

Supplementary material

N-Alkylamides from *Piper longum* L. and their stimulative effects on the melanin content and tyrosinase activity in B16 melanoma cells

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Abstract: *Piper longum* L., known as long pepper, is an edible and medicinal plant used as spice and for the treatment of stomach disease and analgesia in traditional Chinese medicine. *N*-Alkylamides are the major secondary metabolites in this plant. Sixteen known *N*-alkylamides were isolated from *P. longum*. Their structures were established on the basis of spectroscopic data and comparison to reported literatures. Among them, five compounds were isolated from this plant for the first time. Ethanol extract, compounds **1**, **2**, **3**, **7** and **11** exhibited potent ability to increase the melanin content and weak stimulative effect on the tyrosinase activity in a concentration-dependent manner. Moreover, compound **2** also presented strong capacity to increase the tyrosinase activity in a concentration-dependent manner. These results indicated that *P. longum* might be a good natural source of lead compound for skin disorder diseases.

Key words: *Piper longum* L.; *N*-alkylamides; melanin content; tyrosinase activity

Experimental

General procedures

NMR spectra were recorded on Varian MR-400 or 600 (400 or 600 MHz for ^1H and 100 or 150 MHz for ^{13}C) spectrometer. Mass spectra were measured on a 2690-ZQ 4000 Water-Alliance LC-MS spectrometer (Applied Biosystems/MDS Sciex Concord, ON, Canada). Column chromatographic separation was performed on Sephadex LH-20 gel (Amersham Pharmacia Biotech, Stockholm, Sweden), ODS-A (YMC CO., LTD., Japan) and silica gel (Qingdao Haiyang Chemical Factory, Qingdao, China). Organic solvents for analytical and semi-preparative HPLC were used for HPLC grade and purchased from Merck (Merck KGaA, Darmstadt, Germany). Dimethylsulfoxide (DMSO), mushroom tyrosinase, and L-3, 4-dihydroxyphenylalanine (L-DOPA), were purchased from Sigma (St. Louis, MO, USA).

Plant material

The dried fruits of *P. longum* were purchased from local Chinese medicinal herb store. The identification of the plant was performed by Prof. Dr. Ying Fen of Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, and voucher specimen (PL 20151219) has been deposited in Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences.

Extraction and isolation procedures

The dried and powdered fruits (4.5 kg) were extracted three times at room temperature with 95% EtOH (each time 20 L), and was concentrated under vacuum at 45°C to give 450 g of ethanol crude extract. The extract was transferred with the mixture of chloroform and methanol from rotary bottle to a plate. Crystals were yielded from the crude extract in the process of the volatilization of organic solvent, then was picked up and cleaned with methanol rapidly to obtain compound **1** (100.0 mg). The crude extract was suspended in H_2O and successively extracted with petroleum ether, ethyl acetate and *n*-butanol. The resulting ethyl acetate fraction (102 g) was subjected to silica gel chromatography eluting with a step gradient solvent system of chloroform–methanol (100:0, 50:1, 20:1, 10:1, 5:1, 1:1) to afford thirty

fractions (Fr1–Fr30). Fractions Fr1 to Fr 8 were combined after developing their thin layer chromatography (TLC) profiles giving fraction F1, which was further separated by silica gel chromatography eluting with a step gradient solvent system of petroleum ether–acetyl acetate (100:0), and then chloroform–methanol (100:0, 100:1, 50:1, 20:1, 10:1, 5:1, 1:1) to afford 99 subfractions (F1-1–F1-99). Compounds **2** (5.2 mg), **3** (20.7 mg) and **9** (40.1 mg) were crystallized from subfractions F1-47, F1-56, and F1-81, respectively. Subfractions F1-33 to F1-35 were combined and then further purified with semi-preparative HPLC [Waters X-Bridge PreC18 150 × 10 mm, 5 µm, an isogradient solvent system of acetonitrile–H₂O in 57:43 (v/v), flow rate 3.0 mL/min] to yield compounds **15** (3.5 mg) and **16** (3.2 mg). Subfractions F1-56 to F1-64 were combined and further purified to generate compounds **6** (1.5 mg) and **10** (12.6 mg). Subfractions F1-71 to F1-74 were combined and subsequently separated by Sephadex LH-20 and semi-preparative HPLC [Waters X-Bridge PreC18 150 × 10 mm, 5 µm, a isogradient solvent system of acetonitrile–H₂O from 42:58 (v/v) to 70:30 (v/v) in 40 min] to obtain compounds **4** (4.9 mg), **8** (16.8 mg), and **11** (18.2 mg). Subfractions F1-81 to F1-88 were combined to give fraction F2, which was subjected to ODS gel chromatography eluting with methanol–H₂O (40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 100:0) to generate 220 subfractions (F2-1–F2-220). Subfractions F2-30 to F2-51 were combined and further purified by semi-preparative HPLC [Waters SunFire TM RP C18, 10 × 150 mm, 5 µm, a linear gradient solvent system of MeOH–H₂O from 58:42 (v/v) to 67:33 (v/v) in 30 min, flow rate 3.0 mL/min] to give compounds **5** (4.9 m), **7** (40.3 mg), **12** (8.7 mg), **13** (9.3 mg), and **14** (6.5 mg).

Melanin content determination

The ethanol extract, compounds **1–3**, **5**, **7–9**, and **11–12** were tested for their effect on melanin content in B16 melanoma cells. Intracellular melanin content was measured using a previously described NaOH dissolution method (Mamat et al. 2017). The murine B16 melanoma cells, which were purchased from the Type Culture Collection of the Chinese Academy of Sciences, were cultured in DMEM (Gibco, Gaithersburg, USA), and then supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 µg/mL streptomycin (Gibco) and 100 U/mL penicillin (Gibco). The cells were maintained in a humidified incubator with 5% CO₂ at 37 °C, which were sub-cultured every two days to maintain logarithmic growth. B16 cells

were seeded in six-well plates at a density of 2×10^5 cells per well. After incubation for 24 h, the cells were treated with different concentrations of the tested samples for 48 h, treated with 50 $\mu\text{mol/L}$ 8-MOP as a positive control, and control group was added 2 μL DMSO. Then, B16 cells were washed twice with PBS (pH = 7.4), and lysed in 100 μL RIPA buffer for 40 min at 4 °C. The lysates were centrifuged at 12,000 $\times g$ for 20 min at 4 °C. The protein content of supernatants was valued by the BCA assay and after the pellets were treated in 190 μL of 1 mol/L NaOH (with 1% DMSO) for 1 h at 80 °C, melanin content was detected at 405 nm by a multi-plate reader (SpectraMax M5/M5e, Molecular Devices, Sunnyvale, CA, USA). The melanin content was calculated and corrected for the concentrations of proteins, using control group as 100%.

Tyrosinase activity determination

The ethanol extract, compounds **1–3**, **7** and **11** were evaluated for their effect on tyrosinase activity in B16 melanoma cells. TYR activity was estimated by measuring the rate of L-DOPA oxidation (Mamat et al. 2017). B16 cells were seeded in a six-well plate at a density of 3×10^5 cells per well. Then, the cells were treated with different concentrations of samples for 24 h, with 50 $\mu\text{mol/L}$ 8-MOP as the positive control, and control group was added 2 μL DMSO. After that, cells were lysed with PBS containing 1% Triton X-100 and 1% sodium deoxycholate for 30 min at -20 °C. The lysates were centrifuged at 12,000 $\times g$ for 15 min. A reaction mixture containing 90 mL of each cell lysate, 10 μL of 10 mmol/L L-DOPA was added to each well of a 96-well plate. Following a 20–60 min (according to the content of dopachrome formation) incubation at 37 °C in the darkness, the product dopachrome was detected at 490 nm by a multi-plate reader (SpectraMax M5/M5e), the tyrosinase activity of each sample was calculated and corrected for the concentrations of proteins, using control groups as 100%.

Results and discussion

Characterization of constituents

Piperine (1)

White amorphous powder. ESI-MS m/z 288.5 ($[M + H]^+$, $C_{17}H_{21}NO_3^+$). 1H (400 MHz, $CDCl_3$): δ = 7.39 (1H, ddd, J = 14.8, 8.0, 1.2 Hz, H-3), 6.97 (1H, d, J = 1.6 Hz, H-2'), 6.87 (1H, dd, J = 8.0, 1.6 Hz, H-6'), 6.76 (1H, d, J = 8.0 Hz, H-5'), 6.74 (1H, d, J = 14.4 Hz, H-5), 6.73 (1H, dd, J = 14.4, 8.0 Hz, H-4), 6.42 (1H, d, J = 14.8 Hz, H-2), 5.96 (2H, s, H₂-7'), 3.57 (4H, m, H₂-1'', H₂-5''), 1.65 (2H, m, H₂-3''), 1.58 (4H, m, H₂-2'', H₂-4''). ^{13}C (100 MHz, $CDCl_3$): δ = 165.5 (C-1), 148.3 (C-3'), 148.2 (C-4'), 142.6 (C-3), 138.3 (C-5), 131.1 (C-1'), 125.5 (C-4), 122.6 (C-6'), 120.2 (C-2), 108.6 (C-5'), 105.8 (C-2'), 101.4 (C-7'), 47.0 (C-5''), 43.4 (C-1''), 26.8 (C-4''), 25.8 (C-2''), 24.8 (C-3'') (DeAraujoJunior et al., 1997).

Isopiperine (2)

White amorphous powder. HR-ESI-MS m/z 286.1431 ($[M + H]^+$, $C_{17}H_{19}NO_3^+$, calc. 286.1438). 1H (400 MHz, $CDCl_3$): δ = 7.42 (1H, ddd, J = 15.6, 11.2, 0.8 Hz, H-3), 7.02 (1H, d, J = 1.2 Hz, H-2'), 6.89 (1H, dd, J = 8.0, 1.2 Hz, H-6'), 6.74 (1H, d, J = 8.0, 1.6 Hz, H-5'), 6.61 (1H, d, J = 15.6 Hz, H-5), 6.49 (1H, dd, J = 11.2, 11.2 Hz, H-3), 5.98 (1H, d, J = 11.2 Hz, H-2), 5.95 (2H, s, H₂-7'), 3.62 (2H, m, H₂-2''), 3.47 (2H, m, H₂-5''), 1.65 (2H, m, H₂-3''), 1.58 (4H, m, H₂-2'', H₂-4''). ^{13}C (100 MHz, $CDCl_3$): δ = 166.2 (C-1), 148.2 (C-3'), 148.1 (C-4'), 138.5 (C-3), 137.7 (C-5), 131.4 (C-1'), 123.9 (C-4), 122.5 (C-6'), 120.0 (C-2), 108.4 (C-5'), 106.3 (C-2'), 101.3 (C-7'), 47.6 (C-5''), 42.8 (C-1''), 26.3 (C-2'', 4''), 24.7 (C-3'') (Hashimoto et al., 1996).

Piperlonguminine (3)

White amorphous powder. HR-ESI-MS m/z 274.1433 ($[M + H]^+$, $C_{16}H_{20}NO_3^+$, calc. 274.1438). 1H (400 MHz, $CDCl_3$): δ = 7.36 (1H, dd, J = 15.2, 10.8 Hz, H-3), 6.97 (1H, d, J = 1.2 Hz, H-2'), 6.88 (1H, dd, J = 8.0, 1.2 Hz, H-6'), 6.77 (1H, d, J = 15.2 Hz, H-5), 6.77 (1H, d, J = 8.0 Hz, H-5'), 6.66 (1H, dd, J = 15.2, 10.8 Hz, H-4), 5.97 (2H, s, H₂-7'), 5.92 (1H, d, J = 15.2 Hz, H-2), 3.19 (2H, t, J = 6.4 Hz, H₂-1''), 1.83 (1H, m, H-2''), 0.94 (6H, d, J = 6.8 Hz, H₃-3'', H₃-4''). ^{13}C (100 MHz, $CDCl_3$): δ = 166.3 (C-1), 148.3 (C-3', 4'), 141.2 (C-3), 138.9 (C-5), 131.0 (C-1'), 124.8 (C-4), 123.3 (C-2), 122.7 (C-6'), 108.6 (C-5'), 105.9 (C-2'), 101.4 (C-7'), 47.2 (C-1''), 28.8 (C-2''), 20.3 (C-3'', 4'') (Wu et al., 2004).

$\Delta^{\alpha,\beta}$ -Dihydropiperine (4)

White amorphous powder. HR-ESI-MS m/z 288.1589 ($[M + H]^+$, $C_{17}H_{21}NO_3^+$, calc. 288.1594). 1H (600 MHz, $CDCl_3$): δ = 6.77 (1H, dt, J = 15.0, 6.6 Hz, H-3), 6.69 (1H, d, J = 8.4, H-5'), 6.64 (1H, d, J = 1.2 Hz, H-2'), 6.59 (1H, dd, J = 8.4, 1.2 Hz, H-6'), 6.18 (1H, d, J = 15.6 Hz, H-2), 5.88 (2H, s, H₂-7'), 3.55 (2H, m, H₂-1''), 3.38 (2H, m, H₂-5''), 2.66 (2H, m, H₂-5), 2.44 (2H, m, H₂-4), 1.61 (2H, m, H₂-3''), 1.52 (4H, m, H₂-2'', H₂-4''). ^{13}C (150 MHz, $CDCl_3$): δ = 165.5 (C-1), 147.6 (C-3'), 145.8 (C-4'), 144.0 (C-3), 135.0 (C-1'), 121.5 (C-2), 121.2 (C-6'), 108.9 (C-2''), 108.2 (C-5''), 100.8 (C-7''), 46.9 (C-5''), 43.1 (C-1''), 34.6 (C-4), 34.5 (C-5), 26.7 (C-4''), 25.6 (C-2''), 24.7 (C-3'') (Pedersen et al., 2009).

Dihydropiperlonguminine (5)

White amorphous powder. HR-ESI-MS m/z 276.1586 ($[M + H]^+$, $C_{16}H_{22}NO_3^+$, calc. 276.1594). 1H (400 MHz, $CDCl_3$): δ = 6.84 (1H, dt, J = 15.6, 6.8 Hz, H-3), 6.72 (1H, d, J = 8.0 Hz, H-5'), 6.66 (1H, d, J = 1.2 Hz, H-2'), 6.62 (1H, dd, J = 8.0, 1.2 Hz, H-6'), 5.92 (2H, s, H₂-7'), 5.76 (1H, d, J = 15.6 Hz, H-2), 3.15 (2H, t, J = 6.4 Hz, H₂-1''), 2.68 (2H, m, H₂-5), 2.45 (2H, m, H₂-4), 1.79 (1H, m, H-2''), 0.92 (6H, d, J = 6.4 Hz, H₃-3'', H₃-4''). ^{13}C (100 MHz, $CDCl_3$): δ = 166.0 (C-1), 147.8 (C-3'), 145.9 (C-4'), 143.4 (C-3), 135.0 (C-1'), 124.4 (C-2), 121.3 (C-6'), 108.9 (C-2''), 108.3 (C-5''), 101.0 (C-7''), 47.0 (C-1''), 34.5 (C-5), 34.2 (C-4), 28.7 (C-2''), 20.3 (C-3'', 4'') (Xu et al., 2016).

Dehydropiperonaline (6)

White amorphous powder. HR-ESI-MS m/z 340.1911 ($[M + H]^+$, $C_{21}H_{25}NO_3^+$, calc. 340.1907). 1H (600 MHz, $CDCl_3$): δ = 7.23 (1H, dd, J = 14.4, 10.8 Hz, H-3), 6.88 (1H, br s, H-2'), 6.75 (1H, br d, J = 7.8 Hz, H-6'), 6.73 (1H, d, J = 7.8 Hz, H-5'), 6.31 (1H, d, J = 16.2 Hz, H-9), 6.27 (1H, d, J = 14.4 Hz, H-2), 6.21 (1H, dd, J = 14.4, 10.8 Hz, H-4), 6.06 (1H, m, H-5), 6.01 (1H, m, H-8), 5.93 (2H, s, H₂-7'), 3.61 (2H, m, H₂-2''), 3.49 (2H, m, H₂-5''), 2.31 (4H, m, H₂-6, H₂-7), 1.65 (2H, m, H₂-3''), 1.57 (4H, m, H₂-2'', H₂-4''). ^{13}C (150 MHz, $CDCl_3$): δ = 165.6 (C-1), 147.9 (C-3'), 146.7 (C-4'), 142.7 (C-5), 141.1 (C-3), 132.1 (C-1'), 130.1 (C-9), 129.4 (C-4), 127.8 (C-8), 119.1 (C-2), 120.5 (C-6'), 108.2 (C-5'), 105.4 (C-2'), 100.9 (C-7'), 46.9 (C-5''), 43.2 (C-1''), 32.9 (C-6), 32.2 (C-7), 26.7 (C-4''), 25.6 (C-2''), 24.7 (C-3'') (Shoji et al., 1986).

Retrofractamide A (7)

White amorphous powder. HR-ESI-MS m/z 328.1909 ($[M + H]^+$, $C_{20}H_{26}NO_3^+$, calc. 328.1907). 1H (400 MHz, $CDCl_3$): δ = 7.18 (1H, dd, J = 14.8, 10.4 Hz, H-3), 6.87 (1H, br s, H-2'), 6.74 (1H, br d, J = 8.0, H-6'), 6.72 (1H, d, J = 8.0 Hz, H-5'), 6.30 (1H, d, J = 16.0 Hz, H-9), 6.16 (1H, dd, J = 14.8, 10.4 Hz, H-4), 6.06 (1H, m, H-5), 6.01 (1H, m, H-8), 5.92 (2H, s, H₂-7'), 5.78 (1H, d, J = 14.8 Hz, H-2), 3.15 (2H, t, J = 6.4 Hz, H₂-1''), 2.30 (4H, m, H₂-6, H₂-7), 1.79 (1H, m, H-2''), 0.91 (6H, d, J = 6.8 Hz, H₃-3'', H₃-4''). ^{13}C (100 MHz, $CDCl_3$): δ = 166.4 (C-1), 148.0 (C-3'), 146.8 (C-4'), 141.8 (C-5), 141.0 (C-3), 132.2 (C-1'), 130.3 (C-9), 128.9 (C-4), 127.8 (C-8), 122.4 (C-2), 120.5 (C-6'), 108.3 (C-5'), 105.5 (C-2'), 101.1 (C-7'), 47.1 (C-1''), 33.0 (C-6), 32.3 (C-7), 28.7 (C-2''), 20.2 (C-3'', 4'') (Park et al., 2002).

Pipercede (8)

White amorphous powder. HR-ESI-MS m/z 356.2212 ($[M + H]^+$, $C_{22}H_{29}NO_3^+$, calc. 356.2202). 1H (400 MHz, $CDCl_3$): δ = 7.18 (1H, dd, J = 14.8, 10.8 Hz, H-3), 6.88 (1H, br s, H-2'), 6.75 (1H, br d, J = 8.0, H-6'), 6.72 (1H, d, J = 8.0 Hz, H-5'), 6.28 (1H, d, J = 16.0 Hz, H-11), 6.12 (1H, dd, J = 14.8, 10.4 Hz, H-4), 6.07 (1H, m, H-5), 6.02 (1H, m, H-10), 5.92 (2H, s, H₂-7'), 5.76 (1H, d, J = 14.8 Hz, H-2), 3.15 (2H, t, J = 6.4 Hz, H₂-1''), 2.17 (4H, m, H₂-7, H₂-8), 1.79 (1H, m, H-2''), 1.46 (4H, m, H₂-6, H₂-9), 0.91 (6H, d, J = 6.8 Hz, H₃-3'', H₃-4''). ^{13}C (100 MHz, $CDCl_3$): δ = 166.5 (C-1), 148.0 (C-3'), 146.7 (C-4'), 142.8 (C-5), 141.3 (C-3), 132.5 (C-1'), 129.7 (C-11), 129.1 (C-10), 128.5 (C-4), 122.1 (C-2), 120.3 (C-6'), 108.3 (C-5'), 105.5 (C-2'), 101.0 (C-7'), 47.1 (C-1''), 32.9 (C-6), 32.8 (C-9), 29.1 (C-8), 28.8 (C-2''), 28.4 (C-7), 20.3 (C-3'', 4'') (Park et al., 2002).

Guineensine (9)

White amorphous powder. HR-ESI-MS m/z 384.2529 ($[M + H]^+$, $C_{24}H_{34}NO_3^+$, calc. 384.2533). 1H (400 MHz, $CDCl_3$): δ = 7.18 (1H, dd, J = 15.2, 10.0 Hz, H-3), 6.88 (1H, br s, H-2'), 6.75 (1H, br d, J = 8.0, H-6'), 6.73 (1H, d, J = 8.0 Hz, H-5'), 6.27 (1H, d, J = 15.2 Hz, H-13), 6.12 (1H, dd, J = 15.2, 12.0 Hz, H-4), 6.08 (1H, m, H-5), 6.05 (1H, m, H-12), 5.92 (2H, s, H₂-7'), 5.75 (1H, d, J = 15.2 Hz, H-2), 3.16 (2H, t, J = 6.4 Hz, H₂-1''), 2.16 (4H, m, H₂-6, H₂-11), 1.79 (1H, m, H-2''), 1.43 (4H, m, H₂-7, H₂-10), 1.33 (4H, m, H₂-8, H₂-9), 0.92 (6H, d, J = 6.4 Hz, H₃-3'', H₃-4''). ^{13}C (100 MHz, $CDCl_3$): δ = 166.5 (C-1), 148.0 (C-3'), 146.6 (C-4'), 143.1 (C-5), 141.3 (C-3), 132.6 (C-1'), 129.5 (C-12), 129.4 (C-13), 128.4 (C-4), 121.9 (C-2), 120.3 (C-6'),

108.3 (C-5'), 105.5 (C-2'), 101.0 (C-7'), 47.1 (C-1''), 33.0 (C-6, 11), 29.5 (C-10), 29.1 (C-8, 9), 28.8 (C-7, 2''), 20.3 (C-3'', 4'') (Park et al., 2002).

Pipernonaline (10)

White amorphous powder. HR-ESI-MS m/z 342.2050 ($[M + H]^+$, $C_{21}H_{27}NO_3^+$, calc. 342.2064). 1H (600 MHz, $CDCl_3$): δ = 6.88 (1H, d, J = 1.2 Hz, H-2'), 6.82 (1H, dt, J = 16.2, 7.2 Hz, H-3), 6.75 (1H, dd, J = 8.4, 1.2 Hz, H-6'), 6.72 (1H, d, J = 8.4 Hz, H-5'), 6.28 (1H, d, J = 15.6 Hz, H-9), 6.24 (1H, dt, J = 16.2, 11.2 Hz, H-2), 6.02 (1H, dt, J = 15.6, 7.2 Hz, H-2), 5.92 (2H, s, H₂-7'), 3.59 (2H, m, H₂-2''), 3.47 (2H, m, H₂-5''), 2.22 (2H, m, H₂-4), 1.64 (2H, m, H₂-3''), 1.56 (4H, m, H₂-2'', H₂-4''), 1.50 (2H, m, H₂-5), 1.49 (2H, m, H₂-6). ^{13}C (150 MHz, $CDCl_3$): δ = 165.7 (C-1), 148.0 (C-3'), 146.7 (C-4'), 145.7 (C-3), 132.5 (C-1'), 129.7 (C-9), 129.0 (C-8), 120.7 (C-2), 120.3 (C-6'), 108.3 (C-5'), 105.5 (C-2'), 101.0 (C-7'), 47.0 (C-5''), 43.2 (C-1''), 32.8 (C-7), 32.5 (C-4), 29.1 (C-6), 28.1 (C-5), 26.8 (C-4''), 25.7 (C-2''), 24.8 (C-3'') (Lee et al., 2001).

Retrofractamide C (11)

White amorphous powder. HR-ESI-MS m/z 330.2055 ($[M + H]^+$, $C_{20}H_{28}NO_3^+$, calc. 330.2064). 1H (400 MHz, $CDCl_3$): δ = 6.88 (1H, br s, H-2'), 6.83 (1H, dt, J = 15.2, 6.8 Hz, H-3), 6.74 (1H, br d, J = 8.0, H-6'), 6.73 (1H, d, J = 8.0 Hz, H-5'), 6.27 (1H, d, J = 15.6 Hz, H-9), 6.02 (1H, dt, J = 15.6, 6.8 Hz, H-8), 5.92 (2H, s, H₂-7'), 5.76 (1H, dt, J = 15.2, 1.2 Hz, H-2), 3.14 (2H, t, J = 6.4 Hz, H₂-1''), 2.19 (2H, m, H₂-4), 2.17 (2H, m, H₂-7), 1.79 (1H, m, H-2''), 1.50 (2H, m, H₂-5), 1.48 (2H, m, H₂-6), 0.92 (6H, d, J = 6.8 Hz, H₃-3'', H₃-4''). ^{13}C (100 MHz, $CDCl_3$): δ = 166.2 (C-1), 148.1 (C-3'), 146.7 (C-4'), 144.5 (C-3), 132.4 (C-1'), 129.7 (C-9), 129.0 (C-8), 123.9 (C-2), 120.3 (C-6'), 108.3 (C-5'), 105.5 (C-2'), 101.0 (C-7'), 47.0 (C-1''), 32.8 (C-7), 32.0 (C-4), 29.1 (C-6), 28.7 (C-2''), 27.9 (C-5), 20.3 (C-3'', 4'') (Banerji et al., 1985).

trans-Fagaramide (12)

White amorphous powder. HR-ESI-MS m/z 248.1283 ($[M + H]^+$, $C_{14}H_{18}NO_3^+$, calc. 248.1281). 1H (400 MHz, $CDCl_3$): δ = 7.52 (1H, d, J = 15.6 Hz, H-3), 6.98 (1H, d, J = 1.2 Hz, H-2'), 6.95 (1H, dd, J = 8.0, 1.2 Hz, H-6'), 6.76 (1H, d, J = 8.0 Hz, H-5'), 6.25 (1H, d, J = 15.2 Hz, H-2), 5.97 (2H, s, H₂-7'), 3.20 (2H, t, J = 6.4 Hz, H₂-1''), 1.83 (1H, m, H-2''), 0.94 (6H, d, J = 6.8 Hz, H₃-3'', H₃-4''). ^{13}C (100 MHz, $CDCl_3$): δ

= 166.2 (C-1), 149.0 (C-4'), 148.3 (C-3'), 140.6 (C-3), 129.4 (C-1'), 123.8 (C-6'), 119.1 (C-2), 108.6 (C-5'), 106.4 (C-2'), 101.5 (C-7'), 47.2 (C-1''), 28.8 (C-2''), 20.3 (C-3'', 4'') (Cheng et al., 2002).

cis-Fagaramide (13)

White amorphous powder. HR-ESI-MS m/z 248.1284 ([M + H]⁺, C₁₄H₁₈NO₃⁺, calc. 248.1281). ¹H (400 MHz, CDCl₃): δ = 7.11 (1H, d, *J* = 1.6 Hz, H-2'), 6.94 (1H, dd, *J* = 8.0, 1.6 Hz, H-6'), 6.77 (1H, d, *J* = 8.0 Hz, H-5'), 6.63 (1H, d, *J* = 12.4 Hz, H-3), 5.96 (2H, s, H₂-7'), 5.88 (1H, d, *J* = 12.4 Hz, H-2), 3.09 (2H, t, *J* = 6.4 Hz, H₂-1''), 1.73 (1H, m, H-2''), 0.84 (6H, d, *J* = 6.8 Hz, H₃-3'', H₃-4''). ¹³C (100 MHz, CDCl₃): δ = 167.3 (C-1), 148.1 (C-4'), 147.9 (C-3'), 136.1 (C-3), 129.2 (C-1'), 124.0 (C-6'), 123.6 (C-2), 109.3 (C-2'), 108.3 (C-5'), 101.4 (C-7'), 47.0 (C-1''), 28.4 (C-2''), 20.3 (C-3'', 4'') (Cheng et al., 2002).

Piperodione (14)

White amorphous powder. HR-ESI-MS m/z 318.1339 ([M + H]⁺, C₁₇H₁₉NO₅⁺, calc. 318.1336). ¹H (400 MHz, CDCl₃): δ = 7.59 (1H, dd, *J* = 8.0, 1.6 Hz, H-6'), 7.42 (1H, d, *J* = 1.6 Hz, H-2'), 6.85 (1H, d, *J* = 8.0 Hz, H-5'), 6.03 (2H, s, H₂-7'), 3.56 (2H, m, H₂-2''), 3.45 (2H, m, H₂-5''), 3.31 (2H, m, H₂-4), 3.11 (2H, m, H₂-3), 1.66 (2H, m, H₂-3''), 1.65 (2H, m, H₂-4''), 1.61 (2H, m, H₂-2''). ¹³C (100 MHz, CDCl₃): δ = 200.2 (C-2), 196.2 (C-5), 165.8 (C-1), 152.1 (C-4'), 148.3 (C-3'), 131.3 (C-1'), 124.5 (C-6'), 108.0 (C-5'), 107.9 (C-2'), 102.0 (C-7'), 47.0 (C-5''), 42.5 (C-1''), 34.0 (C-3), 32.0 (C-4), 26.5 (C-4''), 25.5 (C-2''), 24.6 (C-3'') (Kubo et al., 2013).

Pellitorine (15)

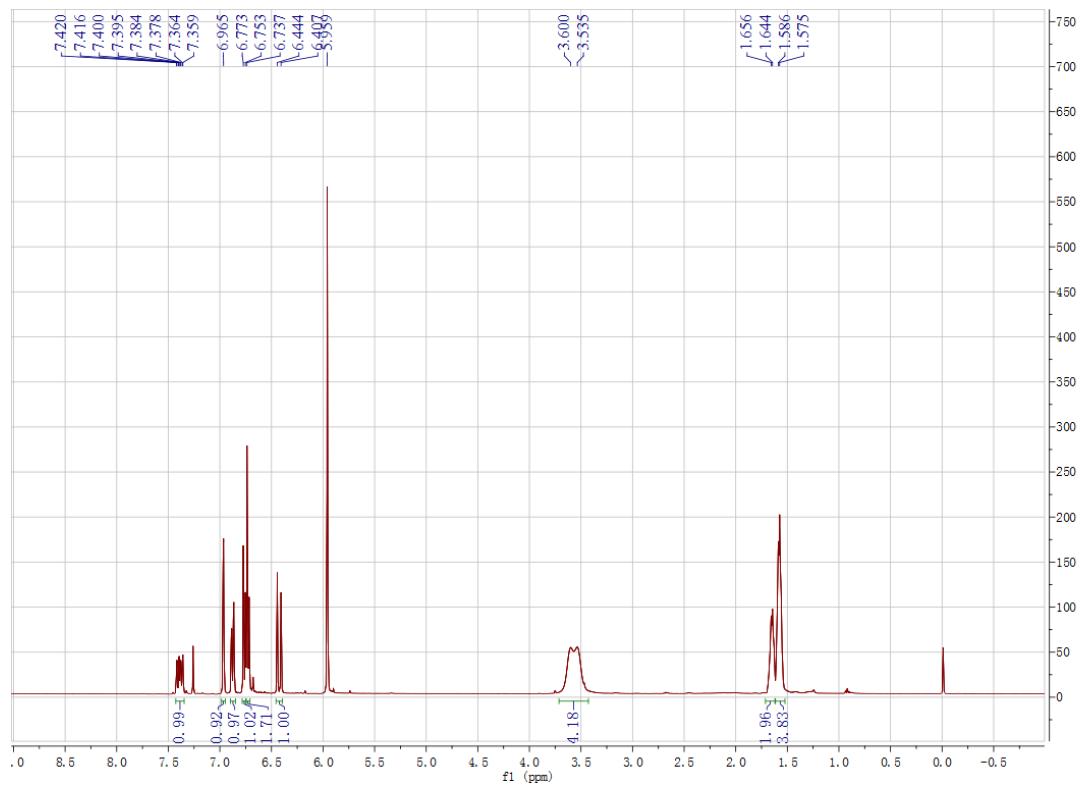
Yellow oil. ¹H (400 MHz, CDCl₃): δ = 7.10 (1H, dd, *J* = 15.2, 10.8 Hz, H-3), 6.06 (1H, dd, *J* = 15.2, 10.8 Hz, H-4), 5.95 (1H, m, H-5), 5.85 (1H, d, *J* = 15.2 Hz, H-2), 3.07 (2H, m, H₂-1'), 2.06 (2H, m, H₂-6), 1.74 (1H, m, H-2'), 1.34 (2H, m, H₂-7), 1.21 (2H, m, H₂-9), 1.19 (2H, m, H₂-8), 0.85 (3H, d, *J* = 6.42 Hz, H₃-3'), 0.83 (3H, d, *J* = 6.4 Hz, H₃-4'), 0.80 (3H, m, H₃-10). ¹³C (100 MHz, CDCl₃): δ = 166.9 (C-1), 142.8 (C-5), 140.9 (C-3), 128.4 (C-4), 122.2 (C-2), 47.0 (C-1'), 32.9 (C-6), 31.3 (C-8), 28.6 (C-2'), 28.5 (C-7), 22.4 (C-9), 20.2 (C-3', 4'), 14.0 (C-10) (Park et al., 2002).

(2E,4E,15Z)-N-isobutyl-eicosa-2,4,15-trienamide (16)

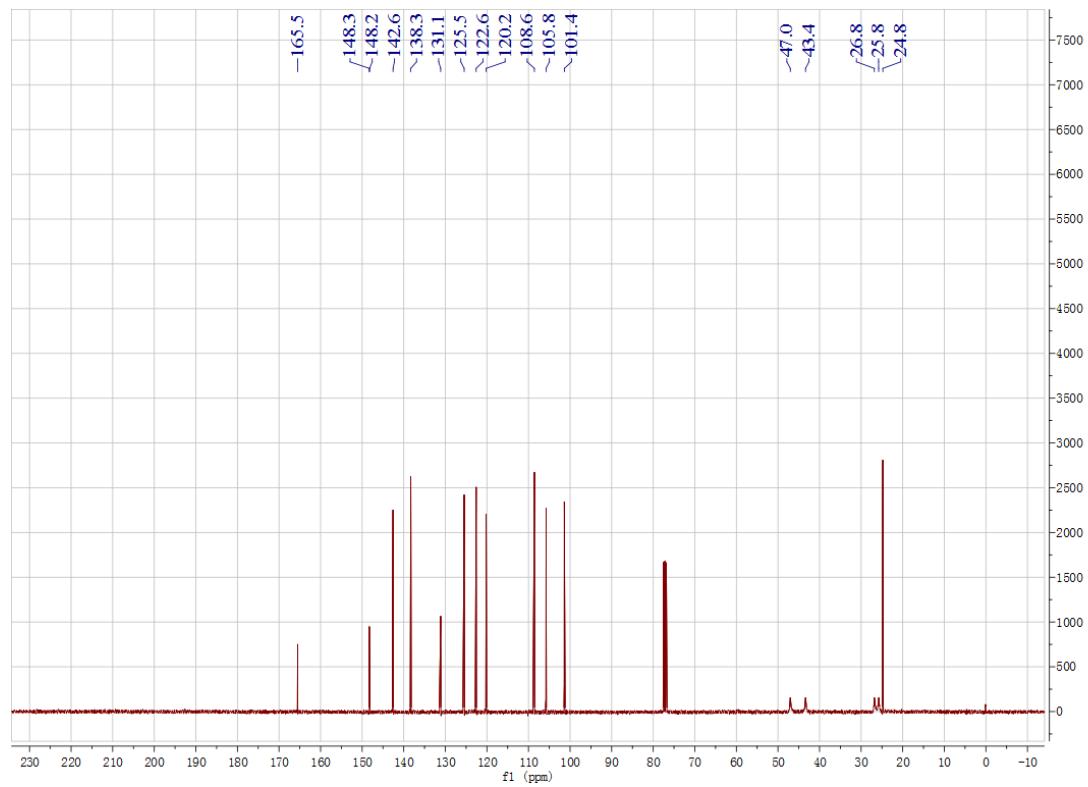
Yellow oil. HR-ESI-MS m/z 362.3406 ([M + H]⁺, C₂₄H₄₃NO⁺, calc. 362.3417).

¹H (600 MHz, DMSO-*d*₆): δ = 7.92 (1H, dd, *J* = 6.6, 6.6 Hz, NH), 6.97 (1H, dd, *J* = 15.6, 10.8 Hz, H-3), 6.16 (1H, dd, *J* = 15.6, 10.8 Hz, H-4), 6.05 (1H, m, H-5), 5.93 (1H, d, *J* = 15.6 Hz, H-2), 5.32 (2H, t, *J* = 5.4 Hz, H-15, 16), 2.93 (1H, t, *J* = 6.6 Hz, H-1'), 2.11 (2H, m, H₂-6), 1.98 (4H, m, H₂-14, H₂-17), 1.69 (1H, m, H-2'), 1.36 (2H, m, H₂-7), 1.29 (2H, m, H₂-9), 1.28 (2H, m, H₂-19), 1.27 (2H, m, H₂-18), 1.23~1.27 (8H, H₂-10, H₂-11, H₂-12, H₂-13), 1.26 (2H, m, H₂-8), 0.86 (3H, m, H₃-20), 0.84 (6H, d, *J* = 6.6 Hz, H₃-3', H₃-4'). ¹³C (150 MHz, DMSO-*d*₆): δ = 165.2 (C-1), 141.5 (C-5), 139.0 (C-3), 129.6 (C-15, 16), 128.6 (C-4), 123.4 (C-2), 46.1 (C-1'), 32.2 (C-6), 31.3 (C-18), 29.1 (C-9), 28.5~28.9 (C-10, 11, 12, 13), 28.5 (C-8), 28.3 (C-7), 28.1 (C-2'), 26.6 (C-14), 26.3 (C-17), 21.7 (C-19), 20.1 (C-3', 4'), 13.8 (C-20) (Dawid et al., 2012).

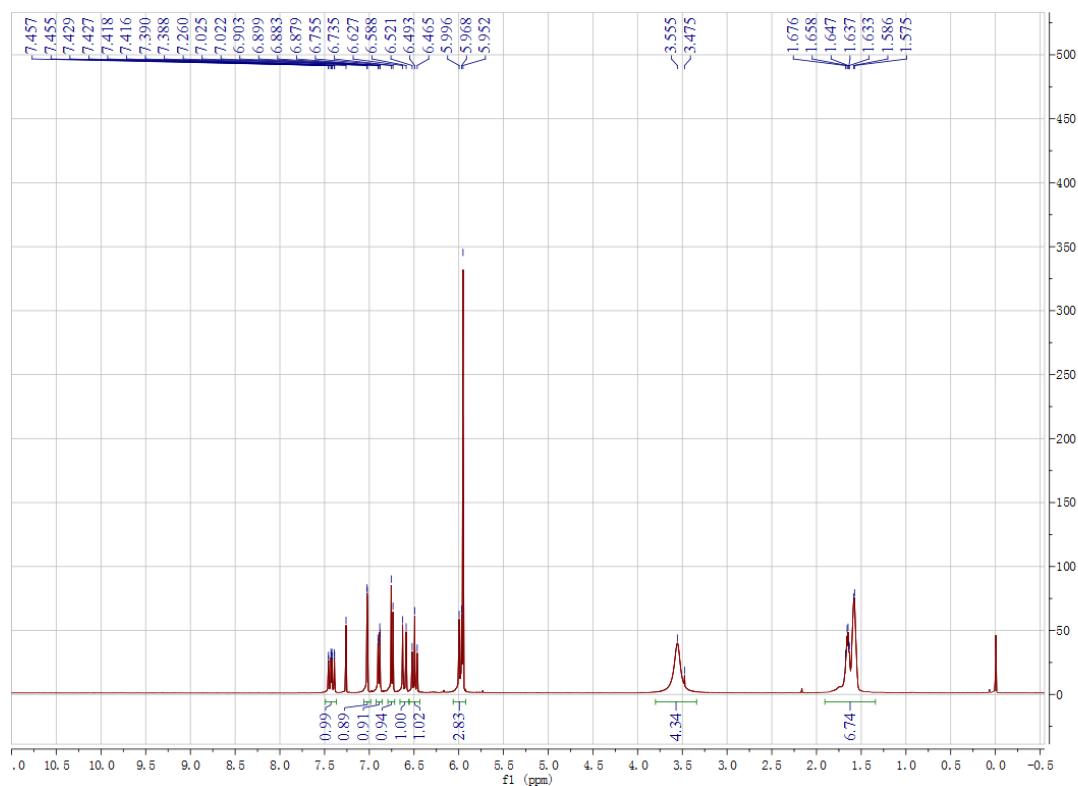
¹H NMR (400 MHz) spectrum of piperine (**1**) in CDCl₃



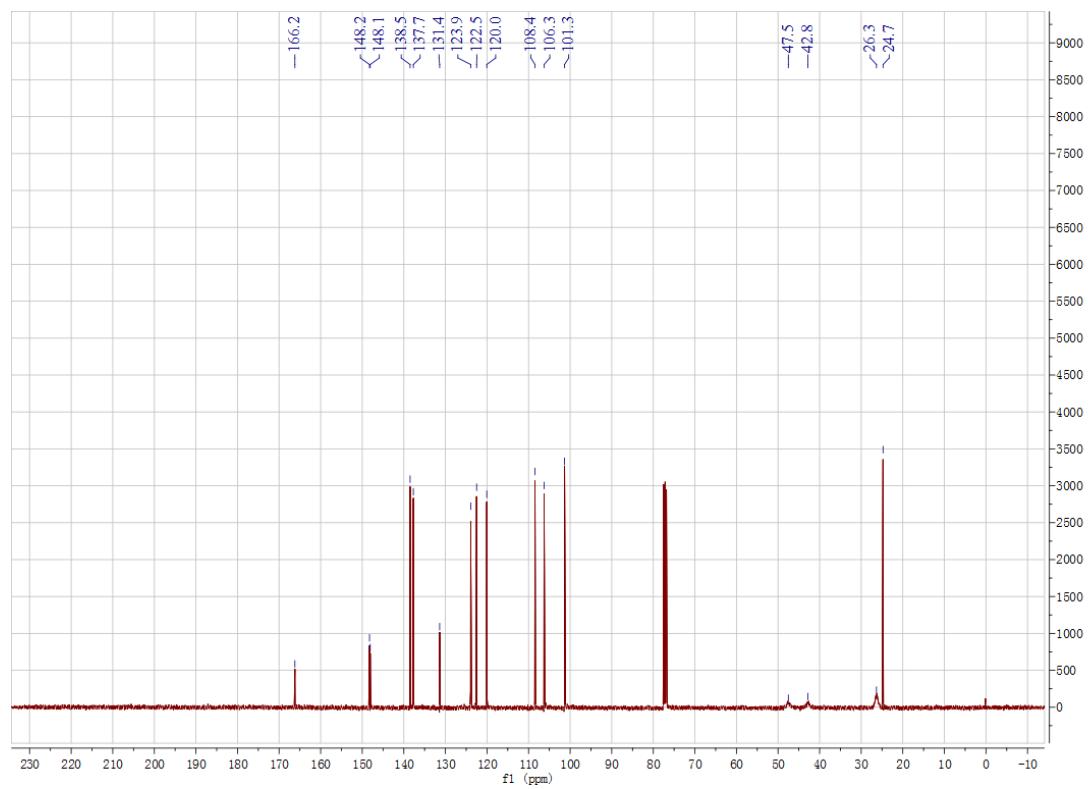
¹³C NMR (100 MHz) spectrum of piperine (**1**) in CDCl₃



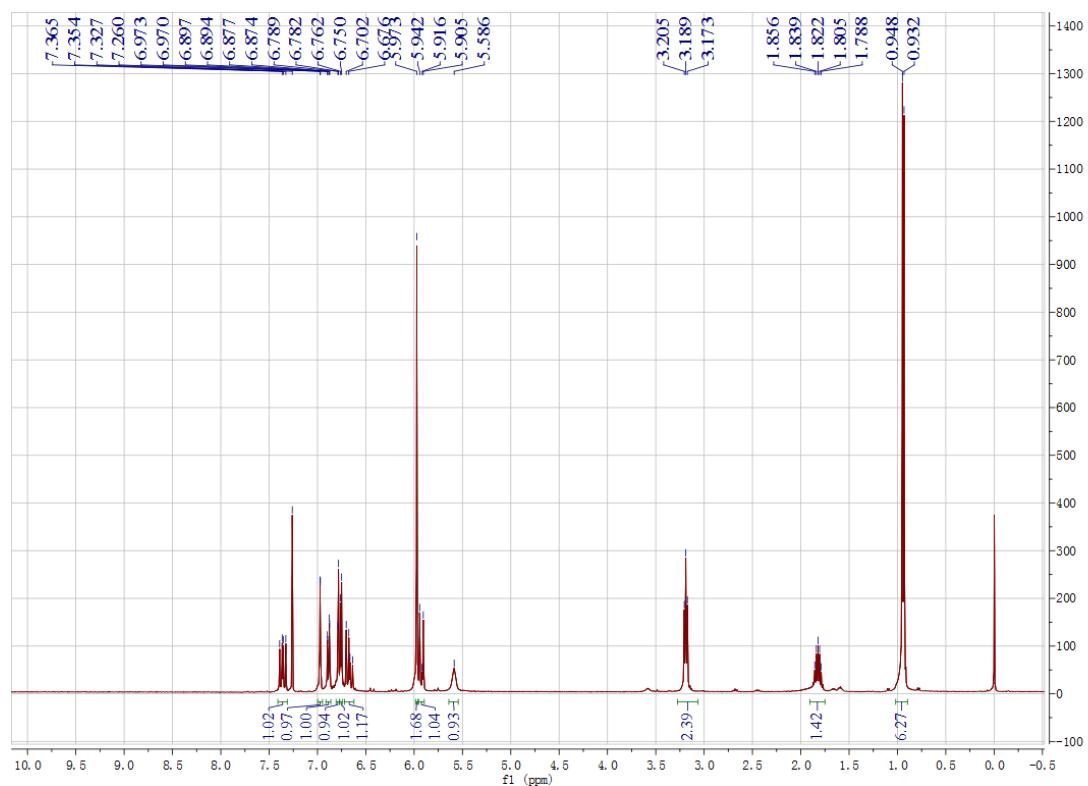
¹H NMR (400 MHz) spectrum of isopiperine (**2**) in CDCl₃



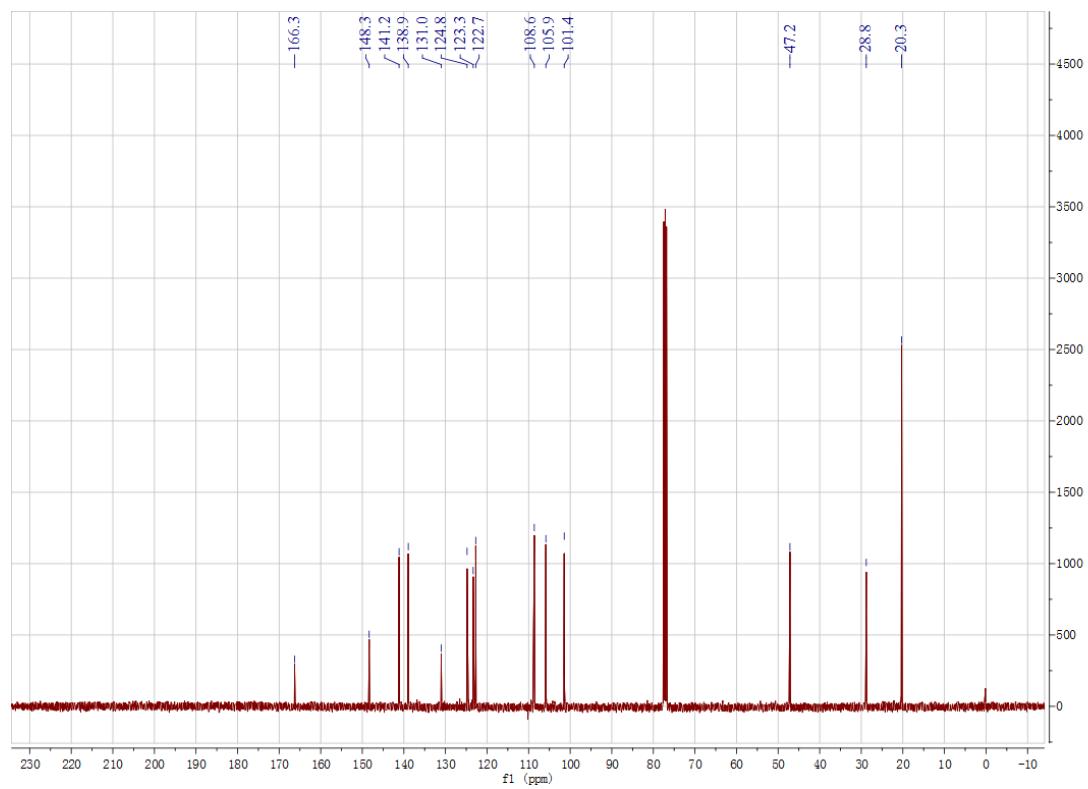
¹³C NMR (100 MHz) spectrum of isopiperine (**2**) in CDCl₃



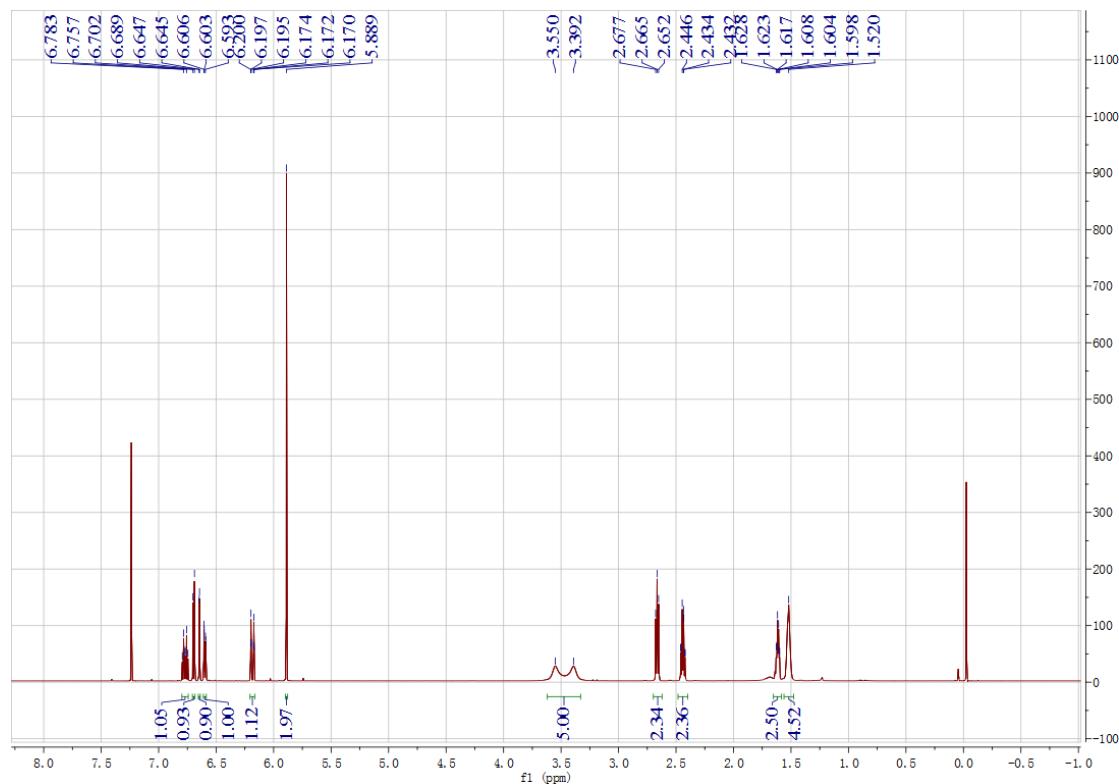
¹H NMR (400 MHz) spectrum of piperlonguminine (**3**) in CDCl₃



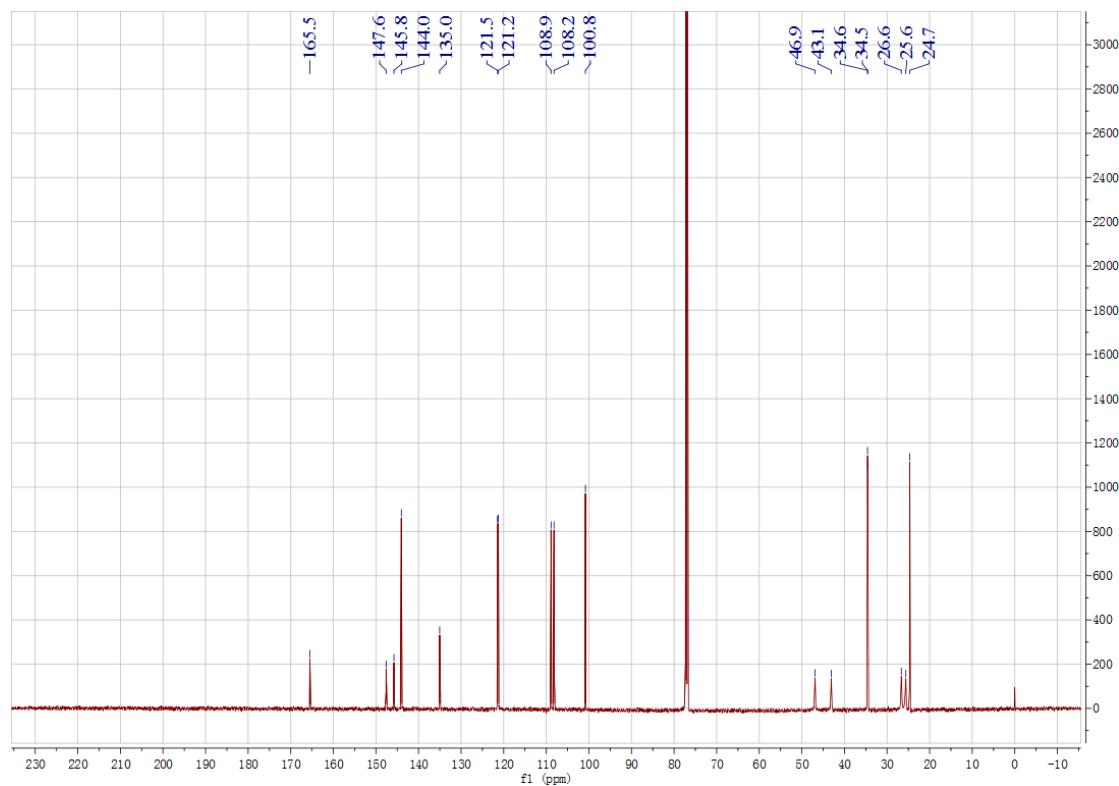
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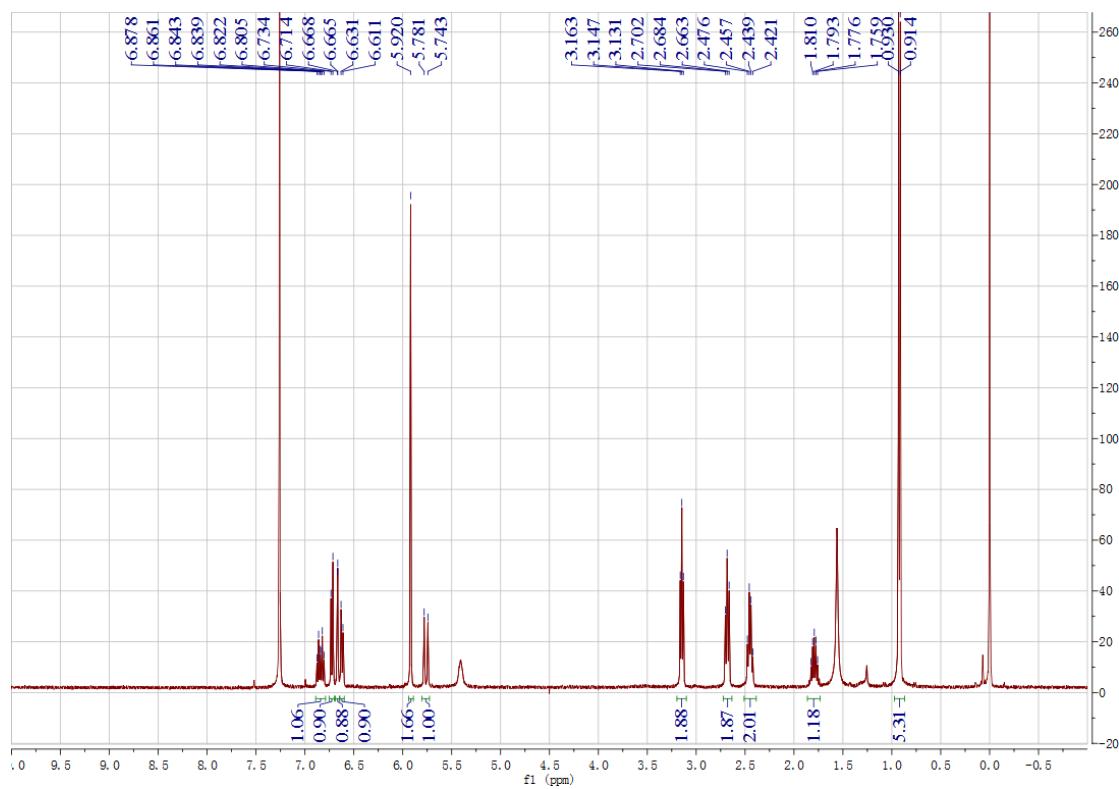
^1H NMR (600 MHz) spectrum of $\Delta^{\alpha,\beta}$ -dihydropiperine (**4**) in CDCl_3



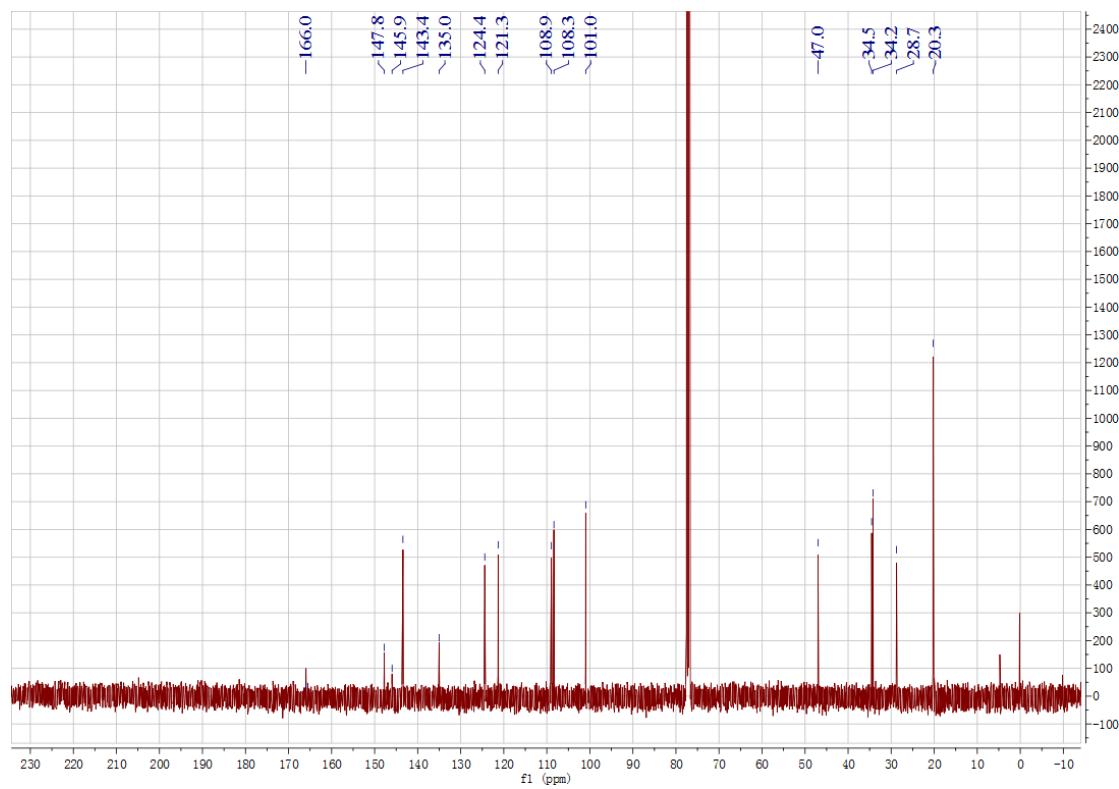
^{13}C NMR (150 MHz) spectrum of $\Delta^{\alpha,\beta}$ -dihydropiperine (**4**) in CDCl_3



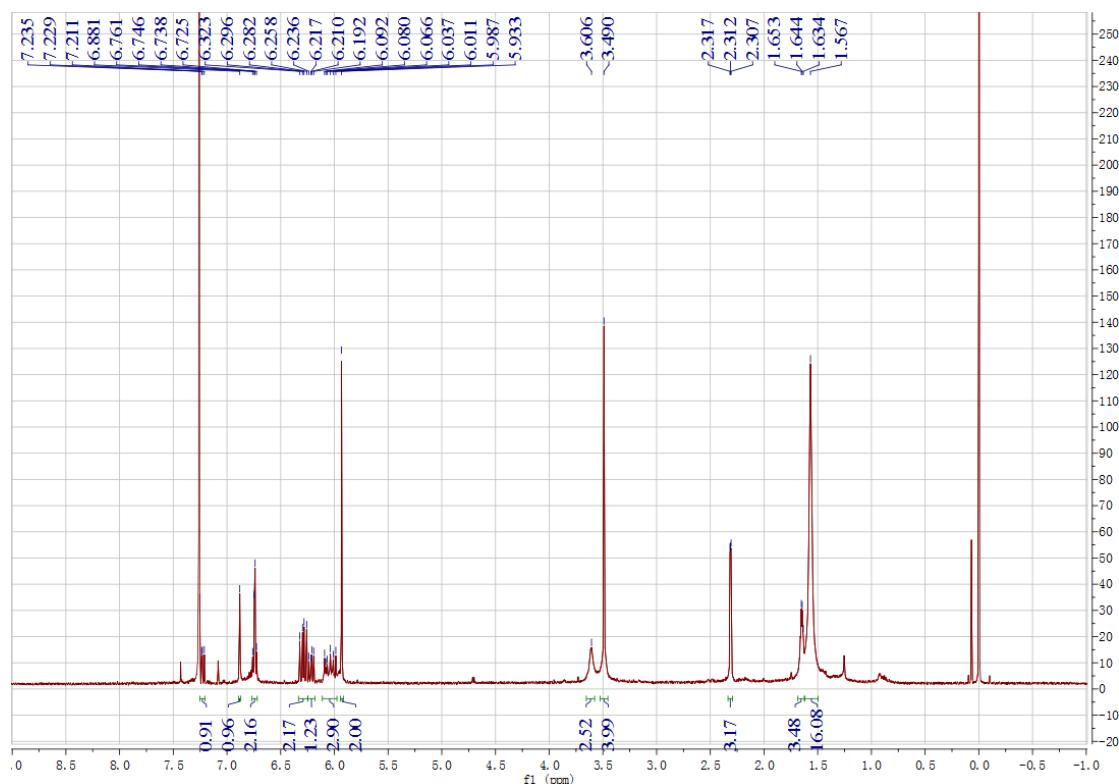
¹H NMR (400 MHz) spectrum of dihydropiperlonguminine (**5**) in CDCl₃



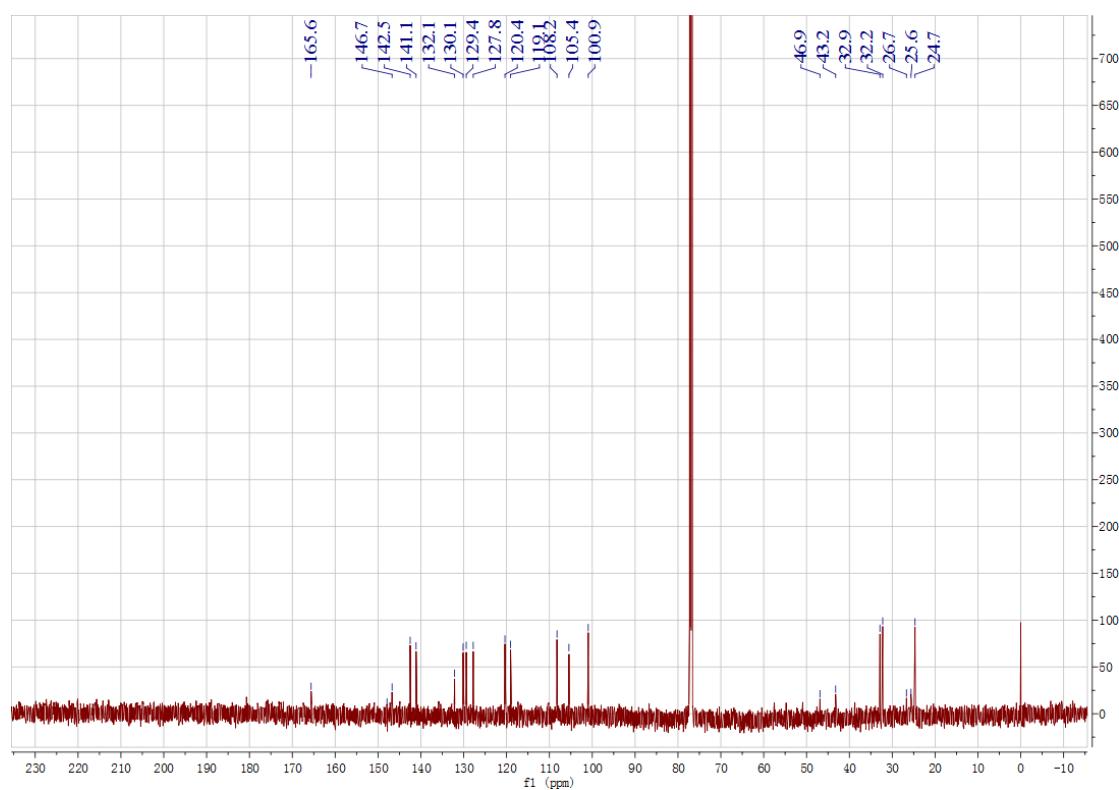
¹³C NMR (100 MHz) spectrum of dihydropiperlonguminine (**5**) in CDCl₃



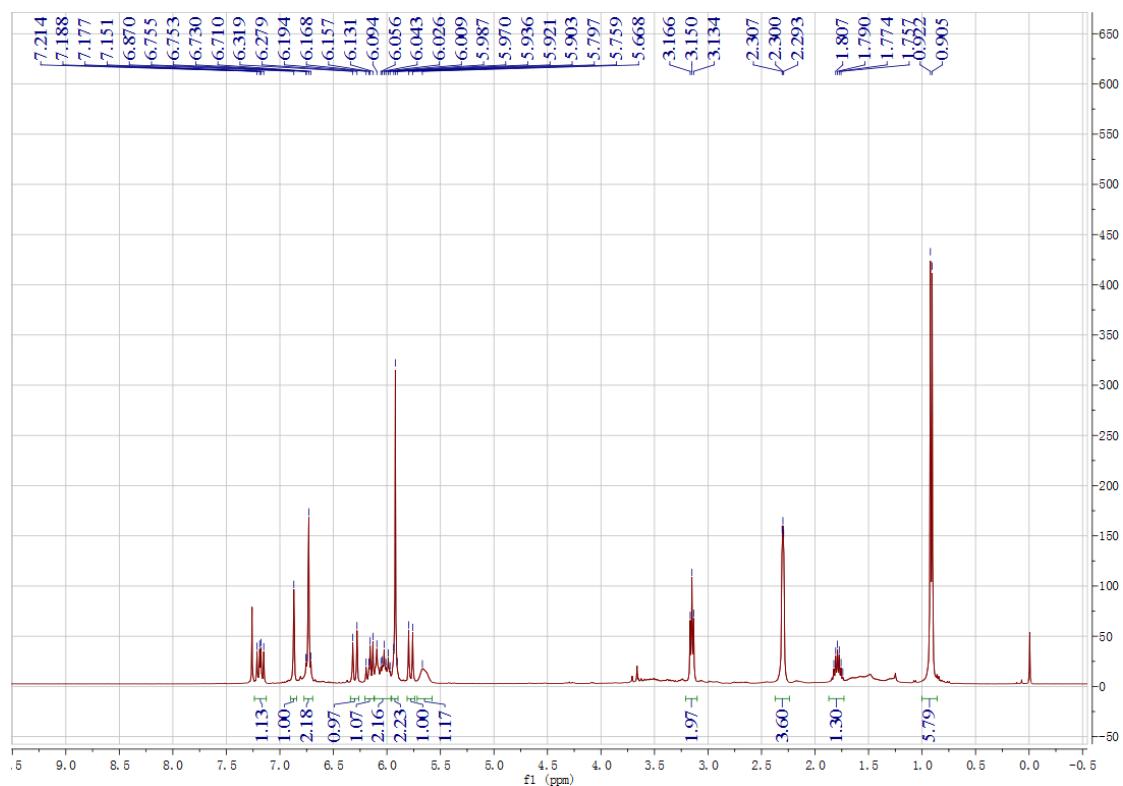
¹H NMR (600 MHz) spectrum of dehydropipernonaline (**6**) in CDCl₃



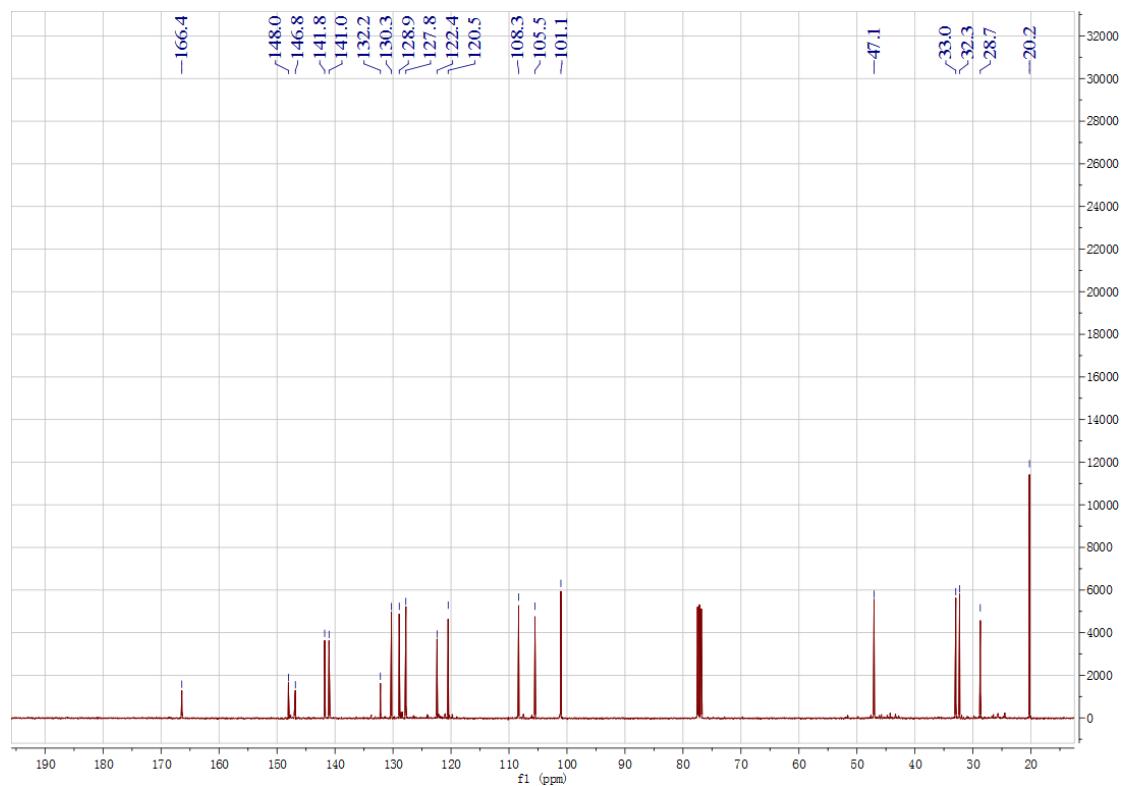
¹³C NMR (150 MHz) spectrum of dehydropipernonaline (**6**) in CDCl₃



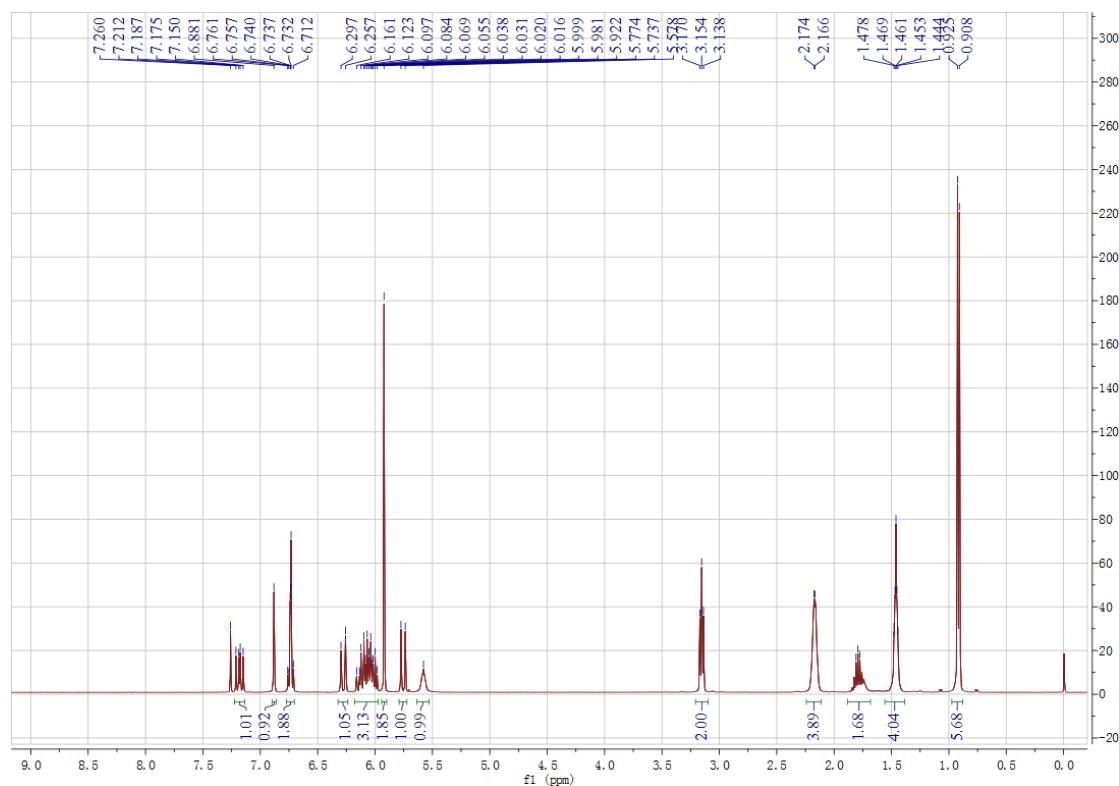
¹H NMR (400 MHz) spectrum of retrofractamide A (**7**) in CDCl₃



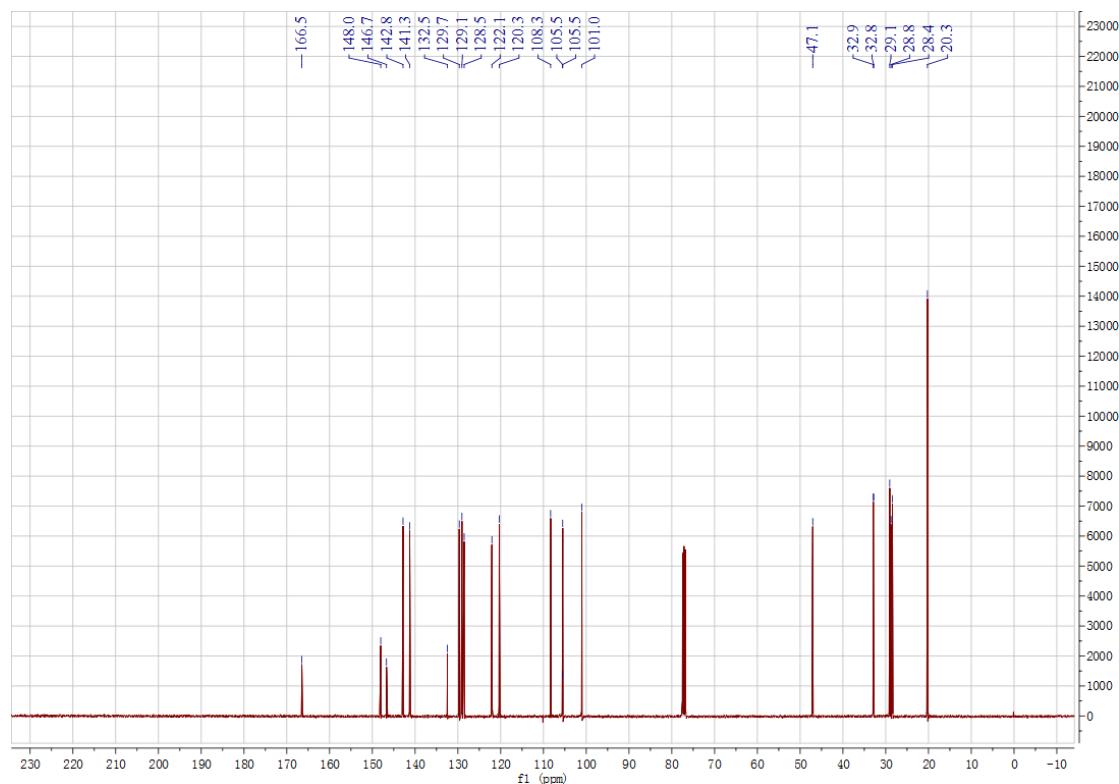
¹³C NMR (100 MHz) spectrum of retrofractamide A (**7**) in CDCl₃



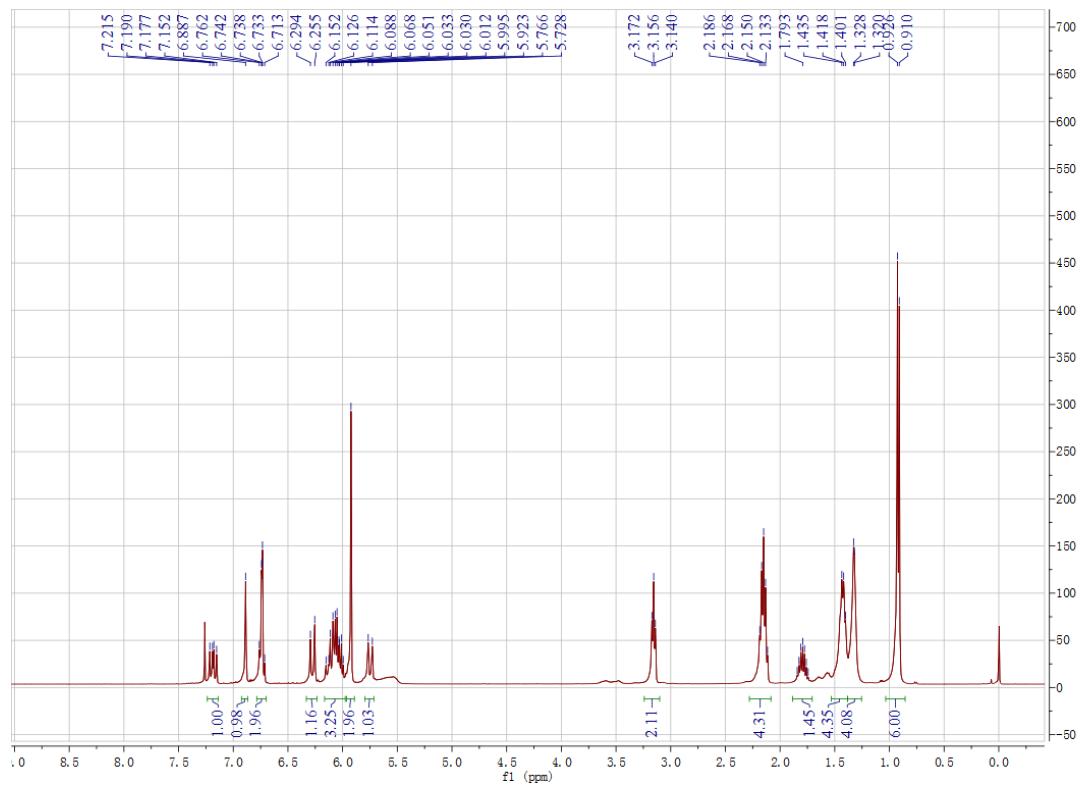
¹H NMR (400 MHz) spectrum of pipercede (**8**) in CDCl₃



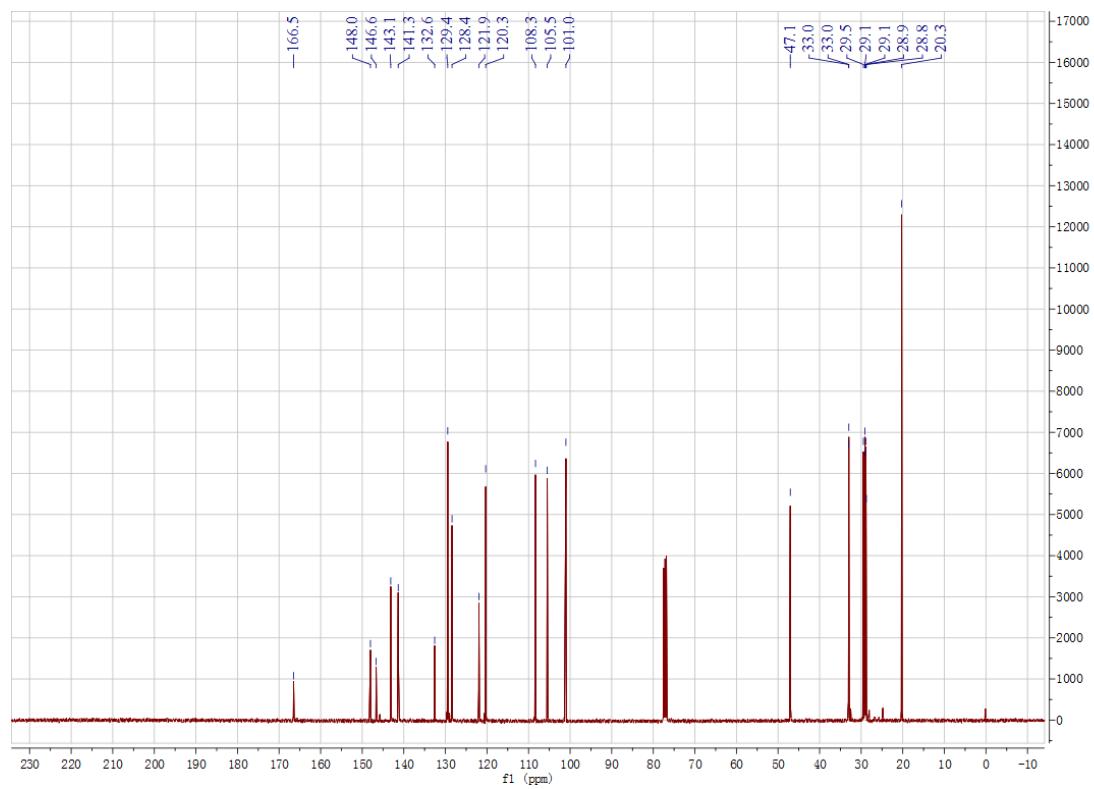
¹³C NMR (100 MHz) spectrum of pipercede (**8**) in CDCl₃



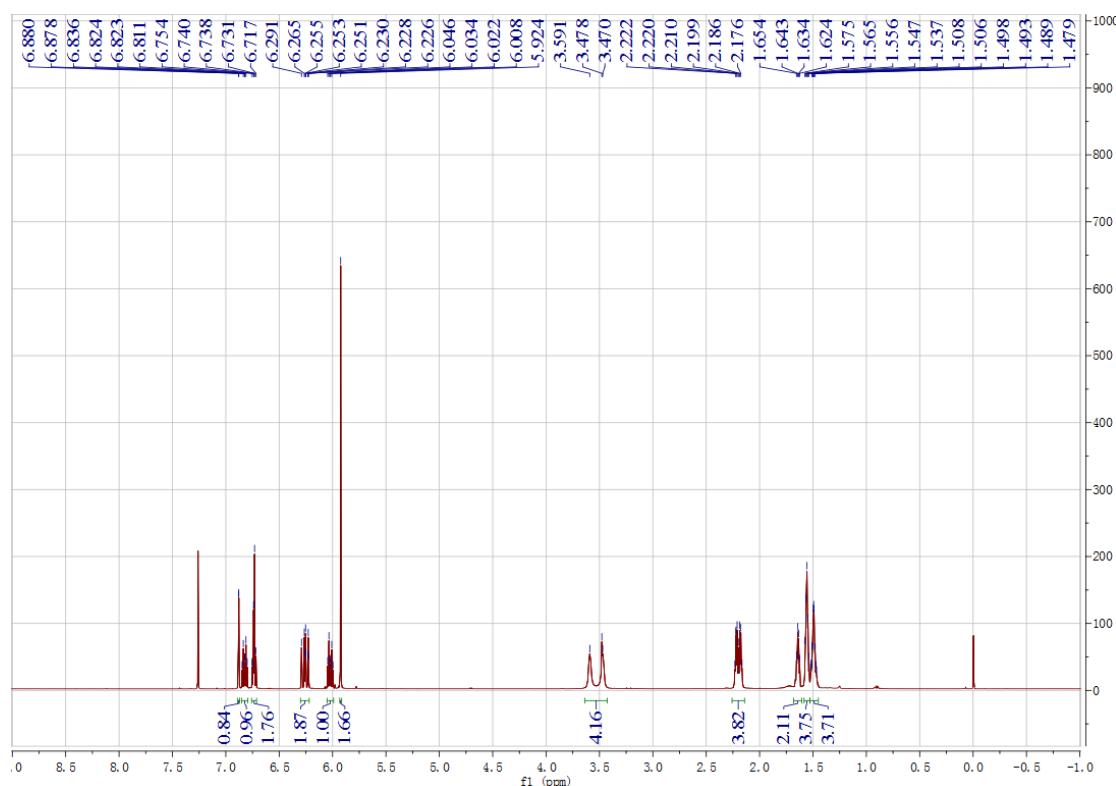
¹H NMR (400 MHz) spectrum of guineensine (**9**) in CDCl₃



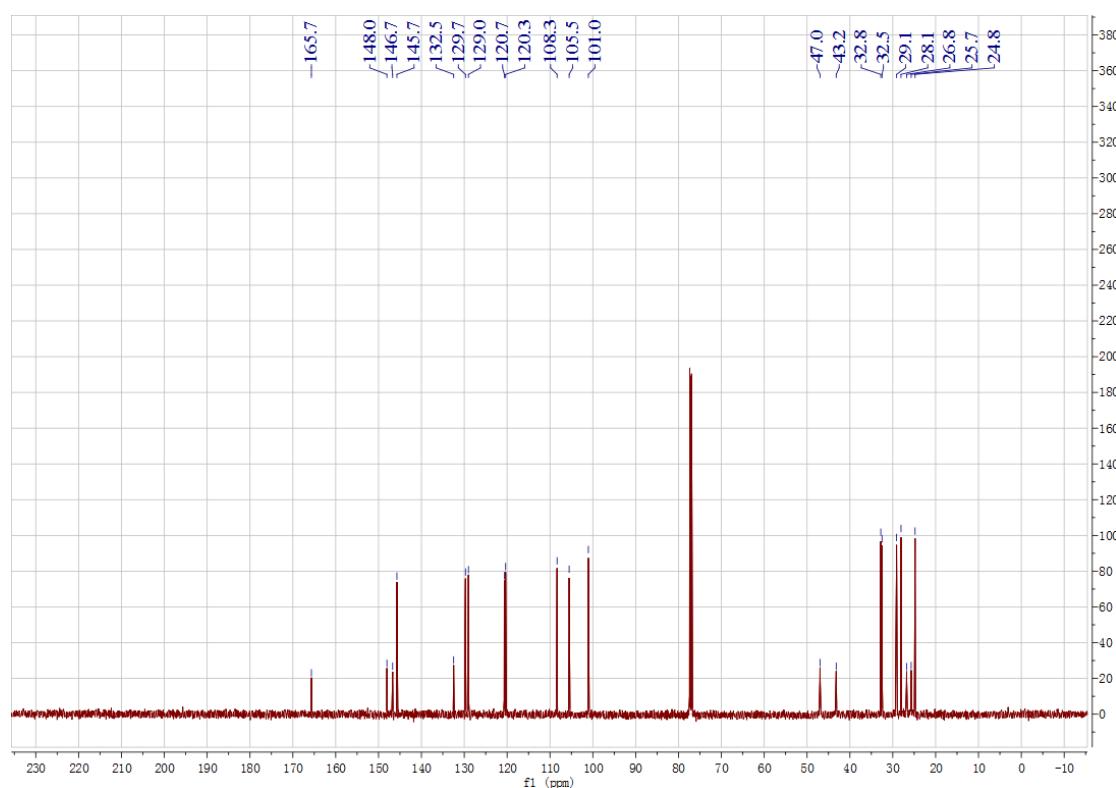
¹³C NMR (100 MHz) spectrum of guineensine (**9**) in CDCl₃



¹H NMR (600 MHz) spectrum of pipernonaline (**10**) in CDCl₃



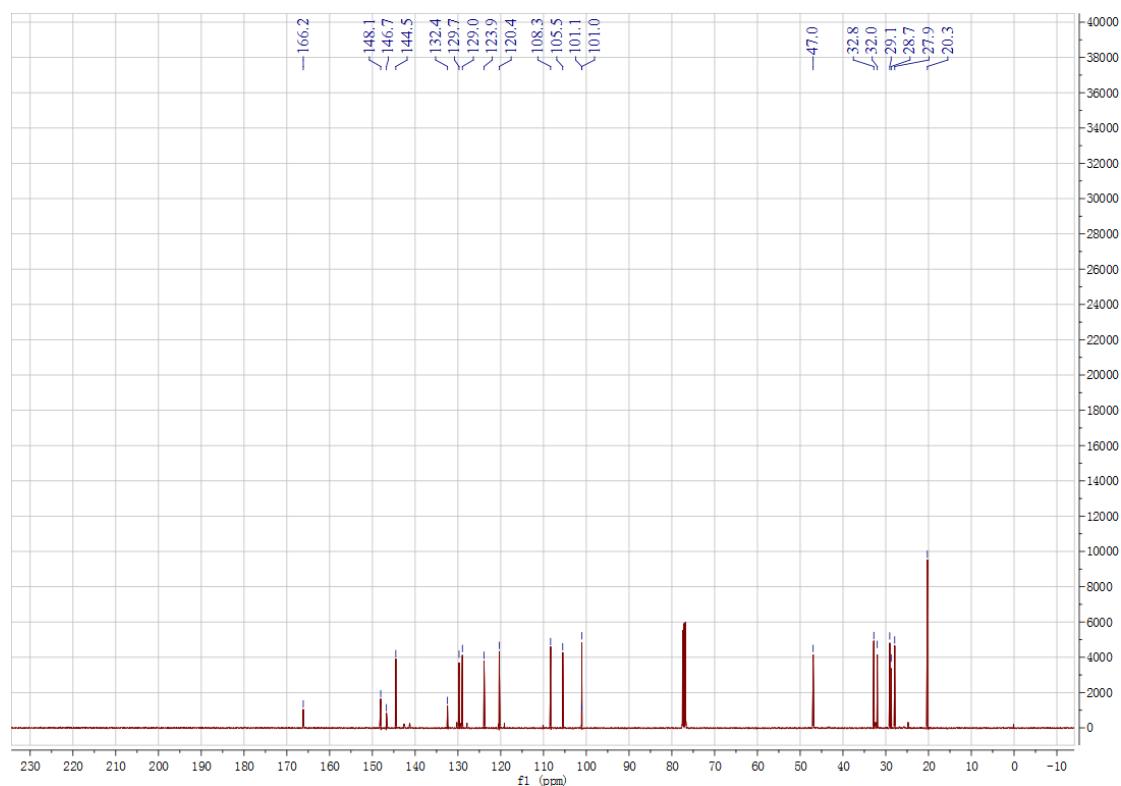
¹³C NMR (150 MHz) spectrum of pipernonaline (**10**) in CDCl₃



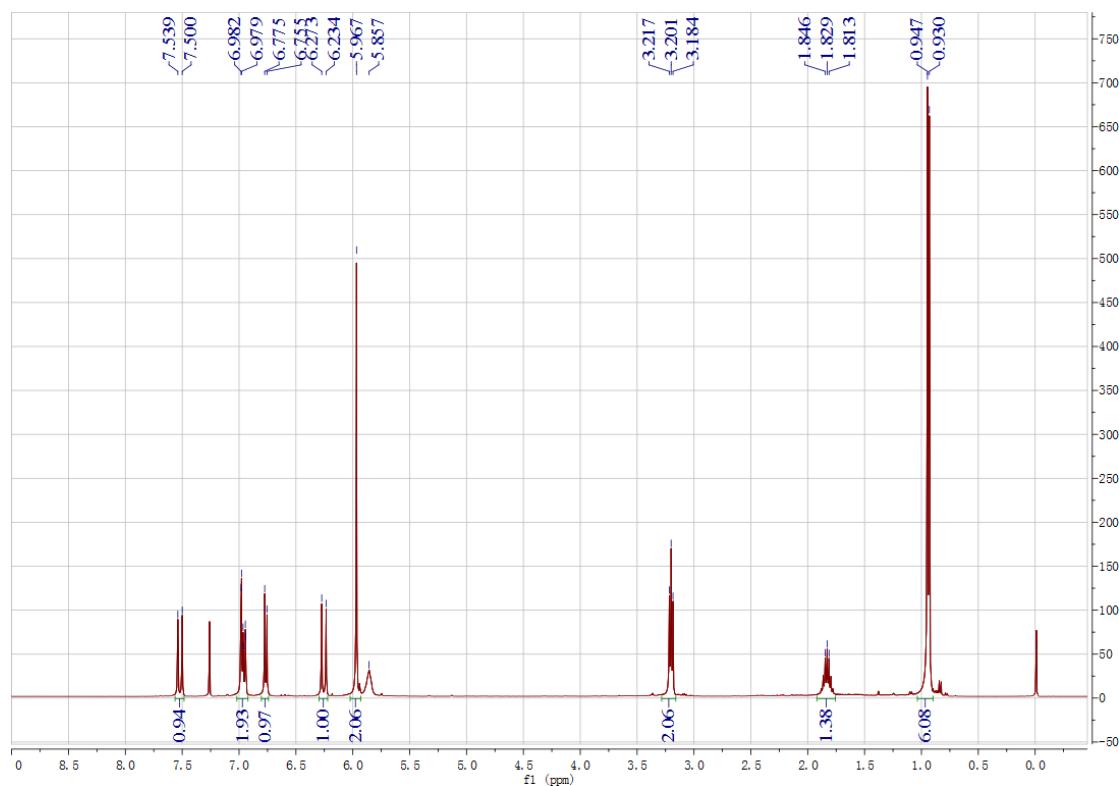
¹H NMR (400 MHz) spectrum of retrofractamide C (**11**) in CDCl₃



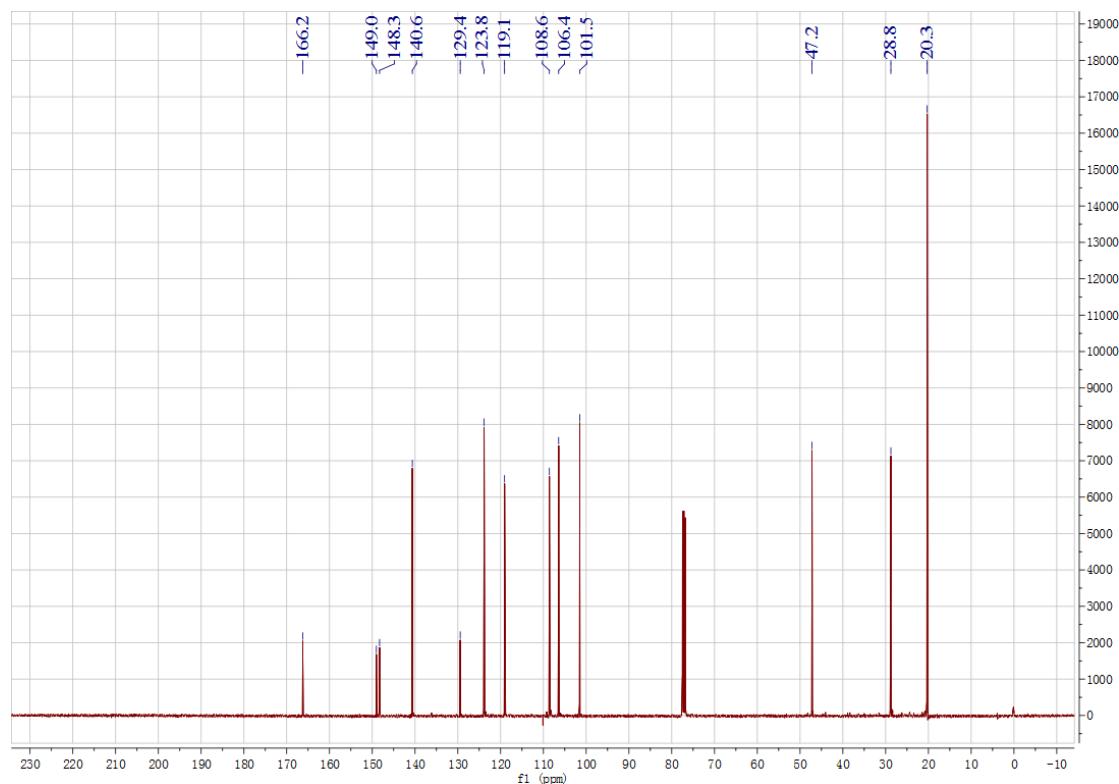
¹³C NMR (100 MHz) spectrum of retrofractamide C (**11**) in CDCl₃



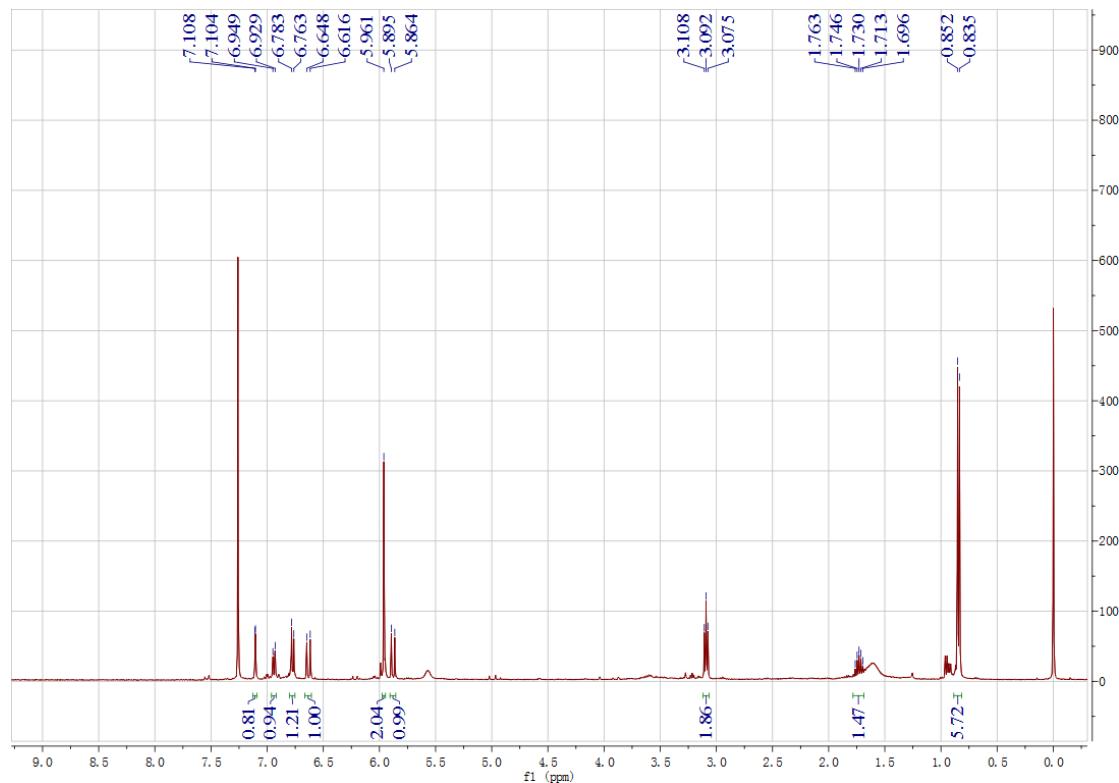
^1H NMR (400 MHz) spectrum of *trans*-fagaramide (**12**) in CDCl_3



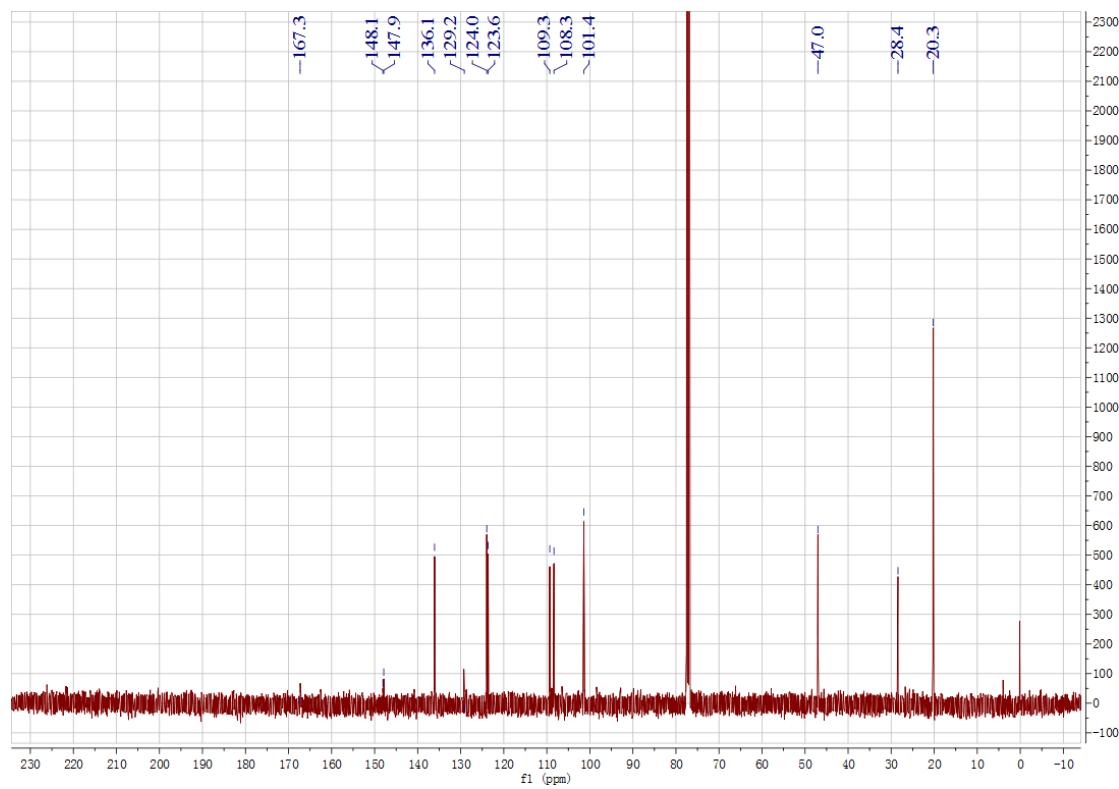
^{13}C NMR (100 MHz) spectrum of *trans*-fagaramide (**12**) in CDCl_3



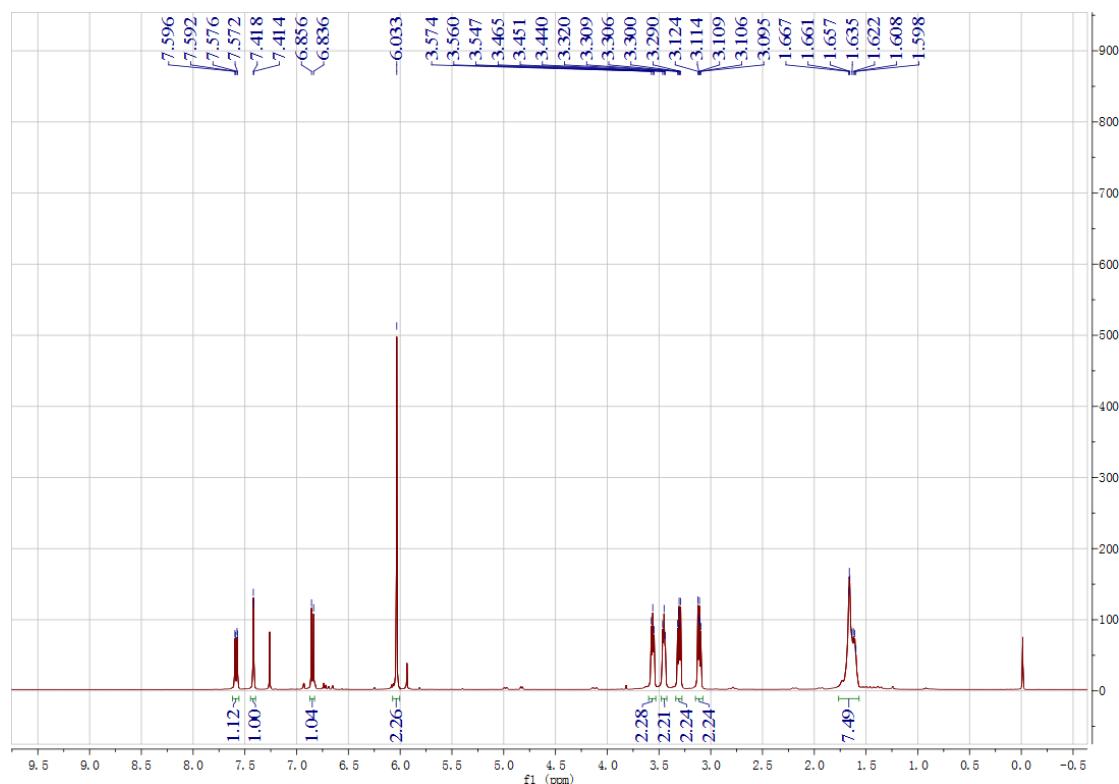
¹H NMR (400 MHz) spectrum of *cis*-fagaramide (**13**) in CDCl₃



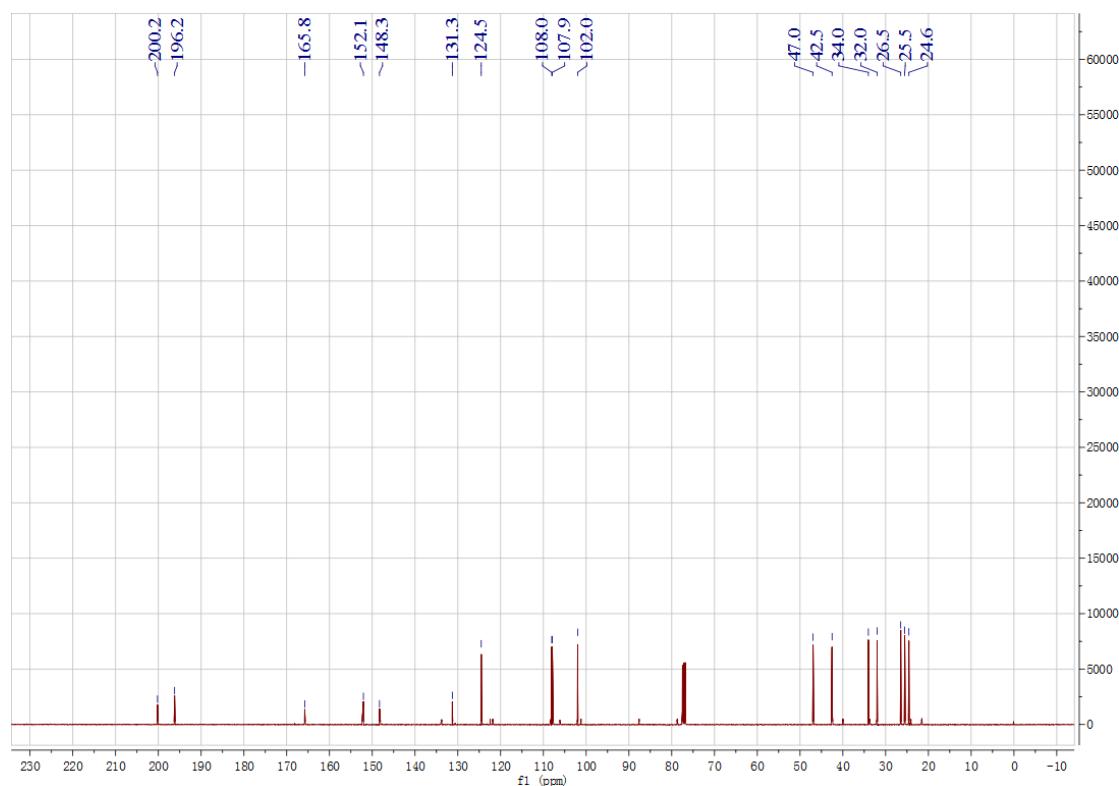
¹³C NMR (100 MHz) spectrum of *cis*-fagaramide (**13**) in CDCl₃



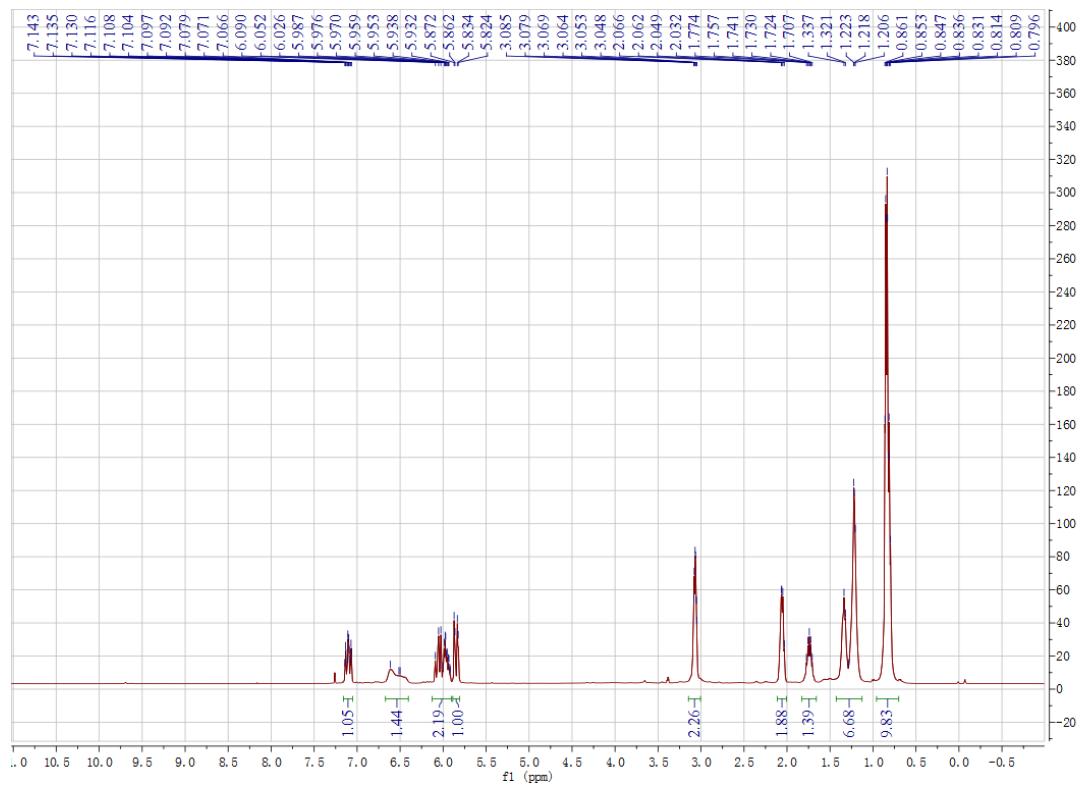
¹H NMR (400 MHz) spectrum of piperodione (**14**) in CDCl₃



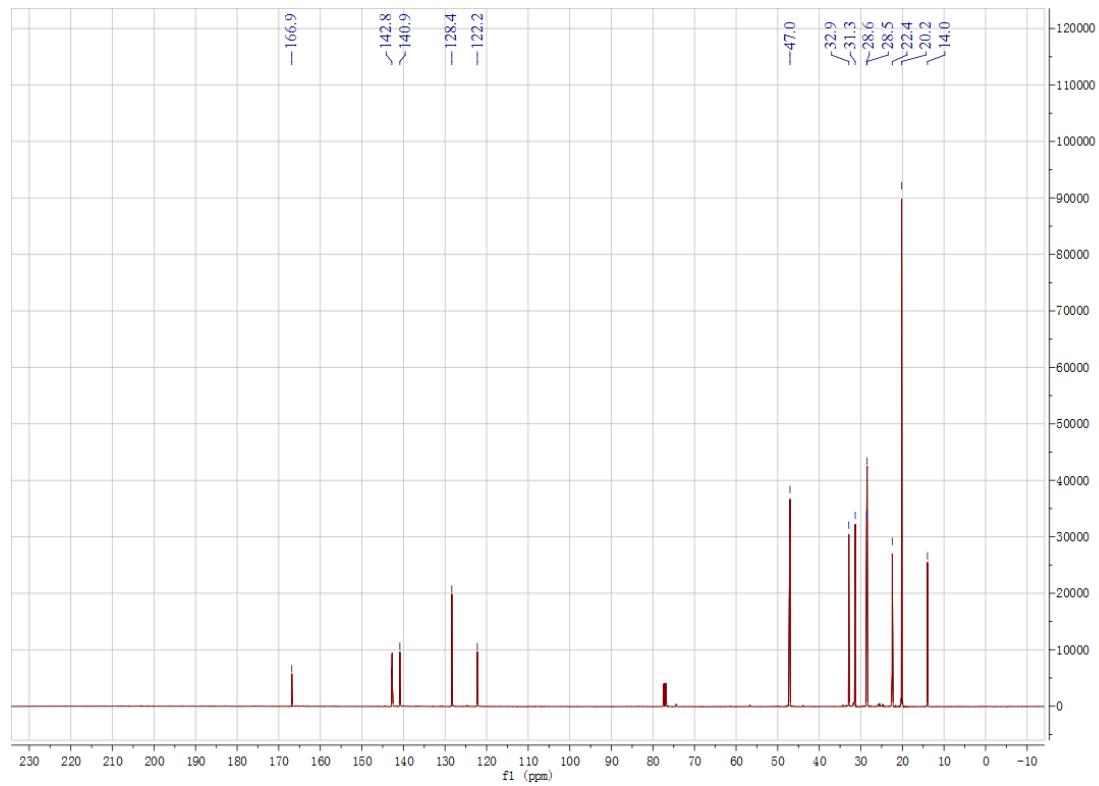
¹³C NMR (100 MHz) spectrum of piperodione (**14**) in CDCl₃



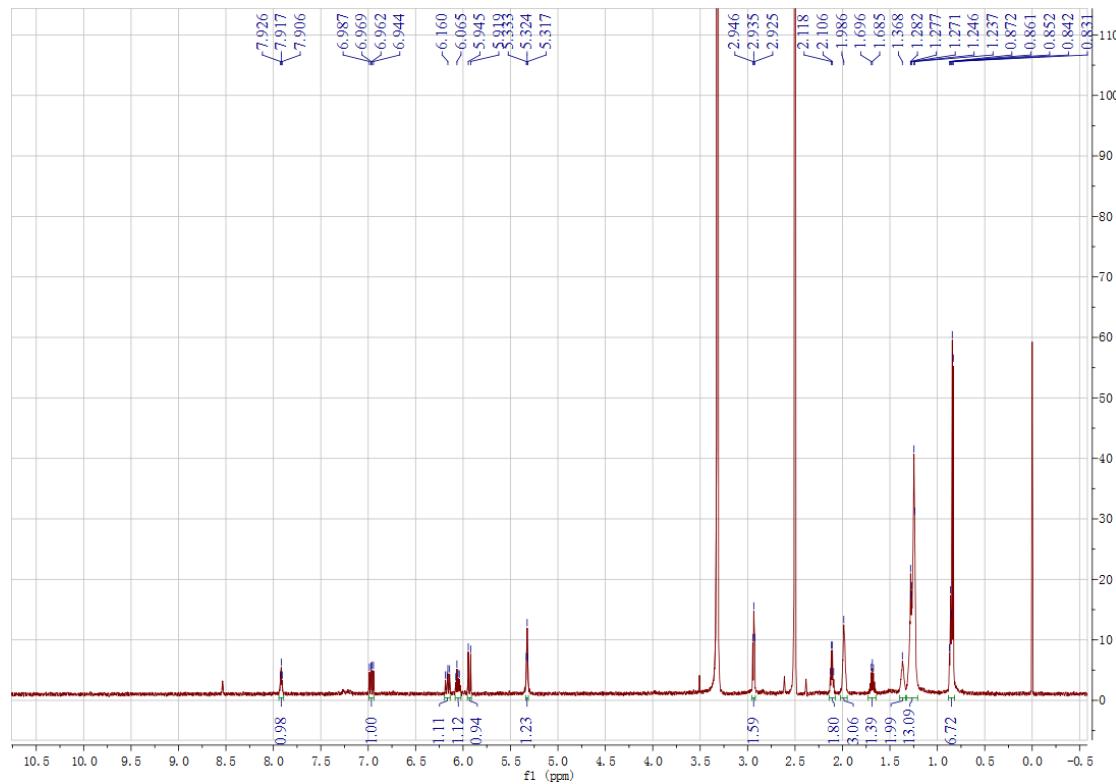
¹H NMR (400 MHz) spectrum of pellitorine (**15**) in CDCl₃



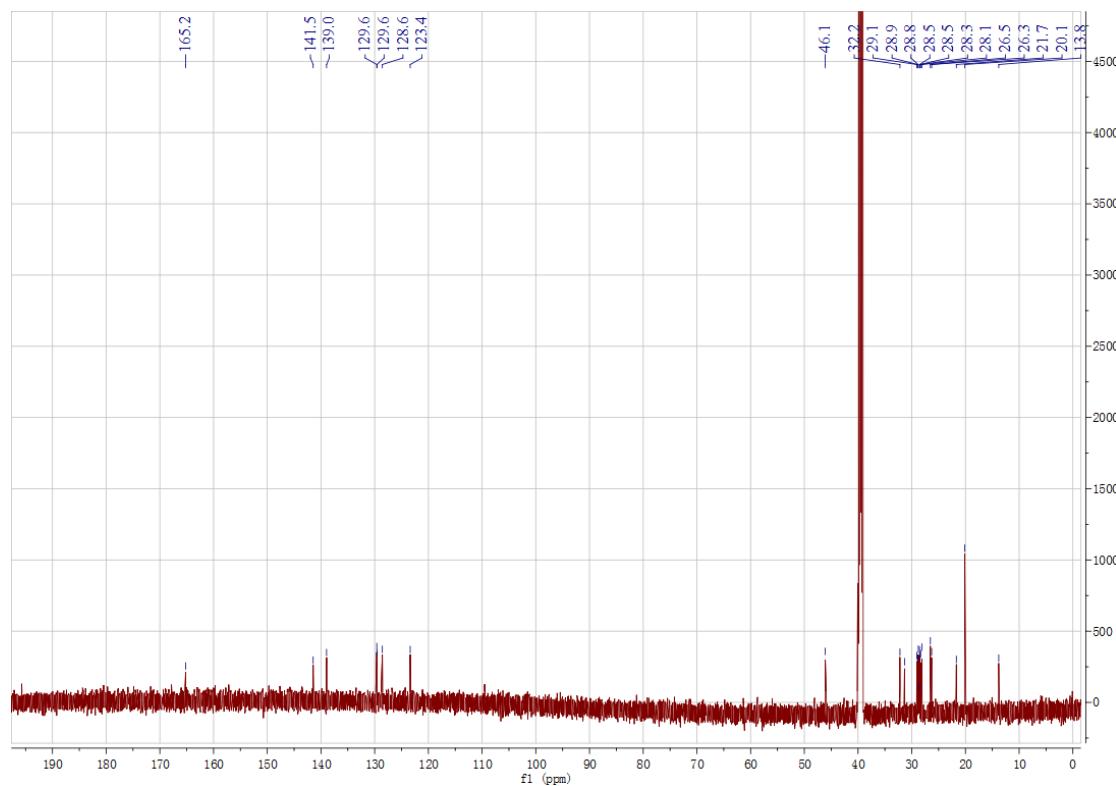
¹³C NMR (100 MHz) spectrum of pellitorine (**15**) in CDCl₃



¹H NMR (600 MHz) spectrum of (2E,4E,15Z)-N-isobutyl-eicosa-2,4,15-trienamide (**16**) in DMSO-*d*₆



¹³C NMR (150 MHz) spectrum of (2E,4E,15Z)-N-isobutyl-eicosa-2,4,15-trienamide (**16**) in DMSO-*d*₆



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