Total Synthesis and Biological Evaluation of (5*Z*,9*Z*)-5,9-Hexadecadienoic Acid, an Inhibitor of Human Topoisomerase I

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The naturally occurring (5*Z*,9*Z*)-5,9-hexadecadienoic acid was synthesized stereochemically pure in six steps starting with commercially available 1,5-hexadiyne. The title compound was antimicrobial against the Gram-positive bacteria *Staphylococcus aureus* (MIC 80 μM) and *Streptococcus faecalis* (MIC 200 μM), but inactive against Gram-negative bacteria such as *Pseudomonas aeruginosa*. In addition, the (5*Z*,9*Z*)-5,9-hexadecadienoic acid completely inhibits human topoisomerase I at a concentration of 800 μM, while 5,9-hexadecadiynoic acid and hexadecanoic acid do not inhibit topoisomerase I (>1000 μM). This comparison reveals that the *cis* double bond geometry in the title compound is required for topoisomerase I inhibition. Moreover, these results suggest that the antimicrobial activity of (5*Z*,9*Z*)-5,9-hexadecadienoic acid against either *S. aureus* or *S. faecalis* could be a result, at least in part, of the inhibitory activity of the acid against topoisomerases.

Fatty acids with the $\Delta^{5,9}$ diunsaturation are an interesting group of lipids with a variety of reported biological activities. Among these compounds the long-chain $\Delta^{5,9}$ fatty acids (C_{27}–C_{30}), typical of marine sponges, are known inhibitors of human topoisomerase I.[1] For example, (5*Z*,9*Z*)-5,9-heptacosadienoic acid effectively inhibits human topoisomerase I with an IC_{50} of 0.9 μM.[1] Some of these fatty acids are also cytotoxic inasmuch as a mixture of (5*Z*,9*Z*)-23-methyl-5,9-tetracosadienoic acid and (5*Z*,9*Z*)-22-methyl-5,9-tetracosadienoic acid are cytotoxic against mouse Ehrlich carcinoma cells with an ED_{50} of 1.8 μg/mL and show a hemolytic effect on mouse erythrocytes.[2] On the other hand, the short-chain $\Delta^{5,9}$ fatty acids (C_{16}–C_{19}) are antimicrobial against Gram-positive bacteria. For example, the branched fatty acid (5*Z*,9*Z*)-14-methyl-5,9-pentadecadienoic acid, first isolated from the Caribbean gorgonian *Eunicea succinea*, displays antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus* (MIC 240 μM) and *Streptococcus faecalis* (MIC 160 μM), but is inactive against Gram-negative bacteria.[3] These findings clearly demonstrate the biomedical potential of marine $\Delta^{5,9}$ fatty acids. Therefore, in an effort to expand our present understanding of the bioactivity of $\Delta^{5,9}$ fatty acids we have undertaken the total synthesis and biological evaluation of the shortest $\Delta^{5,9}$ fatty acid known, namely, (5*Z*,9*Z*)-5,9-hexadecadienoic acid (**1**). We were particularly interested in studying the antimicrobial and topoisomerase I inhibitory activities of **1** so as to gain more insight into the structural features required for bioactivity, since these bioactivities have not been explored for **1**. A working hypothesis is that the short-chain $\Delta^{5,9}$ fatty acids are antimicrobial because they inhibit the bacterial topoisomerases of the Gram-positive organisms, in addition to other possible mechanisms of action, such as DNA gyrase inhibition or changes in membrane fluidity.[4−6]

(5*Z*,9*Z*)-5,9-Hexadecadienoic acid (**1**) is a rare fatty acid first isolated from the cellular slime mold *Dictyostelium discoideum*,[7] but later identified in a few marine sponges such as *Chondrilla nucula*.[8] There is only one four-step synthesis reported for **1**, but it yielded a 10:1 mixture of (5*Z*,9*Z*)- and (5*E*,9*Z*)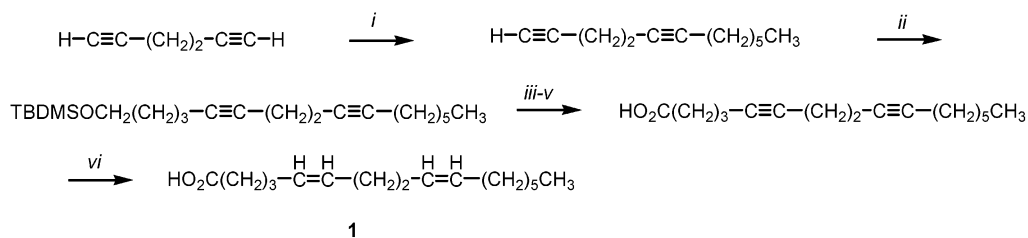-5,9-hexadecadienoic acids.[9] This isomeric 5*Z*/5*E* mixture cannot be used for topoisomerase I inhibition studies since it is known that *trans* fatty acids, such as elaidic acid (9*t*-18:1), do not inhibit topoisomerase I (IC_{50} > 1000 μM), while *cis* fatty acids such as oleic acid (9*c*-18:1) do inhibit the enzyme (IC_{50} 31 μM).[10] Therefore, herein we report the first 100% stereoselective synthesis for (5*Z*,9*Z*)-5,9-hexadecadienoic acid (**1**) together with its antimicrobial and topoisomerase I inhibitory activities.

The synthesis of (5*Z*,9*Z*)-5,9-hexadecadienoic acid (**1**) started with commercially available 1,5-hexadiyne (50% in pentane), which was coupled with 1-bromohexane and *n*-BuLi, affording a 3:1 mixture of 1,5-dodecadiyne and the dialkylated adduct, which were separated by fractional distillation using a Büchi glass oven (Scheme 1). The isolated 1,5-dodecadiyne was then alkylated with (4-bromobutoxy)-*tert*-butyldimethylsilane (prepared from commercially available 4-bromobutanol) and *n*-BuLi, resulting in a 35% isolated yield of 1-(*tert*-butyldimethylsilyloxy)-5,9-hexadecadiyne. Deprotection of the silyl group with tetrabutylammonium fluoride (TBAF) and oxidation of the alcohol with pyridinium chlorochromate (PCC) resulted in a combined 77% yield of 5,9-hexadecadiynal. The aldehyde was then oxidized with $NaClO_2$ in *t*-BuOH, affording the 5,9-hexadecadiynoic acid in an 81% isolated yield. Final hydrogenation over Lindlar's catalyst yielded the stereochemically pure (5*Z*,9*Z*)-5,9-hexadecadienoic acid (**1**) in an 80% isolated yield. No double-bond isomerization was observed. The total overall yield in this six-step synthesis starting from 1,5-hexadiyne was 8%.

The (5*Z*,9*Z*)-5,9-hexadecadienoic acid (**1**) displayed moderate antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus* (MIC 80 μM) and *Streptococcus faecalis* (MIC 200 μM). This fatty acid was not active against the Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*. It is important to mention that hexadecanoic acid (16:0) showed no activity (MIC > 400 μM) against any of these four microorganisms. Therefore, the double bonds in **1** are required for the antimicrobial activity.

In an effort to broaden the scope of the biological potential of the parent (5*Z*,9*Z*)-5,9-hexadecadienoic acid (**1**), we studied its inhibitory activity against human topoisomerase I (from freshly extracted human placenta). We

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Scheme 1[a]

$$H-C{\equiv}C-(CH_2)_2-C{\equiv}C-H \xrightarrow{i} H-C{\equiv}C-(CH_2)_2-C{\equiv}C-(CH_2)_5CH_3 \xrightarrow{ii}$$

$$TBDMSOCH_2(CH_2)_3-C{\equiv}C-(CH_2)_2-C{\equiv}C-(CH_2)_5CH_3 \xrightarrow{iii-v} HO_2C(CH_2)_3-C{\equiv}C-(CH_2)_2-C{\equiv}C-(CH_2)_5CH_3$$

$$\xrightarrow{vi} HO_2C(CH_2)_3-\overset{H}{C}{=}\overset{H}{C}-(CH_2)_2-\overset{H}{C}{=}\overset{H}{C}-(CH_2)_5CH_3$$

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[a] (*i*) n-BuLi, 1-bromohexane, THF, −78 °C; (*ii*) n-BuLi, (4-bromobutoxy)-*tert*-butyldimethylsilane, THF−HMPA, −78 °C; (*iii*) TBAF, THF, rt; (*iv*) PCC, CH₂Cl₂, rt; (*v*) NaClO₂, t-BuOH, 48 h, rt; (*vi*) H₂, Lindlar.
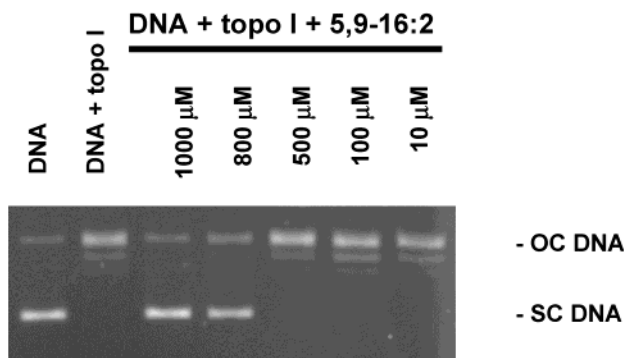

Figure 1. Agarose gel stained with ethidium bromide showing the inhibitory effect of (5*Z*,9*Z*)-5,9-hexadecadienoic acid at 800 and 1000 μM. OC DNA stands for open circular DNA and SC DNA for supercoiled DNA.

found that acid **1** completely inhibits topoisomerase I at a concentration of 800 μM (Figure 1). However, the synthetic precursor 5,9-hexadecadiynoic acid did not show any inhibition of topoisomerase I (>1000 μM). This comparison confirms that the *cis* double bond geometries in **1** are essential for effective topoisomerase I inhibition. It is also important to mention that hexadecanoic acid does not inhibit topoisomerase I, even at concentrations as high as 2000 μM.[10] However, long-chain $\Delta^{5,9}$ fatty acids (C$_{27}$−C$_{28}$) are more effective topoisomerase I inhibitors (IC$_{50}$'s 1−3 μM) than **1**, implying that carbon chain length also plays an important role in topoisomerase I inhibition.[1]

In summary, we have developed the first 100% stereoselective synthesis for (5*Z*,9*Z*)-5,9-hexadecadienoic acid (**1**), utilizing as the key step the selective double alkylation of 1,5-hexadiyne. We have shown that **1** is moderately antimicrobial against Gram-positive bacteria, such as *S. aureus*, but the double bonds in **1** are required for the activity. In addition, **1** inhibits human topoisomerase I at high concentrations, whereas the corresponding 5,9-dialkynoic acid does not. This indicates that the *cis*-unsaturations in **1** are required for topoisomerase I inhibition. In addition, hexadecanoic acid is inactive in both antimicrobial and topoisomerase I bioassays, thus confirming that a specific structural geometry is needed for the bioactivity. Moreover, when compared with the recent literature, carbon chain length also plays an important role in topoisomerase I inhibition, inasmuch as fatty acids with long carbon chains (C$_{27}$−C$_{30}$) are more inhibitory than those with short chains (C$_{16}$).[1]

Before a precise correlation between antimicrobial activity and topoisomerase inhibition can be established, the inhibitory effect of **1** against the topoisomerase IV from *S. aureus* should be determined.[4−6] Topoisomerase IV appears to be the preferential antibacterial target for the quinolones in Gram-positive organisms.[4] It is possible that human topoisomerase I and bacterial topoisomerase IV might share a similar structural homology for a binding pocket

where the fatty acids bind. Such a similarity has been established for other systems. For example, it is also known that *cis*-unsaturated fatty acids (C$_{18}$ or higher) inhibit mammalian DNA polymerase β and human DNA topoisomerase II.[11] In fact, computer modeling analysis has revealed that the amino acids Thr, His, Leu, and Lys form a pocket, in both enzymes, where the fatty acid molecule binds.[11] Such a model could also be operative in other topoisomerases such as I and IV. It is possible that these $\Delta^{5,9}$ fatty acids may also have practical applications in the synthesis of novel lipidic prodrugs aimed at antibiotic-resistant *S. aureus*.[12]

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Nicolet 600 FT-IR spectrophotometer. ¹H and ¹³C NMR were recorded on a General Electric QE-300 or Bruker DPX-300 spectrometer. ¹H NMR chemical shifts are reported with respect to internal Me₄Si, and ¹³C NMR chemical shifts are reported in parts per million relative to CDCl₃ (77.0 ppm). GC−MS analyses were recorded at 70 eV using a Hewlett-Packard 5972A MS ChemStation equipped with a 30 m × 0.25 mm special performance capillary column (HP-5MS) of polymethyl siloxane cross-linked with 5% phenylmethyl-polysiloxane.

1,5-Dodecadiyne. Into a 100 mL round-bottom flask, equipped with a magnetic stirrer, was placed 1.0 g (12.8 mmol) of 1,5-hexadiyne (50% in pentane, AlfaAesar) and 75 mL of dry THF under a nitrogen atmosphere. The temperature was then lowered to −78 °C, and *n*-butyllithium (2.5 M, 4.6 mL, 11.5 mmol) in hexane was slowly added. After 45 min 1-bromohexane (1.8 mL, 12.8 mmol) was added in 5 mL of HMPA. Stirring was continued for 24 h, and the reaction mixture was finally quenched with ice water. The organic products were extracted with diethyl ether (3 × 15 mL), and the organic layer was dried over Na₂SO₄. The solvent was removed in vacuo, affording a 3:1 mixture of the monoalkylated versus the dialkylated adducts. The 1,5-dodecadiyne (0.89 g, 43% yield) was obtained pure by fractional distillation: IR (neat) ν_{max} 3313, 2956, 2932, 2851, 2204, 1460, 1379, 1259, 1078, 797 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz) δ 2.35 (4H, m, H-3, H-4), 2.12 (2H, brt, J = 6.9 Hz, H-7), 1.98 (1H, brt, J = 2.4 Hz, H-1), 1.47−1.23 (8H, m, H-8, H-9, H-10, H-11), 0.86 (3H, t, J = 7.0 Hz, H-12); ¹³C NMR (CDCl₃, 75 MHz) δ 83.0 (s, C-2), 81.5 (s, C-5), 78.0 (s, C-6), 68.9 (d, C-1), 31.3 (t, C-10), 28.9 (t), 28.4 (t), 22.5 (t), 19.1 (t), 18.8 (t), 18.6 (t), 14.0 (q, C-12); GC−MS (70 eV) *m/z* 162 [M⁺] (0.1), 147 (1), 133 (6), 123 (6), 120 (2), 119 (11), 106 (10), 105 (29), 103 (5), 95 (5), 92 (25), 91 (100), 81 (26), 79 (26), 77 (18), 69 (7), 67 (26), 65 (17), 55 (17), 53 (14).

1-(*tert*-Butyldimethylsilyloxy)-5,9-hexadecadiyne. Into a 50 mL round-bottom flask, equipped with a magnetic stirrer, was placed 0.5 g (3.1 mmol) of 1,5-dodecadiyne in 25 mL of tetrahydrofuran under a nitrogen atmosphere. The temperature was then lowered to −78 °C, and *n*-butyllithium (2.5 M, 1.2 mL, 3.1 mmol) in hexane was slowly added. After 45 min (4-bromobutoxy)-*tert*-butyldimethylsilane (0.82 g, 3.1 mmol) was added in 5 mL of HMPA. Stirring was continued for 24 h, and the reaction mixture was finally quenched with ice water.

The organic products were extracted with diethyl ether (3 × 15 mL), and the organic layer was dried over Na_2SO_4. The solvent was removed in vacuo, and the crude product was purified by fractional distillation, affording 0.38 g of the 1-(*tert*-butyldimethylsilyloxy)-5,9-hexadecadiyne for a 35% yield. The product was used for the next step without further purification: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.62 (2H, t, J = 6.9 Hz, H-1), 2.31 (4H, m, H-7, H-8), 2.15 (4H, m, H-4, H-11), 1.62–1.26 (12H, m, H-2, H-3, H-12, H-13, H-14, H-15), 0.85 (12H, brs, −Si−C(CH_3)$_3$ and −CH_3), 0.03 (6H, s, Si−CH_3); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 81.5 (s), 81.1 (s), 78.9 (s), 78.6 (s), 62.7 (t, C-1), 31.8 (t, C-2), 31.3 (t, C-14), 28.9 (t), 28.5 (t), 25.9 (t), 25.3 (q, Si−C(CH$_3$)$_3$), 19.4 (t), 18.7 (t), 18.5 (t), 18.3 (t), 14.0 (q, C-16), −5.4 (q, Si−CH$_3$); GC−MS (70 eV) m/z 349 [M$^+$] (0.1), 291 (6), 217 (3), 215 (7), 207 (1), 193 (3), 187 (2), 173 (2), 171 (2), 161 (2), 155 (2), 145 (11), 133 (5), 131 (19), 119 (19), 117 (13), 105 (8), 101 (6), 97 (6), 91 (17), 85 (3), 81 (6), 79 (9), 77 (9), 75 (100), 59 (10), 55 (10).

5,9-Hexadecadiyn-1-ol. Into a 35 mL round-bottom flask, equipped with a magnetic stirrer, was placed 0.38 g (1.08 mmol) of 1-(*tert*-butyldimethylsilyloxy)-5,9-hexadecadiyne in 20 mL of THF. To this solution was added dropwise 1.1 mL (1.08 mmol) of tetrabutylammonium fluoride (1 M) in THF. After 24 h the reaction mixture was poured into H_2O and extracted with diethyl ether (3 × 15 mL). The organic extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo, affording 0.2 g of 5,9-hexadecadiyn-1-ol for a 78% yield. The product was used for the next step without further purification: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.62 (2H, t, J = 6.3 Hz, H-1), 2.30 (4H, m, H-7, H-8), 2.14 (4H, m, H-4, H-11), 1.93 (1H, brs, OH), 1.65–1.26 (12H, m, H-2, H-3, H-12, H-13, H-14, H-15), 0.87 (3H, t, J = 7.1 Hz, −CH_3); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 81.2 (s), 80.6 (s), 79.2 (s), 78.6 (s), 62.3 (t, C-1), 31.7 (t, C-2), 31.3 (t, C-14), 28.9 (t), 28.4 (t), 25.6 (t), 25.1 (t), 22.5 (t), 19.4 (t), 18.6 (t), 18.4 (t), 14.0 (q, C-16); GC−MS (70 eV) m/z 234 [M$^+$] (0.1), 205 (4), 203 (3), 191 (3), 189 (7), 175 (11), 163 (17), 149 (27), 145 (15), 137 (8), 135 (12), 133 (16), 131 (29), 121 (15), 119 (50), 117 (49), 115 (17), 110 (20), 107 (20), 105 (64), 103 (11), 95 (15), 93 (40), 91 (100), 81 (33), 79 (62), 77 (51), 67 (47), 65 (27), 57 (14), 55 (51).

5,9-Hexadecadiynal. To a stirred solution of 5,9-hexadecadiyn-1-ol (0.2 g, 0.8 mmol) in 15 mL of dichloromethane was slowly added pyridinium chlorochromate (0.2 g, 0.8 mmol) at room temperature. After 24 h the reaction mixture was filtered through Florisil and washed with diethyl ether (50 mL). After evaporation of the solvent 0.2 g of 5,9-hexadecadiynal was obtained for a 99% yield. The product was used for the next step without further purification: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.78 (1H, t, J = 1.2 Hz, H-1), 2.57 (2H, dt, $J_{2,1}$ = 1.2 Hz and $J_{2,3}$ = 7.2 Hz, H-2), 2.30 (4H, m, H-7, H-8), 2.21 (2H, brt, J = 6.8 Hz, H-4), 2.11 (2H, brt, J = 7.0 Hz, H-11), 1.79 (2H, q, J = 6.9 Hz, H-3), 1.52–1.24 (8H, m, H-12, H-13, H-14, H-15), 0.86 (3H, t, J = 7.1 Hz, −CH_3); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 202.1 (s, C-1), 81.2 (s, C-6), 80.1 (s, C-9), 79.6 (s, C-5), 78.5 (s, C-10), 42.7 (t, C-2), 31.3 (t, C-14), 28.9 (t), 28.5 (t), 22.5 (t), 21.3 (t), 19.35 (t), 19.32 (t), 18.7 (t), 18.1 (t), 14.0 (q, C-16); GC−MS (70 eV) m/z 231 [M$^+$ − 1] (1), 217 (1), 204 (3), 203 (6), 189 (7), 175 (20), 162 (9), 161 (28), 149 (5), 147 (24), 143 (9), 136 (5), 133 (31), 131 (21), 129 (18), 119 (45), 117 (51), 115 (20), 105 (63), 103 (12), 95 (10), 93 (23), 91 (100), 81 (38), 79 (62), 77 (37), 69 (10), 67 (40), 65 (33), 55 (51).

5,9-Hexadecadiynoic Acid. To a stirred solution of 5,9-hexadecadiynal (0.2 g, 0.8 mmol) in *t*-BuOH (20 mL) was added a solution of NaClO$_2$ (2.5 mmol) and NaHPO$_4$ (3 mmol) in 3 mL of water over a period of 10 min. The pale yellow solution was stirred at room temperature for 48 h. The mixture was diluted with water and extracted with diethyl ether (3 × 15 mL). The combined extracts were washed with a saturated brine solution, dried over Na_2SO_4, and concentrated. The resulting product was purified by column chromatography on silica gel using hexane−EtOAc (3:1), which afforded 0.17 g of 5,9-hexadecadiynoic acid for an 81% yield: IR (neat) ν_{max} 2957, 2929, 2855, 2217, 1687, 1458, 1438, 1411, 1339, 1262, 1207, 1155, 1060, 1028, 918, 746, 729 cm^{-1}; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 2.50 (2H, t, J = 7.4 Hz, H-2), 2.32 (4H, m, H-7, H-8),

2.24 (2H, brt, J = 6.9 Hz, H-4), 2.13 (2H, brt, J = 6.9 Hz, H-11), 1.80 (2H, q, J = 7.0 Hz, H-3), 1.48−1.27 (8H, m, H-12, H-13, H-14, H-15), 0.88 (3H, t, J = 7.0 Hz, −CH_3); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 179.5 (s, C-1), 81.3 (s, C-6), 80.0 (s, C-9), 79.5 (s, C-5), 78.6 (s, C-10), 32.7 (t, C-2), 31.3 (t, C-14), 28.9 (t), 28.5 (t), 23.7 (t), 22.5 (t), 19.40 (t), 19.37 (t), 18.7 (t), 18.1 (t), 14.0 (q, C-16); GC−MS (70 eV) m/z 248 [M$^+$] (0.1), 219 (4), 213 (5), 205 (8), 191 (13), 187 (4), 177 (17), 175 (21), 161 (11), 159 (11), 145 (24), 135 (13), 133 (29), 131 (58), 129 (12), 124 (12), 119 (64), 117 (100), 115 (22), 105 (93), 97 (10), 95 (16), 91 (98), 81 (41), 79 (70), 77 (53), 69 (21), 67 (49), 65 (32), 57 (13), 55 (69).

(5*Z*,9*Z*)-5,9-Hexadecadienoic Acid. Into a 15 mL round-bottom flask, equipped with a magnetic stirrer and 10 mL of dry hexane, were placed 0.015 g (0.04 mmol) of 5,9-hexadecadiynoic acid, 0.2 equiv of quinoline, and 0.010 g of Lindlar's catalyst. After a couple of purging cycles, hydrogen was added until a volume equivalent to the initial amount of alkynes was consumed. After filtration and removal of the solvent in vacuo, 0.012 g (80% yield) of the previously reported (5*Z*,9*Z*)-5,9-hexadecadienoic acid was obtained.[9]

Antibacterial Activity. Antibacterial activity against *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), and *S. faecalis* − group D (ATCC 29212) was determined following National Committee of Clinical Laboratory Standards (NCCLS).[15] A 200 μL solution of the hexadecadienoic acid in Mueller-Hinton broth was inoculated with 10^5 colony-forming units in a 96-well plate. The minimal inhibitory concentration (MIC) was determined after an overnight incubation of the fatty acid and the microorganisms at 37 °C. The MIC was determined by observing the highest dilution of the fatty acid that inhibited growth when compared to an uninoculated chemical-control well. The generated data were taken from at least three separate experiments in duplicate.

DNA Topoisomerase I Assay. The enzyme activity of topoisomerase I was assessed with a Topoisomerase I Drug Screening Kit (TopoGEN, Inc., Columbus, OH) using topoisomerase I from human placenta (1 unit relaxes 0.5 μg of DNA in 15 min at 37 °C) and 0.25 μg of supercoiled pHOT1 plasmid DNA. Fatty acids were dissolved in DMSO and were tested at a final concentration of 1000, 800, 500, 100, and 10 μM. Reactions (final volume 20 μL) were carried out for 30 min at 37 °C, after which 2 μL of 10% sodium dodecyl sulfate (SDS) was added. Bound protein was digested by incubation with proteinase K (final concentration 0.05 mg/mL) for 30 min at 37 °C. Reactions were stopped by adding 5 μL of electrophoresis loading buffer (0.25% bromophenol blue, 50% glycerol) and then were electrophoresed in a 1% agarose gel (70 V/105 min). To visualize the reaction products, the gel was stained with 0.5 μg/mL ethidium bromide for 45 min and destained for 30 min in distilled water. DNA bands were detected and quantitated in a Versa Doc imaging system (model 1000, Bio Rad).

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