Synthesis of the novel (±)-2-methoxy-6-icosynoic acid – a fatty acid that induces death of neuroblastoma cells

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Abstract

The first total synthesis for the novel fatty acid (±)-2-methoxy-6-icosynoic acid was accomplished in seven steps and in a 14% overall yield starting from 2-(4-bromobutoxy)- tetrahydro-2H-pyran. The title compound displayed an $EC_{50} = 23 \pm 1$ μM against the human SHSY5Y neuroblastoma cell line and an $EC_{50} = 26 \pm 1$ μM against the human adenocarcinoma cervix cell line (HeLa) after 48 h of exposure. The corresponding non-methoxylated analog 6- icosynoic acid did not display cytotoxicity ($EC_{50} > 500$ μM) towards the studied cell lines as well as the 2-methoxyicosanoic acid ($EC_{50} > 300$ μM). The critical micelle concentration (CMC = 20–30 μM) for the (±)-2- methoxy-6-icosynoic acid was also determined. It was found that α- methoxylation decreases the CMC of a fatty acid.

Keywords

Cancer; Neuroblastoma; Methoxylated Fatty Acids; Micelles; Synthesis

1. Introduction

Neuronal cells are the main functional and structural unit of the nervous system. Defects on the normal neural-crest cell development program in the embryo promote disorders such as neuroblastoma. Neuroblastoma is a neoplasm of the sympathetic nervous system which is the second most common extracranial malignant tumor of childhood and the most common solid tumor of infancy as well as the source of 15% of all cancers related to death of children (Park etal., 2010). This mortal disorder is one of the outcomes of the loss of homeostasis between differentiation, proliferation, and cell death in the nervous system. Accordingly, this deregulation results in a characteristic heterogeneous population of cells ranging from undifferentiated neuroblasts to fully differentiated neurons. Moreover, the continuous failure to differentiate and to stop growing without responding to cell death programs promotes the metastatic disease. Despite the fact that a growing body of evidence has demonstrated

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prominent advances against neuroblastoma, resistance remains the hallmark of failure of chemotherapy reporting 25–30% of survival for high-risk patients (Park et al., 2010; Kushner, 2004). Given the significance of the nervous system and the lack of successful treatments, it is imperative the constant search and assessment of novel chemotherapeutic agents.

Fatty acids are a group of lipids that have been reported to affect a diversity of physiological processes as well as pathological conditions. Previous studies have identified fatty acids as drug candidates against viruses, bacteria, fungi, and cancerous cells (Parang et al., 1997; Carballeira et al., 2005; Carballeira, 2008). In particular, fatty acids have been identified as possible chemotherapeutic agents for inducing death responses against cancerous cells (Hardy et al., 2000; Schwarz et al., 2006; Lin et al., 2012). Thus, fatty acids play a key role regulating the characteristic loss of homeostasis in cancer. For example, palmitic acid has been documented to induce cell death in breast cancer cells (Hardy et al., 2000). In aqueous solutions, at physiological environments, the amphiphilic character of fatty acids allows for versatile structural arrangements and integration into cell membranes. Studies have shown that fatty acids may be integrated into cell membranes by extrinsic supplementation to *in vitro* cell models, and subsequently alter cell responses and functions such as membrane fluidity (Reynolds et al., 2001; Yang et al., 2011). Recently, the self-association of amphiphilic compounds as polymeric micelles has been explored for cancer therapy. Structurally, the hydrophilic segment forms a coronal layer while the hydrophobic portion forms a functional reservoir for lipophilic anticancer drugs (Blanco et al., 2009). Given the amphiphilic nature of fatty acids, they may be interesting dietary supplements to promote differential and specific responses such as cell death.

Previous studies from our laboratory have shown that α-methoxylated fatty acids displayed enhanced anticancer properties when compared to their corresponding non-methoxylated fatty acids by promoting cell death in human chronic myelogenous leukemia (K- 562), histiocytic lymphoma (U-937), and acute promielocytic leukemia (HL-60) (Carballeira et al., 2003). By reasons that are still unknown, the α-methoxylated fatty acids display a better anticancer profile than their corresponding non-methoxylated analogs. However, the activities displayed so far by the studied α-methoxylated fatty acids are in the range of EC_{50}'s between 230 and 470 μM (Carballeira et al., 2003). Therefore, it became of interest to us to synthesize a more efficient anticancer fatty acid by placing together functional groups from other fatty acids that have been reported to display interesting biological activities. Based on previous findings whereby many marine naturally occurring α-methoxylated fatty acids do have a double bond unsaturation at carbon 6 of the acyl chain (Carballeira, 2002), and motivated by the fact that the 6-icosynoic acid displays strong antiprotozoal activity towards *Leishmania donovani* (EC_{50} = 1.1 μg/mL) (Carballeira et al., 2009), we envisaged the possibility of synthesizing a fatty acid that could contain, in the same molecule, the α-methoxy functional group, a triple bond at C-6 and a C_{20} alkyl chain. Therefore, the synthesis of the novel (±)-2-methoxy-6-icosynoic acid (**1**) was envisaged as a possible fatty acid that could display a better anticancer activity than the α- methoxylated fatty acids studied so far.

In the present study we synthesized **1** and indeed demonstrate that the α-methoxy functionality in **1** enhances the fatty acid-induced cell death responses of our *in vitro* cell model system of neuronal cancer. The potential of **1** to form micelles was also studied, suggesting that the α-methoxy functionality enhances micelle formation in this type of fatty acids. These results could be important for future research work, providing insights into strategies whereby the α- methoxylated fatty acids could be used in the fight against cancer.

2. Materials and methods

2.1. Instrumentation

IR spectra were recorded on a Bruker FT-IR spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DRX-500 spectrometer. ^{1}H-NMR chemical shifts are reported with respect to internal Me_4Si and ^{13}C-NMR chemical shifts are reported in parts per million (ppm) relative to $CDCl_3$ (77.0 ppm). Mass spectra data was acquired using a GC-MS (Agilent 5975)C MS ChemStation; Agilent, Palo Alto, CA, USA) at 70 eV equipped with a 30 m×0.25 mm special performance capillary column (HP-5MS) of polymethysiloxane cross-linked with 5 % phenyl methylpolysiloxane. UV-Vis data were determined on a Shimadzu UV-2555 spectrophotometer.

2.2. 2-(Nonadec-5-ynyloxy)-tetrahydro-2H-pyran (3)

Into a 100-mL round-bottomed flask at 0°C containing dry THF (8.0 mL) was added 1-pentadecyne (1.9 mL, 7.2 mmol) followed by the dropwise addition of 2.5M *n*-BuLi (14.4 mmol). The mixture was stirred at 0°C for 80 min. The temperature was then lowered to −60°C and HMPA (16.0 mL) was added followed by the addition of 2-(4-bromobutoxy)-tetrahydro-2H-pyran (1.71 g, 7.2 mmol). The mixture was stirred for 24h and then washed with brine (2×25mL), extracted with hexane (2×25mL) and dried over Na_2SO_4 affording 1.47 g (64 % yield) of **3** after purification by Kugelrohr distillation at 170°C/3 mm Hg; IR (neat) ν_{max}: 2924,2854, 1456, 1380, 1350, 1119, 1076,1034 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 4.56 (1H, t), 3.87-3.71 (2H, m), 3.50-3.37 (2H, m), 2.15- 2.10 (4H, m), 1.80-1.42 (6H, m), 1.24 (22H, brs, $-CH_2-$), 0.86 ppm (3H, t, $-CH_3$); ^{13}C-NMR ($CDCl_3$, 125MHz) δ 98.96 (C-20), 80.72 (C-6), 79.99 (C-5), 67.27 (C-1), 62.45 (C- 24), 32.13 (C-17), 30.94 (C-21), 29.90 (C-15), 29.88 (C-14), 29.85 (C-13), 29.77 (C-12), 29.57 (C- 11), 29.39 (C-10), 29.13 (C-2), 29.10 (C-9, C-10), 26.14 (C-23), 25.71 (C-3), 22.89 (C-18), 19.82 (C- 22), 18.96 (C-4), 18.82 (C-7), 14.31 (C-19); GC-MS (70eV) *m/z* (relative intensity) 364 (*M*$^+$, 2), 296 (16), 181 (11), 137 (3), 123 (3), 111 (7), 110 (7), 109 (6), 100 (7), 97 (8), 95 (18), 93 (10), 85 (100), 81 (14), 80 (12), 79 (17), 67 (19), 57 (13), 56 (16), 55 (20). HRMS Calcd for $C_{24}H_{44}O_2$ [M+H]$^+$ 365.3414, found 365.3411.

2.3. 5-Nonadecynol (4)

To a mixture of methanol (25 mL) and **3** (2.08 g, 5.71 mmol) was added catalytic amounts of *p*-toluenesulfonic acid (*p*-TSA) and the reaction mixture was stirred at 60°C for 24h. After this time the solvent was rotoevaporated and the organic extract was washed with a saturated solution of sodium bicarbonate (3×25mL), dried over Na_2SO_4, filtered and evaporated *in vacuo*, affording 1.57 g (98% yield) of **5** as a pale yellow solid; IR (neat) ν_{max}: 3400, 2926, 2855,1457, 1445, 1381, 1350,1119, 1076, 755 cm^{-1}; ^{1}H-NMR ($CDCl_3$,

500 MHz) δ 3.66 (2H, t), 2.20 (2H, t), 2.12 (2H, t), 1.67 (2H, m), 1.56 (4H, m), 1.22 (20H, brs, -CH$_2$-), 0.86 ppm (3H, t, -CH$_3$); ^{13}C-NMR (CDCl$_3$, 125MHz) δ 80.97 (C-6), 79.91 (C-5), 62.74 (C-1), 32.14 (C-17), 32.09 (C-2), 29.91 (C-15), 29.88 (C-14), 29.86 (C-13), 29.85 (C-12), 29.77 (C-11), 29.57 (C-16), 29.38 (C-8), 29.35 (C-10), 29.11 (C-9), 25.57 (C-3), 22.90 (C-18), 18.95 (C-7), 18.74 (C-4), 14.33 (C-19); GC-MS (70eV) *m/z* (relative intensity) 280 (*M*$^+$), 149 (6), 135 (5), 121 (7), 112 (32), 111 (39), 110 (10), 107 (8), 98 (23), 97 (69), 96 (22), 95 (29), 94 (59), 93 (26), 83 (29), 82 (30), 81 (38), 79 (100), 77 (17), 69 (27), 68 (49), 67 (49), 57 (37), 55 (54). HRMS Calcd for C$_{19}$H$_{36}$O [M+H]$^+$ 281.2839, found 281.2837.

2.4. 5-Nonadecynal (5)

To a stirred solution of pyridinium chlorochromate (1.22 g, 5.54 mmol) in 60 mL of CH$_2$Cl$_2$ was added dropwise **4** (0.518 g, 1.85 mmol) in 13 mL of CH$_2$Cl$_2$ at room temperature. After 24 h the reaction mixture was filtered through Florisil and washed with diethyl ether. Evaporation of the solvent afforded **5** as a pale yellow solid in a 98% yield; IR (neat) ν$_{max}$: 2953, 2918, 2849, 2714, 1726, 1464, 1340, 722 cm^{-1}; ^{1}H-NMR (CDCl$_3$, 500 MHz) δ 9.80 (1H, brs), 2.56 (2H, t), 2.22 – 2.11 (4H, m), 1.80 (2H, q), 1.45 (2H, q), 1.34 - 1.24 (20H, brs, -CH$_2$-), 0.87 ppm (3H, t, -CH$_3$); ^{13}C-NMR (CDCl$_3$, 125MHz) δ 202.42 (C-1), 81.89 (C-6), 78.87 (C-5), 43.05 (C-2), 32.14 (C-17), 29.90 (C-15), 29.88 (C-14), 29.86 (C-13), 29.77 (C-12), 29.57 (C-11, 16), 29.37 (C-8), 29.25 (C-10), 29.11 (C-9), 22.91 (C-18), 21.77 (C-3), 18.91 (C-7), 18.44 (C-4), 14.34 (C-19); GC-MS (70eV) *m/z* (relative intensity) 278 (*M*$^+$, 1), 151 (21), 137 (26), 133 (16), 123 (30), 119 (35), 111 (36), 110 (58), 109 (38), 107 (22), 106 (17), 97 (26), 96 (27), 95 (70), 93 (45), 92 (75), 91 (43), 83 (62), 82 (100), 81 (67), 80 (33), 79 (84), 69 (32), 68 (36), 67 (86), 65 (18), 57 (30), 55 (87). HRMS Calcd for C$_{19}$H$_{34}$O [M+H]$^+$ 279.2682, found 279.2681.

2.5. 2-(Trimethylsilyloxy)-icos-6-ynenitrile (6)

To a solution of **5** (0.428 g, 1.54mmol) in CH$_2$Cl$_2$ (26mL) at 0°C was added trimethylsilyl cyanide (TMSCN) (0.23 mL, 1.70 mmol), and then catalytic amounts of triethylamine. The mixture was stirred for 3h. The solvent was removed *in vacuo* and the crude reaction mixture was washed with water (2×25mL), extracted with ether (2×25mL) and dried over Na$_2$SO$_4$, affording **6** (0.560g) in a 97% yield, which was used for the next step without further purification; ^{1}H-NMR (CDCl$_3$, 500 MHz) δ 4.42 (1H, m), 2.19 (2H, t), 2.10 (2H, t), 1.88 (2H, q), 1.62 (2H, m), 1.44 (2H, m), 1.23 (20H, brs, -CH$_2$-), 0.84 ppm (3H, t, -CH$_3$), 0.21 (9H, s); ^{13}C-NMR (CDCl$_3$, 125MHz) δ 119.82 (C-1), 81.36 (C-7), 78.56 (C-6), 61.06 (C-2), 35.15 (C-3), 31.85 (C-18), 30.83 (C-16), 29.62 (C-15), 29.58 (C-14), 29.48 (C-13), 29.34 (C-12), 29.28 (C-17), 29.09 (C-9), 29.00 (C-11), 28.84 (C-10), 23.92 (C-19), 22.57 (C-4), 18.63 (C-8), 18.08 (C-5), 14.03 (C-20), -0.48 (C-21, 22, 23); GC-MS (70eV) *m/z* (relative intensity) 377 (*M*$^+$, 2), 362 (16), 208 (22), 195 (10), 194 (21), 167 (11), 133 (16), 129 (16), 121 (10), 120 (11), 119 (59), 118 (18), 116 (28), 107 (11), 100 (13), 95 (24), 93 (24), 92 (60), 91 (26), 84 (18), 82 (15), 81 (26), 80 (15), 79 (39), 77 (13), 75 (56), 73 (100), 67 (33), 66 (20), 57 (24), 55 (35).

2.6. (±)-2-Hydroxy-6-icosynoic acid (7)

To a solution of **6** (0.49 g, 1.29 mmol) in 2-methyltetrahydrofuran (19.0 mL) was added concentrated HCl (7.6 mL). The solution was stirred at 60°C for 4 h. The reaction mixture was extracted with ether (2×20 mL), dried over MgSO$_4$, and the crude product was purified by Kugelrohr distillation at 135°C/3mmHg. Acid **7** was obtained in a 73% yield as an oil; IR (neat) ν_{max}: 3412, 2925, 2854, 1731, 1457, 1379, 1260, 1211, 1175, 1105 cm^{-1}; ^{1}H-NMR (CDCl$_3$, 500 MHz) δ 4.10 (1H, m), 2.21 (2H, t), 2.12 (2H, t), 1.88 – 1.40 (6H, m), 1.25 (20H, brs, -CH$_2$-), 0.88 ppm (3H, t, -CH$_3$); ^{13}C-NMR (CDCl$_3$, 125MHz) δ 173.26 (C-1), 81.34 (C-7), 78.68 (C-6), 69.98 (C-2), 31.91 (C-18, C-3), 29.68 (C-16), 29.64 (C-15), 29.55 (C-14), 29.34 (C-14), 29.15 (C-12), 29.07 (C-17), 28.89 (C-9), 28.51 (C-10, C-11), 22.68 (C-4, C-19), 18.71 (C-5), 18.23 (C-8), 14.11 (C-20). GCMS (70ev) *m/z* (relative intensity) for the methyl ester derivative; 338 (*M*$^+$, 3), 279 (11), 261 (10), 207 (13), 169 (23), 165 (12), 156 (37), 151 (23), 135 (16), 132 (16), 128 (40), 123 (17), 121 (21), 117 (11), 115 (13), 113 (18), 111 (23), 110 (15), 109 (35), 107 (18), 105 (11), 103 (30), 102 (31), 101 (16), 97 (44), 95 (52), 90 (87), 87 (19), 84 (25), 83 (41), 82 (30), 81 (64), 79 (46), 69 (54), 67 (64), 57 (82), 55 (100). HRMS Calcd for C$_{20}$H$_{36}$O$_3$ [M+H]$^+$ 325.2737, found 325.2734.

2.7. (±)-2-Methoxy-6-icosynoic acid (1)

To a solution of NaH (0.057 g, 1.83 mmol) in dry THF (10 mL) under argon was added a solution of **7** (0.178 g, 0.523 mmol) in THF (6 mL). The reaction was stirred for 15 min and then methyl iodide (0.1mL, 1.57 mmol) was added dropwise at 0°C. The reaction was stirred at rt for 2h. Then, concentrated HCl was added until the pH was acidic. The mixture was extracted with ether (2×25 mL) and dried over Na$_2$SO$_4$. The product thus obtained was purified by silica gel column chromatography first eluting with hexane/ether (9:1), and then with ether affording 0.068 g of methyl 2-methoxy-6-icosynoate for a 48% yield. The methyl 2-methoxy-6-icosynoate (0.068 g, 0.193 mmol) was placed in a round-bottomed flask together with 22.2 mL of 1M KOH/EtOH and the reaction mixture was refluxed for 2 h. After this time the reaction was cooled to 25°C and the solvent evaporated, hexane (11.9 mL) was added, and the mixture acidified with 6M HCl (7.4 mL). The desired final acid was extracted with ether (2×25 mL), dried over Na$_2$SO$_4$, filtered and evaporated *in vacuo* affording 68% yield (0.044 g) of the desired acid **1** as an oil; IR (neat) ν_{max}: 3411, 2923, 2853, 1717, 1457, 1377, 1201, 1114 cm^{-1}; ^{1}H-NMR (CDCl$_3$, 500 MHz) δ: 3.83 (1H, t), 3.45 (3H, s, -OCH$_3$), 2.21 (2H, t), 2.12 (2H, t), 1.65 (2H, q), 1.24 (24H, brs, -CH$_2$-), 0.87 ppm (3H, t); ^{13}C-NMR (CDCl$_3$, 125 MHz) δ 176.40 (C-1), 81.05 (C-7), 79.73 (C-2), 79.02 (C-6), 58.22 (C-2'), 31.89 (C-18), 31.32 (C-16), 29.63 (c-14, C-15), 29.52 (C-12, C-13), 29.33 (C-17), 29.14 (C-9), 29.08 (C-3), 28.88 (C-10, C-11), 24.43 (C-19), 22.66 (C-4), 18.70 (C-5), 18.44 (C-8), 14.09 (C-20). GC-MS (70ev) *m/z* (relative intensity) for methyl 2-methoxy-6-icosynoate; 352 (*M*$^+$), 293 (33), 261 (12), 152 (35), 151 (19), 139 (12), 138 (13), 137 (20), 135 (34), 129 (30), 125 (29), 123 (27), 121 (43), 120 (25), 111 (52), 109 (46), 107 (38), 104 (100), 97 (52), 96 (24), 95 (75), 94 (19), 93 (81), 92 (99), 91 (47), 83 (40), 81 (91), 79 (88), 77 (23), 71 (74), 69 (39), 68 (22), 67 (83), 57 (41), 55 (62). HRMS Calcd for C$_{21}$H$_{38}$O$_3$ [M+H]$^+$ 339.2894, found 339.2899.

2.8. Cell Cultures

Human SH-SY5Y neuroblastoma cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's/F12 medium (DMEM/F-12; Invitrogen Corp.) supplemented with 10% (v/v) fetal clone III serum (FCS III; Invitrogen Corp.), penicillin (100 U/mL), and streptomycin (100 μg/ml). Human HeLa cervix adenocarcinoma cells (American Type Culture Collection, Manassas, VA) were cultured in Eagle's Minimum Essential Medium (EMEM; Invitrogen Corp.) supplemented with 10% (v/v) fetal bovine serum (FB; Invitrogen Corp.), penicillin (100 U/mL), and 1% glutamine. Both cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

2.9. Sulforhodamine B colorimetric assay

Cell growth experiments were performed following the protocol and analyzed as previously described (Carballeira et al., 2003; Orellano et al., 2010). Cultures were grown to 80% confluency onto 96-well plates. Then, cells were subjected to medium replacement containing 1% (v/v) of FCS III before treatment. The experimental day, cultures were treated with a range of concentrations between 0.1 and 1000 μM of the experimental agents (first dissolved in less than 2% DMSO and then diluted with the testing medium until the final concentrations were reached) for 24 h, 48 h, and 72 h. Thereafter, cells were fixed in situ for 90 min at 4 °C with 50% (w/v) trichloroacetic acid (TCA) to produce a final concentration of 16% (w/v) TCA. Then, cells were washed five times with water and air-dried. Subsequently, cells were stained with a solution of 0.4% (w/v) sulforhodamine B in 1% (v/v) acetic acid for 15 min, washed with 1% (v/v) acetic acid, air-dried, and incubated with 10 mM Tris-base for 15 min at room temperature. The results were based on the relative absorbance at 490 nm of the solubilized stain measured on a microplate reader (MRX II; Dynex Technologies, Chantilly, VA).

2.10. Determination of the critical micelle concentration (CMC)

The CMC of the fatty acids was assessed as previously described by Courtney et al., 1986. Briefly, the fatty acids were first dissolved in less than 2% DMSO and then diluted with phosphate-buffered saline (PBS) affording fatty acid concentrations between 0.1 and 1000 μM and containing rhodamine 6G at 2.5×10^{-6} M. The wavelength of maximum absorption was determined for each dilution using a Shimadzu spectrophotometer and plotted as a function of the fatty acid concentration. The CMC value was described as the point at which the wavelength of maximum absorption first deviated from linearity.

2.11. Statistical Analyses

All analyses were performed with Prism software, version 4.0 (San Diego CA). The EC_{50} value, which is the effective concentration of experimental agent required to achieve half-maximal degree of inhibition of cell growth, was calculated from titration curves.

3. Results and Discussion

The successful synthesis of **1** was accomplished in seven steps with an overall yield of 14% (Scheme 1). The synthesis started with the 2-(4-bromobutoxy)-tetrahydro-*2H*-pyran (**2**), which was prepared as previously described (Carballeira et al. 2012). The first step was the

coupling of commercially available 1-pentadecyne with **2** in the presence of *n*-BuLi in THFHMPA at −70°C, which resulted in the 2-(nonadec-5-ynyloxy)-tetrahydro-2*H*-pyran (**3**) in a 64% yield. The deprotection of **3** was successfully achieved with *p*-TSA in methanol at 60°C, resulting in the desired 5-nonadecynol (**4**) in a 98% yield. Alkynol **4** was then oxidized using pyridinium chlorochromate (PCC) in dichloromethane (CH_2Cl_2) affording 5-nonadecynal (**5**) in a 98% yield. Alkynal **5** was subsequently reacted with trimethylsilyl cyanide (TMSCN) in CH_2Cl_2 and catalytic amounts of triethylamine (Et_3N) at 0°C resulting in the desired 2-(trimethylsilyloxy)-icos-6-ynenitrile (**6**) in a 97% yield, which was then converted, in a 73% yield, into the (±)-2-hydroxy-6- icosynoic acid (**7**) by acid hydrolysis of nitrile **6** in 2-methyltetrahydrofuran (2-MeTHF) as solvent. Methylation of both the acid and the free hydroxy group in **7** was achieved using sodium hydride (NaH) and methyl iodide (CH_3I) in THF as solvent, which was followed by saponification of the ester using KOH/ EtOH under reflux, which afforded the desired acid **1** in a combined 32% yield for the last two steps.

The most significant absorptions in the ^{1}H-NMR spectrum of **1** are for the carbon and hydrogens bearing the methoxy group. The methoxy protons resonated at δ 3.45 ppm, and the methine hydrogen resonated at δ 3.83 ppm. In the ^{13}C-NMR spectrum of **1** the most significant carbons were observed at δ 58.22 ppm for the carbon of the methoxy group, at δ 79.73 ppm for the C-2 methine carbon, the C-6 and C-7 acetylenic carbons were observed at δ 79.02 ppm and at δ 81.05 ppm, respectively, while at δ 176.40 ppm resonated the C-1 carbon.

Aimed at exploring the cytotoxicity of the novel α-methoxylated acid **1** we used the well-establish human SH-SY5Y neuroblastoma cell line as a simplified environment of cancer of the nervous system to examine cell death responses promoted by **1**, its corresponding nonmethoxylated analog 6-icosynoic acid, and the saturated 2-methoxyicosanoic acid. In order to quantify the cell death effects of these fatty acids, experiments were performed using a sulforhodamine B colorimetric assay and analyzed as a function of cell growth inhibition for 24 h, 48 h, and 72 h. Control cells grew progressively and continuously over the experimental time (from 24 h to 72 h) without any sign of contact inhibition or nutrient depletion. SH-SY5Y neuroblastoma cells supplemented with **1** displayed significant cell death (EC_{50}'s between 23 and 103 μM) when compared to cells supplemented with the 6-icosynoic acid ($EC_{50} > 500$ μM) at all the time intervals evaluated (24 h, 48 h, and 72 h). However, supplementation with **1** for 48 h promoted the major cell death, as demonstrated by the lowest EC_{50} value of 23 ± 1 μM obtained (Table 1). Based on our statistical analyses we can also conclude that acid **1** is significantly toxic over the time period evaluated and its toxicity is not significantly different between 48 h and 72 h. We should mention that the 2-methoxyicosanoic acid was not as toxic ($EC_{50} > 300$ μM) to the SH-SY5Y neuroblastoma cells as **1** (data not shown), thus indicating that the triple bond in **1** at C-6 is also needed for the displayed cytotoxicity. As a control, palmitic acid was studied and it was not effective against the neuroblastoma cells ($EC_{50} > 500$ μM).

To further characterize the cytotoxic effect promoted by the α-methoxy functionality in **1** and to investigate whether **1** induces cell death in other cancer cell lines, we used the HeLa adenocarcinoma cell line as an *in vitro* cell model of human cervix cancer. Supplemented

HeLa cell cultures with **1** showed significant cell death (EC_{50}'s between 16 and 26 μM) in contrast to cell cultures supplemented with the 6-icosynoic acid at all the time intervals evaluated (24 h, 48 h, and 72 h) (Table 1). Thus, acid **1** is comparably effective against both cancerous cells. It is important to note that our *in vitro* cell models are from human origin, thereby, different tissue types, such as cervix and brain, could have different susceptibilities responding dissimilar to anticancer drugs. This difference was observed for palmitic acid; whereby it was not toxic ($IC_{50} > 500$ μM) to our SH-SY5Y cells, it was toxic (IC_{50}'s between 60 and 64 μM) to the HeLa cells after 48 h (Table 1). The toxicity of **1** towards SH-SY5Y and HeLa cells was statistically significant for all the times evaluated in comparison to the 6-icosynoic acid. However, the relative sensibility over time between both cancerous cells was not statistically significant. We should also mention that acid **1** does not display toxicity towards BALB/c murine macrophages ($IC_{50} > 100$ μM; data not shown).

The question arises as to why **1** is more effective than the 6-icosynoic acid against the studied cancer cell lines. Aimed at finding a plausible explanation we studied the critical micelle concentrations (CMCs) of our fatty acids. This is a worthwhile study by itself since, to the best of our knowledge, it is not known how α-methoxylation changes the CMC of a fatty acid. Previous studies have demonstrated in numerous occasions a relationship between the CMC of amphiphilic compounds and their biological activities (Courtney et al., 1986). Following this reasoning, we determined the CMC of our fatty acids as a function of their concentration (Figure 1). Acid **1** displayed a CMC of 20–30 μM, while the CMC of the 6-icosynoic acid was calculated to be greater than 500 μM (Table 2). Therefore, the α-methoxy functionality tends to lower the CMC of a fatty acid. Whether this observation can be correlated to our observed EC_{50}'s is debatable although plausible. However, it is clear that the different biophysical properties of **1**, as compared to the 6-icosynoic acid, might be responsible for the difference in cytotoxicity.

The proper functioning of the nervous system depends on a regulated homeostasis among differentiation, proliferation, and cell death. Malfunction in any of these processes may contribute to pathological disorders such as neuroblastoma. Fatty acids play a key role regulating this characteristic loss of homeostasis in cancer (Gleissman et al., 2010). Previous studies from our laboratory have shown that α-methoxylated fatty acids have anticancer properties promoting cell death of human leukemia and histiocytic lymphoma (Carballeira et al., 2003). However, the toxicity of α-methoxylated fatty acids against cancer of the nervous system was not explored. The present study has clearly demonstrated that the α-methoxy functionality in **1** is important for its toxicity towards human neuroblastoma cells.

Cell death is also essential in cancer to safeguard the organism against unwanted proliferating cells. Following such reasoning, we aimed to assess **1** and its non-methoxylated analog 6-icosynoic acid with respect to cell death of proliferating human SH-SY5Y neuroblastoma cells. Our results showed that the added α-methoxy functionality in **1** induces a significant cell death at all the time intervals evaluated (24 h, 48 h, and 72 h) in comparison to the 6-icosynoic acid (Table 1), but supplementation with **1** for 48 h promoted the major neuroblastoma cell death ($EC_{50} = 23 \pm 1$ μM). Etoposide treatment for 48 h, a well-documented and FDA approved anticancer drug, promoted significant neuroblastoma cell death with an $EC_{50} = 9 \pm 2$ μM. Based on these results, and previous findings where

etoposide was reported to exert its anticancer effects in the micromolar range (Rodríguez-López et al., 2000), we identified supplementation of **1** for 48 h as the optimal conditions to study the effects of the α-methoxy functionality in fatty acids against this *in vitro* cell model of cancer.

The fatty acid composition of cell membranes has pivotal physiological as well as pathological roles that have been well-documented to promote profound differential functional effects according to the cell state. Thereby, exogenous supplementation has been identified as a bright strategy to integrate fatty acids into cell membranes, which affects intrinsic fatty acid composition and subsequently cell responses (Hardy et al., 2000; Reynolds et al., 2001). Previous studies have shown that the fatty acid composition of cells has a key role in pathological conditions such as neurodegenerative diseases and cancer. For example, recent studies focused on the prevention of Alzheimer's disease have shown that fatty acids may be integrated into cell membranes to increase membrane fluidity and lead to increase in alpha-secretase-cleaved soluble amyloid precursor protein secretion (Yang et al., 2011). Moreover, cancer studies using murine lymphoma cells have identified that an increase in *de novo* lipid synthesis promotes lipid droplets accumulation during cell death (Boren and Brindle, 2012). Hence, such studies have highlighted the relevance of the structure of lipids under pathological conditions. One possibility is for the cell death response induced by **1** to be mediated by micelle formation. Similar cytotoxic effect for lipoteichoic acid on human heart cells has been reported to be mediated by micelle formation (Courtney et al., 1986). In our *in vitro* studies, acid **1** could be operating in a similar fashion but additional experimentation is needed to unequivocally prove this hypothesis. Despite the fact that all fatty acids have the ability of forming micelles, the α-methoxy functionality in **1** remarkably decreases its CMC. This is the first time that a CMC has been determined for a α-methoxylated fatty acid. It should be noted that previous studies have established the relevance of the relationship between spatial structure and electrostatic properties with a high compatibility of micelles with cell membranes (Xu et al., 2011).

In conclusion, we developed a synthesis of seven steps (14% overall yield) for the novel acid **1**. The α-methoxy functionality in **1**, in combination with the C-6 triple bond, are responsible for the toxicity displayed towards the SH-SY5Y neuroblastoma cell line and the human adenocarcinoma cervix cell line (HeLa). Based on these results, and our previous work with other α-methoxylated fatty acids (Carballeira et al., 2003), we identify the α-methoxy functionality in **1** as a specific structural modification that enhances its cytotoxicity towards human *in vitro* models of neuroblastoma. Therefore, the present research work will surely expand our understanding of the multiplicity of roles mediated by the α-methoxylated fatty acids.

Acknowledgments

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Highlights

- The α-methoxy functionality enhances the cytotoxicity of fatty acids towards *in vitro* models of neuroblastoma.

- The α-methoxylated acetylenic fatty acids form micelles more readily than their nonmethoxylated ones.

- These results provide insights into strategies whereby the α-methoxylated fatty acids could be used in micelles to transport drugs in the fight against cancer.
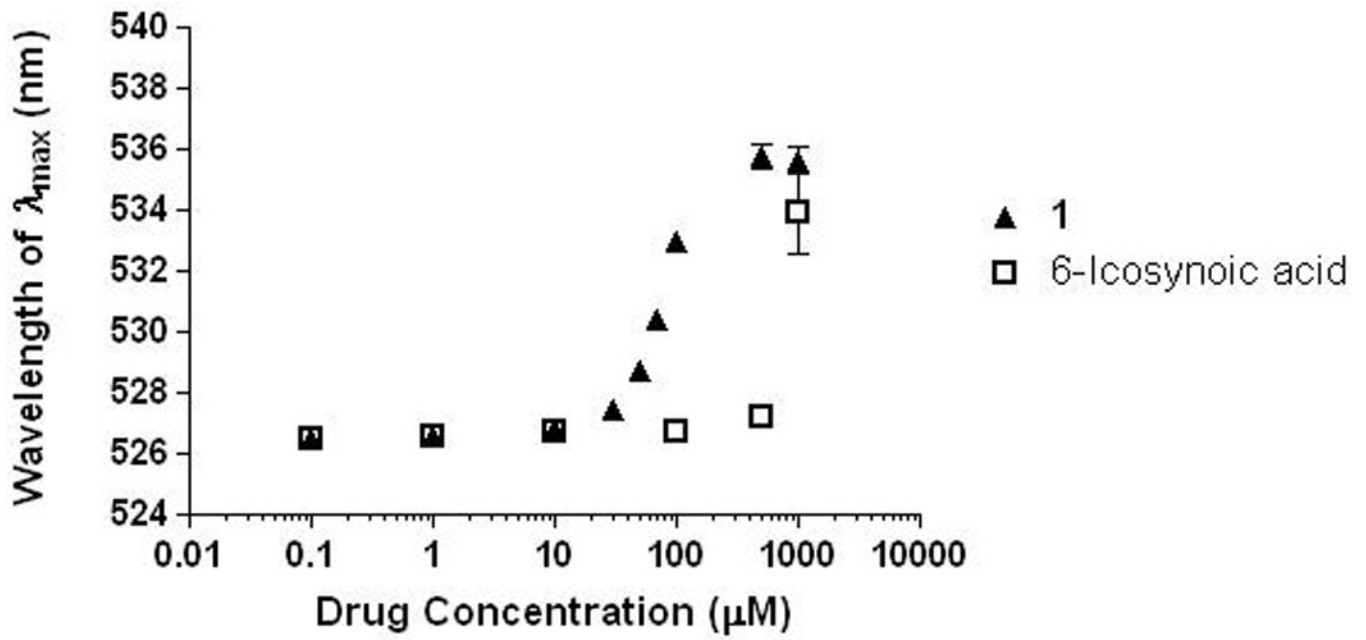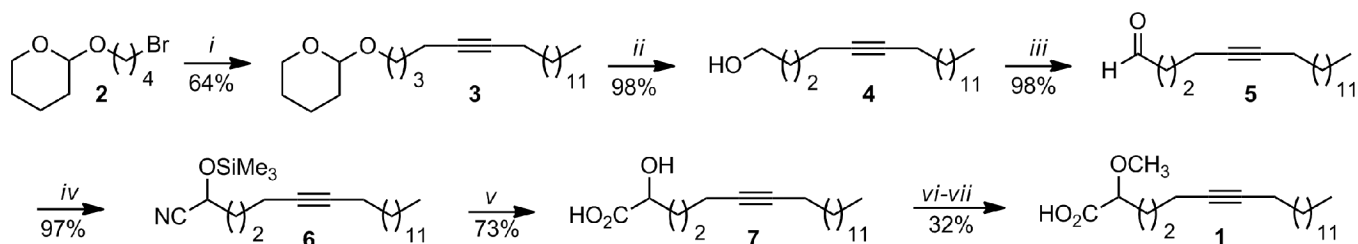
Fig. 1.
The critical micelle concentration (CMC) of the studied fatty acids. The CMC was determined as described in materials and methods. Data are the means ± SEM of values (n=3).

Scheme 1.

Synthesis of the (±)-2-methoxy-6-icosynoic acid (**1**). *i*) 1-pentadecyne, *n*-BuLi, THF-HMPA, −70°C, 24h; *ii*) PTSA, MeOH, 60°C, 24h; *iii*) PCC, CH$_2$Cl$_2$, 24h, rt; *iv*) TMSCN, Et$_3$N, CH$_2$Cl$_2$, 0°C, 3h; *v*) HCl (conc.), 2-MeTHF, 60°C, 4h; *vi*) HCl, MeOH, reflux then NaH, THF, CH$_3$I, 0°C, 2h; *vii*) KOH/ EtOH (1M), reflux, 2h.

Table 1

Anticancer activity of the studied fatty acids against the human neuroblastoma SH-SY5Y and cervix adenocarcinoma (HeLa) cell lines.

Experimental agents	24 h EC$_{50}$ (µM)	48 h EC$_{50}$ (µM)	72 h EC$_{50}$ (µM)
6-Icosynoic acid	> 500	> 500	> 500
6-Icosynoic acid	> 500[a]	> 500[a]	> 500[a]
1	103 ± 1	23 ± 1	58 ± 2
1	16 ± 5[a]	26 ± 1[a]	18 ± 1[a]
Palmitic acid	> 500	> 500	> 500
Palmitic acid	> 500[a]	61 ± 1[a]	64 ± 1[a]
Etoposide	ND	9 ± 2	8 ± 1
Etoposide	ND	4 ± 1[a]	4 ± 1[a]
2% DMSO	> 100	> 100	> 100

[a]HeLa cell line. Cultures were treated with several concentrations of the corresponding fatty acids (the concentration of DMSO was less than 2%) for 24 h, 48 h, and 72 h. On the experimental day, inhibition of cell growth was assessed by SRB assays as described in the materials and methods section. The EC$_{50}$ value, which is the effective concentration of experimental agent required to achieve half-maximal degree of inhibition of cell growth, was calculated using Prism Software 4.0 from titration curves generated from at least six experimental values. ND = not determined.

Table 2

Relationship between the anticancer activities of the studied fatty acids and their CMC.

Fatty Acids	EC$_{50}$ (µM)	CMC (µM)
6-Icosynoic acid	> 500	> 500
1	23 ± 1	20–30

The human neuroblastoma SH-SY5Y cells were treated with several concentrations of the corresponding fatty acid (first dissolved in less than 2% DMSO) for 48 h. On the experimental day, inhibition of cell growth was assessed by SRB assays. The EC$_{50}$ was calculated using Prism Software 4.0 from titration curves generated from at least six experimental values. The CMC was determined as described in materials and methods. Data are the means ± SEM of values (n=3).