Orbicularisine: A Spiro-Indolothiazine Isolated from Gills of the Tropical Bivalve Codakia orbicularis

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ABSTRACT: A novel spiro-indolofuranone fused to a thiazine skeleton, orbicularisine (**1**), was isolated from gills of the mollusk *Codakia orbicularis*. The isolation, structure elucidation using spectroscopic evidence including mass and NMR spectroscopy are described. The final structure of **1** was supported key HMBC correlation.

Mollusks are the second largest animal phylum on Earth constituting approximately 7% of living animals.[1] For 52,000 named species of marine mollusks described to date,[2] no more than 1000 have been subjected to chemical studies leading to the description and characterization of about 1000 secondary metabolites.[3] Thereby, marine mollusks have become the focus of many natural products studies aimed at the isolation of new metabolites.1 Some of the metabolites have therapeutic value, such as ziconotide, which was isolated from cone snails and is used for the treatment of chronic pain.[4] Kahalalide F (ES-285), isolated from a bivalve, has succeeded in Phase I of clinical trials. Finally, dolastatin 10, described from a sea-hare, showed anticancer activity and is currently in Phase II of clinical trials.[1,5] A synthetic analog of dolastatin 10 linked to an anti-CD30 antibody, Adcetris, that is known as brentuximab vedotin, is the latest marine drug to successfully enter into the market.

We herein describe the isolation of a novel metabolite orbicularisine (**1)** from the tropical bivalve *Codakia orbicularis* living in seagrass beds. While this mollusk has been studied for years as a biological model for shallow water thio-autotrophic bacterial symbiosis,[6] as far as we are concerned, no chemical researches were conducted up now. This clam harbors intracellular sulfur-oxidizing bacteria within its gill tissues[7] and such bacterial population is probably regulated by the host itself, using specific molecules with bacteriostatic activities.

RESULTS AND DISCUSSION

Gill filaments were extracted with EtOAc. From this latter EtOAc extract, 20 g of elemental yellow elemental sulfur S_8 precipitated.[8] After filtration and solvent evaporation of the supernatant, 25 g of extract were obtained. The residue was chromatographed on silica gel column using a gradient of solvent mixtures starting from hexane-EtOAc, and then EtOAc-MeOH. The most antibacterial fractions according to disc agar-plate method[9] were subjected to repeated separation and purification by HPLC (Sunfire C-18 columns) to provide a total of 8.1 mg of pure orbicularisine (**1**) (Figure 1).

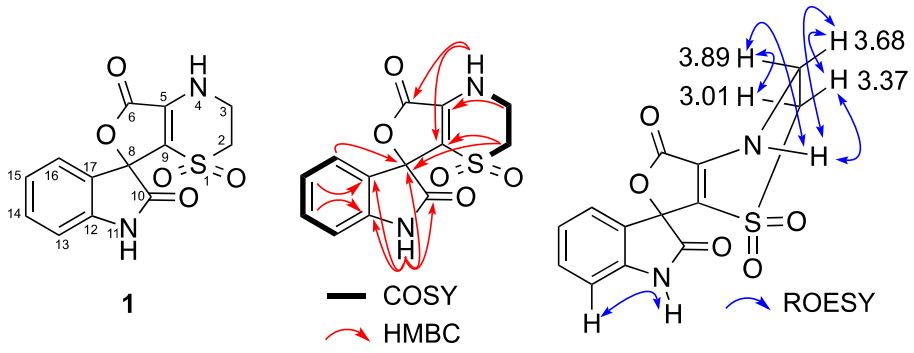

Figure 1. Structure of orbicularisine (**1**), COSY, key HMBC and ROESY correlations.

The molecular formula of compound **1** was suggested to be $C_{13}H_{11}N_2O_5S$ by HRESIMS which displayed a protonated molecule at *m/z* 307.0391 $[M+H]^+$, appropriate for 10 degrees of unsaturations. ^{1}H NMR and HSQC spectra revealed two methylenes at δ_H 3.01 (H-2b)/3.37 (H-2a) correlated to the carbon at δ_C 48.1 (C-2), and δ_H 3.68 (H-3b)/3.89 (H-3a) correlated to δ_C 40.8 (C-3). The COSY correlations sequence between H-2, H-3 and H-4 (NH) (Figure 1), and the characteristic ^{13}C chemical shifts at δ_C 48.1 and 40.8 (Table 1) suggested a part of the thiazine SO_2–C-2–C-3–N-4.[10,11] The characteristic strong IR absorbance at 1129 cm^{-1} supported the sulfone function. Further HSQC analysis (Table 1) showed clearly an aromatic part where the protons at δ_H 7.39 (H-16), 7.36 (H-14), 7.02 (H-15) and 6.91 (H-13) were attached to carbons resonating at δ_C 126.6 (C-16), 132.1 (C-14), 122.6 (C-15) and 110.8 (C-13) respectively.

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compound **1** in DMSO-d_6 (^{1}H 500 MHz, ^{13}C 125 MHz)

atom	Orbicularisine (**1**)	
	δ_C / δ_N, type	δ_H, mult (*J* in Hz)
2a	48.1, CH_2	3.37, m
2b		3.01, ddd (13.6, 12.6, 4.0)
3a	40.8, CH_2	3.89, m
3b		3.68, ddd (13.8, 12.6, 2.0)
4	74.1, NH	8.47, brs
5	138.3, C	-
6	165.1, C	-
8	82.2, C	-
9	111.2, C	-
10	170.9, C	-
11	133.8, NH	10.96, brs
12	142.8, C	-
13	110.8, CH	6.91, d (7.6)
14	132.1, CH	7.36, dt (7.6, 1.1)
15	122.6, CH	7.02, dt (7.6, 1.1)
16	126.6, CH	7.39, d (7.6)
17	122.4, C	-

The remaining exchangeable proton signals (δ_H 10.96 (H-11)/8.47 (H-4)) were attributed by ^{1}H-^{15}N HSQC, as an amide (δ_N 133.8, N-11) and a secondary amine (δ_N 74.1, N-4). The UV spectrum (λ_{max} (MeOH): 210, 250 and 292 nm) suggested the presence of an indoline chromophore group.[12] Moreover, strong HMBC correlations were observed from the amide proton H-11 at δ_H 10.96 to the spiro-carbon C-8 at δ_C 82.2 and the non-protonated carbon C-17 at δ_C 122.4. In addition, weak correlations were observed between H-11 and the non-protonated carbons C-12 (δ_C 142.8) and C-10 (δ_C 170.9). The HMBC correlations between the protons H-14 / H-15 and the carbons C-12 (δ_C 142.8) / C-17 (δ_C 122.4) respectively suggested the indolone partial structure.[13,14,15] These results suggested a lactam connected to the aromatic spin system C-

16–C-15–C-14–C-13. Fusion of this moiety to the remaining unassigned carbons δ_C 111.2 (C-9), 138.3 (C-5) and 165.1 (C-6) was deduced from the long-range HMBC correlations from the proton at δ_H 3.37 (H-2a) to the spiro-carbon at δ_C 82.2 (C-8) and the non-protonated carbon at δ_C 111.2 (C-9). Likewise the proton at δ_H 3.89 (H-3a) showed correlations with the non-protonated carbon at δ_C 138.3 (C-5). In addition H-4 of the thiazine NH showed a correlation with C-6 and confirms the orientation of the thiazine.

These latter were together attached to form a double bond and a cyclic thiazine.[16] According to Davis and co-workers, thiaplakortone displayed a double bond in a quinone-thiazine bicyclic system with similar chemical shifts.[17] In accordance with the HRMS data, the two carbonyl oxygens completed the formula. Moreover, on the basis of HMBC data, the amine proton H-4 of the cyclic thiazine showed correlations with the lactone carbonyl at δ_C 165.1 (C-6) and the non-protonated carbons at 111.2 (C-9) and 138.3 (C-5). Thus, the cyclic thiazine was found to be fused with the furanone, which is connected to the indolone through the spiro-carbon C-8 at δ_C 82.2. IR data of compound **1** secured this assignment because the vibration frequency of this lactone was 1787 cm^{-1} corresponding to a furanone.[18] It is interesting to note that the IR spectrum confirmed the existence of the carbonyl at 1740 cm^{-1}, secondary amide and amine at 3308 cm^{-1}. The structural fragments were validated by LTQ-Orbitrap-SM^2 due to the formation of sodium fragment at ions *m/z* 193, 220, 257 and 285(Figure 2).[19]

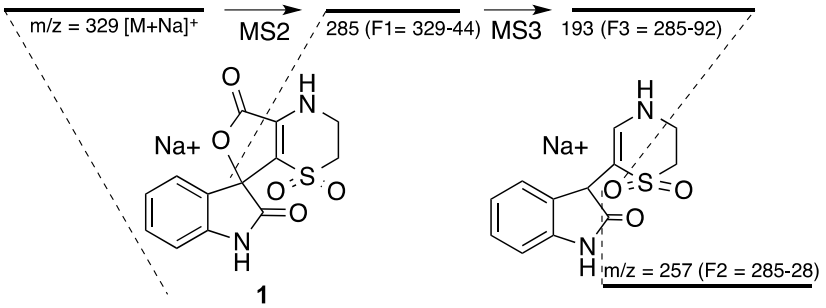

Figure 2. LTQ-Orbitrap-MS^2 and LTQ-Orbitrap-SM^3 fragmentations from the sodium adduct ion $[M+Na]^+$ of compound **1**.

At this step, all atoms had been accounted for the appropriate structure **1-A** (Figure 3). Although the regioisomer 1-B had been considered, the HMBC correlations from the NH of the thiazine to the lactone carbonyl carbon and the proton at δ_H 3.37 (H-2a) to the spiro-carbon at δ_C 82.2 (C-8) and the non-protonated carbon at δ_C 111.2 (C-9) correlated to the regioisomer **1-A.**

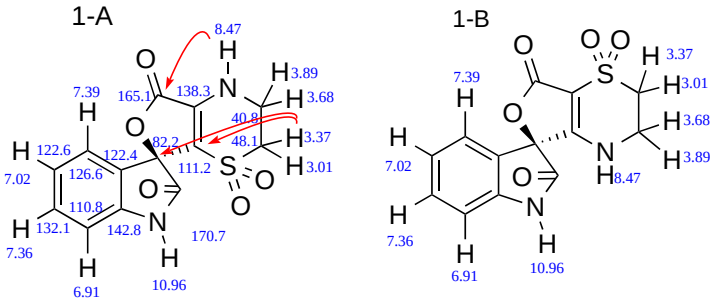

Figure 3. Discriminatory HMBC correlations corroborating the structure 1-A for compound 1 instead of 1-B

Orbicularisine (**1**) contains one single stereocenter. The zero value of the optical rotation and the flat ECD spectrum indicated the racemic nature of the compound.

The spiro-indolo-thiazine skeleton of orbicularisine (**1**) could be derived from tryptophan and cysteine or their close derivatives indole-3-propionic acid and taurine deamination.

Orbicularisine (**1**) was subjected to various bioassays. Despite the antibacterial activity of the original fraction on Gram negative and Gram positive panel of bacteria, orbicularisine (**1**) which was the major compound of the fraction, was totally inactive against *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumonia*, *Klebsiella pneumonia* ATCC 700603, *Escherichia coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 25922.

Inhibition assays against a panel of kinases including Hs_CDK2/CyclinA, Hs_CDK5/p25, Hs_CDK9/CyclinT, Hs_RIPK3, Hs_Haspin, Hs_AuroraB, Ld_TLK, Hs-Pim1, Ssc_GSK3 a/b, Lm_CK1 and Rn_Dyrk1A showed residual activities > 60% for 16 μM/mL of orbicularisine (**1**). Finally, the treatment of HCT116 colon cancer cells and U87-MG glioblastoma cancer cells with concentrations up to 100 μM showed no activity. Further bioassays using various targets are in progress. It is intriguing to speculate about the role of orbicularisine (**1**) within the host bivalve. This issue is also central themes in the research efforts, which we will continue by additional chemical and biological investigations.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotations $[\alpha]_D$ was measured using an Anton Paar MCP-300 polarimeter. Electronic Circular Dichroism (ECD) experiments were performed on a Jasco J-810 spectropolarimeter. The IR spectrum was recorded on a Perkin Elmer BX FT-IR spectrometer. 1D and 2D NMR spectra were recorded on a Bruker Avance 500 MHz or a Bruker Avance 600 MHz (TXI 1.7 mm probe) (CNRS-ICSN). The chemical shifts are relative to the residual signal solvent (CD_3OD: δ_H 3.31; δ_C 49.20; DMSO-d_6: δ_H 2.5; δ_C 39.5). High-resolution mass spectra were obtained on a Waters LCT Premier XE spectrometer in electrospray ionization mode by direct infusion of the pure compounds. Fragmentations were conducted in electrospray and nanospray ionization mode, on a Thermo Scientific LTQ-Orbitrap connected with a chromatographic system Dionex Ultimate 3000. Column chromatography (63*3 cm) was performed on silica gel (MERCK silica gel, 70-230 mesh ASTM) and flash chromatography was performed on Redisep Rf (220-gram, 69-2203-422 TELEDYNE Isco). These were carried out using hexane, EtOAc and MeOH. Thin layer chromatography (TLC) was performed on commercial TLC plates (pre-coated Kiesegel 60 F254 TLC, 20 x 20 mm, thickness 0.25 mm,

Merck) and was visualized under UV (254nm and 366nm). Analytical HPLC analyses were performed using Waters Alliance 2695 separation module equipped with mass spectrometer (Waters ZQ 2000 with a single-quadrupole and electrospray ionization source), ELS detector (Waters 2420) and photodiode array detection DAD (Waters 996). Semi-preparative HPLC was performed using an Auto Prep system (Waters 600 controller and Waters 600 pump, equipped with a Waters 996 Photodiode Array Detector).

Biological Material. Adult *Codakia orbicularis* (Linné, 1758) individuals (40-60 mm shell length) were collected in May 2015 by hand from *Thalassia testudinum* sea-grass sediments in Guadeloupe (lat 16°9'0.596"N, long 61°33'41.797"W). The fresh material was identified by one of the authors (O. Gros). Fresh gills were dissected and stored at -20°C until chemical extraction.

Extraction and Isolation of Compound 1. Gills (3 kg) of gills were extracted with EtOAc at room temperature under agitation for 2 days. The EtOAc solution was stored at 4 °C for 12 h. The residue formed by precipitation, which is pure elemental sulfur $S_{8,}$ was separated from the EtOAc solution by filtration. The EtOAc fraction was evaporated under reduced pressure then under nitrogen purge. The residue (25 g) was fractionated by chromatography on a silica gel column using a solvent mixture of increasing polarity (EtOAc-hexane, EtOAc, EtOAc-MeOH and MeOH), to yield sixteen fractions (F_1-F_{16}). Fractions F_1 to F_{12} were eluted with a hexane-EtOAC gradient (20-100%) and fractions F_{13} to F_{16} with a EtOAc-MeOH gradient (10-100%). All 16 fractions were tested on Micrococcus luteus ATCC 10240 (Gram positive bacterium) and Escherichia coli ATCC 35218 (Gram negative bacterium) for qualitative antimicrobial activity using the disc diffusion assay and nine of them showed encouraging (up to 18 mm diameter) bioactivity. Fraction F_{14} (70 mg, eluted with EtOAc-MeOH/90-10%) was the most active. The HPLC profile of F_{14} was recorded using a stepwise gradient of milliQ H_2O and 2-100% MeCN,

both with 0.1% HCOOH (flow rate: 1mL/min; Waters Sunfire C-18 column, 150 x 4.6mm, 5µm). Semi-preparative HPLC of this fraction, (Waters Sunfire C-18 column, 150 x 10mm, 5µm; flow rate 4.5 mL/min) with the same transposed gradient, gave five sub-fractions $F_{14\text{-}1}$ (10 mg), $F_{14\text{-}2}$ (10.7 mg), $F_{14\text{-}3}$ (11.8 mg), $F_{14\text{-}4}$ (17,4 mg) and $F_{14\text{-}5}$ (6.8 mg). The major compound in $F_{14\text{-}1}$ at retention time 12.52 min was detected and was slowly precipitating to give 3.9 mg of the pure compound **1** (noted $F_{14\text{-}1res}$). Semi-preparative HPLC in the same conditions described above was performed on supernatant of $F_{14\text{-}1}$ and led to 1.9 mg of the pure compound **1**. Fraction $F_{14\text{-}2}$ gave equally 0.7 mg of **1**. Similar protocol applied to the next fraction F_{15} (184 mg, eluted with EtOAc-MeOH/80-20%) led to 1.6 mg of **1**. Finally, a total of 8.1 mg of this compound (**1**) named orbicularisine was isolated.

Orbicularisine (**1**): yellow powder; $[\alpha]_{25D}$ 0.0 (*c* 3.7, MeOH); UV (MeOH) λ_{max} (log ε) 210 (1.32), 292 (0.41) nm; IR (neat) υ_{max} 3308, 2934, 1788, 1740, 1663, 1622, 1474, 1293, 1181, 1129, 1029, 799, 684 cm^{-1}; ^{1}H NMR (DMSO, 500 MHz) and ^{13}C NMR (DMSO, 125 MHz), see Table 1; HRESIMS *m/z* 307.0391 $[M+H]^+$ (calcd. for $C_{13}H_{11}N_2O_5S$, 307.0389).

Bacterial Strains. A panel of bacterial strains were used for bioassays: *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 10240 and *Streptococcus pneumonia* ATCC 49619 belonging to Gram positive bacteria and *Klebsiella pneumonia* ATCC 700603, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 25922 belonging to Gram negative bacteria. These strains were obtained from the collection of the Pasteur Institute (Guadeloupe) and were cultivated on agar plates with Mueller-Hinton medium nutrient medium at 37 °C for all bioassay experiments.

Bioassay using disc diffusion assays. Individual chromatographic fractions were evaluated on *Micrococcus luteus* (ATCC 10240) and *Escherichia coli (*ATCC 35218). An agar diffusion assay

modified according to Brinkhoff *et al.* [20] was used . Inocula were obtained using nutrient medium #1(meat extract [5 g], agar [7.5 g], tryptic pepton [7.5 g], NaCl [2.5 g] and milliQ water [500 mL]; pH = 7) incubated at 37 °C for 24 hours. One hundred microliters of a 10^{-2} dilution culture was spread on an agar plate with nutrient agar medium #1. Concerning the antimicrobial assay on Gram negative bacteria, 50 µg-amoxicillin antibiotic disc (Biomérieux, France) was used as positive control while on Gram positive bacteria, 50 µg-vancomycin antibiotic disc was used. Moreover, two sterile antibiotic assay discs (5 mm-diameter, Dutscher) were placed by plate after spreading. One of them received twenty microliters of EtOAc, as negative control. Fractions were solubilized by EtOAc and the remaining sterile discs received twenty microliters of the prepared solution. The plates were then incubated for 24 hours at 37 °C. Inhibition zones of bacterial development around the impregnated discs containing the fractions will appear if they are active fractions. Inhibition of a target strain was determined as positive when the diameter of the inhibition zone was at least 2 mm greater than the negative control. The experiments were performed in three replicates from three different extractions.

Bioassay using MIC method in liquid culture. Compound **1** (2.7 mg) was dissolved in DMSO (100 µL) and then diluted with distilled H_2O (900 µL). From this solution, eight diluted solutions were prepared: 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL and 2 µg/mL. Microbial suspensions were prepared in sterile 96-well cell culture plates (Cat.-No; 655180, CELLSTAR). Each well received 178 µL of Mueller-Hinton medium, 20 µL of one of solutions S2-S9 and 2 µL of an overnight culture of bacteria corresponding to 0.5 McFarland[21] scale, namely $1.5*10^8$ CFU/mL. Two controls were prepared. The first one was used in order to know if the DMSO inhibited bacterial growth. This control was composed of 2 µL of the overnight culture bacteria ($1.5*10^8$ CFU/mL), 178 µL of Mueller-Hinton medium and 20 µL from a solution containing 100 µL DMSO and 900 µL H_2O. To ensure that working conditions were sterile (no contamination by environmental bacteria), a second control was used (180 µL of Mueller-Hinton medium + 20 µL from a solution consisting of 100 µL DMSO and 900 µL H_2O).

The same protocol was applied for each bacterial strain. The plate was incubated for 24 hours at 37°C and inspected for any inhibition (clear) or growth of bacteria (turbid).[22]

ASSOCIATED CONTENT

Supporting Information. "The Supporting Information is available free of charge on the ACS Publications website at DOI:"

Spectroscopic data, ^{1}H, ^{13}C, NMR, COSY, HSQC, HMBC, ROSY, ^{1}H-^{15}N HSQC, ^{1}H-^{15}N HMBC, IR, UV, HRMS and EDC for orbicularisine (**1**) (.pdf).

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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TOC Graphic

A spiro-indolofuranone fused to a thiazine skeleton, orbicularisine (**1**), was isolated from gills of the mollusk *Codakia orbicularis*.

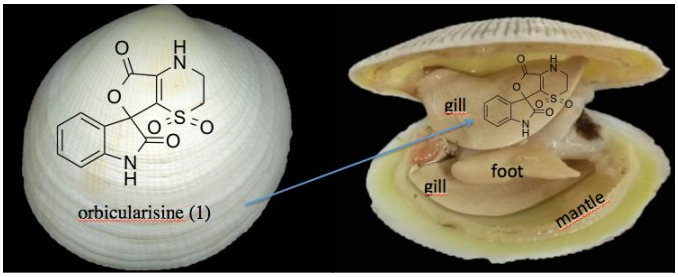

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Figure S1: ^{1}H NMR spectrum of the fraction F_{14} in MeOD (500 MHz).

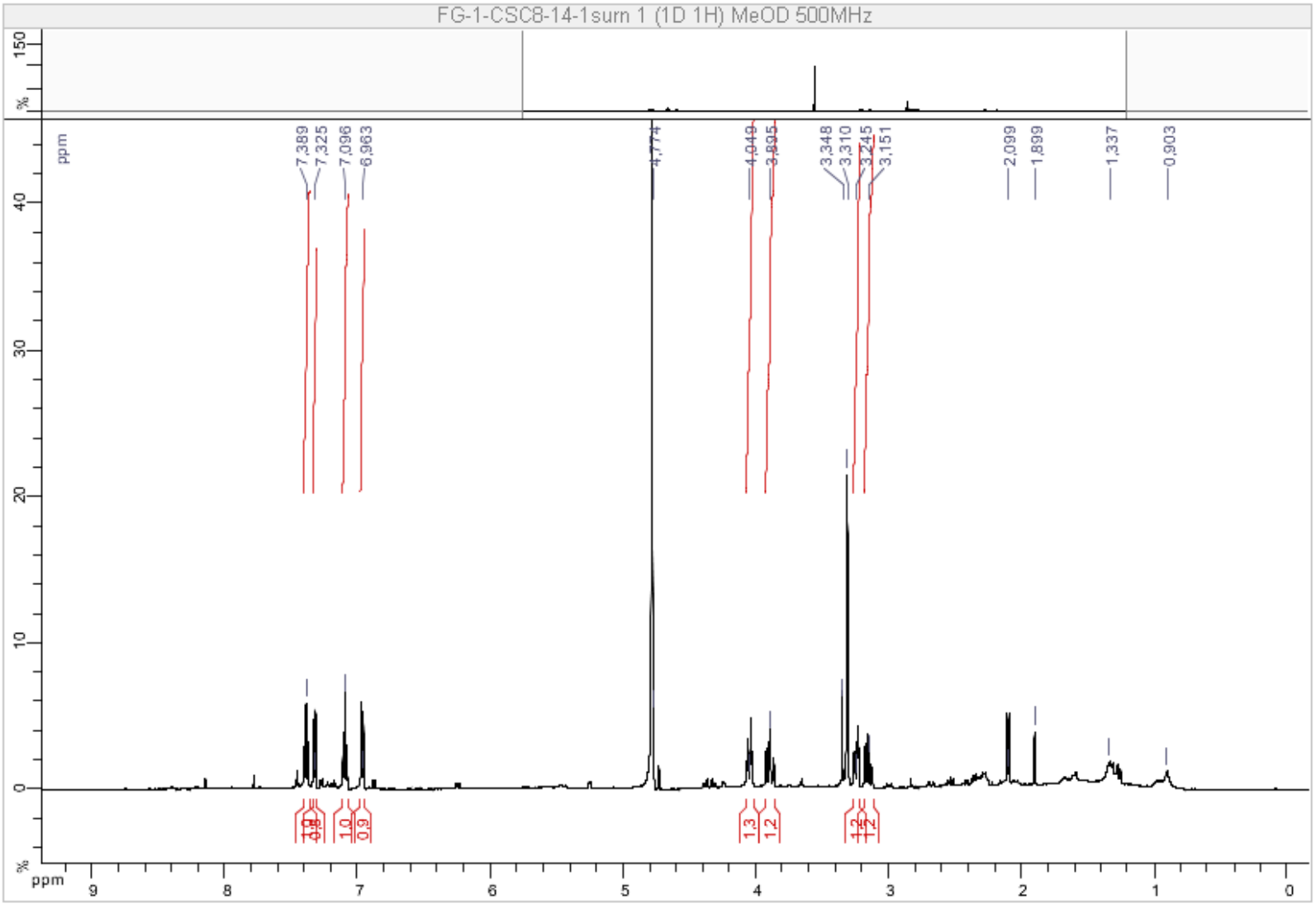


Figure S2: ^{1}H NMR spectrum of compound **1** in DMSO (500 MHz).

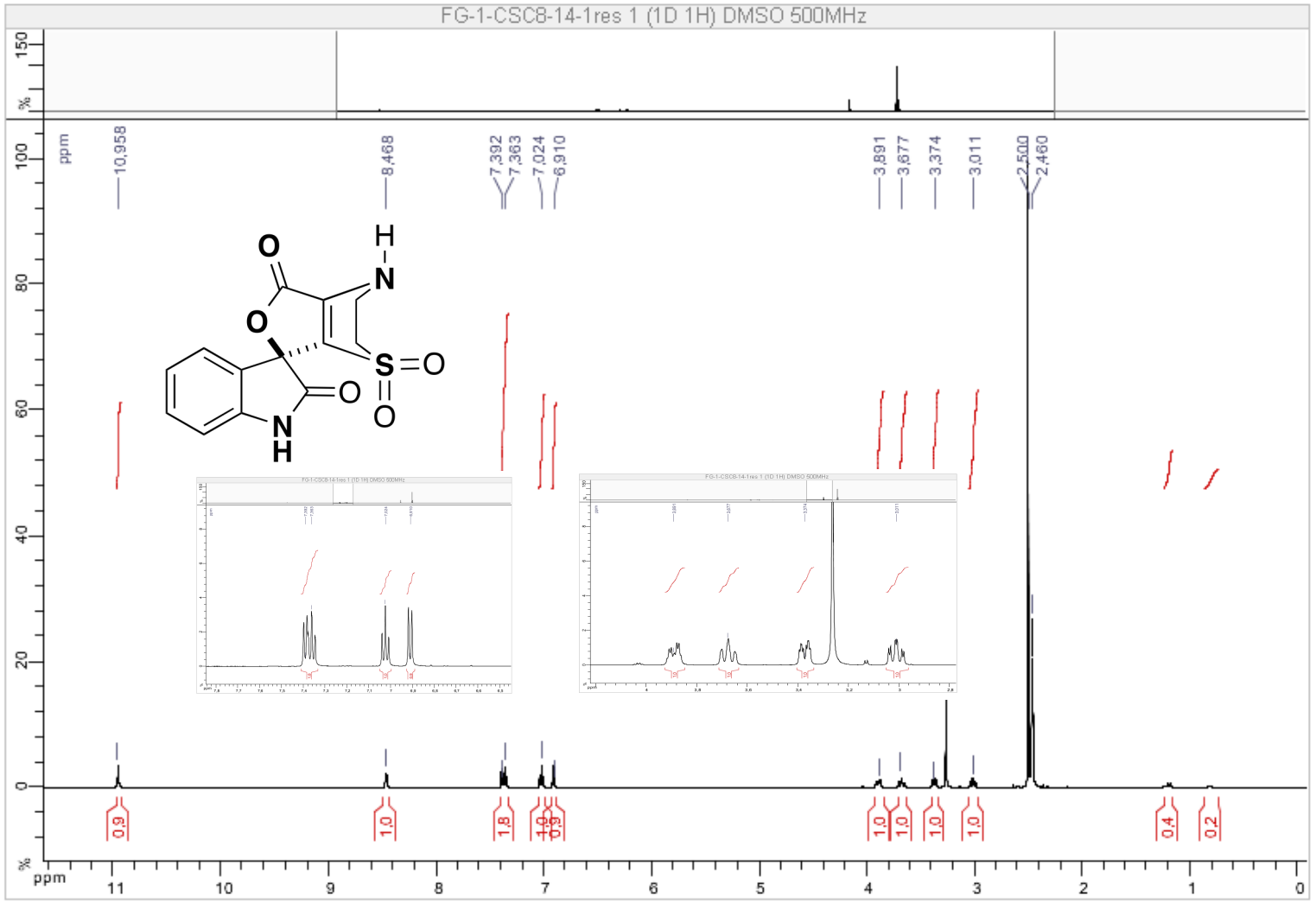


Figure S3: ^{13}C spectrum of compound **1** in DMSO (125 MHz).

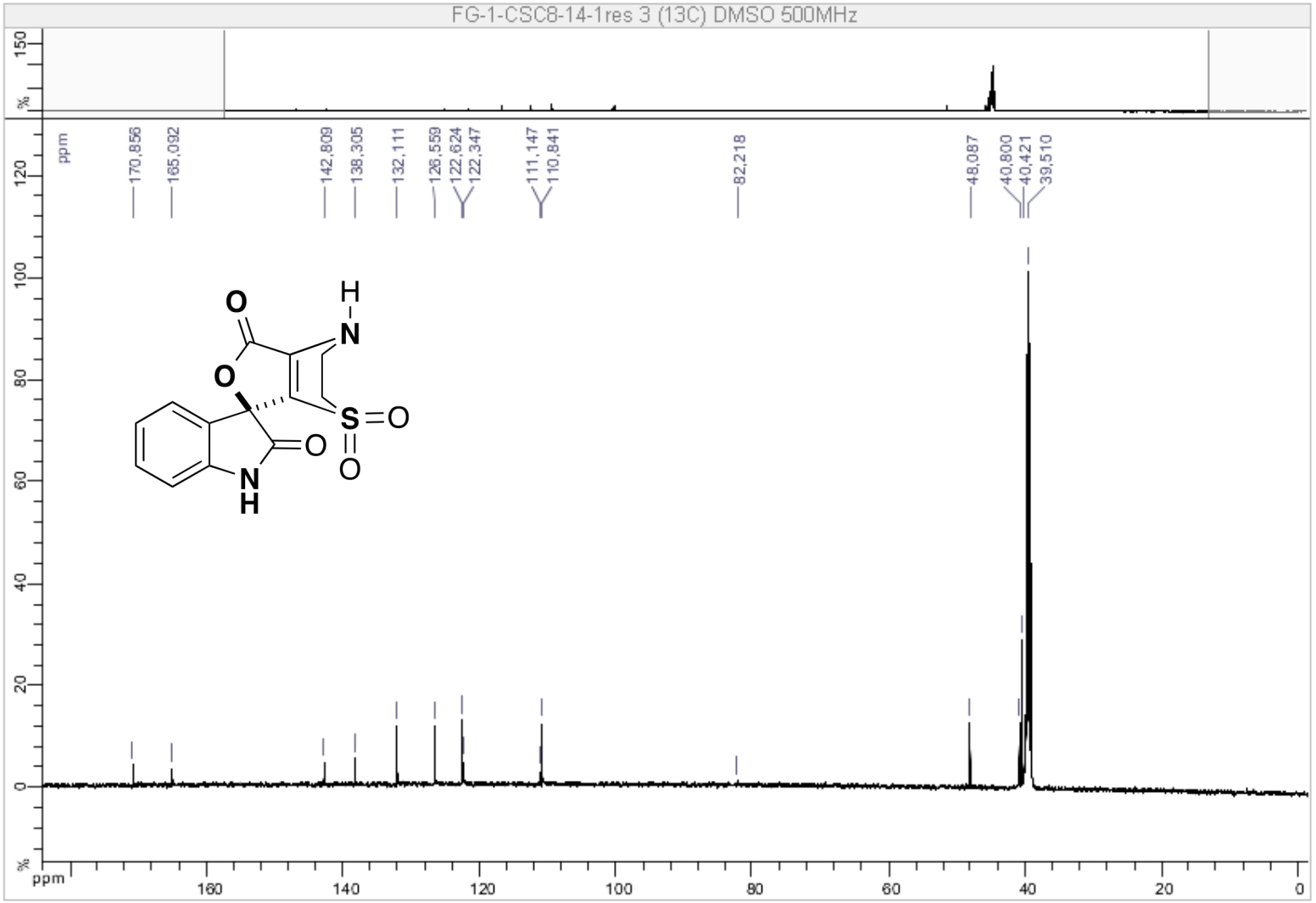


Figure S4: ^{1}H-^{1}H COSY spectrum of compound **1** in DMSO (600 MHz).

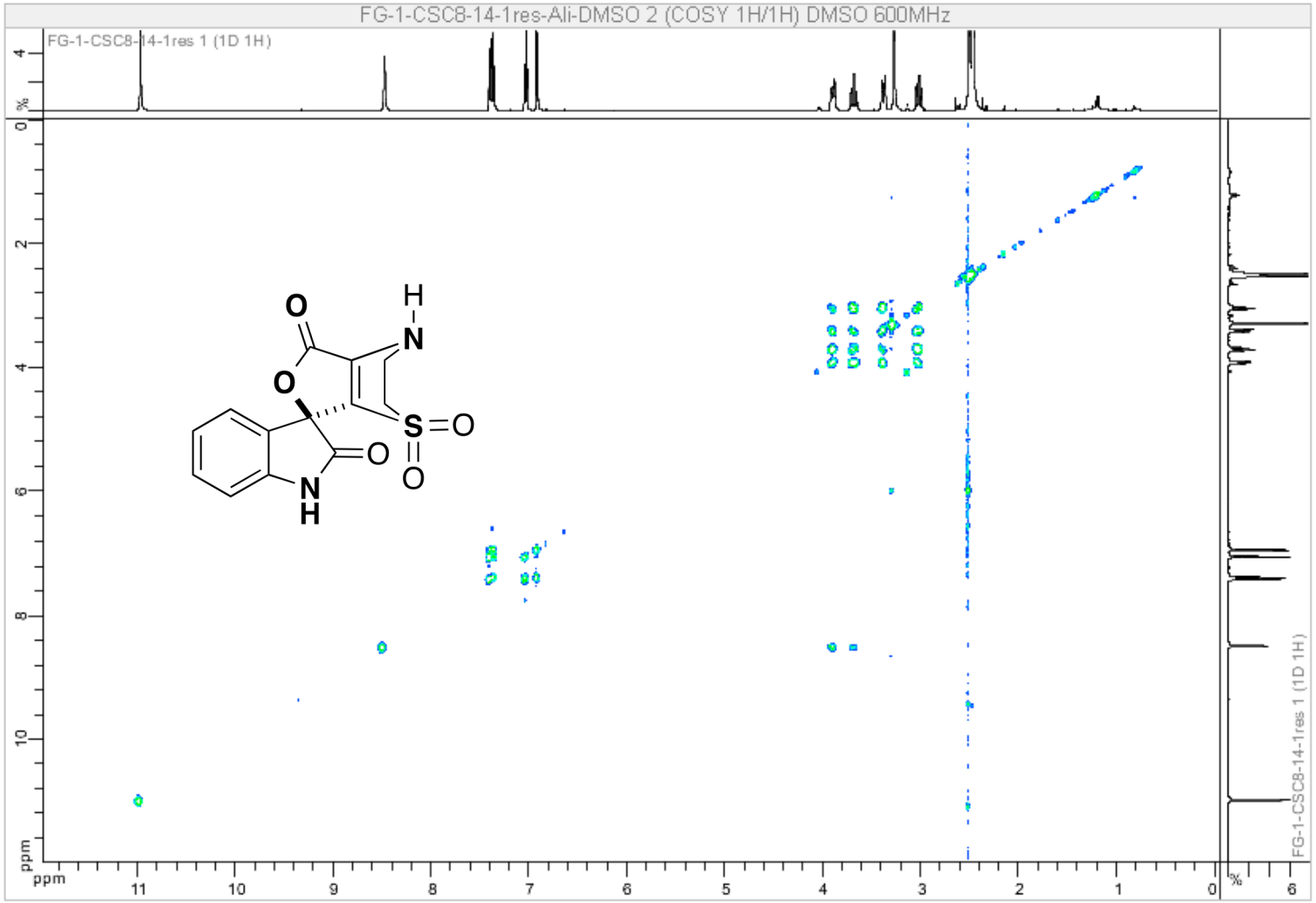


Figure S5: ^{1}H-^{13}C HSQC spectrum of compound **1** in DMSO (500 MHz).

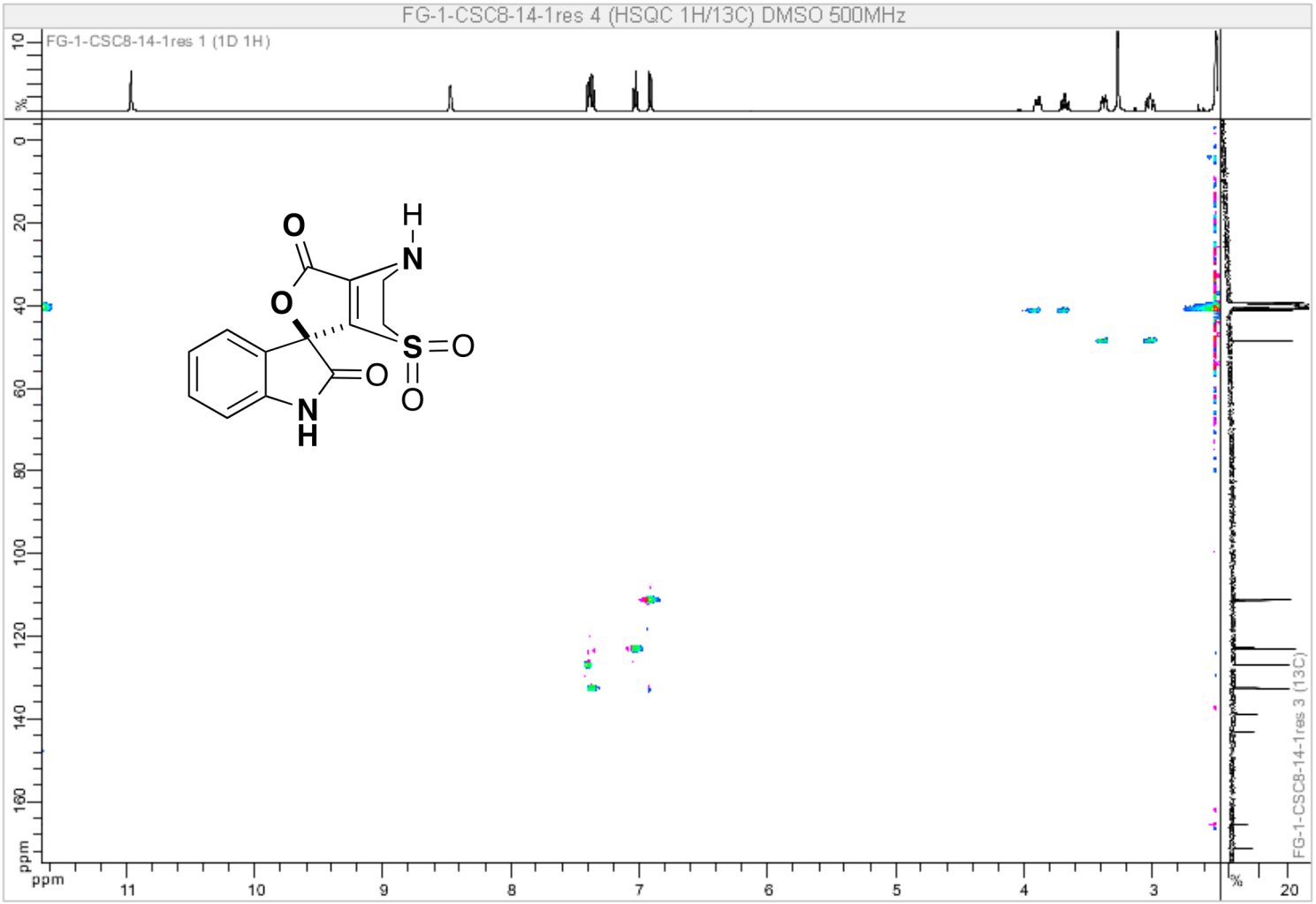


Figure S6: ^{1}H-^{13}C HMBC spectrum of compound **1** in DMSO (600 MHz).

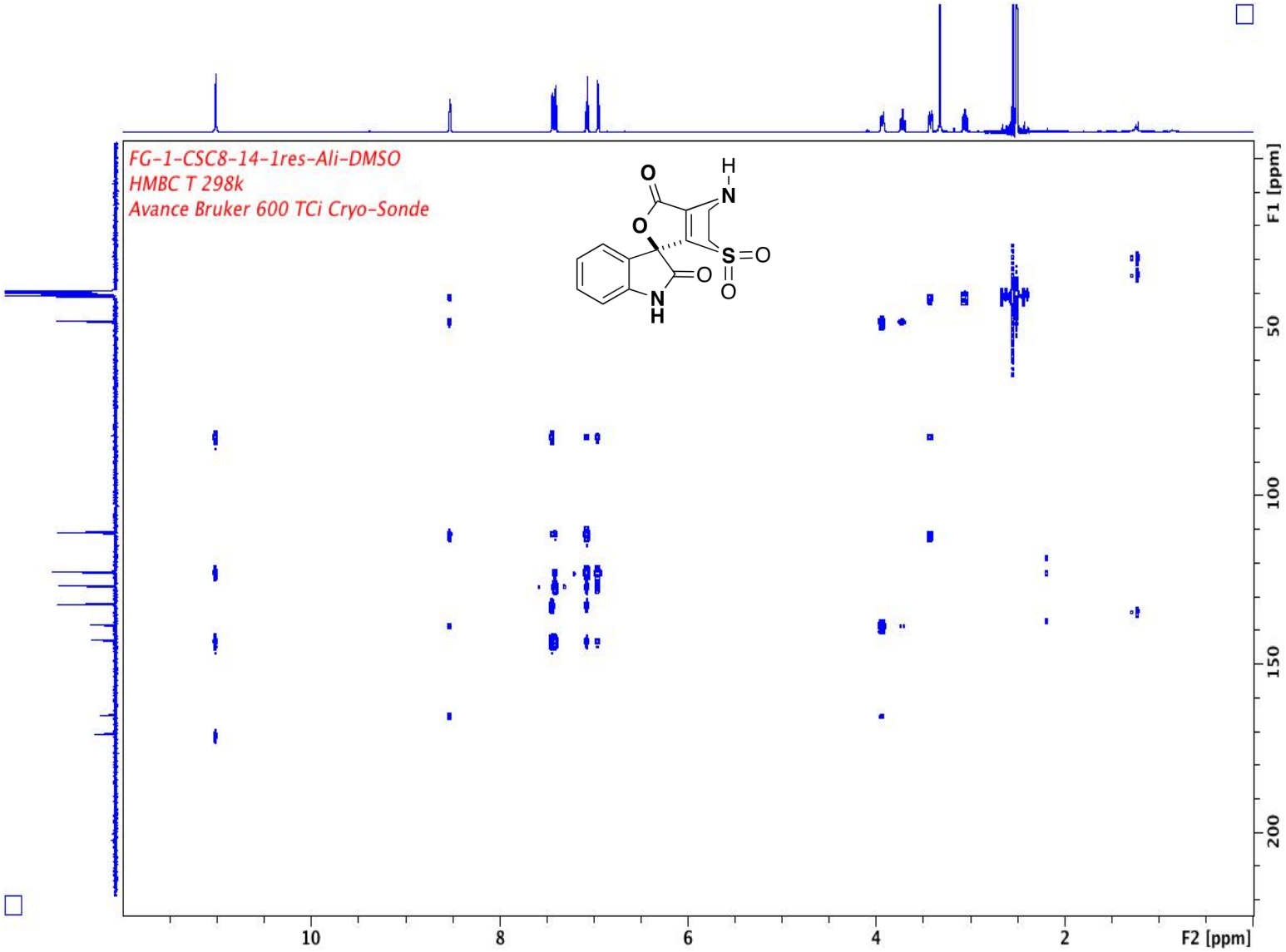


Figure S7: ^{1}H-^{15}N HSQC spectrum of compound **1** in DMSO (600 MHz).

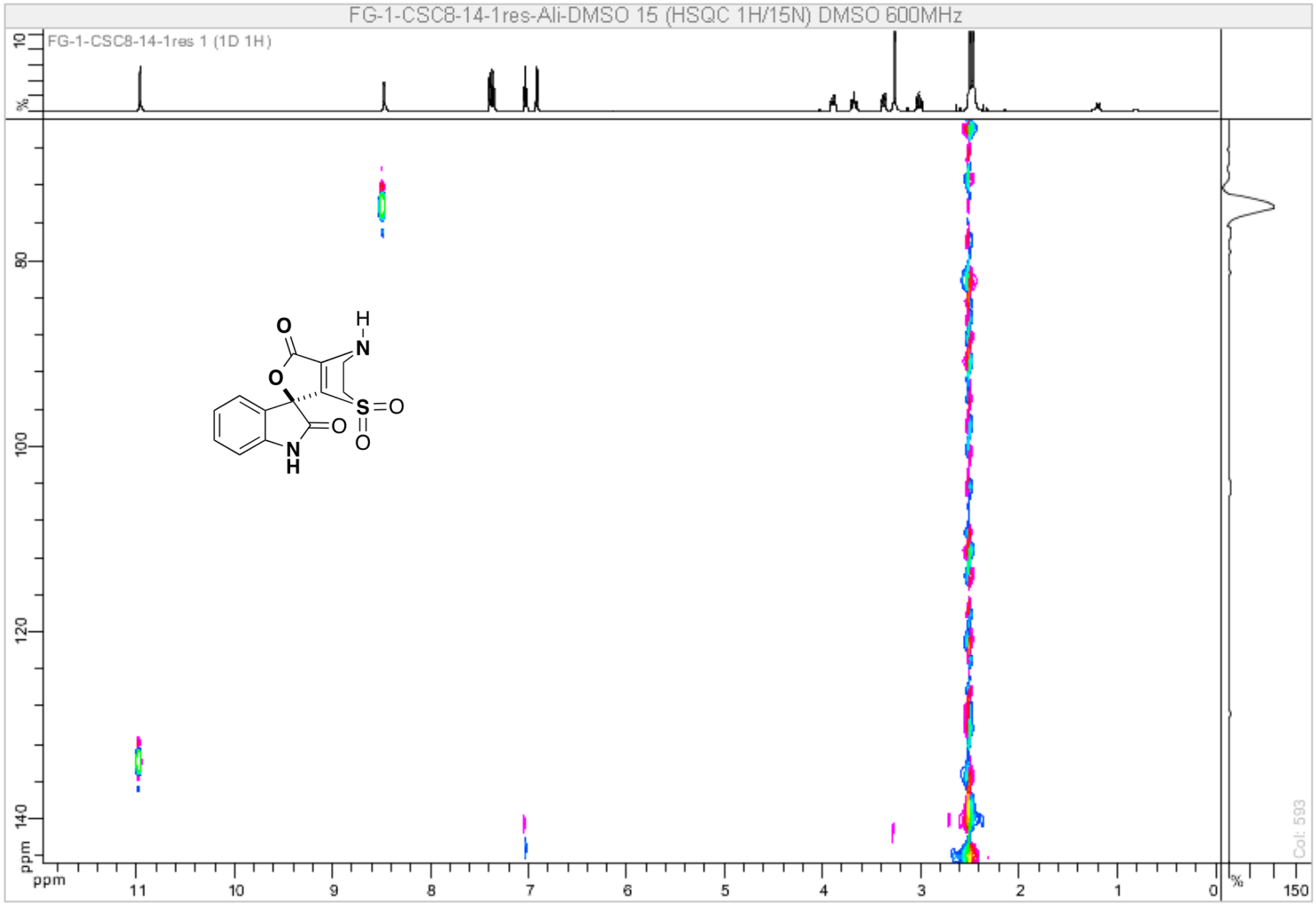


Figure S8: ^{1}H-^{15}N HMBC spectrum of compound **1** in DMSO (600 MHz).

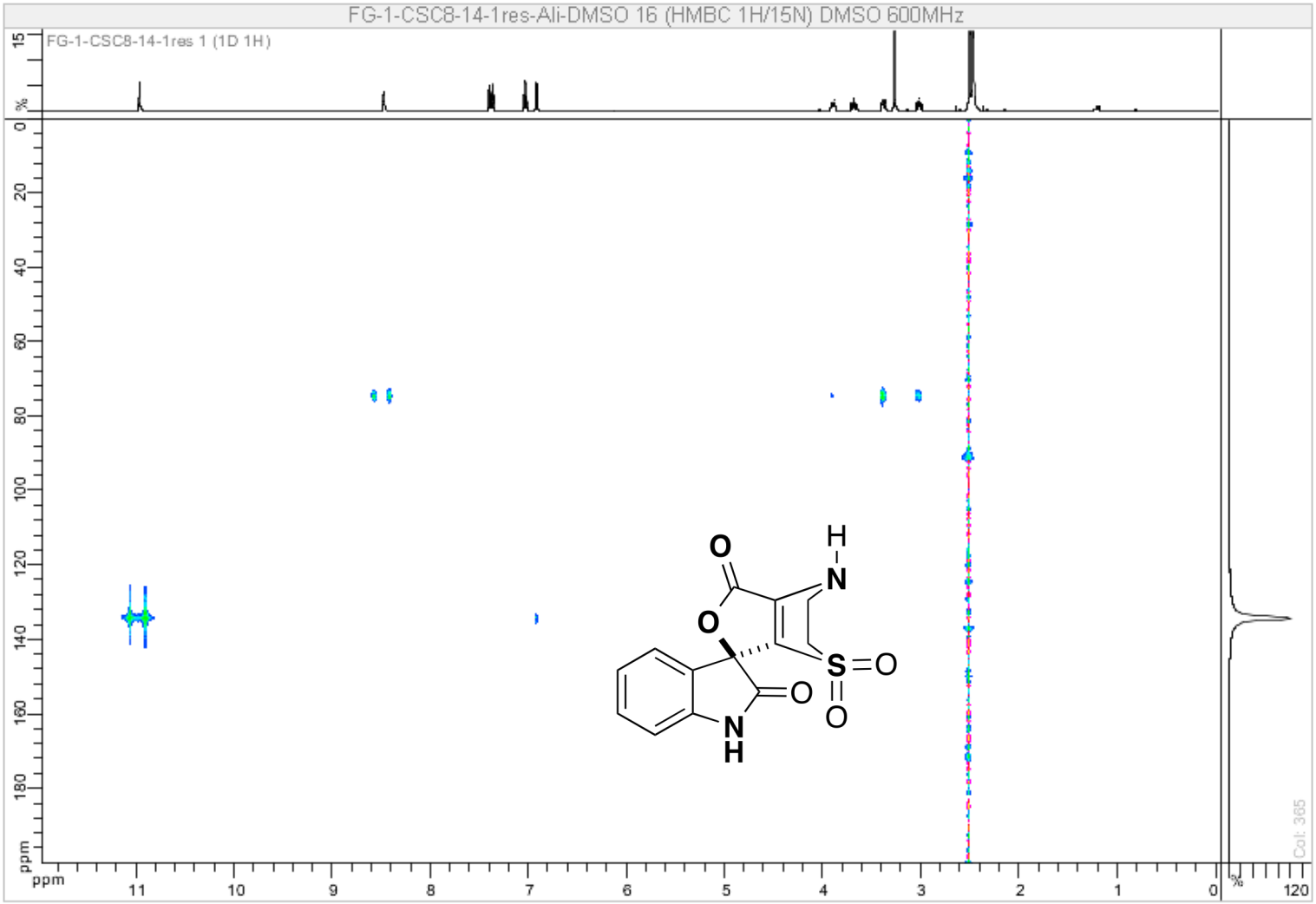


Figure S9: ^{1}H-^{1}H ROESY spectrum of compound **1.**

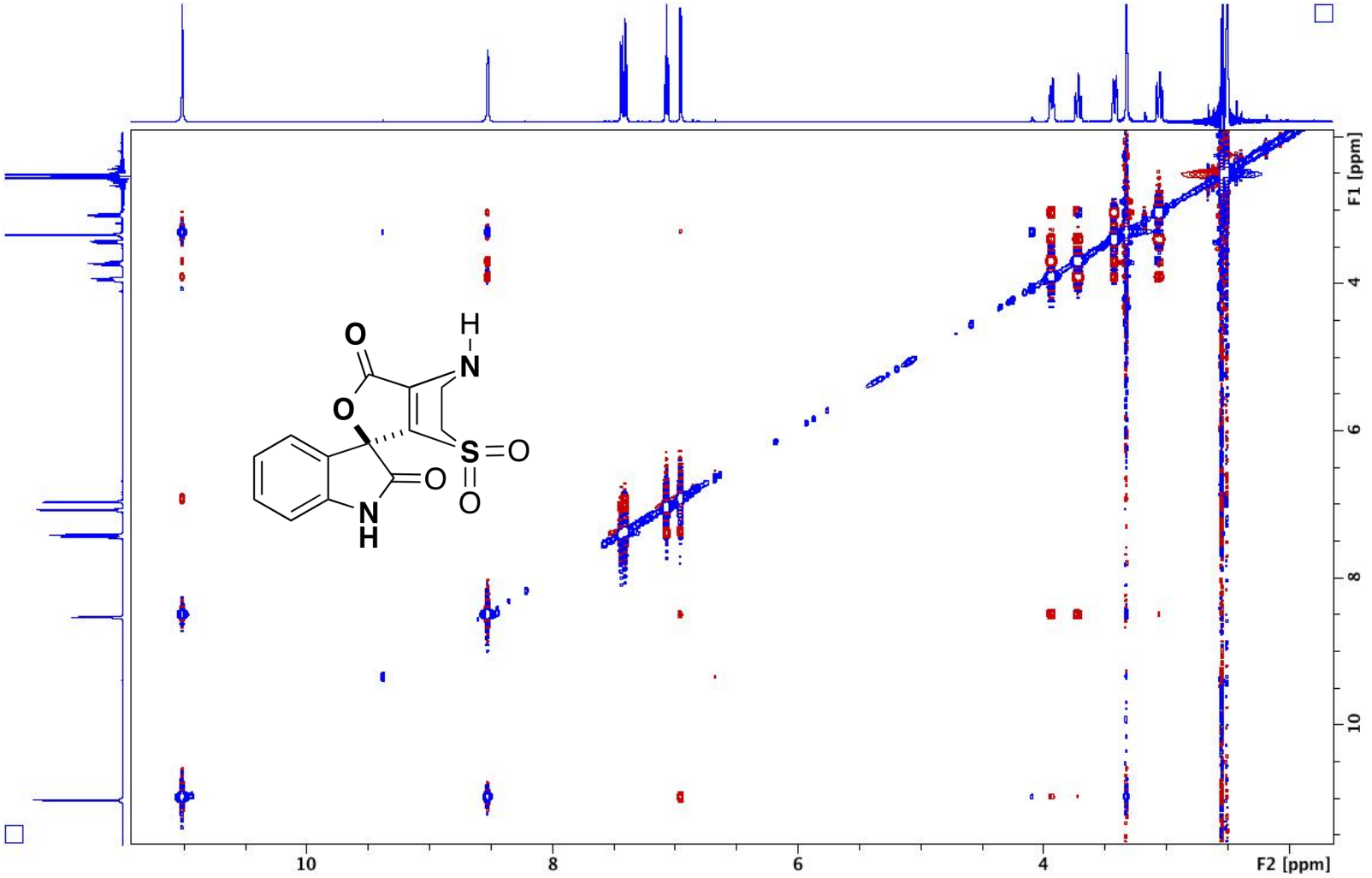


Figure S10: IR spectrum of compound **1.**

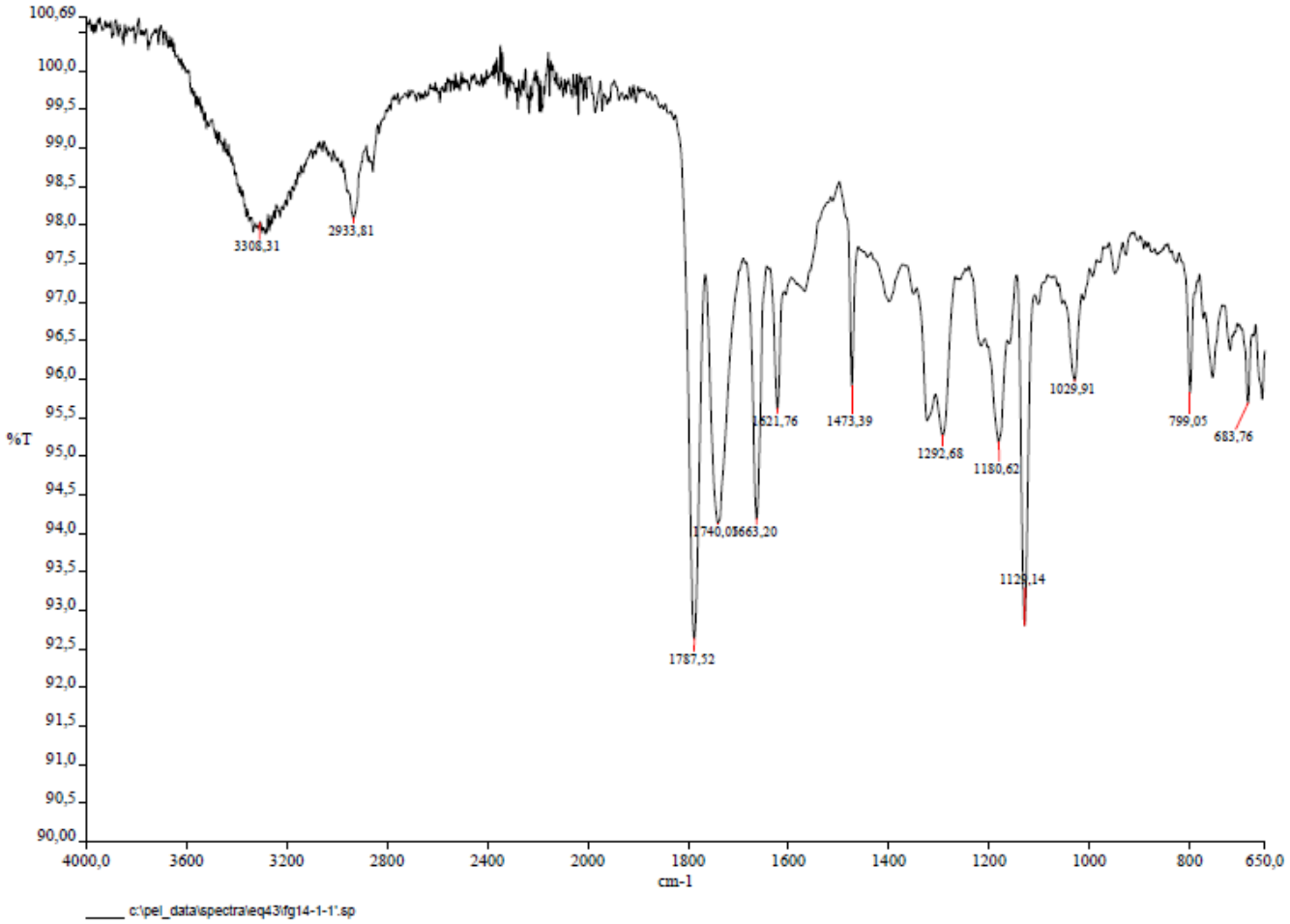


Figure S11: UV spectrum of compound **1** in MeOH.

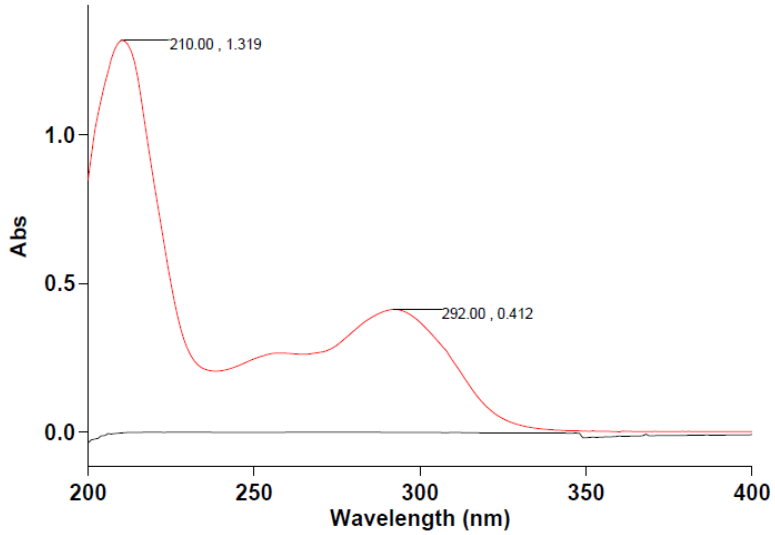


Figure S12: HR-MS of compound **1.**

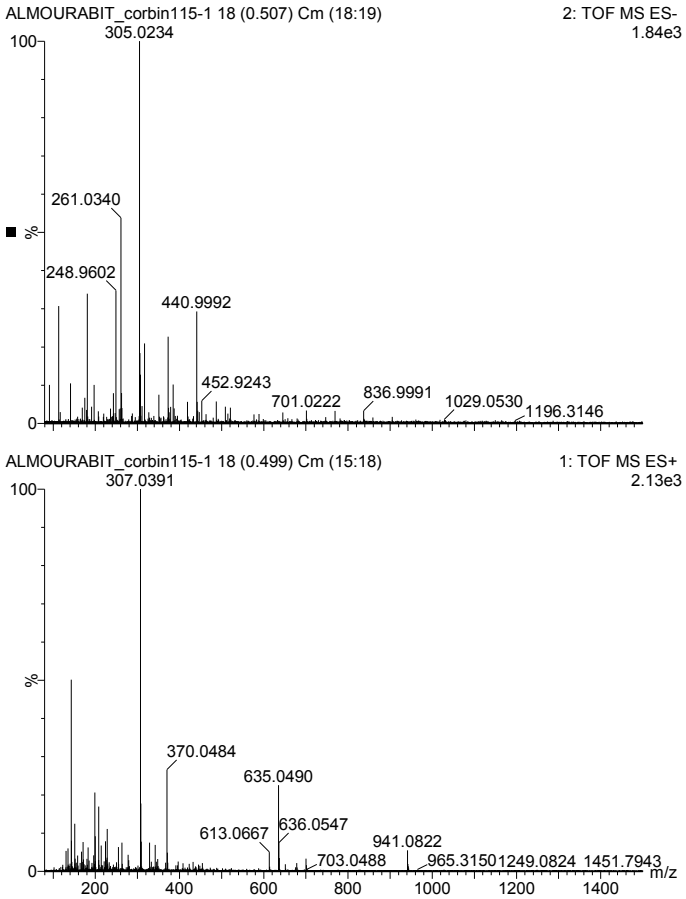
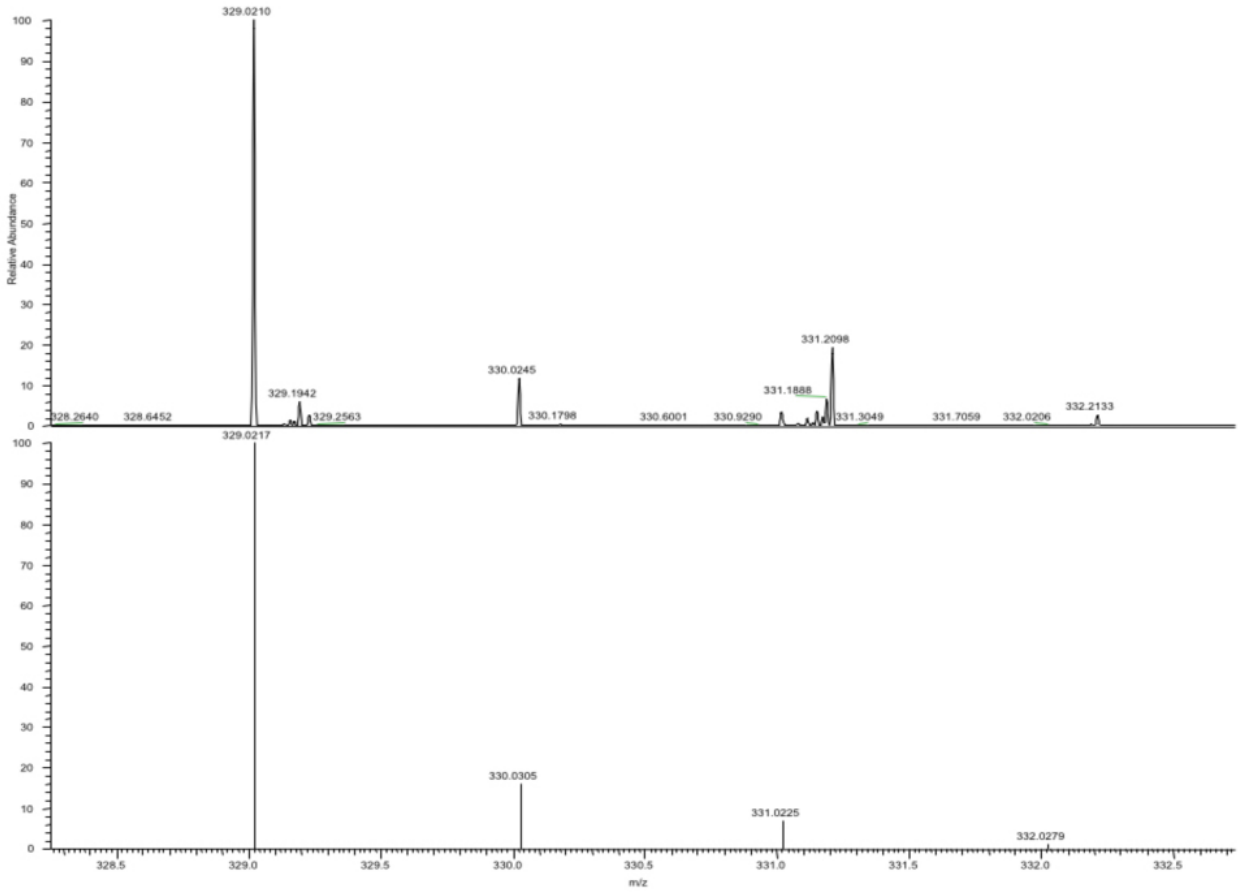


Figure S13: Experimental (a) and theoretical (b) isotopic profile of compound 1.


329.0210
NL:
1.14E7
MSMS_306_Ali_Francesca
#118-166 RT: 1.84-2.59
AV: 49 F: FTMS + p ESI Full
ms [100.00-500.00]
331.2098
330.0245
329.1942
331.1888
332.2133
328.2640
328.6452
329.2563
330.1798
330.6001
330.9290
331.3049
331.7059
332.0206
Relative Abundance
329.0217
NL:
8.11E5
C H N O S +Na :
C H N O S Na
c (gss ,s ,p:40)(Val) Chrg 1
R: 1 Da @10%
330.0305
331.0225
332.0279
m/z

(a)

(b)

Fragmentations MSn Protocol

A solution of 200µg of compound **1** in of MeOH (100 µL) for the injections (5µL/min). The spray voltage was 5 kV, capillary voltage was 25 V and the tube lens was 120 V. Fragmentations MS^n were obtained in linear ion Trap and the acquisitions in an Orbitrap analyzer with a resolution of 30000 at *m/z* = 400 (Figures S18, S19 and S20).

Figure S14: MS^2 spectrum of compound **1.**

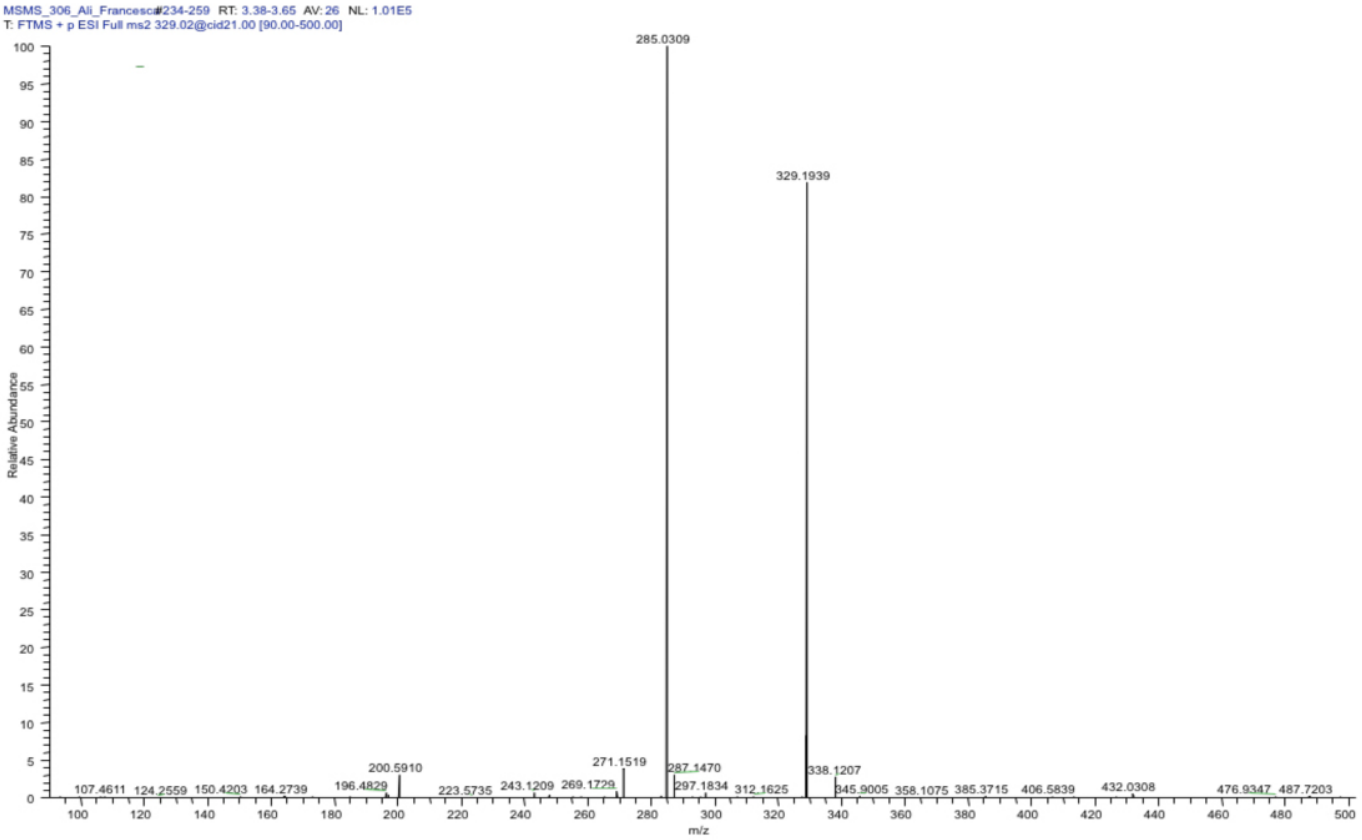


Figure S15: MS^3 spectrum of compound **1.**

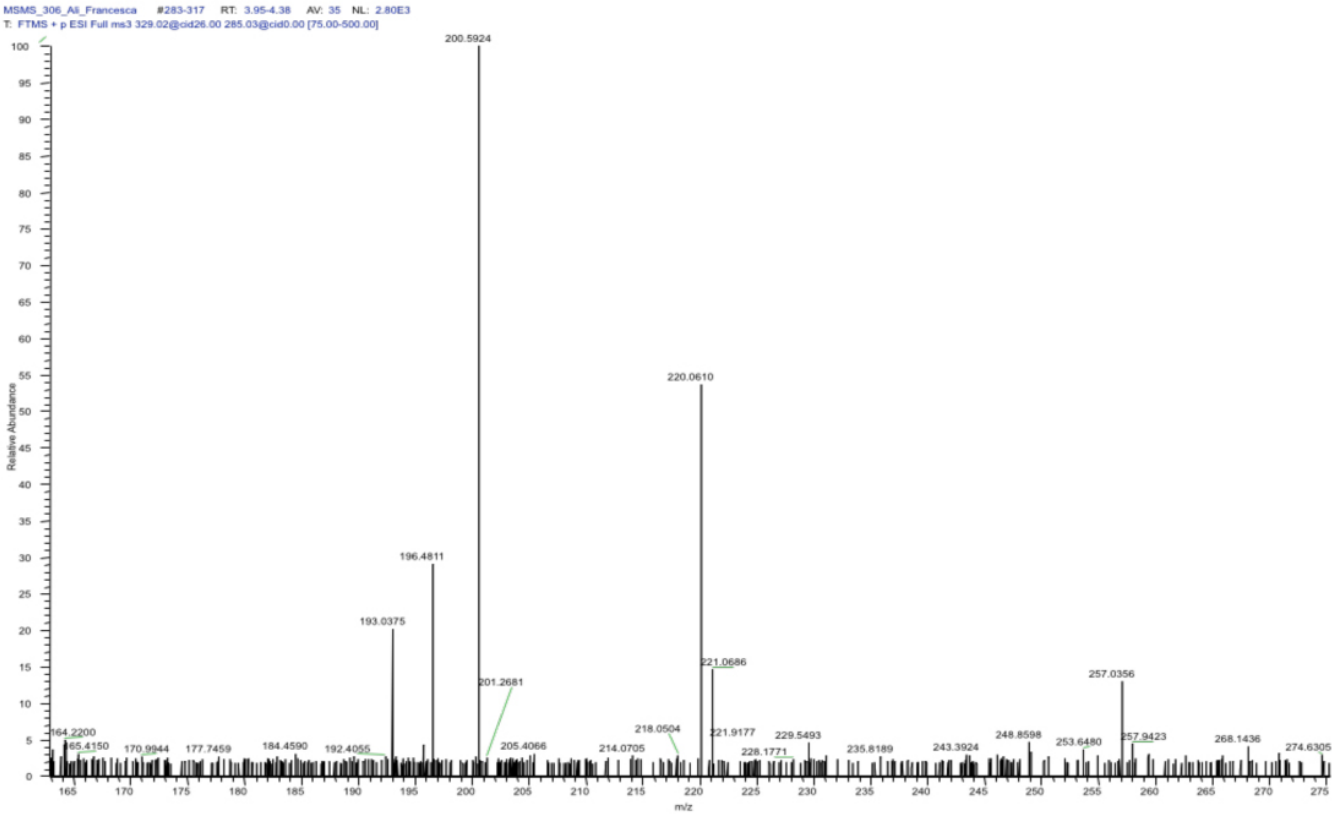
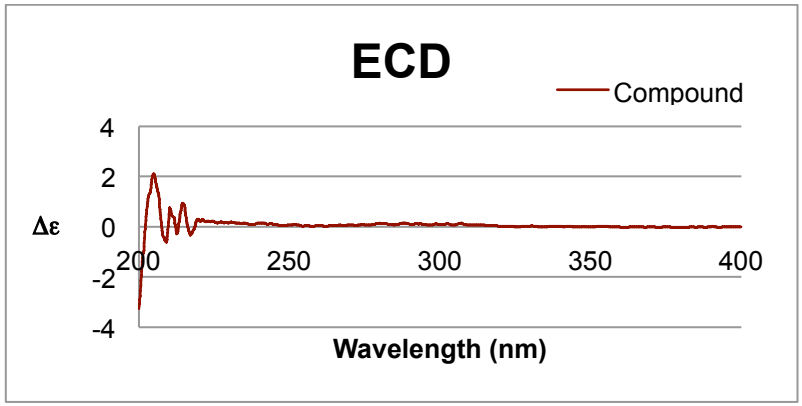


Figure S16: EDC spectrum of compound 1.


ECD
Compound
4
2
0
-2
-4
Δε
200
250
300
350
400
Wavelength (nm)