Electronic Supplementary Information (ESI)

Biocatalyst Mediated Functionalization of Salannin, an Insecticidal Limonoid

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EXPERIMENTAL SECTION

General Experimental Procedures. Thin film IR spectra were recorded in $CHCl_3$ using Perkin Elmer FT-IR spectrophotometer and optical rotations were measured in JASCO (P-2000) polarimeter at 589 nm in $CHCl_3$ (c in g/100 mL). NMR (^1H, ^{13}C, DEPT-135, COSY, NOESY, HSQC and HMBC) data were recorded on Bruker 400 MHz spectrophotometer in $CDCl_3$ and signal for the residual solvent was marked as the reference. HRMS data and quantification of the individual limonoids were performed on Q Exactive Quadrupole/Orbitrap MS system (Thermo Scientific) attached with Accela 1250 pump. Thermo Scientific Hypersil Gold column of particle size 5 μm with a flow rate of 0.5 mL/min and gradient solvent program of 27.0 min (0.0 min, 10% methanol/water; 0.5 min, 10.0% methanol/water; 2.0 min, 45% methanol/water; 10.0 min, 50% methanol/water; 20.0 min, 65% methanol/water; 22.0 min, 90% methanol/water; 24.0 min, 90% methanol/water; 25.0 min, 10% methanol/water; 27.0 min, 10% methanol/water) was used for the resolution of individual components. HRMS experiments were performed in ESI-positive ion mode within the mass range *m/z* 100-1000 using the tune method as sheath gas flow rate 45, auxiliary gas flow rate 10, sweep gas flow rate 2, spray current (μA) 3.70, spray voltage (KV) 3.60, capillary temperature (ºC) 320, heater temperature (ºC) 350, s-lens RF level 50. For LC-ESI-HRMS analyses LC-MS grade solvents were purchased from JT Baker. Technical grade solvents were used for the extraction and preparative chromatography purpose and distilled prior to use. Chemicals for preparing medium were procured from Himedia. Thin layer chromatography (TLC) was carried out on Merck silica gel-G pre-coated plates of thickness 0.25 mm and developed twice using the mobile phase of 3.0% methanol in dichloromethane. Anisaldehyde charring reagent was sprayed on TLC and heated for visualizing the spots. Metabolites were purified over silica-gel (230-400 mesh) flash column chromatography using the gradient mixture of methanol in dichloromethane as the eluent. X-ray intensity data measurements of salanninolide (**2**) was carried out on a Bruker SMART APEX II CCD diffractometer with graphite-monochromatized (MoKα= 0.71073Å) radiation at 90 (2) K. The X-ray generator was operated at 50 kV and 30 mA. A preliminary set of cell constants and an orientation matrix were calculated from three sets of 12 frames. Data were collected with ω scan width of 0.5º at eight different settings of φ and 2θ with a frame time of 10 sec keeping the sample-to-detector distance fixed at 5.00 cm. The X-ray data collection was monitored by APEX2 program.

Substrate, Organisms and Screening. Salannin, isolated from the seed kernel of *Azadirachta indica* (Neem) was used in this study. Micro-organisms used for the screening and preparative scale fermentation purpose were obtained from National Collection of Industrial Microorganisms (NCIM), CSIR-NCL, Pune. Cultures were stored at 4 ºC and propagated at 30 ºC on PDA (Potato dextrose agar) slants. Inoculum (3.0% v/v) prepared from 2 days grown culture was used for the inoculation of 100 mL sterile modified Czapek-dox medium contained in a 500 mL conical flask maintaining aseptic condition. Flasks were further left for orbital shaking on rotary shaker at 30 ºC and 180 rpm for 2

days. To the well grown culture of different fungi in 100 mL of medium, 10 mg salannin (**1**) was added dissolving in 0.2 mL of acetone. Before addition of the substrate pH of the cultured medium was adjusted to 6.0-7.0 using 1 M K_2HPO_4. After incubation at 30 ºC and 180 rpm for 5 days, both the broth and mycelia was extracted separately with ethyl acetate (100 mLx2) and ethyl acetate layer after concentration was analyzed for bioconversion of salannin (**1**) by TLC and LC-ESI-HRMS.

Time-course and Resting Cell Experiment. Time-course experiment with *Cunninghamella echinulata* was carried out by aseptically taking out 10 mL of medium (100 mL containing 10 mg of salannin) in every alternate days and extracting the fermented medium by ethyl acetate (10 mLx2). Progress of the reaction was monitored by injecting concentrated organic layer in LC-ESI-HRMS and the level of metabolites or substrate was quantified using the standard graphs drawn for **1**, **2** and **3** using area under respective peaks. The peak positions of individual components were confirmed by co-injection studies. For the resting cell experiment, well grown culture of *Cunninghamella echinulata* was filtered through muslin cloth and mycelia was washed repeatedly by distilled water followed by phosphate buffer of pH 7.2. Squeezed mycelia (3 g) were added to 50 mL of phosphate buffer (pH 7.2) containing 250 mg dextrose and 2 mg salannin (in 0.2 mL acetone). Reaction mixture was incubated at 30 ºC and 180 rpm for 36 h. After the incubation the reaction mixture was extracted with ethyl acetate (50 mLx2) and analyzed by TLC and LC-ESI-HRMS. Similarly, resting cell experiments were carried out using brown conical flask covered with silver foil under dark condition.

Preparative Scale Biotransformation. Large scale fermentation was carried out by distributing 0.40 g salannin (dissolved in 8.0 mL acetone) in 40 flasks each containing 100 mL well-grown culture of *Cunninghamella echinulata* and incubating under identical conditions. At higher substrate concentration, the level of metabolite formation was reduced. After 8 days of incubation, contents of all the flasks were pooled together and mycelia were separated by filtration. Broth and mycelia were extracted individually using ethyl acetate (4 Lx3 and 500 mLx3 respectively). Both the ethyl acetate layers were concentrated separately and analyzed to check for the metabolites. According to the TLC and LC-ESI-HRMS, metabolites were observed only in the broth extract (0.56 g) and not in the mycelial extract. The brown crude broth extract was further purified by flash column chromatography by gradient elution of increasing percentage of methanol in dichloromethane. Unmetabolized salannin was eluted at 1.0% methanol in dichloromethane followed by salanninolide (**2**) at 1.4% and salanninactam (**3**) at 2.8%. Purified metabolites **2** and **3** were characterized using numerous spectroscopic and crystallographic studies.

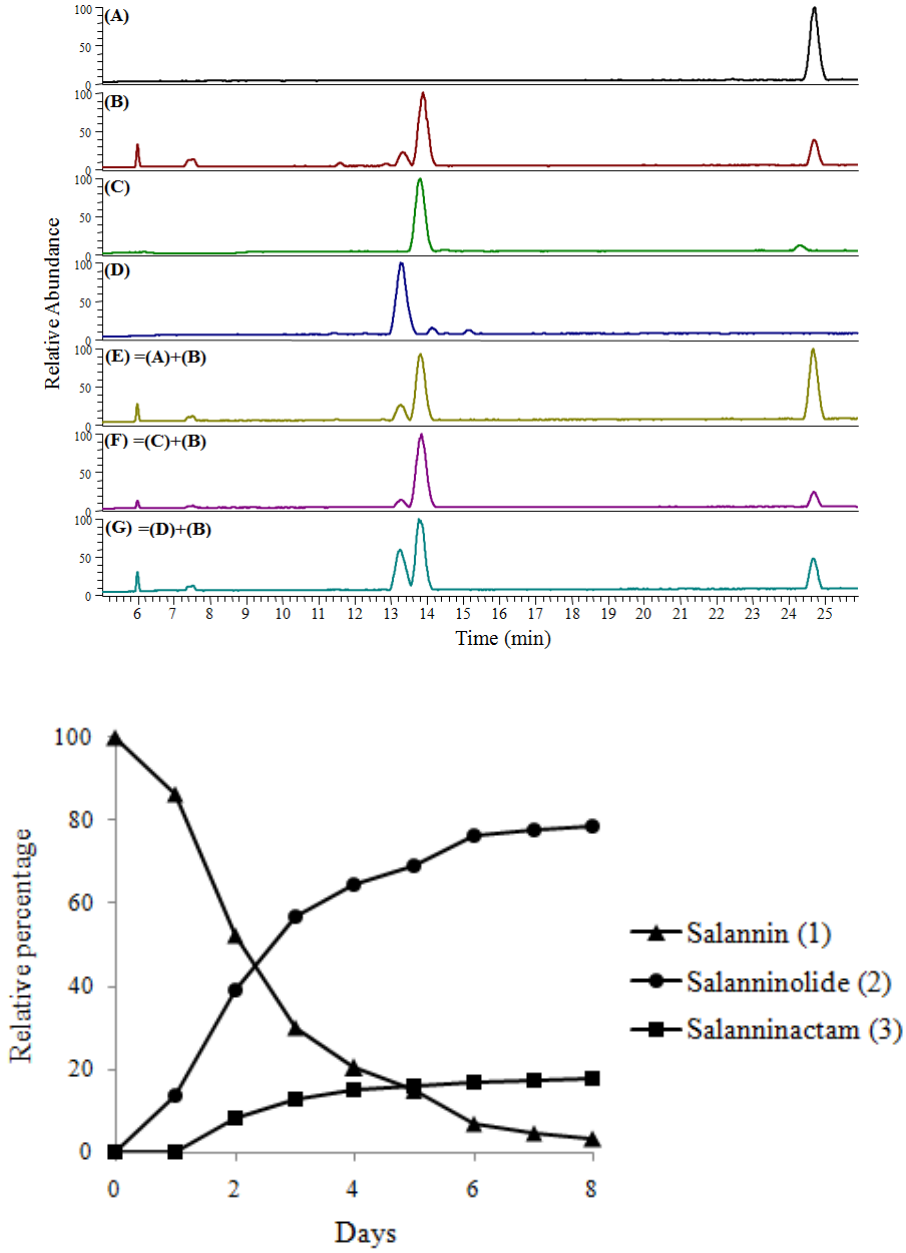


Fig. S1 LC-ESI-HRMS chromatograms of (**A**) standard salanin, (**B**) resting cell experiment of salanin with *Cunninghamella echinulata*, (**C**) purified salanninolide, (**D**) purified salanninactam, (**E**) co-injection of salanin with resting cell experiment, (**F**) co-injection of salanninolide with resting cell experiment, (**G**) co-injection of salanninactam with resting cell experiment; (**H**) Time course experiment (substrate concentration: 0.1 g l^{-1}).

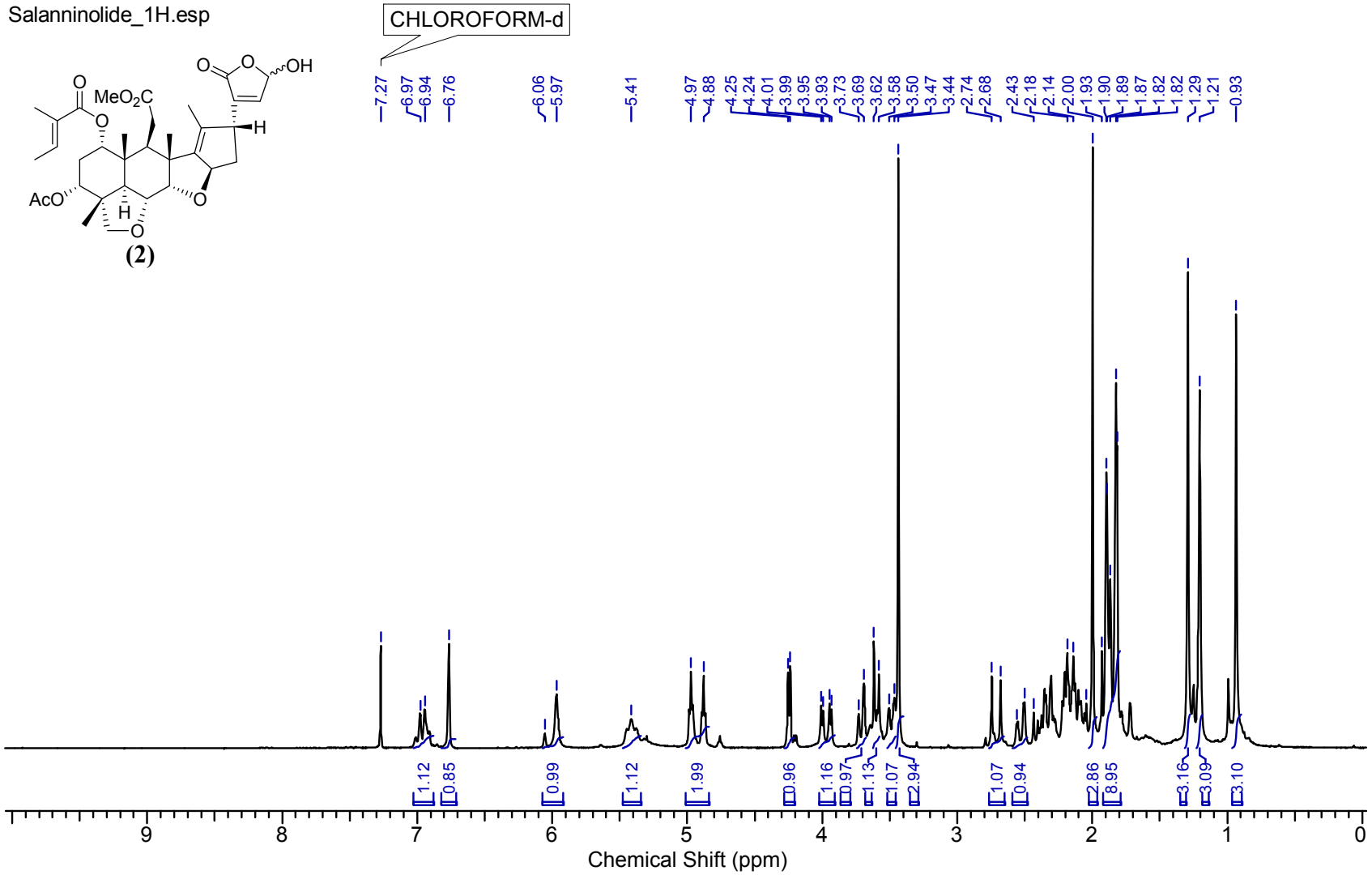
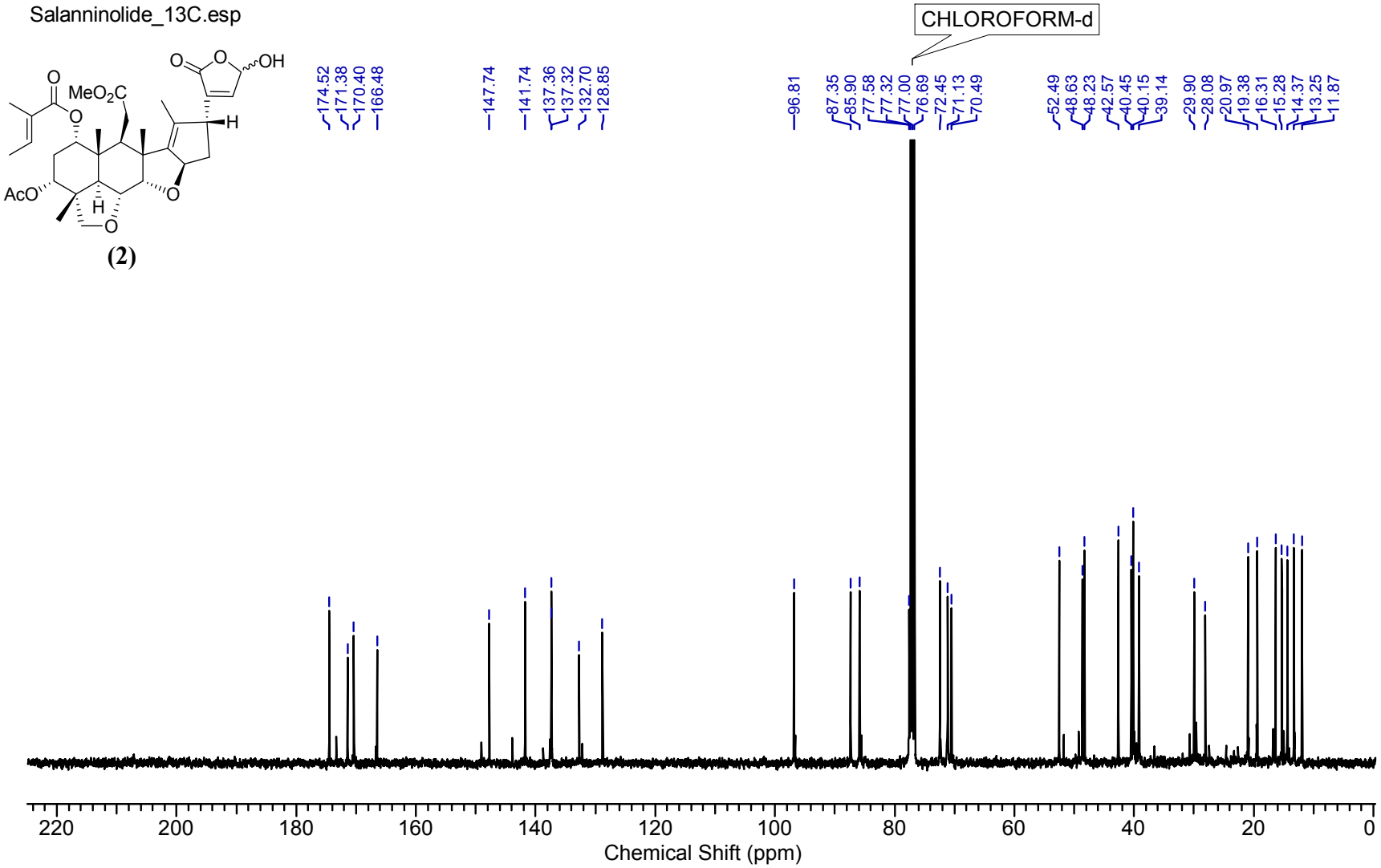


Fig. S2 ^{1}H NMR spectrum of salanninolide (**2**) in $CDCl_3$.



(2)

Fig. S3 ^{13}C NMR spectrum of salanninolide (**2**) in $CDCl_3$.

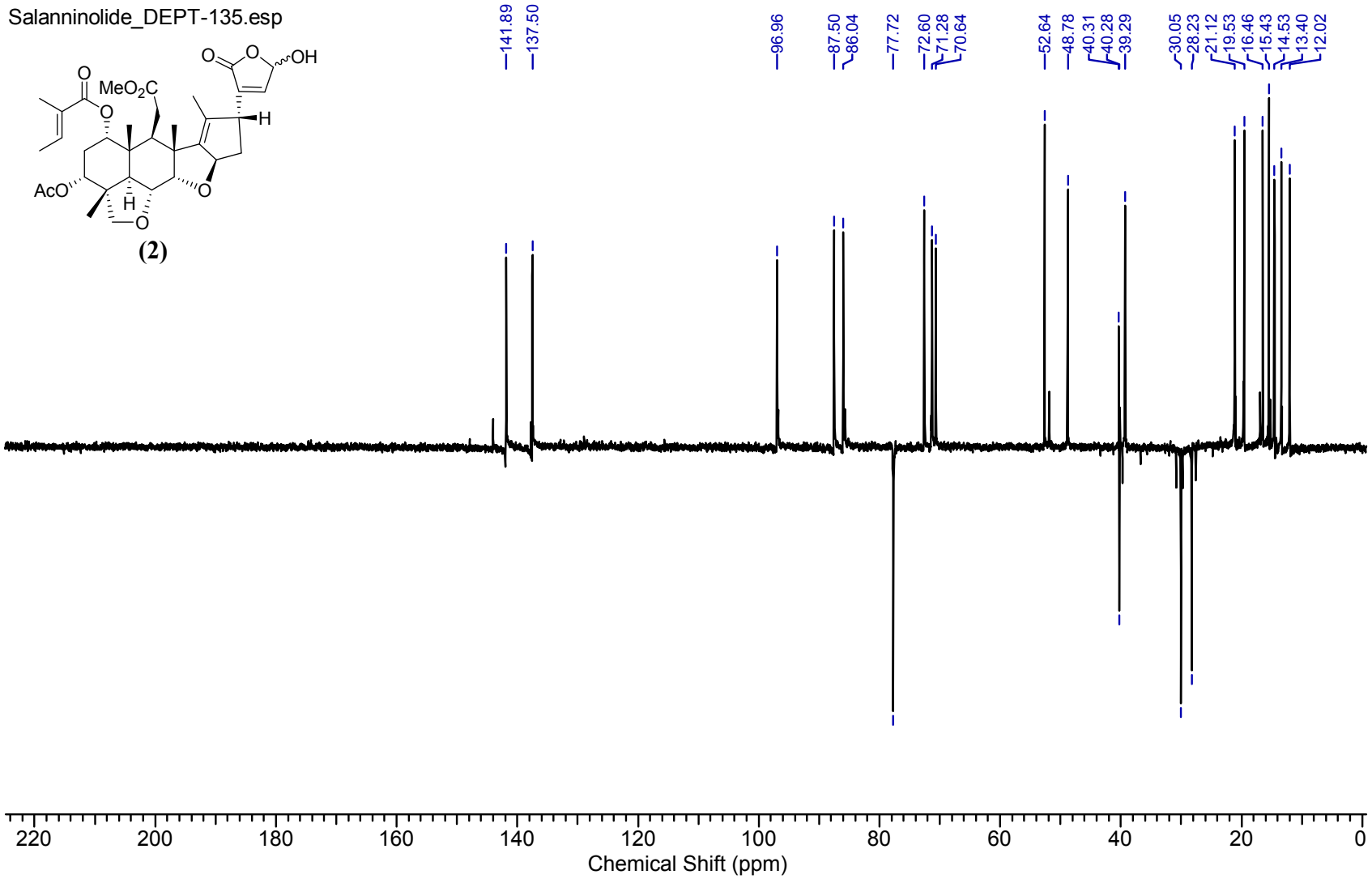


Fig. S4 DEPT-135 NMR spectrum of salanninolide (**2**) in $CDCl_3$.

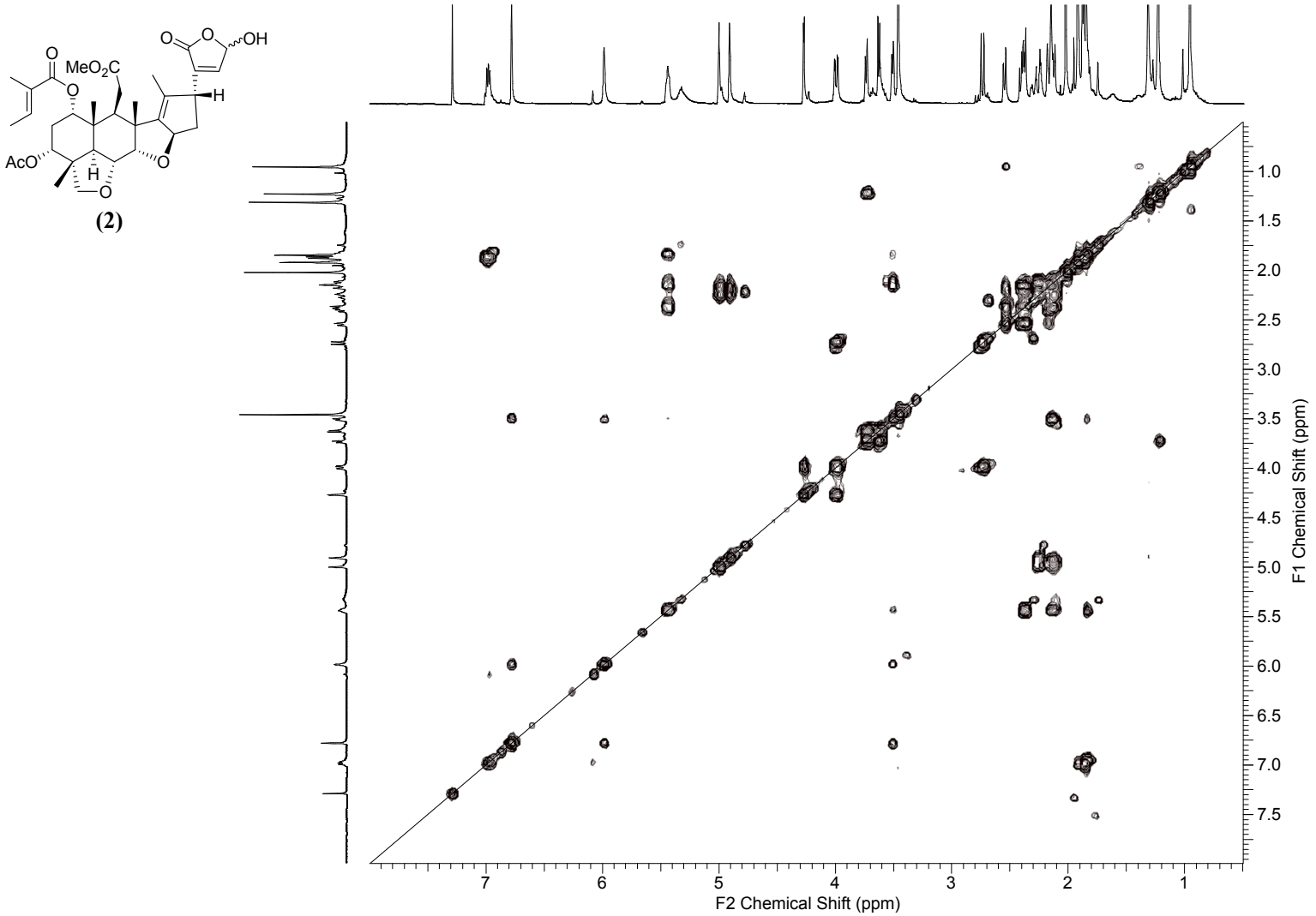


Fig. S5 COSY spectrum of salanninolide (**2**) in $CDCl_3$.

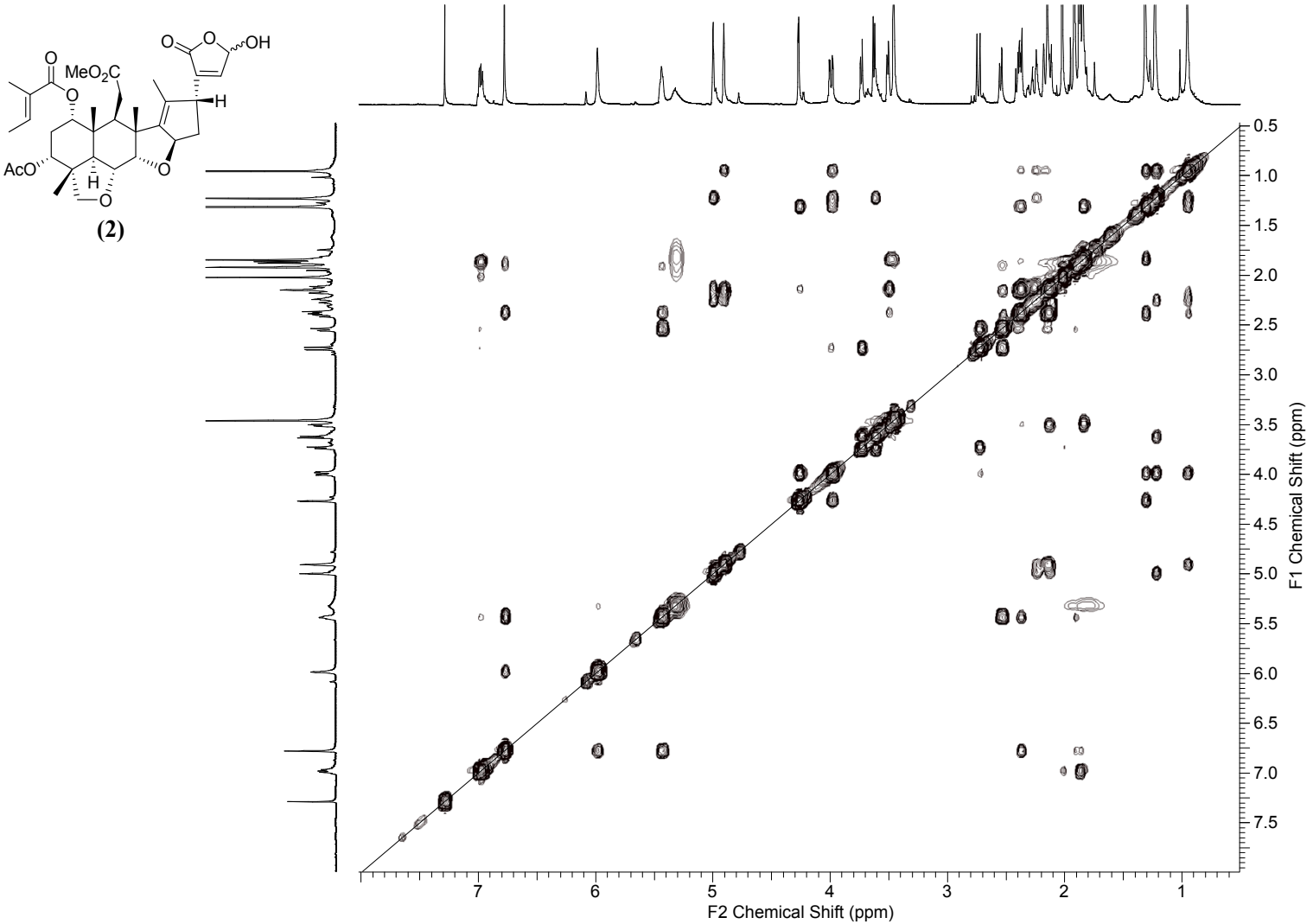


Fig. S6 NOESY spectrum of salanninolide (**2**) in $CDCl_3$.

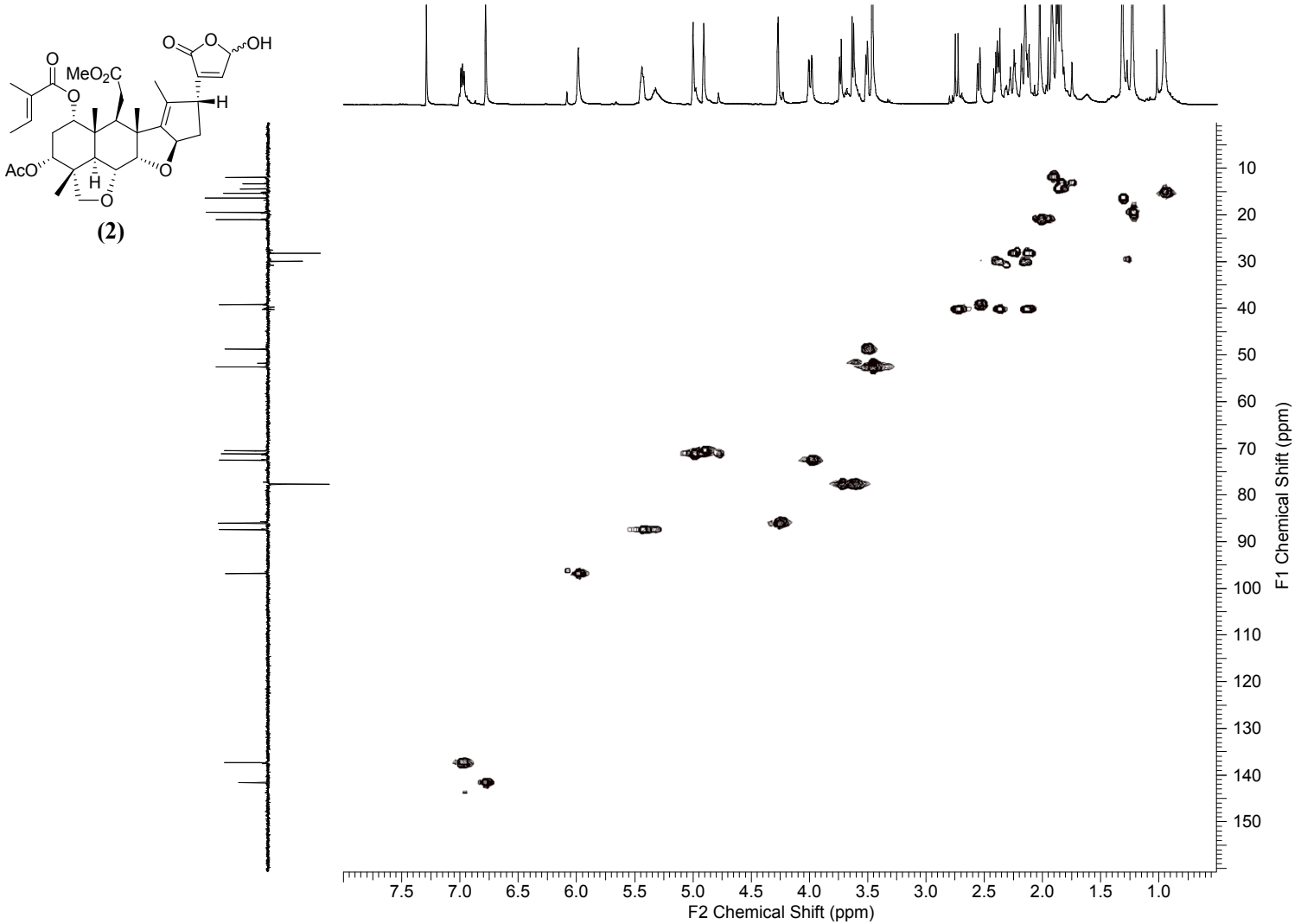


Fig. S7 HSQC spectrum of salanninolide (**2**) in $CDCl_3$.

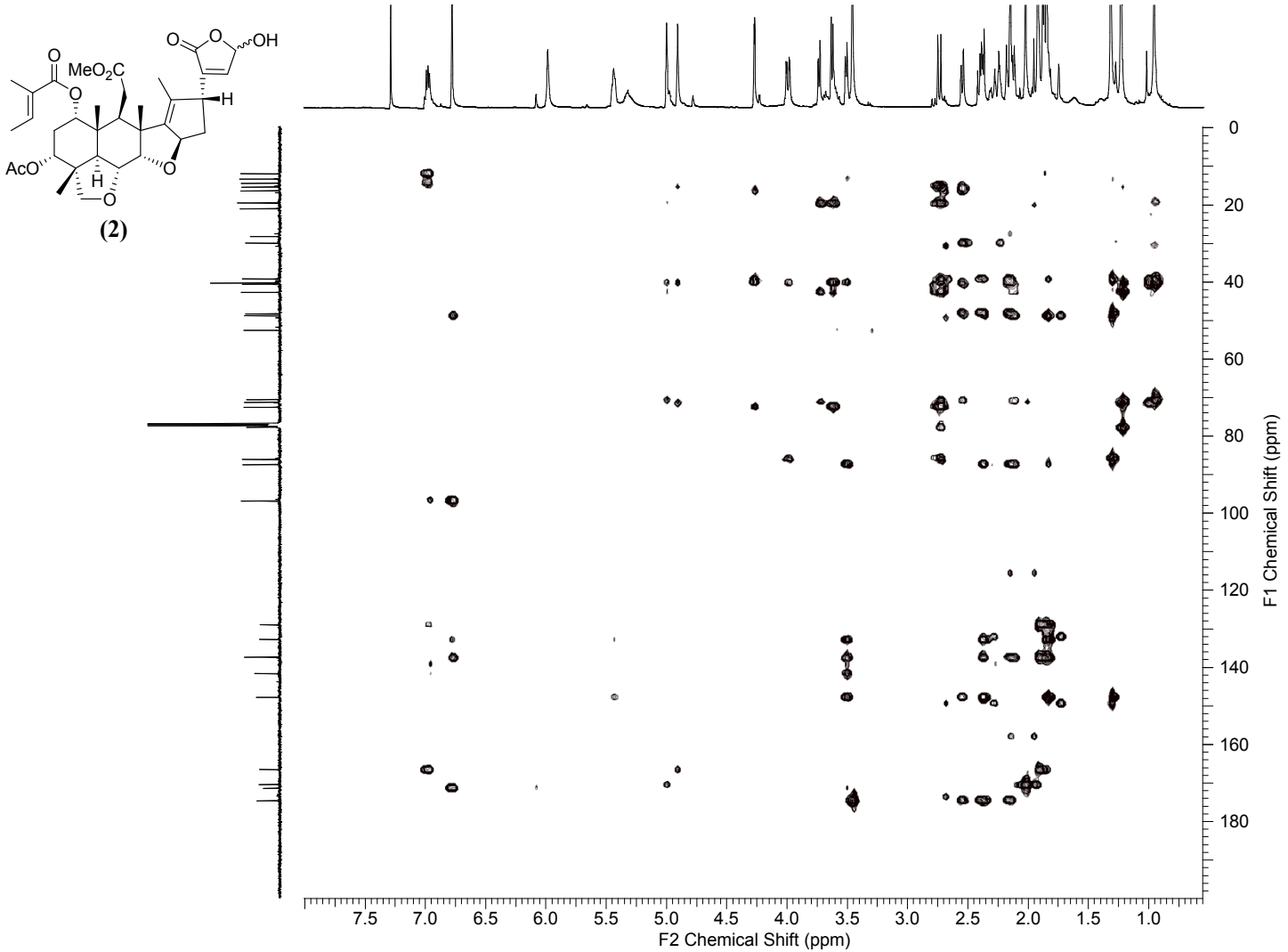


Fig. S8 HMBC spectrum of salanninolide (**2**) in $CDCl_3$.

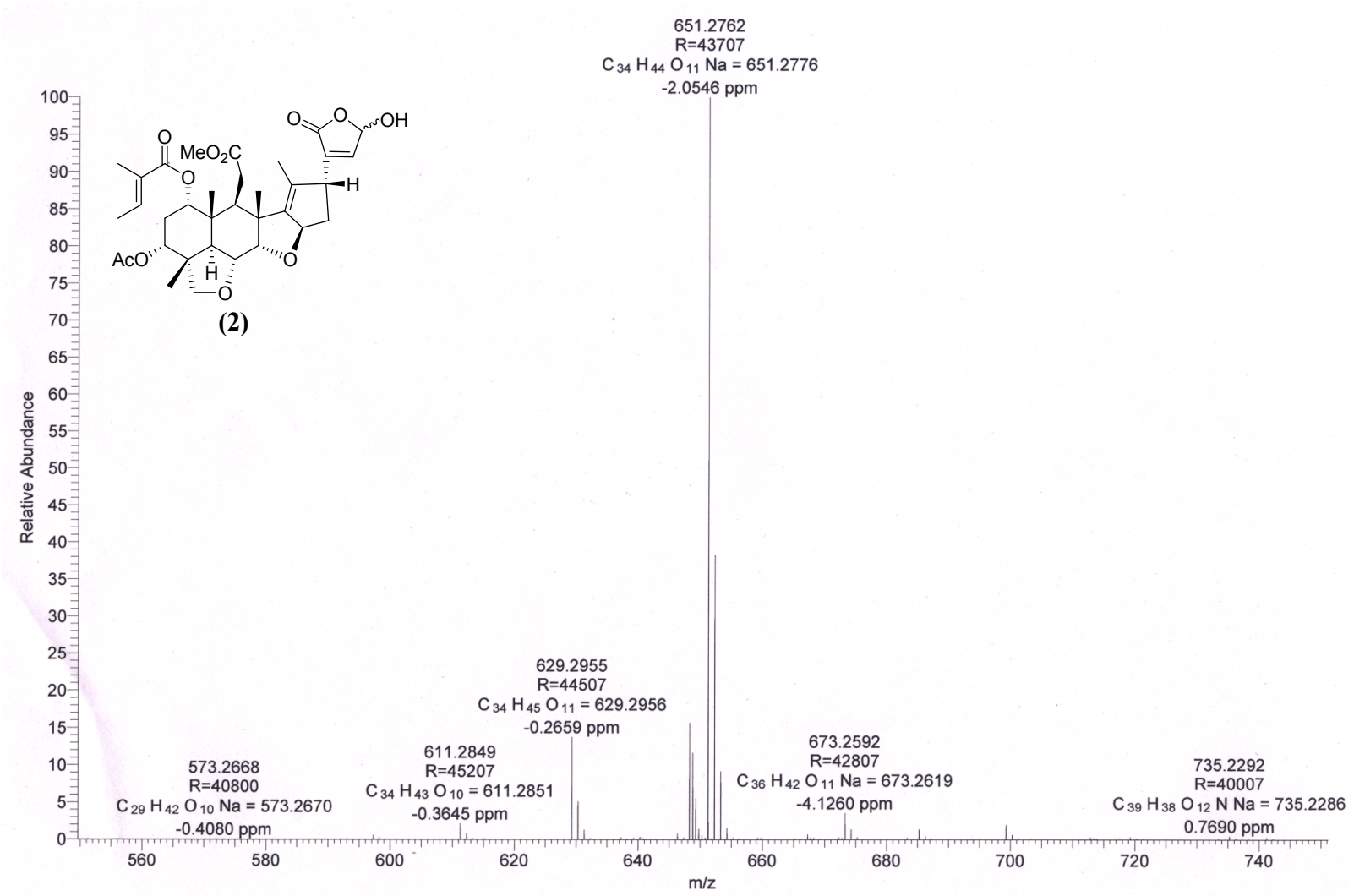


Fig. S9 LC-ESI-HRMS spectrum of salanninolide (**2**).

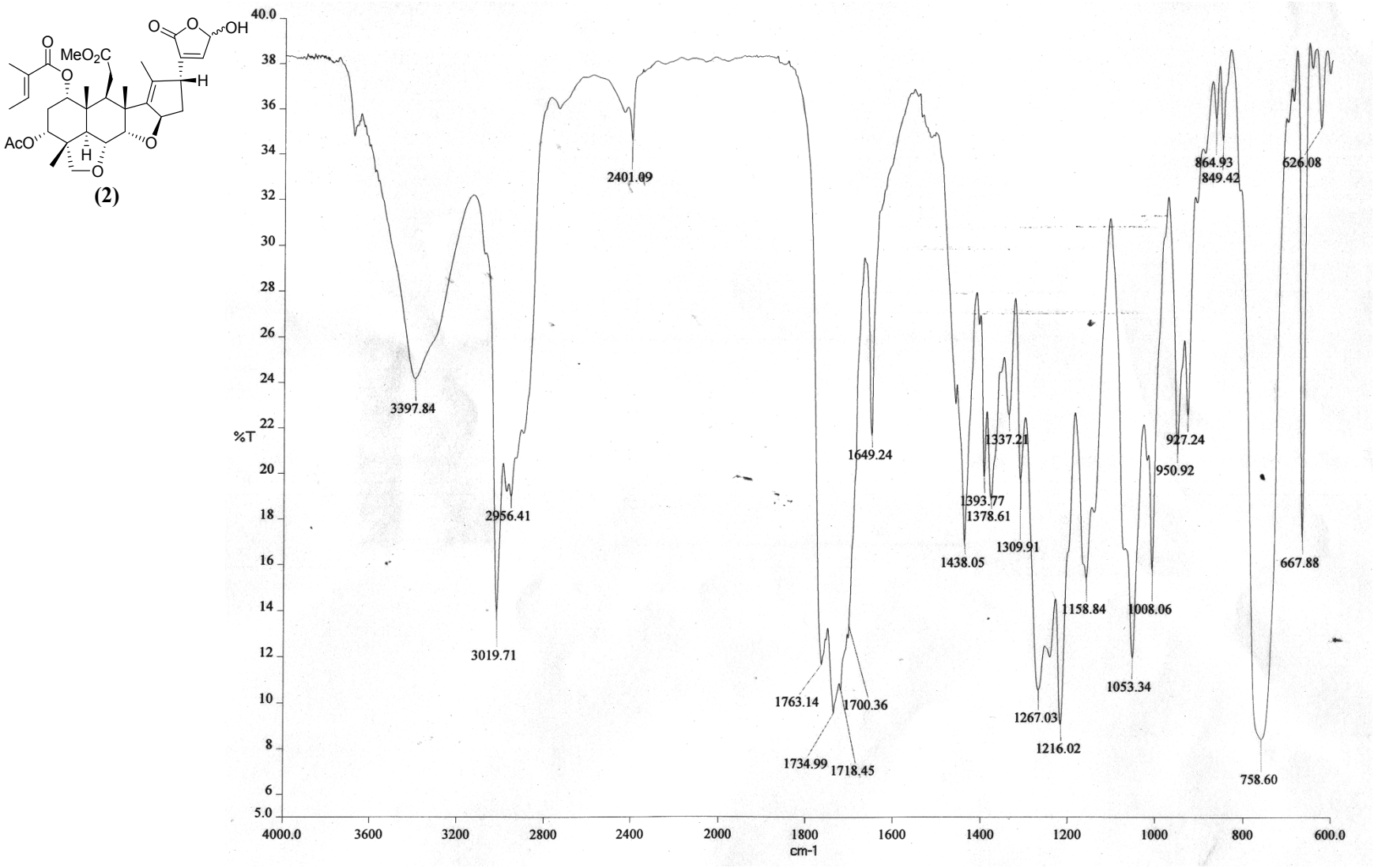


Fig. S10 IR spectrum of salanninolide (**2**) in $CHCl_3$.

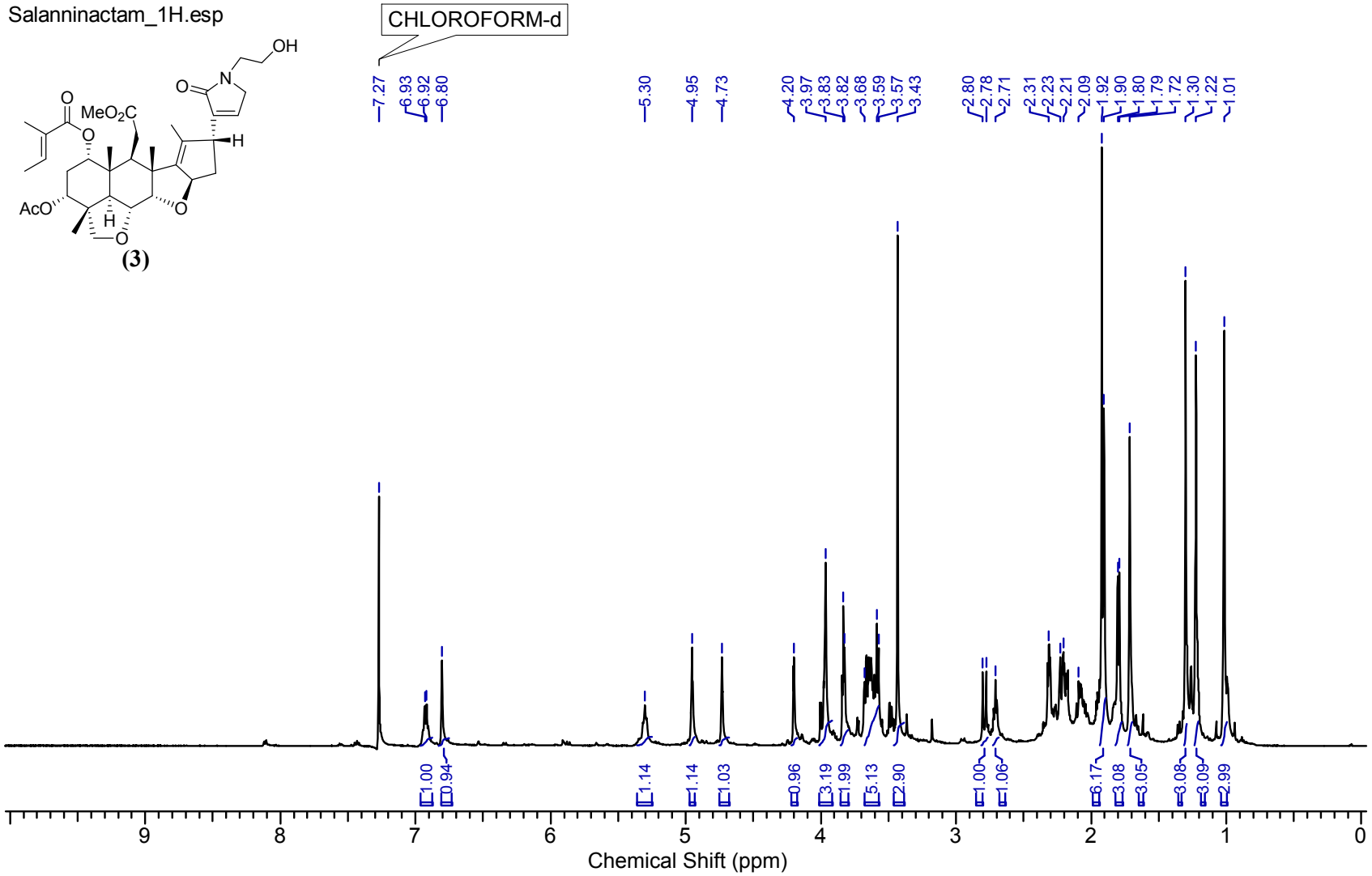


Fig. S11 ^{1}H NMR spectrum of salanninactam (**3**) in $CDCl_3$.

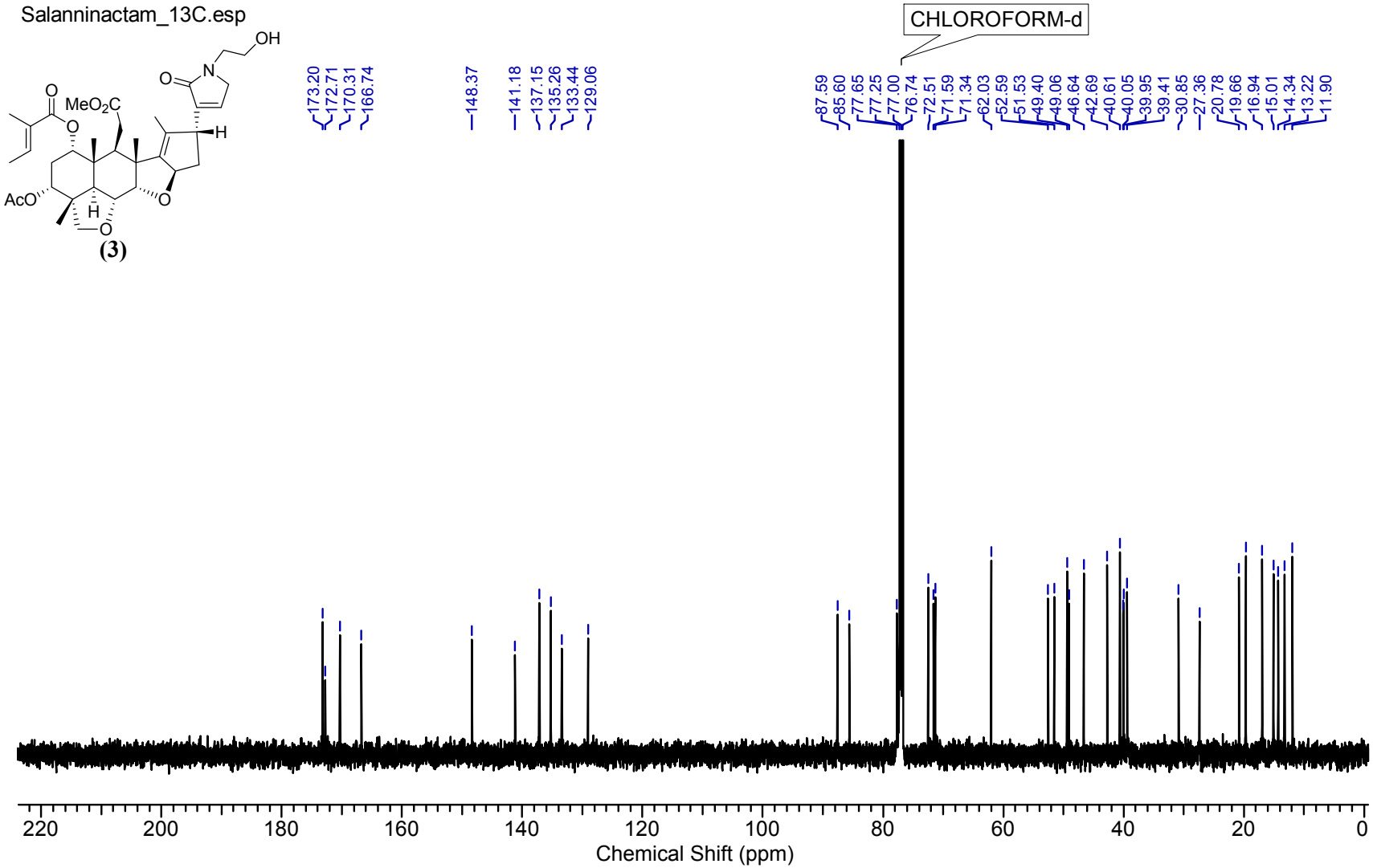


Fig. S12 ^{13}C NMR spectrum of salanninactam (**3**) in $CDCl_3$.

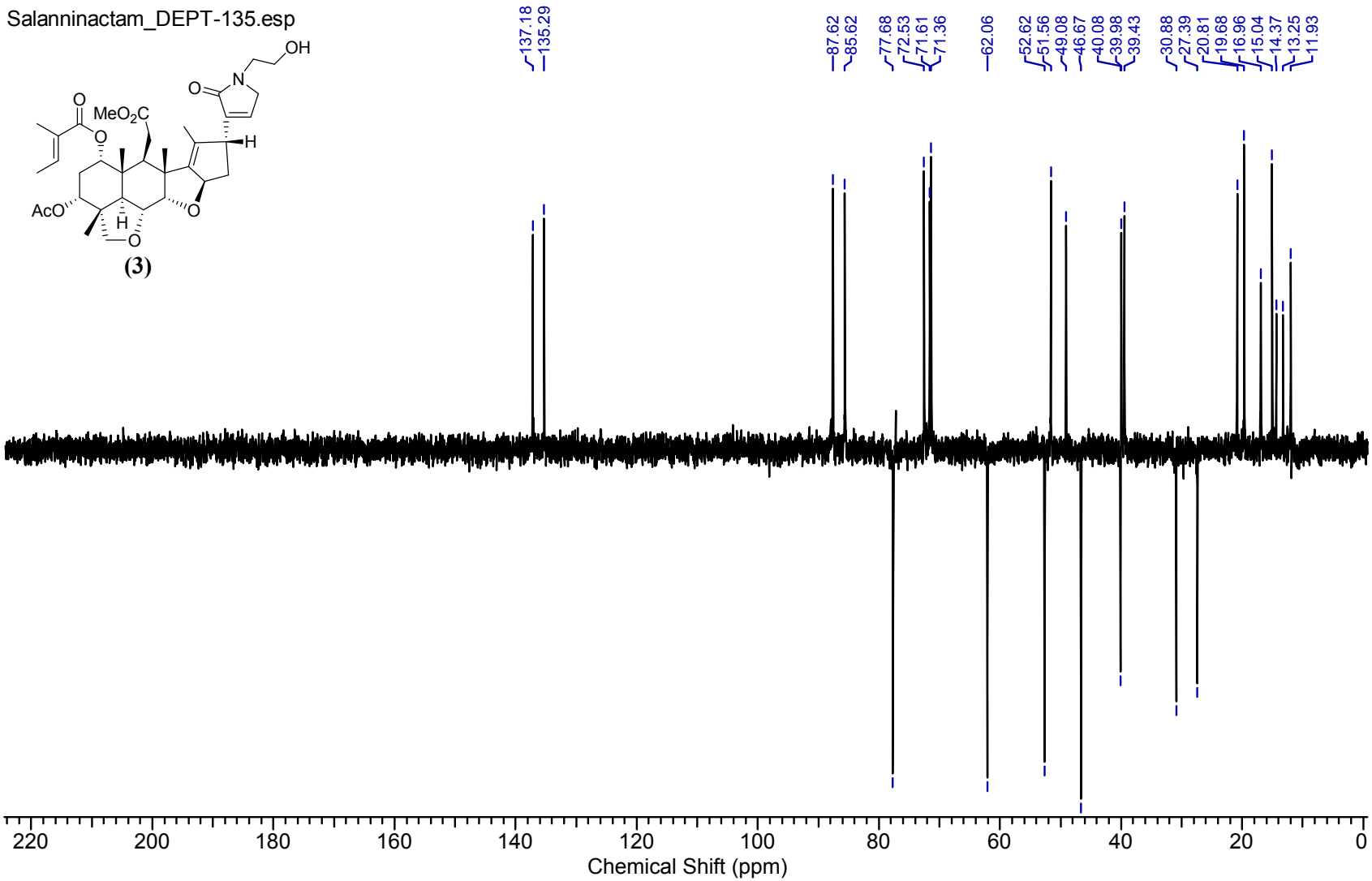


Fig. S13 DEPT-135 NMR spectrum of salanninactam (**3**) in $CDCl_3$.

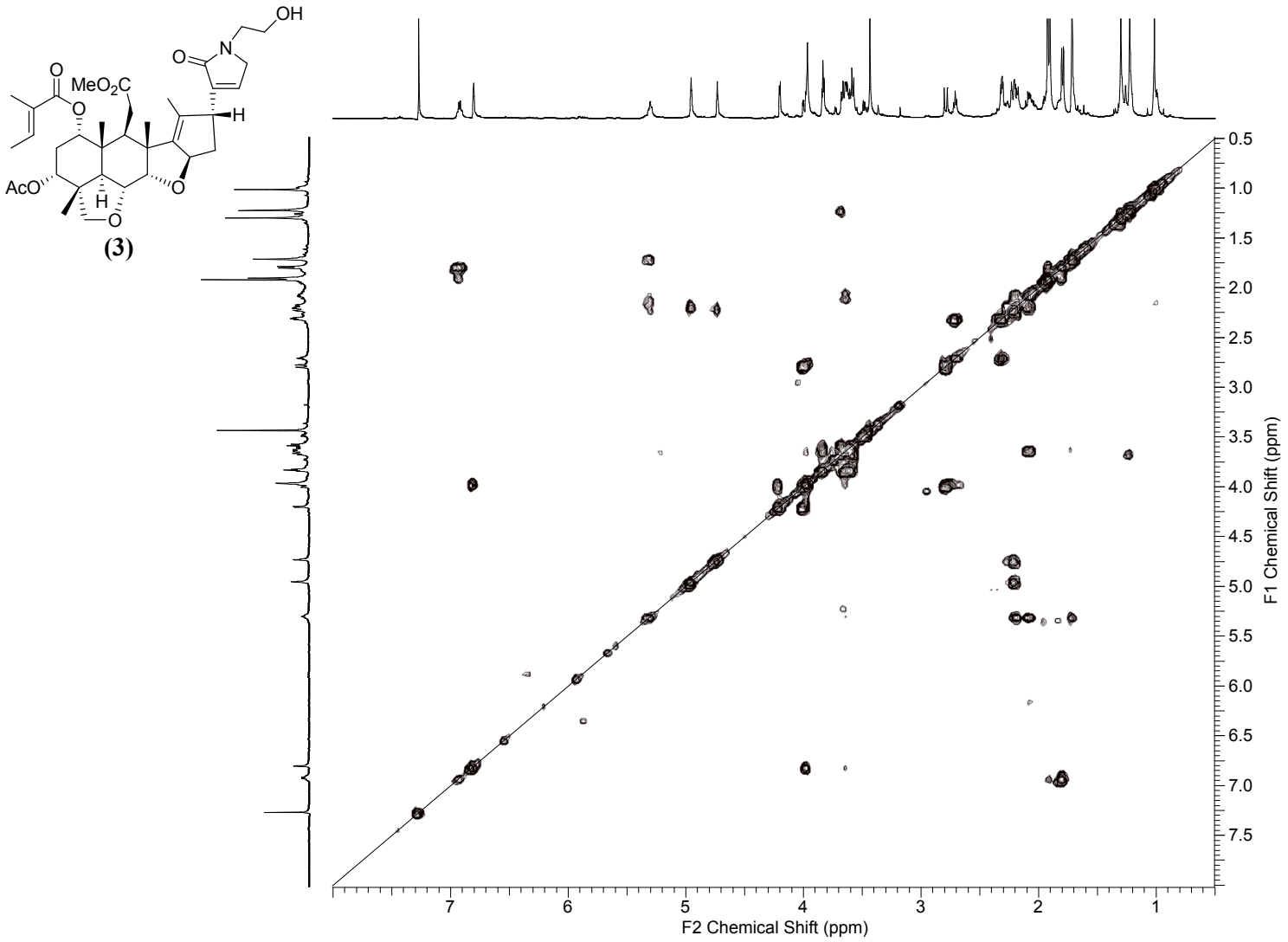


Fig. S14 COSY spectrum of salanninactam (**3**) in $CDCl_3$.

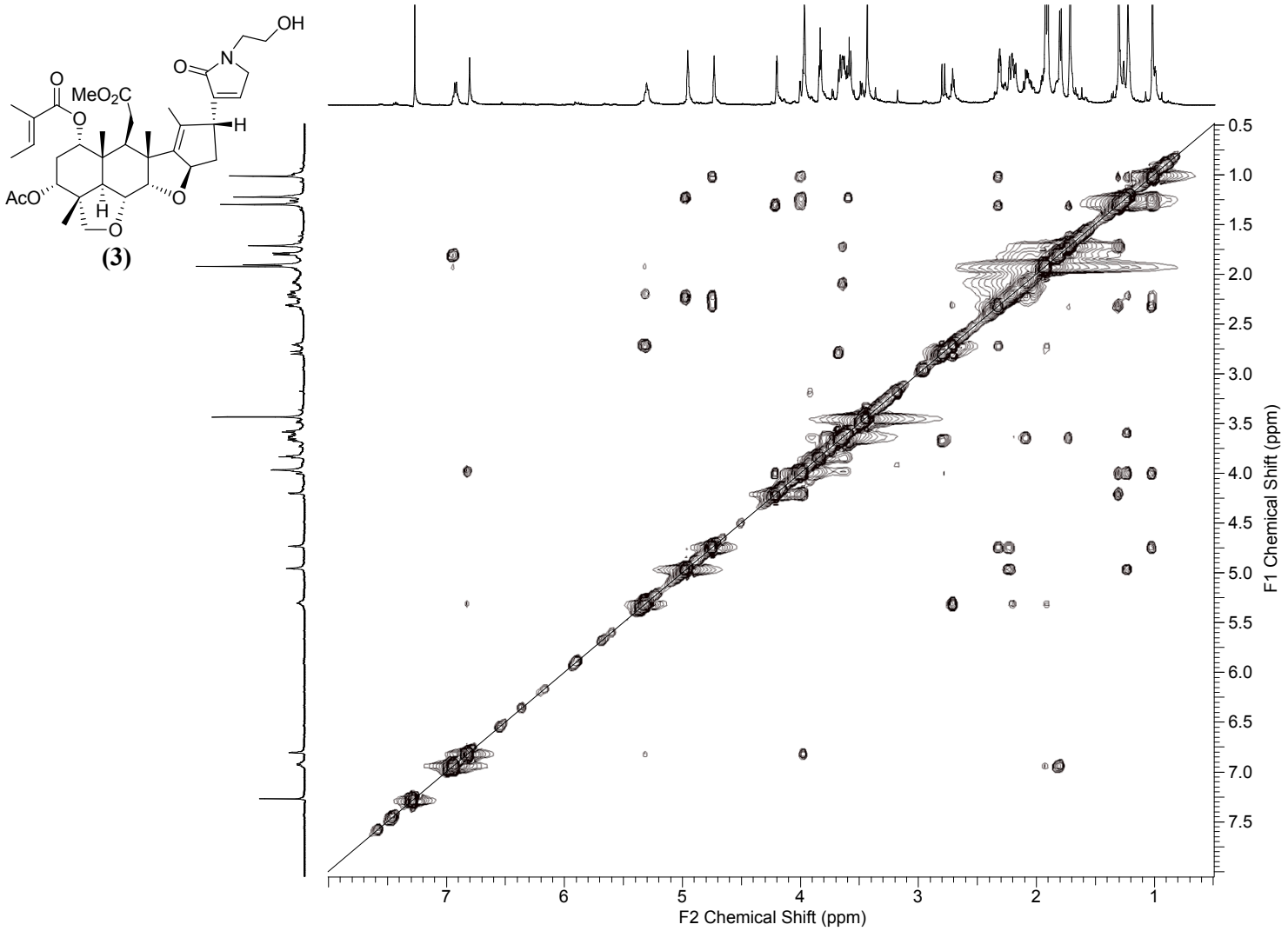


Fig. S15 NOESY spectrum of salanninactam (**3**) in $CDCl_3$.

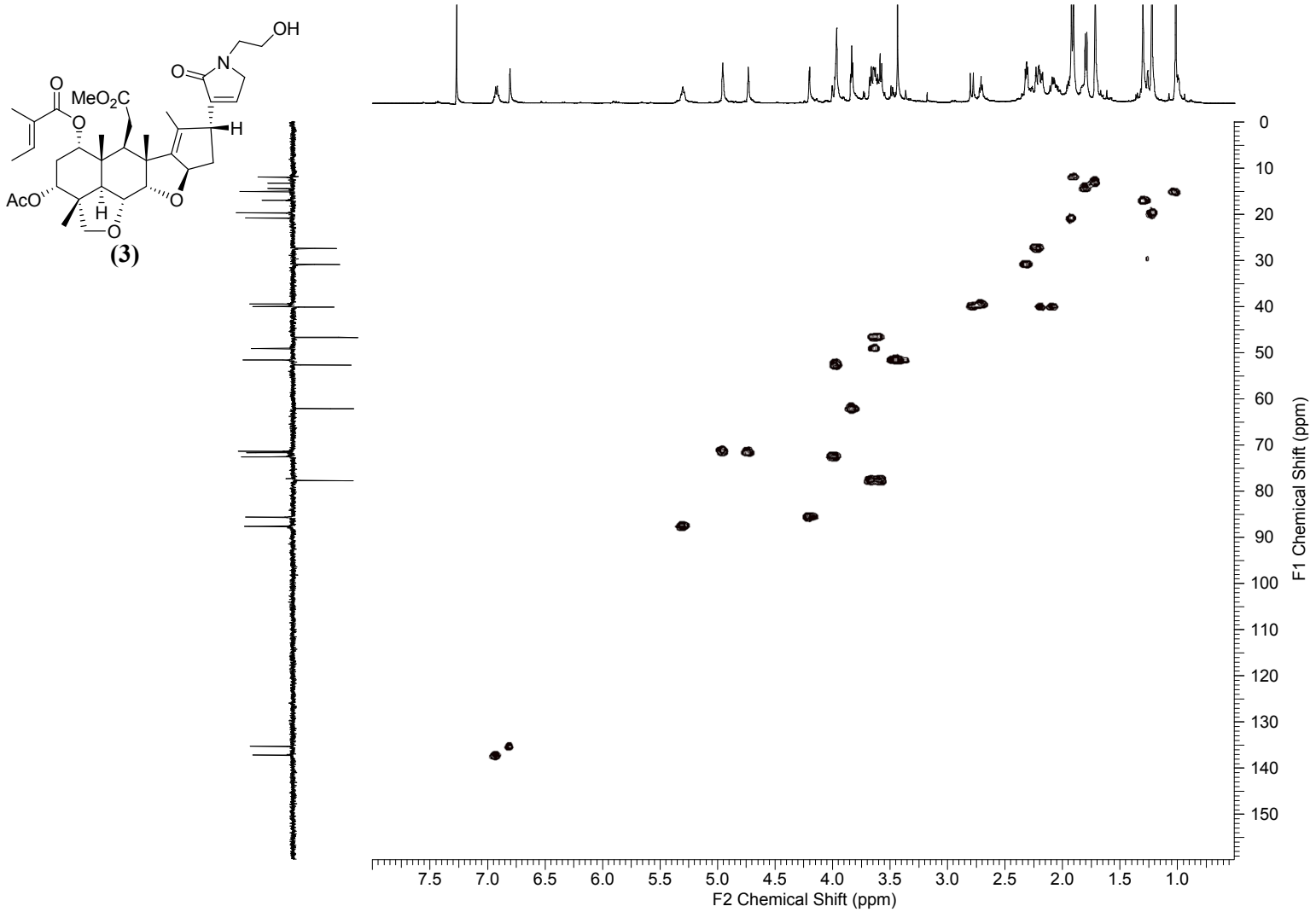


Fig. S16 HSQC spectrum of salanninactam (**3**) in $CDCl_3$.

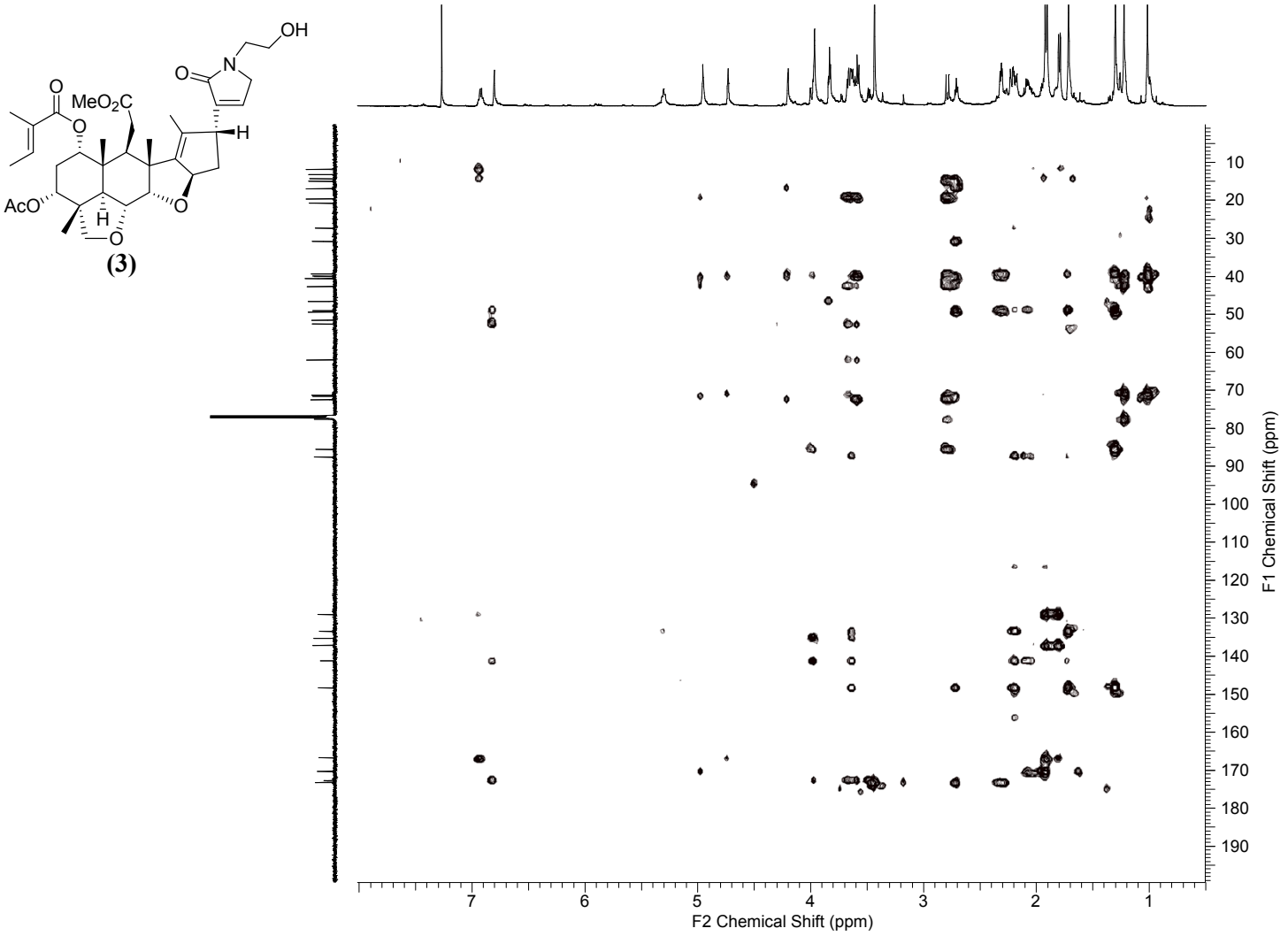


Fig. S17 HMBC spectrum of salanninactam (**3**) in $CDCl_3$.

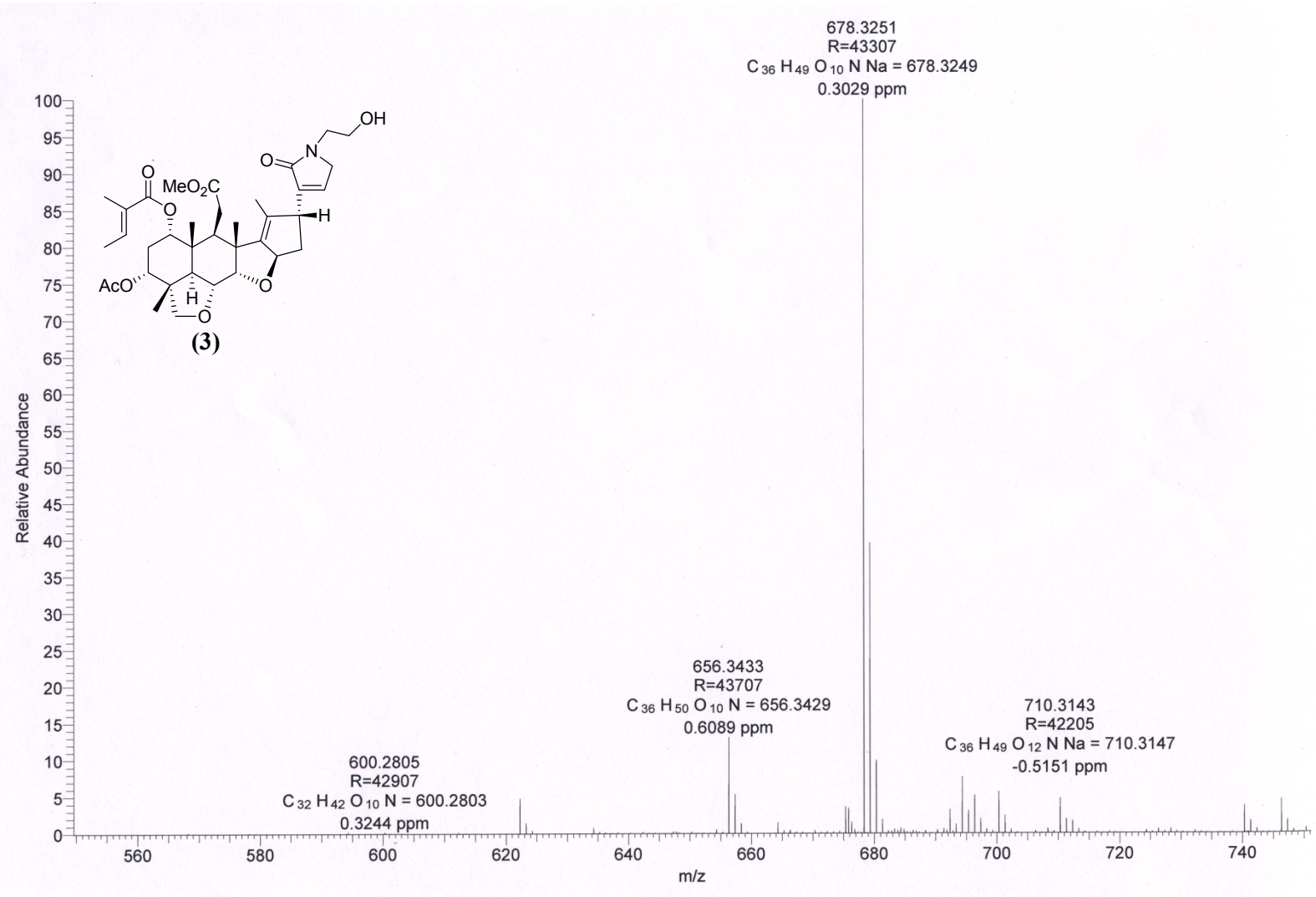


Fig. S18 LC-ESI-HRMS spectrum of salanninactam (**3**).

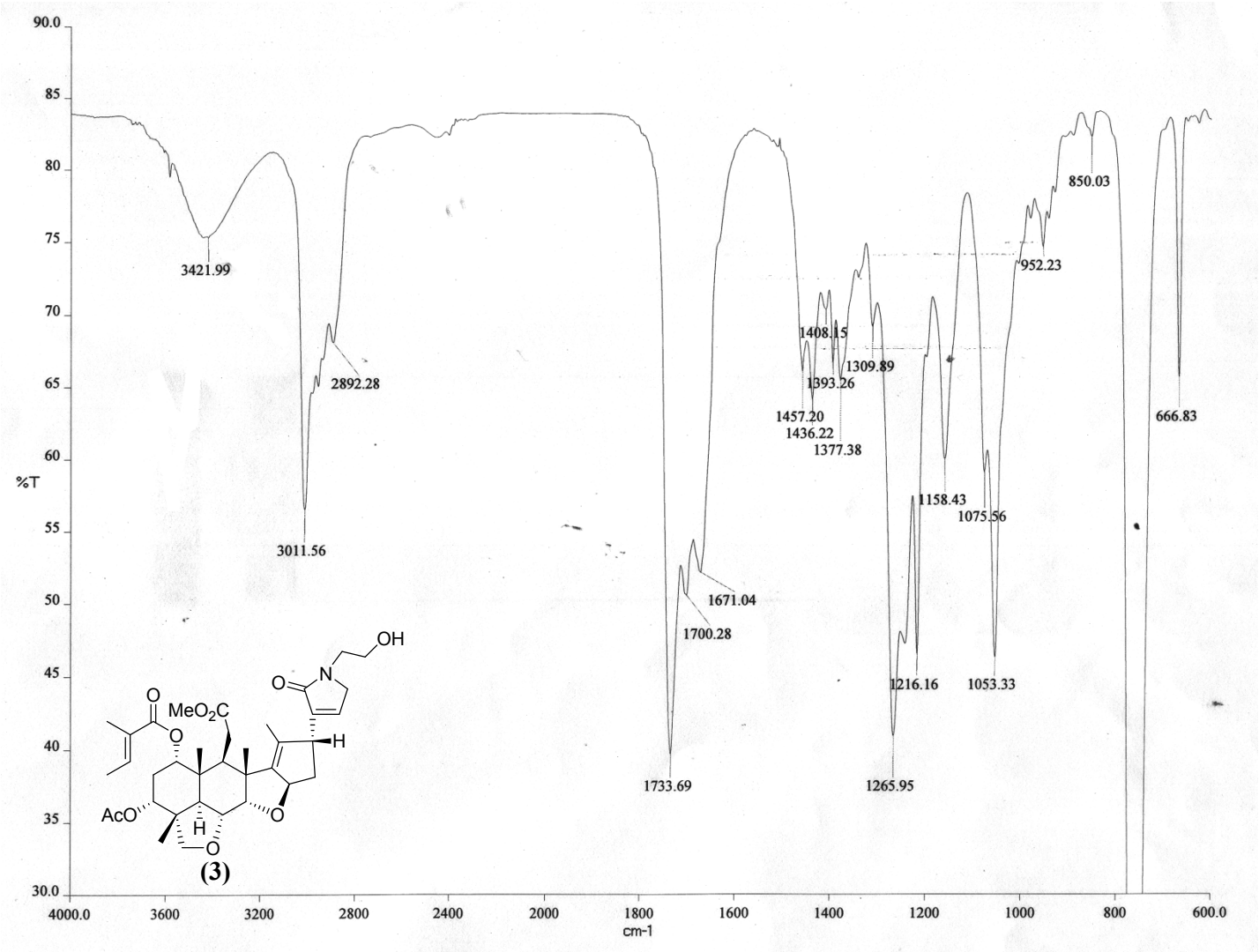


Fig. S19 IR spectrum of salanninactam (**3**) in $CHCl_3$.