SUPPLEMENTARY MATERIAL

Continental and Antarctic Lichens: Isolation, identification and molecular modeling of the depside tenuiorin from the Antarctic lichen *Umbilicaria antarctica* as tau protein inhibitor

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Abstract: Alzheimer´s disease (AD) is the most common form of dementia involving Aβ and tau protein. So far, AD cure remains elusive, but considering that AD progresses throughout tau pathology, which turns tau protein an appropriate target, besides tau is also included in other neurodegenerative disorders named as tauopathies. Here, we have isolated seventeen compounds belonging to six lichens species. Due to scarce of spectroscopic data of the compound 5,7-dihydroxy-6-methylphthalide, we explained their structural elucidation based on NMR data. In this study, we show that only tenuiorin from *Umbilicaria antarctica* inhibited 50 % of tau 4R at 100 μM. Then, we shown that molecular interactions of tenuiorin with the steric zipper model of the hexapeptide ^{306}VQIVYK^{311} were studied by docking calculations and the results suggested that tenuiorin forms both hydrogen bonds with lysine and glutamine side chains and forms several hydrophobic interactions with valine and lysine from ^{306}VQIVYK^{311} motif in the same way as observed for orange G and other known drugs.

Keywords: Alzheimer´s disease; Tau protein; Lichens.

Experimental part

General procedures

TLC was performed on Kieselgel 60 GF254 using *n*-hexane/EtOAc (6:4 v/v) as mobile phase. TLC spots were visualized by spraying the chromatograms with H_2SO_4-MeOH (5:95, v/v) and heating at 120°C for 2-3 min. Column chromatography (CC) was performed over Merck Kieselgel 60, particle size 0.063-0.200 mm. All solvents were dried and purified before use according to standard procedures.

Instrumentation

Measurements of NMR spectra used a Bruker Avance AM-400 spectrometer equipped with 5 mm probes. All lichen substances were individually dissolved in 0.8 ml of $CDCl_3$ or CD_3COCD_3 containing tetramethylsilane (TMS) as internal standard. Chemical shifts (δ) were reported in ppm and coupling constants (*J*) in Hertz. Mass spectra acquired using a Thermo Q EXACTIVE model spectrometer.

Material

Umbilicaria antarctica, Stereocaulon ramulosum, Usnea antarctica and *Catillaria corimbosa* were collected in "Peninsula Fildes", King George Island, Antarctic during March, 2014. Carefully we removed lichens from the rocks by abrasion. Samples were identified with both traditional methods and specialized literature. Vouchers specimens were deposited at the Extreme natural product laboratory, Universidad de Chile with the reference numbers: UA-010414, SR-010414, USA-010414, and CC-010414 respectively. *Everniopsis trulla* and *Usnea barbata* were collected at Huaraz, Perú, in 2011 (altitude of 3427 m) and at Chillan, VIII Region, Chile, in 2015 respectively. A voucher specimen Nº ET-15032011 and UB-06092015 was generated, respectively.

Extraction and isolation

Umbilicaria antarctica (50g) was dried, powdered and extracted with methanol (MeOH, 3 x 0.5L). After concentration under reduced pressure below 40 ºC, 3.0 g of extract was obtained. The MeOH extract was subjected to Sephadex LH-20 using as mobile phase MeOH. Some 180 fractions (10mL) were monitored by TLC and combined to give three fractions A-C. Fraction A (1.0 g) was chromatographed on silica gel (150g, 63-200µm) using *n*-hexane/EtOAc mixtures (0 up to 100%) to yield 120 mg of tenuiorin **1**, and 600 mg of usnic acid **2**. Fraction B (0.5 g) and C (3.0 g) were chromatographed on silica gel (SiO_2, 63-200µm) using DCM/MeOH mixtures (0 up to 100%) giving

unsuccessful results. Fraction B (100 mg) was then subjected to preparative HPLC (Prepchrom C-700, Büchi, Switzerland) using water/MeOH as mobile phase (gradient 9:1; 8:2; 7:3; 6:4; 1:1; 4:6; 3:7; 2:8; 1:9; 0:10) yielding 30 mg of gyrophoric acid **3** and 50 mg of methyl orsenillate **4**. Fraction C (100 mg) was submitted to preparative HPLC. The HPLC gradient was water/MeOH (9:1; 8:2; 7:3; 6:4; 1:1; 4:6; 3:7; 2:8; 1:9; 0:10) and led to the isolation of 20 mg of compound **4** and 60 mg of lobaric acid (**5**).

S. ramulosum (100g) was dried, powdered and extracted with methanol (MeOH, 3 x 1L). After concentration under reduced pressure below 40 ºC, 6.0 g of extract was obtained. Following the same procedure showed above, the compounds usnic acid (800mg; **2**), atranorin (900mg; **6**) and methyl orsenillate (50mg; **4**) were isolated.

E. trulla (60g) was dried, powdered and extracted with methanol (MeOH, 3 x 0.5L). After concentration under reduced pressure below 40 ºC, 4.0 g of extract was obtained. Following the same procedure showed above, the compounds methyl haematommate (10 mg; **7**), ethyl haematommate (30 mg; **8**), atranol (7 mg; **9**), usnic acid (500 mg; **2**), atranorin (600 mg; **6**), chloroatranorin (12mg; **10**) and gyrophoric acid (70mg; **3**) were isolated.

Usnea antarctica (100g) was dried, powdered and extracted with methanol (MeOH, 3 x 1L). After concentration under reduced pressure below 40 ºC, 5.0 g of extract was obtained. Following the same procedure showed above, the compounds usnic acid (1.5 g; **2**), 5,7-dihydroxy-6-methylphthalide (11 mg; **11**), fumarprotocetraric acid (20 mg; **12**), and lobaric acid (45 mg; **5**) were isolated. Compound **11**: ^{1}H NMR (400 MHz, CD_3COCD_3): 6.59 (1H, s, H-4), 5.19 (2H, s, H-3), 2.05 (3H, s, H-8). ^{13}C NMR (100 MHz, CD_3COCD_3): 173.0 (C-1), 164.1 (C-5), 156.2 (C-7), 147.3 (C-3a), 111.5 (C-6), 103.7 (C-7a), 101.4 (C-4), 70.8 (C-3), 7.8 (C-8). HRESIMS (M-H): calculated for $C_9H_7O_4$: 179.0350, found: 179.0342.

C. corimbosa (10g) was dried, powdered and extracted with methanol (MeOH, 3 x 0.2L). After concentration under reduced pressure below 40 ºC, 0.5 g of extract was obtained. Following the same procedure showed above, the compounds atranorin (6 mg; **6**), and protolichesterinic acid (21 mg; **13**) were isolated.

Usnea barbata (200g) was dried, powdered and extracted with methanol (MeOH, 3 x 1L). After concentration under reduced pressure below 40 ºC, 8.0 g of extract was obtained. Following the same procedure showed above, the compounds usnic acid (3.5 g; **2**), galbinic acid (14 mg; **14**), norstictic

acid (19 mg; **15**) divaricatic acid (130 mg; **16**), and salazinic acid (29 mg; **17**) were isolated. All known compounds (Figure S1) were isolated and identified on the basis of their spectroscopic data (Huneck and Yoshimura 1996).

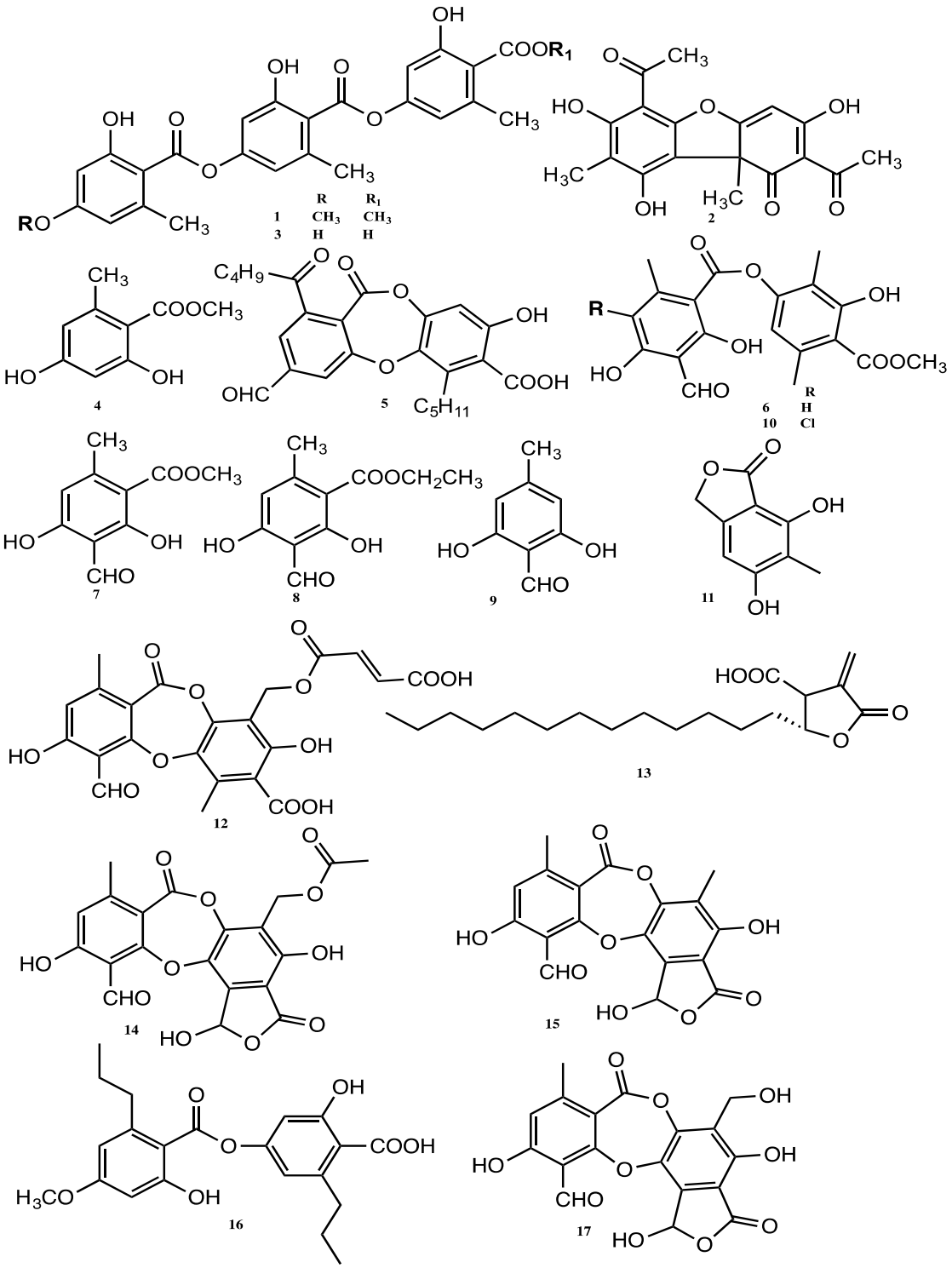

Figure S1. Secondary metabolites (**1-17**) isolated from lichens.

Tau protein production

Tau fragment 4R ($htau_{244-372}$) was amplified by using the plasmid for htau40 as a template. The PCR sequence was subcloned into pET-28a vector (Novagen) to produce a His-tagged protein. The recombinant fragment 4RMBD was expressed in *Escherichia coli* strain BL21 (DE3) as described (Cornejo et al. 2011). LB medium containing kanamycin was inoculated with a stationary overnight culture. Bacterial culture was grown at 37°C to OD_{600} of 0.5-0.6 and protein expression was induced by addition of 1 mM IPTG for 4 h; and cells were pelleted and sonicated. Recombinant tau was purified via a succession of Ni-Sepharose chromatography (equilibrated in 20 mM NaH_2PO_4, 500 mM NaCl, and 20 mM imidazole, pH 7.4, elution with buffer 200 mM imidazole) and side exclusion chromatography coupled to HPLC in a Proteema 100 column (PSS, Germany) with buffer 50 mM NaH_2PO_4 , 300 mM NaCl, pH 6.5. The purity of the protein was verified on a Coomassie Brilliant Blue-stained SDS-polyacrylamide gel. The protein was concentrated and stored at -80°C until use. The concentration of purified 4RMBD was determined using the extinction coefficient at 280 nm.

Thioflavin T assay

The ThT fluorescence assay was performed as reported by Cornejo et al. 2016. Briefly, to examine the inhibition of tau aggregation, the total volume of the reaction mixture was 100 µL, which included 20 µM 4RMBD, 5 µM heparin in 100 mM sodium acetate, pH 7.0 with the compound isolated **1-17** at different concentrations. After 20h of incubation at 37°C, addition of 100 µL of a 25 µM solution of ThT was performed and the incubation continued for an additional hour at room temperature prior to fluorescence reading. Then, the fluorescence was measured in a Biotek H1 spectrofluorimeter (Biotek Instruments, USA) with an excitation wavelength at 440 nm and emission wavelength of 485 nm in a 96-well plate. Each experiment was replicated at least three times and background fluorescence was subtracted when needed.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 6 software. Data is presented as Mean ± SE by using Paired t-test. Significance was determined as $p < 0.05$.

Docking method

The only crystal structure of tau protein was reported by Landau et al. 2011 and is in Protein Data Bank with the code 3OVL; this structure is the hexapeptide ^{306}VQIVYK^{311} forming complex with orange-G. Other reports have suggested that phenolic compounds could form similar complexes with this tau fragment (Cornejo et al. 2016, Mohamed et al. 2013). In this work, the structure of the hexapeptide ^{306}VQIVYK^{311} was used as the receptor of docking experiments and the model of tenuiorin was sketched using the molecular editor of the software Maestro (Maestro, version 10.1, Schrodinger, LLC, New York, NY, 2015). The Protein Preparation Wizard module also included in Maestro was employed for preparing the protein structure and for obtaining three-dimensional coordinates for the tenuiorin. We docked the tenuiorin structure inside a cavity formed between two steric zippers (named model A) and on the surface of one steric zipper (named model B) replicating the protocol reported (Cornejo et al. 2016).

Docking was performed by using the Glide method (Alzate-Morales et al. 2010, Friesner et al. 2004). A grid box of 18Å × 10Å × 10Å covered the whole cavity in model A and whole surface in model B. Docking parameters were used as in previous work (Alzate-Morales et al. 2010); Glide extra-precision (XP) mode was explored during the search. Glide uses hierarchical filters to explore ligand conformations and place it in the receptor site. The hierarchy includes a site-point search and identification of atoms capable of making hydrogen bonds. Poses with good interactions with the receptor are preliminarily scored, but they are refined and minimized on the van der Waals and electrostatic grids of the receptor, leading to a re-scoring with the *Glide-Score XP* scoring function (Friesner et al. 2006). After, performing this process, the more energetically favourable conformation was selected as the final solution of the docking for both models A and B (Figure 2S).

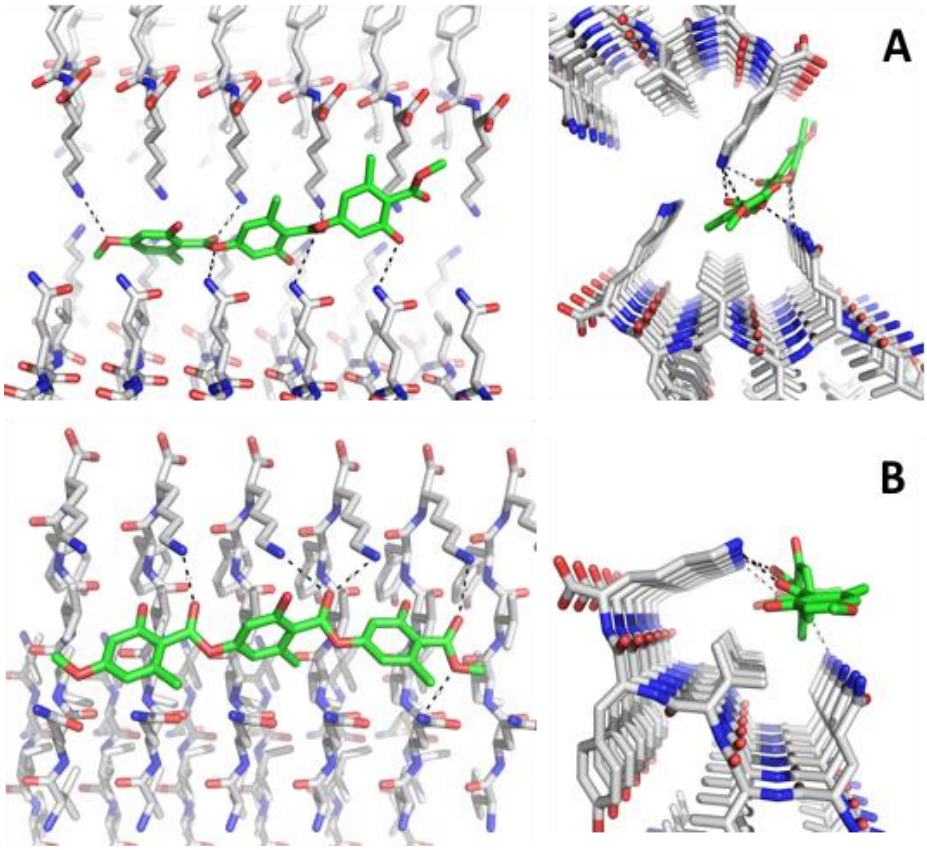

Figure S2. Docking images of tenuiorin in the interface of two poly-^{306}VQIVYK^{311} hexapeptide zippers (**A**) and on the surface of one steric zipper (**B**). In both A and B: *left*: the view is perpendicular to the fiber axis; *right-top*: view looks down the fiber axis. Hydrogen bounds (HBs) between tenuiorin and lysine and glutamine residues of the hexapeptide are represented as dashed lines.

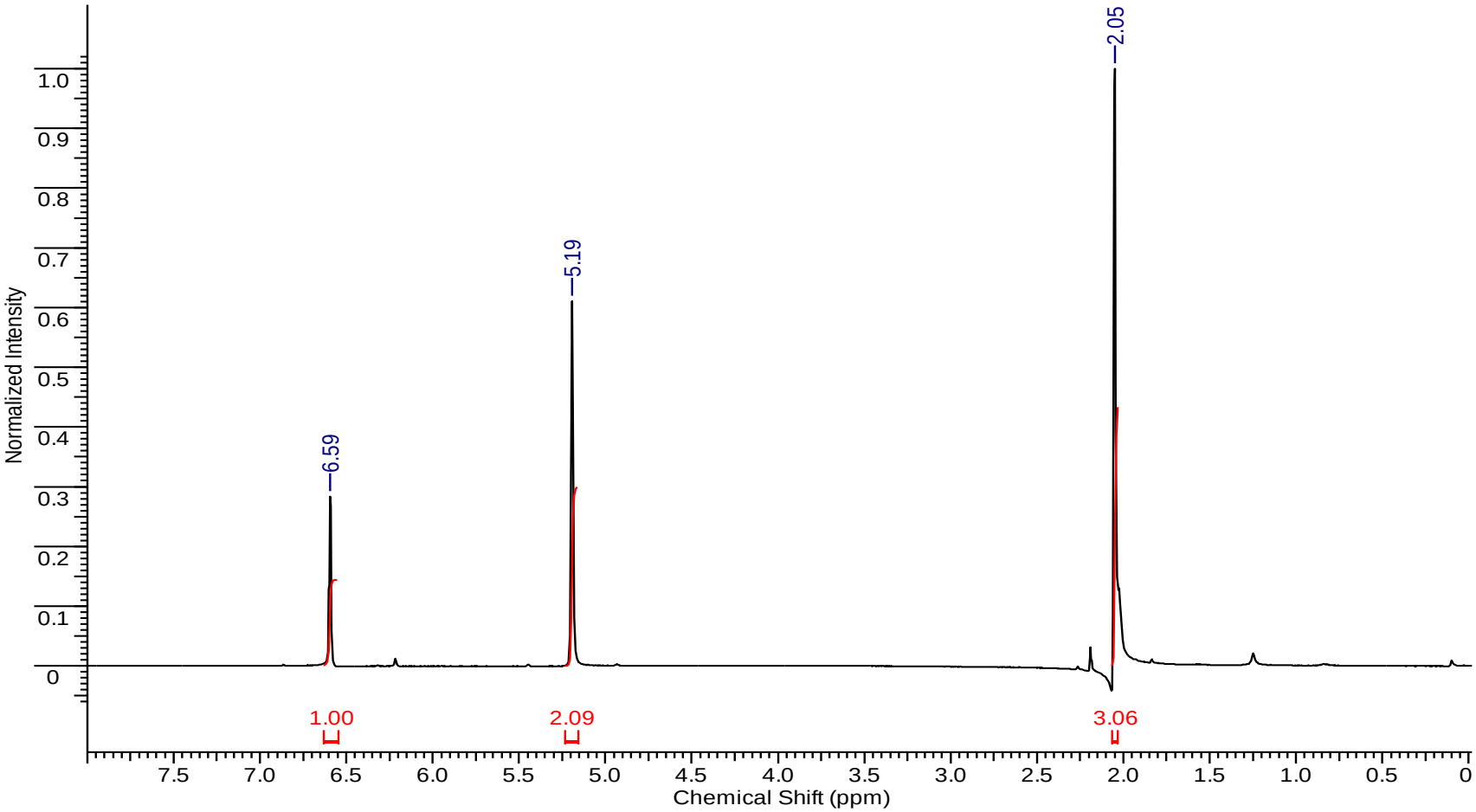

Figure S3. ^{1}H-NMR spectrum of compound **11** (CD_3COCD_3)

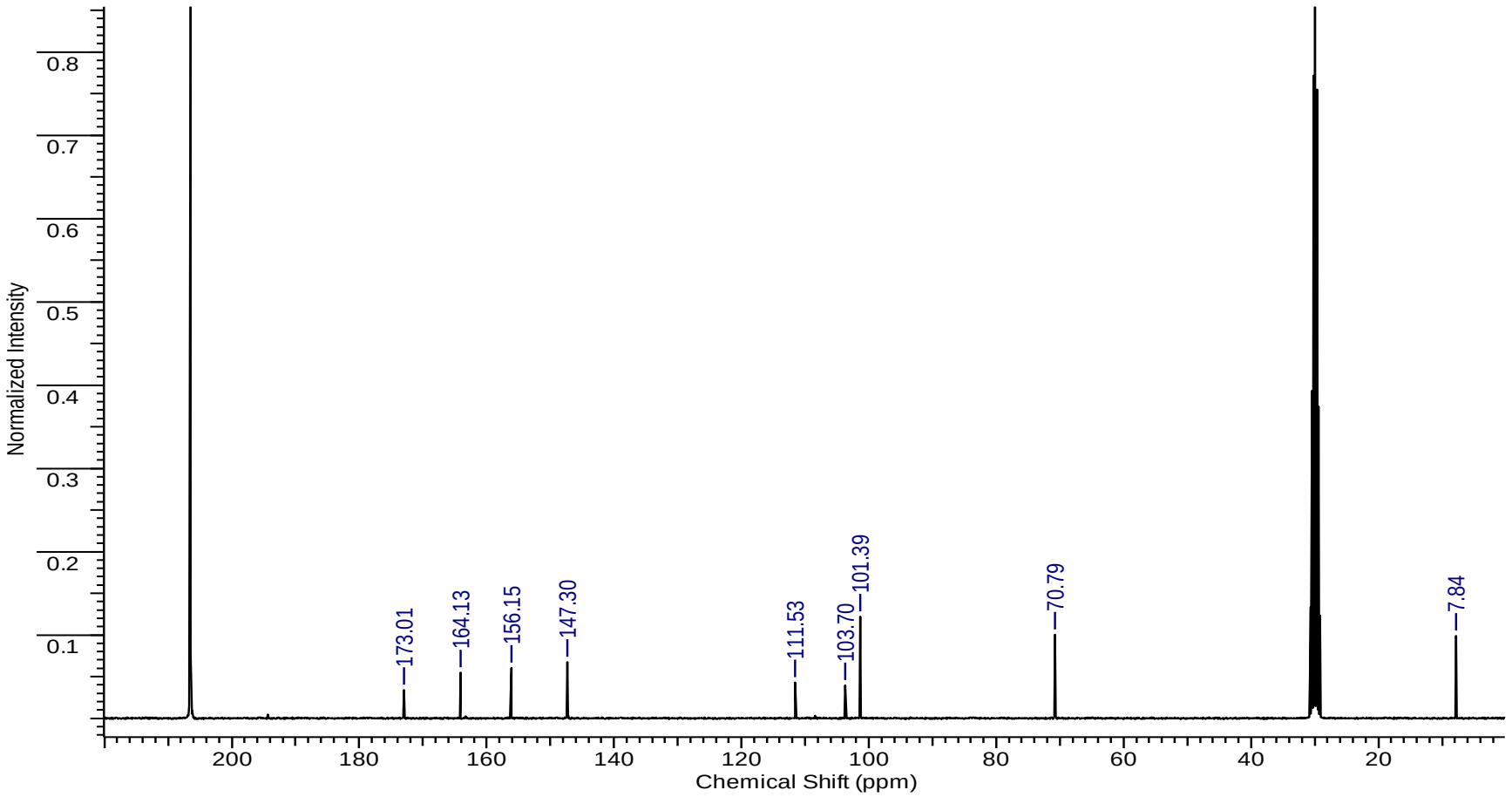

Figure S4. ^{13}C-NMR spectrum of compound **11** (CD_3COCD_3)

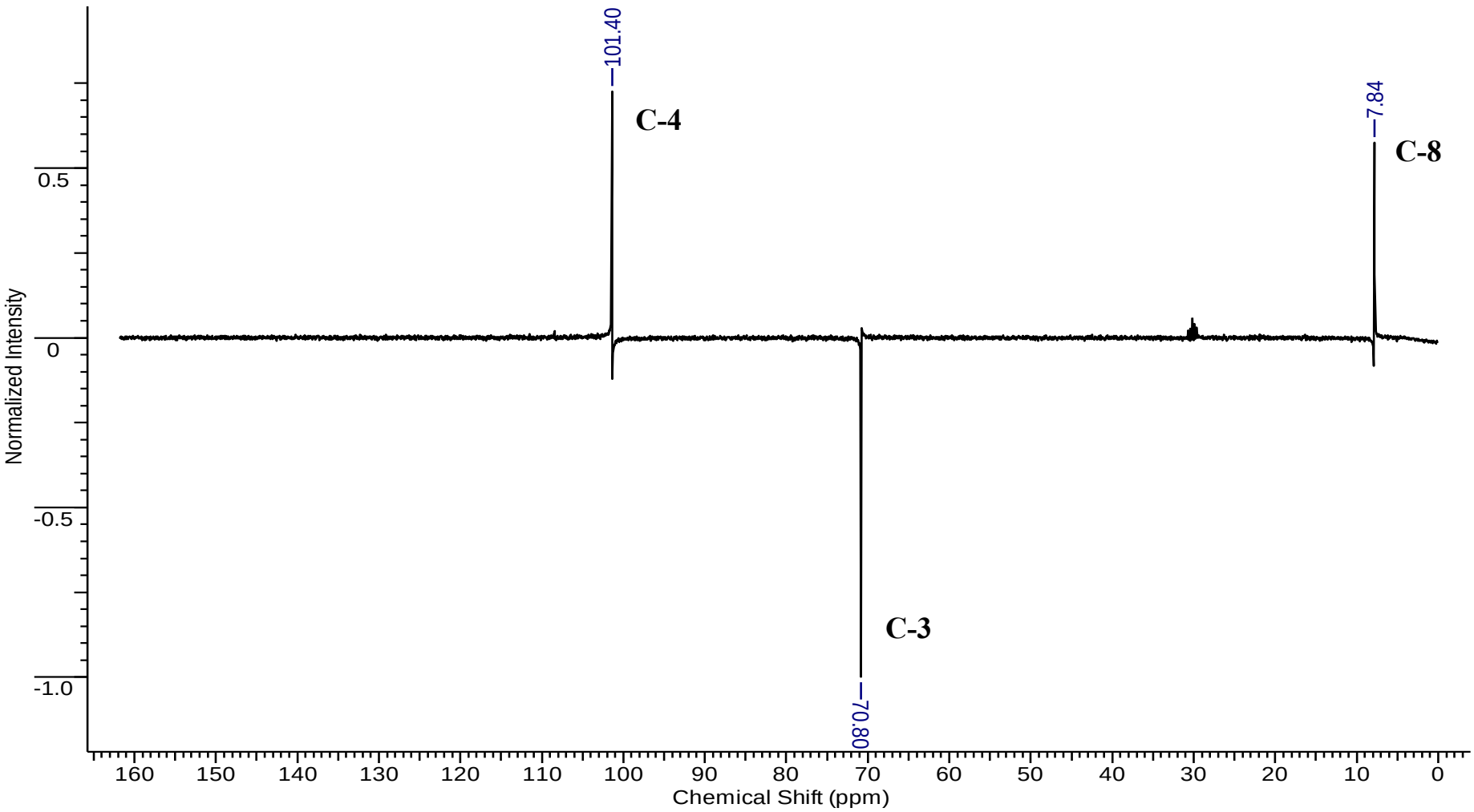

Figure S5. DEPT 135 spectrum of compound **11** (CD_3COCD_3)

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