol) was used to confirm the R absolute configuration at C-2. The CD spectrum displayed a negative Cotton effect at $\lambda_{max} = 221$ nm ($\Delta\varepsilon = -13.3$). This result is consistent with the absolute configuration reported by Djerassi *et al.* on the first α-methoxylated FA isolated from a sponge [22].

Once the structures of the new acids **1a**–**1b** were secured, it became of interest to find out if these free FA were also present in the phospholipids of the sponge. In fact, there is no report of the actual structure of a phospholipid from sponges containing α-methoxylated FA, only an indirect proof that they might be there. Therefore, we undertook a typical Bligh and Dyer [23] extraction of the phospholipids from *A. niger*. The phospholipid composition consisted of phosphatidylcholine (PtdCho) and phosphatidylinositol (PtdIns) as determined by thin-layer chromatography of the isolated methanol fraction. The FA of the phospholipids (Table 2) were obtained as methyl esters by transesterification of the whole phospholipid mixture as previously described [3]. As can be seen from Table 2 the phospholipids contained a series of methyl-branched *iso*/*anteiso* FA with the $C_{15}$–$C_{17}$ *iso*/*anteiso* series predominating (28 mol%). Their presence is quite consistent with what is needed for the biosynthesis of very long-chain *iso*/*anteiso* Δ5,9 FA, inasmuch as the latter are supposed to be biosynthesized from these bacterial precursors [8]. Although in lesser amounts, a complete series of $C_{17}$–$C_{19}$ and $C_{26}$–$C_{29}$ 2-methoxylated Δ5,9 fatty acids were also detected in the mixture with 2-OMe-5,9-18:2 predominating in the $C_{17}$–$C_{19}$ series (80 % relative abundance among the short-chain series) and **1a**–**1b** predominating in the $C_{26}$–$C_{29}$ series (62 % relative abundance among the long-chain series) (Table 3).

Aiming at characterizing the phospholipids containing the novel **1a**–**1b**, the phospholipid fraction was submitted to a sort of lipidomics type of analysis using ultra high performance liquid chromatography coupled to mass spectrometry (UPLC-MS). Samples were analyzed using a Quadrupole time-of-flight mass spectrometer with electrospray ionization (see "Materials and Methods"). While we were able to confirm the presence of the free 2-methoxylated FA described in Table 3, no phospholipids containing these 2-methoxylated FA were detected. Presumably, a trace amount of the free 2-methoxylated FA co-eluted with the phospholipid fraction in the isolation procedure. However, we were able to identify two peaks in the UPLC-MS analysis that seem to correspond to glycosylceramides.

**Table 2** Principal non-methoxylated FA from the phospholipids of *A. niger*

| Fatty acid | Relative abundance mol (%) |
|---|---|
| *i*-14:0 | 0.5 |
| *n*-14:0 | 2.7 |
| 4-Me-14:0 | 0.8 |
| *i*-15:0 | 9.6 |
| *ai*-15:0 | 4.6 |
| *n*-15:0 | 0.2 |
| 3-Me-15:0 | 0.2 |
| *i*-16:0 | 3.8 |
| *n*-16:0 | 11.0 |
| 5,9-16:2 | 0.2 |
| 11-16:1 | 1.4 |
| 16:1 | 0.8 |
| *i*-9-17:1 | 4.8 |
| *br*-17:0 | 6.0 |
| *i*-5,9-17:2 | 1.7 |
| *i*-17:0 | 5.9 |
| *ai*-17:0 | 7.8 |
| 5,9-17:2 | 0.2 |
| 17:1 | 2.0 |
| 17:0 | 2.3 |
| *br*-18:0 | 1.3 |
| 2-OH-16:0 | 0.6 |
| *br*-18:0 | 0.4 |
| 5,9-18:2 | 1.1 |
| *i*-18:0 | 0.6 |
| 9-18:1 | 0.5 |
| 11-18:1 | 0.7 |
| *n*-18:0 | 7.8 |
| *i*-11-19:1 | 0.6 |
| 11/12-Me-18:0 | 9.7 |
| 19:0 | 1.3 |
| *br*-21:0 | 0.4 |
| n-22:0 | 0.2 |
| *br*-23:0 | 0.2 |
| *br*-25:0 | 0.3 |
| n-25:0 | 0.2 |
| *br*-27:0 | 0.2 |

*br* methyl branched

One of them was related to an ion at *m/z* 880.7256 corresponding to the molecular formula $C_{52}H_{98}NO_9$ (Calc. *m/z* 880.7242), which can be correlated to a glycosylceramide bearing either **1a** or **1b** as the fatty amide residue together with a $C_{16}$ sphingosine. In the case of the second peak, an ion at *m/z* 866.7054 corresponding to the molecular formula $C_{51}H_{96}NO_9$ (Calc. *m/z* 866.7085) can very well fit the structure of another glycosylceramide bearing in this case a $C_{26}$ 2-methoxylated Δ5,9 FA as the fatty amide residue and the same $C_{16}$ sphingosine. In addition, an assigned MS/MS spectrum indicated a neutral loss of hexose from both $C_{52}H_{98}NO_9$ and $C_{51}H_{96}NO_9$ lipids with the 700.6615 (Calc. *m/z* 700.6686) and 686.6437 (Calc. *m/z* 686.6451) MS/MS product ions, respectively, further confirming the glycosylceramide nature of the identified lipids. Taken together, these results tend to indicate that **1a**–**1b** are either present as free FA in the sponge or bound to other type of lipids, such as glycosylceramides. However, we still cannot totally discard the possibility that phospholipids containing these novel methoxylated FA might have been hydrolyzed by some phospholipases during the storage of the sponge or in the isolation procedures, thus rendering them undetectable.

### Topoisomerase IB Inhibitory Studies

The inhibitory activities of **1a**–**1b** towards either *L*TopIB or *h*TopIB were determined as previously described [13]. One unit of both enzymes was assayed in a plasmid DNA relaxation assay for 30 min at 37 °C in the presence of 0.15–10 mg/mL of **1a**–**1b**. Reaction products were resolved in agarose gel and subsequently visualized by ethidium bromide staining (Fig. 2). Surprisingly, **1a**–**1b** was more efficient towards *h*TopIB ($EC_{50}$ = 1.83 ± 0.06 μg/mL) than towards *L*TopIB ($EC_{50}$ = 5.10 ± 0.34 μg/mL), but in both cases the mixture of FA was quite effective in inhibiting the TopIB enzymes. The mechanism of inhibition of these compounds was also different from the mechanism of inhibition displayed by camptothecin (CPT), as **1a**–**1b** is not capable of stabilizing the cleavable complex (Fig. 3).

### Cytotoxicity Towards THP-1 and HepG2 Cells

Since **1a**–**1b** displayed considerable *h*TopIB inhibition the next logical step was to study the toxicity of the mixture towards human cancer cell lines. For this study we chose the THP-1 human acute leukemia cell line and the HepG2 human hepatocellular carcinoma cell line on the basis of availability. As expected **1a**–**1b** did display moderate toxicity towards THP-1 ($EC_{50}$ = 41.08 ± 2.58 μg/mL) and HepG2 ($EC_{50}$ = 34.59 ± 5.26 μg/mL) (Fig. 4). It was also determined that apoptosis was not the preferred mode of toxicity arising from the exposure of THP-1 to **1a**–**1b** as revealed by following PtdSer externalization using the typical Annexin V-FITC assay (Fig. 5).

### Cytotoxicity Towards *L. infantum* Amastigotes and Promastigotes

Since **1a**–**1b** displayed considerable *L*TopIB inhibition we studied their toxicity towards *ex vivo* mouse splenocytes infected with *L. infantum* amastigotes and promastigotes

**Table 3** Diunsaturated α-methoxylated Δ5,9 FA identified in *A. niger* by UPLC-MS (negative ion mode) and as methyl esters by GC–MS (70 eV)

| [M-H]$^-$ | Experimental | Theoretical | Fatty acid | Key MS fragmentations of the methyl esters *m/z* (rel. abundance) |
|---|---|---|---|---|
| $C_{18}H_{31}O_3$ | 295.2275 | 295.2279 | 2-OMe-17:2[a] | $M^+$ 310(1), 278(4), 251(5), 219(4), 139(26), 104(100) |
| $C_{19}H_{33}O_3$ | 309.2436 | 309.2435 | 2-OMe-18:2[b] | $M^+$ 324(2), 292(6), 265(11), 233(11), 139 (36), 104(100) |
| $C_{20}H_{35}O_3$ | 323.2582 | 323.2592 | 2-OMe-19:2[a,d] | $M^+$ 338(1), 306(3), 279(6), 247(7), 139 (33), 104(100) |
| $C_{27}H_{49}O_3$ | 421.3691 | 421.3687 | 2-OMe-26:2[c,d] | $M^+$ 436(1), 404(3), 377(7), 345(6), 139 (36), 104(100) |
| $C_{28}H_{51}O_3$ | 435.3844 | 435.3844 | 2-OMe-27:2[a,d] | $M^+$ 450(2), 418(5), 391(10), 359(10), 139 (36), 104(100) |
| $C_{29}H_{53}O_3$ | 449.4000 | 449.4000 | 2-OMe-28:2[a,d] | $M^+$ 464(1), 432(3), 405(6), 373(6), 139 (37), 104(100) |
| $C_{30}H_{55}O_3$ | 463.4155 | 463.4157 | 2-OMe-29:2[a,d] | $M^+$ 478(1), 446(1), 419(3), 387(2), 139(31), 104(100) |

[a] As determined by their GC retention times these compounds were a mixture of *iso*/*anteiso* FA

[b] Only the normal chain FA was detected in the mixture

[c] Only the *anteiso* FA was detected in the mixture

[d] These FA are unprecedented in nature

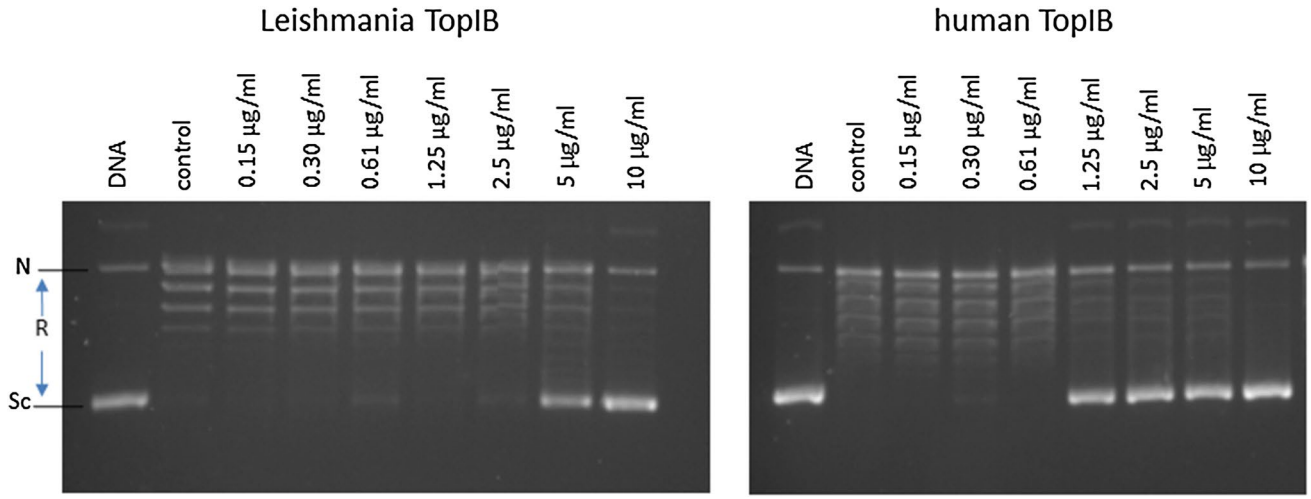


**Fig. 2** Comparison of the inhibition of the relaxation activity of *L*TopIB (*left*) and *h*TopIB (*right*) by **1a**–**1b**. One unit of both enzymes was assayed in a plasmid DNA relaxation assay for 30 min at 37 °C in the presence of 0.15–10 mg/mL of **1a**–**1b**. Reaction products were resolved in agarose gel and subsequently visualized by ethidium bromide staining. The relative position of the negatively supercoiled DNA substrate is indicated by Sc, R is the relaxed DNA, whereas the ladder of relaxed DNA topoisomer bands is shown in between. Reactions were stopped with 1 % SDS. Lane (DNA) contains 0.5 µg of pSK plasmid DNA and lane (control) is the reaction mixture plus 10 % DMSO

(Fig. 6). It was found that **1a**–**1b** was more toxic towards *L. infantum* amastigotes ($IC_{50} = 0.17 \pm 0.01$ mg/mL) than *L. infantum* promastigotes ($IC_{50} = 0.34 \pm 0.01$ mg/mL). However, in either case they were not as effective as the toxicity displayed towards the human THP-1 and HepG2 cell lines.

## Discussion

*Asteropus niger* presented a rather unusual FA composition as judged by the considerable number of methyl-branched FA and the free occurring 2-methoxylated FA, where the key compounds were **1a** and **1b**. It is quite plausible that the great concentration of *iso*/*anteiso* $C_{15}$-$C_{17}$ FA, typical of bacteria, are needed to biosynthesize our new compounds **1a**–**1b** as well as the other *iso*/*anteiso* 2-methoxylated Δ5,9 FA present in trace amounts in the sponge [1, 8]. As to the origin of **1a**–**1b** we can speculate that the biosynthetic pathways *i*-15:0 → *i*-27:0 → *i*-5,9-27:2 → 2-OH-*i*-5,9-27:2 → 2-OMe-*i*-5,9-27:2 and *ai*-15:0 → *ai*-27:0 → *ai*-5,9-27:2 → 2-OH-*ai*-5,9-27:2 → 2-OMe-*ai*-5,9-27:2 might be operative in *A. niger*, which can explain the need for the accumulation of the methyl-branched short precursors. Despite the fact that the methyl branched *i*-5,9-27:2 and *ai*-5,9-27:2 FA have been isolated from several sponges [6, 7],

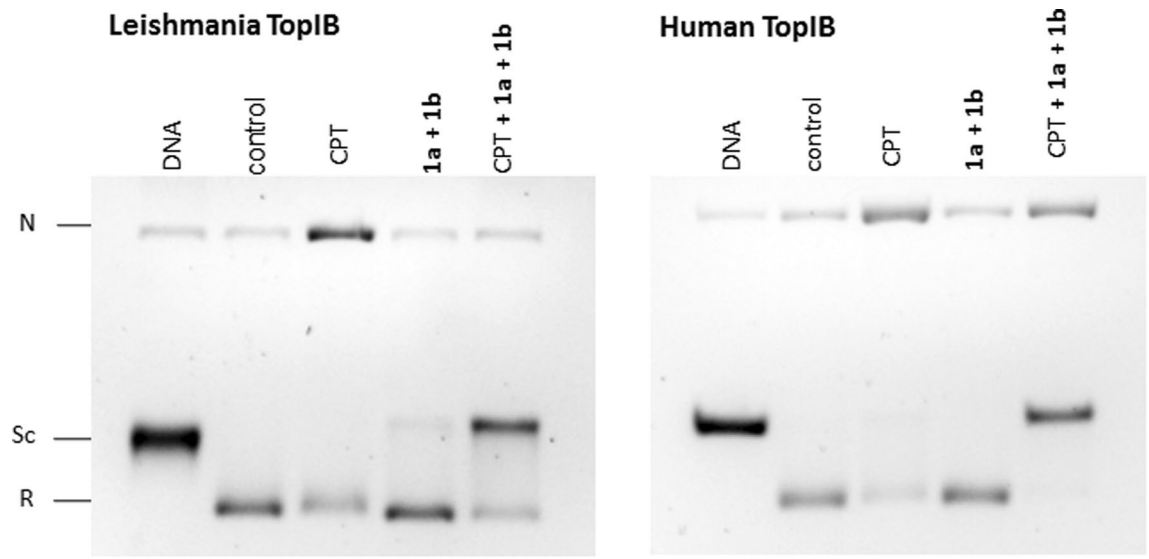


**Fig. 3** Inhibition of *L*TopIB (*left*) and *h*TopIB (*right*) by **1a**–**1b** in a CPT-independent mechanism. Both enzymes were assayed in the presence of DMSO (control), 100 μM CPT, 15 μg/mL and a mixture of **1a**–**1b** at the indicated concentrations. Samples were incubated for 4 min at 25 °C, stopped with 1 % SDS and digested for one extra hour at 37 °C in the presence of 1 mg/mL proteinase K. DNA was extracted with one volume of phenol–chloroform and samples were run on a 1 % agarose gel containing ethidium bromide to a final concentration of 40 μg/mL in order to separate supercoiled (Sc) relaxed DNA. The nicked band (N) corresponds to the stabilized cleavage complexes. The results are representative of three independent trials

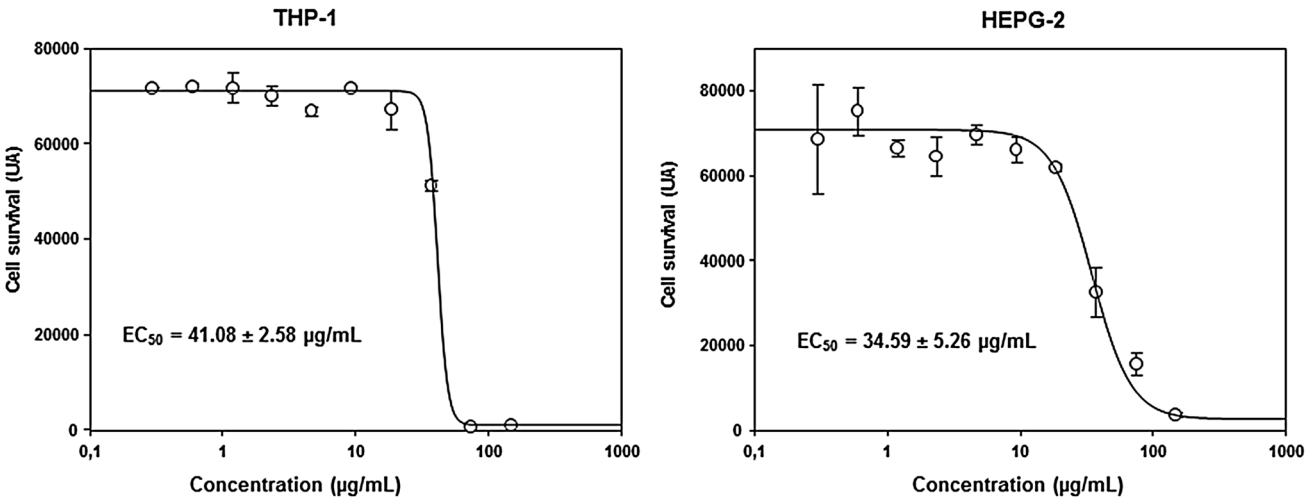


**Fig. 4** Cytotoxicity of **1a** and **1b** towards THP-1 and HepG2 Cells

this is the first time that their 2-methoxylated analogs have been identified in a sponge.

Many 2-methoxylated FA have been isolated from sponges [24] and some of them have been ascribed to phospholipids present in either the sponge or in bacterial symbionts within the sponge [24]. However, no phospholipids containing 2-methoxylated FA have been thoroughly identified in a sponge. Our attempt to identify such a phospholipid in *A. niger* failed. Instead, we isolated the free **1a**–**1b** and what seems to be glycosylceramides containing these FA. This implies that these 2-methoxylated Δ5,9 FA might not be present in conventional phospholipids (PtdCho or PtdIns) but rather as free FA or as part of a glycosylceramide. This is not surprising as glycosylceramides containing very-long chain 2-hydroxy fatty amides have been isolated from several sponges [25, 26]. However, the possibility still exists for phospholipids containing these novel FA to have hydrolyzed (by specific lipases) during

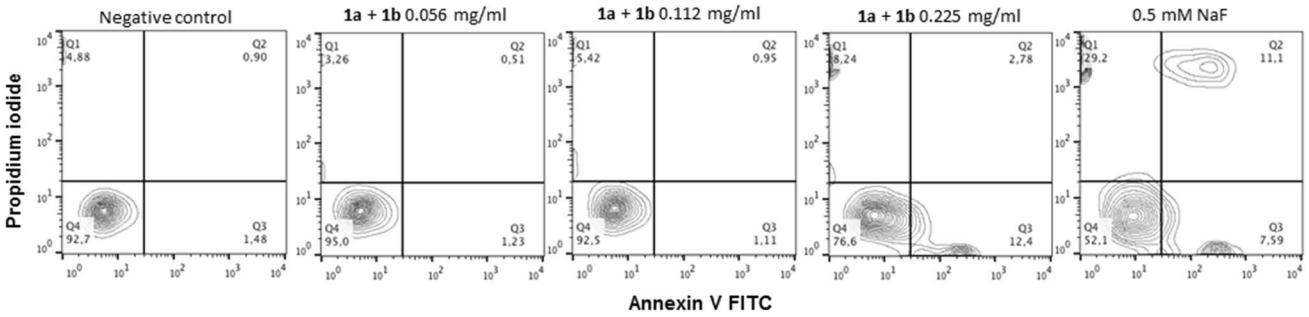


**Fig. 5** Flow cytometry analysis of PtdSer externalization in undifferentiated human monocytes THP-1 exposed to **1a**–**1b**. THP-1 cells were incubated at 37 °C in the absence (*left panel*) and in the presence of growing concentrations of **1a**–**1b** (*three middle panels*) for 15 h or NaF (*right panel*) as a positive control. Q1–Q4 represent necrosis, late apoptosis, early apoptosis and living cells. Dead cells were excluded by propidium iodide incorporation. Surface plots are representative of three independent assays

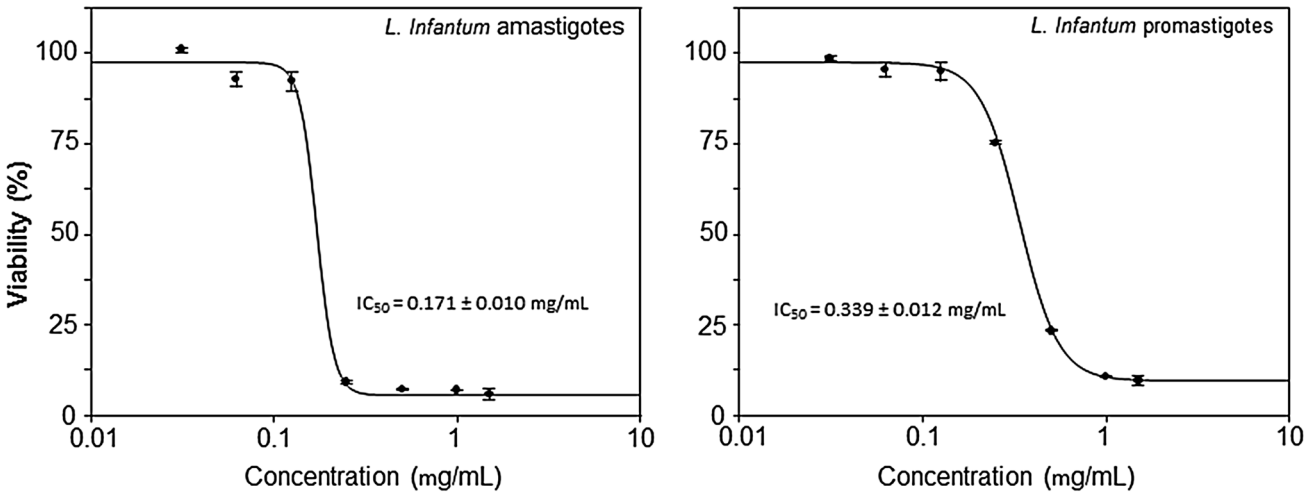


**Fig. 6** $IC_{50}$ calculation after a 72 h period of exposure of **1a**–**1b** to *L. infantum*-iRFP.70 amastigote-infecting mouse splenocytes and *L. infantum*-iRFP.70 promastigotes. Drugs were added in a twofold dilution series, being the highest concentration 1.5 mg/mL. $IC_{50}$ values were calculated from dose–response curves performed in triplicate and repeated twice after nonlinear fitting with the Sigma Plot program. The complete experimental methodology regarding the use, in these assays, of infrared fluorescent *L. infantum* strains (iRFP) was reported in [18]

the storage or isolation procedures, thus rendering them undetectable.

*Asteropus niger* provided enough material of **1a**–**1b** to be able to study their biological properties. Separation of **1a** from **1b** proved to be quite difficult, so we decided to test them together as they were going to provide similar information. Synthesis of either **1a** or **1b** can be a challenging and an expensive task. For example, installing the *iso* and *anteiso* functionalities in a very long chain as well as achieving 100 % enantiopure compounds at C-2 can be quite demanding. Therefore, we were fortunate to have sufficient material of **1a**–**1b** at hand to perform these preliminary bioassays.

As expected from the previous literature, where very long-chain Δ5,9 FA were reported to be good inhibitors of *h*TopI [10–12], acids **1a**–**1b** also proved to be very good inhibitors of *h*TopIB ($EC_{50}$ ~ 4.6 μM). Since we did not have the corresponding $C_{27}$ non-methoxylated Δ5,9 FA at hand, it was not possible to test how the C-2 methoxy group affected the binding to *h*TopIB. However, out of the 2-methoxylated FA that we have tested so far [13], acids **1a**–**1b** are certainly the best inhibitors of *h*TopIB. This result implies that the very long chains are indeed needed for an effective *h*TopIB inhibition. We were also able to show that the mechanism of *h*TopIB inhibition by **1a**–**1b** is quite different from the established mechanism of stabilizing the ternary complex (drug-DNA-enzyme) displayed by CPT (Fig. 3). This means that **1a**–**1b** does not bind directly to the DNA-topoisomerase I binary complex but rather the acids seem to be interacting directly with the enzyme. We

can also hypothesize that **1a–1b** is binding (although non-specifically) in close proximity to the TopIB active site thus preventing the catalytic tyrosine to execute the nucleophilic attack on the DNA phosphate [27].

Since the Δ5,9 FA have only been tested against *h*TopI, with one exception [28], we decided to test them against another important topoisomerase IB, namely *L*TopIB, an important therapeutic target against *L. infantum*, the causative agent of visceral leishmaniasis in the Mediterranean Basin [14]. Previous work from our laboratory has shown that short chain FA are better inhibitors of *L*TopIB than of *h*TopIB [28]. This is not surprising since both enzymes bear significant differences [14]. For example, the synthetic fatty acid (±)-2-OMe-5,9-20:2, which is also a natural FA to be found in the sponge *E. goffrilleri* [3], preferentially inhibits *L*TopIB ($EC_{50}$ = 31 μM) over *h*TopIB ($EC_{50}$ > 100 μM) [28]. However, in the case of **1a–1b** this trend was reversed. For example, despite the fact that **1a–1b** is quite inhibitory towards *L*TopIB ($EC_{50}$ ~ 11.5 μM), there was a 2.5 fold preference towards *h*TopIB ($EC_{50}$ ~ 4.6 μM) (Fig. 2). Nevertheless, the displayed inhibition of **1a–1b** towards *L*TopIB is also the best we have observed so far for any tested 2-methoxylated FA towards a Leishmania topoisomerase IB [13, 28]. Interesting is the fact that by changing the alkyl chain length of a 2-OMe-Δ5,9 FA it is possible to fine-tune the preference of these acids towards one TopIB enzyme over the other.

Since **1a–1b** displayed *h*TopIB and *L*TopIB inhibition, we were able to translate this inhibition into cytotoxicity towards THP-1 and HepG2 cancer cell lines and towards *L. infantum*-iRFP promastigotes and amastigotes (Fig. 6). Despite the fact that other mechanisms of toxicity are possible for **1a–1b**, the observed toxicities followed the same trend as the one observed for the TopIB enzymes, i.e., a 4/8-fold preference for the human cancer cell lines over the Leishmania parasites. Moreover, we were able to show that **1a–1b** did not induce apoptosis in the THP-1 cells (Fig. 5), thus bespeaking of other types of cytotoxicity mechanisms, such as TopIB inhibition or the ability to destabilize the integrity and function of the THP-1 cell membranes [29]. It is known that due to the amphipathic character of FA they can incorporate into lipid membranes creating instability of the lipid bilayer and rendering the lipid membrane more permeable [29]. The (±)-2-OMe-5,9-20:2 acid was not tested in previous studies against cancer cell lines, but it was found that the latter was 2/3-fold more efficient towards *L. infantum* promastigotes [28]. Therefore, until more analogs are tested, we can only speculate at this time that the very-long chain 2-OMe-Δ5,9 FA (e.g., $C_{27:2}$) are more efficient anticancer compounds than antileishmanial agents. This trend seems to be reversed as the chain length is reduced, inasmuch as the shorter-chain 2-OMe-Δ5,9 FA (e.g., $C_{20:2}$) are better antileishmanial agents [28].

Further work will certainly require the enantiomeric synthesis of pure **1a** and **1b**, since in the present work only a mixture of **1a** and **1b** was tested, with a double concentration of the *anteiso* acid **1b** over the *iso* acid **1a**. For example, it will be of interest to find out if there is differential binding towards TopIB enzymes between an *anteiso*-2-OMe-Δ5,9 FA and an *iso*-2-OMe-Δ5,9 FA. Further work in this direction continues in our laboratories.

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