Chemistry–A European Journal

Supporting Information

Late-Stage Chemoenzymatic Installation of Hydroxy-Bearing Allyl Moiety on the Indole Ring of Tryptophan-Containing Peptides

Nagaraju Mupparapu, Lauren Brewster, Katrina F. Ostrom, and Sherif I. Elshahawi*

I- EXPERIMENTAL SECTION

General Methods. Chemicals and reagents were purchased from Sigma-Aldrich or Fisher Scientific and were used without further purification unless otherwise stated. All solvents used were of ACS grade or higher and purchased from Fisher Chemical. Daptomycin was obtained from Acros Organics, cyclo-(L-Trp-L-Trp) from Bachem while cyclo-(L-Trp-L-Tyr) and cyclo-(L-Trp-Gly) were purchased from AkSci. All DNA sequencing was conducted with the primers T7 promoter (5′-TAATACGACTCACTATAGGG-3′) and T7 terminator (5′-GCTAGTTATTGCTCAGCGG-3′) obtained from Integrated DNA Technologies. The pET28a *E. coli* expression vector was purchased from Novagen. *E. coli* 5α and BL21(DE3) competent cells were purchased from New England Biolabs. Analytical TLC was performed on silica gel aluminum TLC plates purchased from Sigma-Aldrich. Visualization was accomplished with UV light (254 nm), staining with potassium permanganate solution or phosphomolybdic acid reagent and heating. All pyrophosphates and intermediates were purified by gravity column chromatography using silica gel (Silicycle) 60–100 or 100–200 mesh. PD-10 columns and Ni-NTA super flow columns were purchased from GE Healthcare. The NMR spectra were recorded at a 400 MHz for ^{1}H, 100 MHz for ^{13}C and 162 MHz for ^{31}P using Bruker NMR spectrometer (Chapman University School of Pharmacy Nuclear Magnetic Resonance facility) and the one-dimensional ^{1}H, ^{31}P, ^{13}C and DEPT as well as two-dimensional ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC and ^{1}H-^{1}H NOESY spectra were recorded at ambient temperature (~25 °C) using 99.8% d_6-DMSO, $CDCl_3$, and D_2O obtained from Cambridge Isotope Laboratories as solvents for cyclo-(L-Trp$_1$-L-Trp$_2$) and daptomycin analogs, synthetic intermediates and pyrophosphate substrates, respectively. Chemical shifts were referenced and calibrated to internal solvent resonances (DMSO, δ_H 2.50 ppm, δ_C 39.52 ppm; $CDCl_3$, δ_H 7.26 ppm, δ_C 77.16 ppm; D_2O, δ_H 4.79 ppm) and are reported in parts per million (ppm) with coupling constants *J* given in Hz. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Spectra were processed with MestreNova (Mestrelab Research). High Performance Liquid Chromatography (HPLC) analysis were performed using Shimadzu Nexera X2 LC-AD UHPLC equipped with a SIL-30AC autosampler and an SPD-M30A diode array detector (HPLC method A) and a Shimadzu HPLC LCMS-2020 equipped with a diode array detector SPD-M20A (HPLC methods B, C and D). Purification of derivatives of daptomycin and cyclo-(L-Trp$_1$-L-Trp$_2$), was performed on Hitachi HPLC equipped with a diode array detector L-2455 (HPLC methods E, F and G). Signals were detected at λ = 230, 254, 280 nm. Low resolution (LR) and high resolution (HR)-ESI-MS experiments were carried out using Bruker Impact II Ultra High Resolution Qq-Time-Of-Flight mass spectrometry in the positive and negative mode equipped with Thermo Scientific DIONEX 3000 UHPLC (HPLC method H).

HPLC Method A: Titan™ C18 80 Å (1.9 μm, 150 mm × 2.1 mm) column (Supelco) [7% B for 2 min, gradient of 2 to 7% over 0.1 min, gradient of 7% B to 100% B over 11 min, 100% B for 2 min 100% B to 7% B over 0.5 min, 7% B for 4.5 min (A = Milli-Q grade H_2O with 0.1% formic acid; B = acetonitrile with 0.1% formic acid, flow rate = 0.3 mL min^{-1}; $A_{254, 280}$)].

HPLC Method B. Ascentis™ C18 (5 μm, 250 mm × 4.6 mm) column (Supelco) [7% B for 4.5 min, 7% to 15% over 0.5 min, gradient of 15% B to 40% B over 25 min, 40% to 100% over 0.5 min, 100% B for 5 min, 100% B to 25% B over 0.5 min, 7% B for 4.5 min (A = Milli-Q grade H_2O with 0.1% formic acid; B = acetonitrile with 0.1% formic acid, flow rate = 1.0 mL min^{-1}; $A_{254, 280}$)].

HPLC Method C. Ascentis™ C18 (5 μm, 250 mm × 4.6 mm) column (Supelco) [7% B for 4 min, 7% to 23% over 0.5 min, gradient of 23% B to 40% B over 25.5 min, 40% to 100% over 0.5 min 100% B for 5 min 100% B to 7% B over 0.1 min, 7% B for 5 min (A = Milli-Q grade H_2O with 0.1% formic acid; B = acetonitrile with 0.1% formic acid, flow rate = 1.0 mL min^{-1}; $A_{254, 280}$)].

HPLC Method D. Ascentis™ C18 (5 μm, 250 mm × 4.6 mm) column (Supelco) [7% B for 4 min, 7% to 23% over 0.5 min, gradient of 23% B to 40% B over 25.5 min, 40% B to 50% B over 5 min, 50% to 100% over 0.5 min, 100% B for 4.5 min, 100% B to 7% B over 0.1 min, 7% B for 5 min (A = Milli-Q grade H_2O with 0.1% formic acid; B = acetonitrile with 0.1% formic acid, flow rate = 1.0 mL min^{-1}; $A_{254, 280}$)].

HPLC Method E. Ascentis™ C18 (5 μm, 250 mm × 10 mm) column (Supelco) [24.5% B for 55 min, gradient of 24.5% B to 100% B over 5 min, 100% B for 10 min, 100% B to 25% B over 0.5 min, 25% B for 9.5 min (A = Milli-Q grade H_2O with 0.1% formic acid; B = acetonitrile with 0.1% formic acid, flow rate = 4.0 mL min^{-1}; $A_{230, 254, 280}$)].

HPLC Method F. Ascentis™ C18 (5 μm, 250 mm × 10 mm) column (Supelco) 25% B for 10 min, gradient of 25% B to 80% B over 60 min, 80% to 100% for 0.5 min, 100% B for 10 min, 100% B to 25% B over 0.5 min, 25% B for 9.5 min (A = Milli-Q grade H_2O with 0.1% trifluoroacetic acid; B = acetonitrile with 0.1% trifluoroacetic acid, flow rate = 4.0 mL min^{-1}; $A_{230, 254, 280}$)].

HPLC Method G. Ascentis™ C18 (5 μm, 250 mm × 10 mm) column (Supelco) 25% B for 10 min, gradient of 25% B to 100% B over 70 min, 100% B for 10 min, 100% B to 25% B over 0.5 min, 25% B for 9.5 min (A = Milli-Q grade H_2O with 0.1% formic acid; B = acetonitrile with 0.1% formic acid, flow rate = 4.0 mL min^{-1}; $A_{230, 254, 280}$)].

HPLC Method H. Titan™ C18 80 Å (1.9 μm, 50 mm × 2.1 mm) column (Supelco) [7% B for 2 min, gradient of 7% B to 100% B over 8 min, 100% B for 2 min, 100% B to 7% B over 0.5 min, 7% B for 2.5 min (A = Milli-Q grade H_2O with 0.1% formic acid; B = acetonitrile with 0.1% formic acid, flow rate = 0.3 mL min^{-1}; A_{254})].

General synthetic procedure for the formation of alkyl pyrophosphates 4. In a 25 ml round-bottomed flask equipped with a magnetic bar containing alkyl chloride **3** (1.0 mmol, 1.0 eq) dissolved in dry acetonitrile (3 mL) and Tris(tetrabutylammonium) hydrogen pyrophosphate (TBAPP) (1.3 mmol, 1.3 eq) were allowed to stir at rt for 2−3 h. The reaction was monitored by TLC in isopropanol/NH_4OH/H_2O in 7:2:1 with phosphomolybdic acid stain solution. After reaction completion, the solvent was removed under reduced pressure and the crude residue was purified on silica gel (60–120 mesh) using isopropanol/NH_4OH/H_2O in 7:2:1 ratio as the mobile phase. The combined fractions containing the Tris(tetrabutylammonium) (TBA) salt of desired products were concentrated under reduced pressure, passed through Dowex-50WX8 ion exchange column (1 × 8 cm) pre-equilibrated with concentrated NH_4OH:H_2O (3:1), flushed with buffer (0.025 M NH_4HCO_3) until the pH was 8.0. The ammonium salt of alkyl pyrophosphate **4** was eluted with two column volumes of 0.025 M NH_4HCO_3 buffer and lyophilized until dryness.

*Ethyl (E)-4-chloro-3-methylbut-2-enoate (**2a**)*: In a 50 mL one-neck round-bottom flask 1-chloropropan-2-one **1** (1.00 g, 10.8 mmol) was dissolved in 15 mL dry dichloromethane. Ethyl 2-(triphenylphosphoranylidene) propionate (4.30 g, 11.9 mmol) was added in one portion. The reaction mixture was stirred for 12 h at rt until TLC indicated full conversion of the starting material. To the mixture 15 mL of water was added followed by extraction of the organic layer (2 × 15 mL). The combined organic layers were washed with brine solution, dried over sodium sulfate, and concentrated under reduced pressure. The product was purified via column chromatography using ethylacetate and hexane (1:9) producing **2a** as colorless liquid of 1.4 g with 80% yield.[1]

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.94 (s, 1H), 4.16 (q, J = 7.1 Hz, 2H), 4.02 (s, 2H), 2.22 (s, 3H), 1.27 (t, J = 7.1 Hz, 4H).

*(E)-4-chloro-3-methylbut-2-en-1-ol (**3a**)*: In a flame dried 50 mL two-neck round-bottom flask with nitrogen inlet, **2a** (0.5 g, 3.08 mmol) was dissolved in 10 mL dry dichloromethane and cooled to −40 °C using an acetone/dry ice bath. 1 M DIBAL-H in hexane (6.2 mL, 6.17 mmol) was added dropwise. The mixture was stirred until the TLC showed full conversion of **2a** for 30 min. The reaction temperature was raised to 0 ºC and quenched with saturated aqueous solution of Rochelle's salt (sodium potassium tartrate, 10 mL) and allowed to stir for 20 min at 22 °C. Different layers were separated, and the aqueous layer was washed with dichloromethane (15 mL × 3). The combined organic layers were washed with brine (1 × 30 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The product was purified via silica gel chromatography using hexane and ethylacetate (4:1). The pure 0.24 g colorless liquid of **3a** was isolated with 66% yield.[2]

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.64 (q, J = 6.2 Hz, 1H), 4.09 (d, J = 6.5 Hz, 2H), 3.96 (s, 2H), 1.71 (s, 3H).

*(E)-4-hydroxy-2-methylbut-2-en-1-yl diphosphate (**4a**):* the pure **3a** was processed for pyrophosphorylation as described above.

white solid (117 mg, 38% yield). ^{1}H NMR (400 MHz, D_2O) δ 5.65 (t, J = 6.9 Hz, 1H), 4.28 (t, J = 7.8 Hz, 2H), 4.11 (t, J = 7.8 Hz, 2H), 1.64 (d, J = 9.1 Hz, 3H). ^{31}P NMR (162 MHz, D_2O) δ –6.45 (d, J = 20.3 Hz), –10.40 (d, J = 20.9 Hz). ^{13}C NMR (100 MHz, D_2O) δ 136.3 (d, J = 7.3 Hz), 123.7, 70.2 (d, J = 5.3 Hz), 57.4, 12.7. HRMS (TOF) *m/z* $[M - H]^-$ Calcd for $C_5H_{11}O_8P_2$ 260.9935, found 260.9912.

*Ethyl (E)-4-hydroxy-2-methylbut-2-enoate (**6**)*: In a 50 mL one-neck round-bottom flask 1,4-dioxane-2,5-diol **5** (2.00 g, 16.6 mmol) was dissolved in 17 mL dichloromethane. Ethyl 2-(triphenyl-λ^5-phosphanylidene) propanoate (6.03 g, 16.6 mmol) was added in one portion. The reaction mixture was heated to 45 °C until TLC indicated full conversion of the starting material **5** after 2 h. Reaction mixture was cooled to room temperature, 15 mL of water was added, followed by extraction of the organic layer (2 × 15 mL). The combined organic layers were washed with brine solution, dried over anhydrous sodium sulfate (Na_2SO_4) and concentrated under reduced pressure. The product was purified via column chromatography using ethylacetate and hexane (1:3) producing the desired product **6** in colorless liquid 2.2 g with good yields (83%).[1]

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.79 (t, J = 6.0 Hz, 1H), 4.31 (d, J = 5.5 Hz, 2H), 4.17 (q, J = 7.1 Hz, 2H), 3.00 (s, 1H), 1.80 (dd, J = 2.2, 1.0 Hz, 3H), 1.27 (t, J = 7.1 Hz, 3H). The recorded spectra are in accordance with the reported in literature.[1]

*Ethyl (E)-4-chloro-2-methylbut-2-enoate (**2b**)*: In a flame dried 50 mL two-neck round-bottom flask with nitrogen inlet, **6** (1.1 g, 7.58 mmol) was dissolved in 36 mL anhydrous dichloromethane and cooled to –20 °C using an acetone/dry ice bath. Dry pyridine (4.29 mL, 4.198 g, 53.1 mmol) and triphenylphosphine PPh_3 (7.95 g, 30.3 mmol) were added, and the clear, colorless solution was stirred for 20 min at –20 °C. *N*-chlorosuccinimide NCS (2.025 g, 15.2 mmol) was added in small portions within 30 min and the reaction solution turned dark brown. After slowly warming the reaction mixture to 22 °C for 2 h, TLC indicated complete consumption of starting material. The reaction mixture was quenched by the addition of 1M HCl (25 mL). The crude was extracted with dichloromethane (2 × 20 mL) and the combined organic layers were washed with brine (1 × 30 mL). The organic phase was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The product was purified via silica gel chromatography and mobile phase as hexane and ethyl acetate (20:1). The pure 0.7 g colorless liquid of **2b** as isolated with 57% yield.[1]

^{1}H NMR: The recorded spectra are in accordance with the reported in literature.[1] ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.84 (td, J = 7.8, 1.5 Hz, 1H), 4.23 (q, J = 7.1 Hz, 2H), 4.18 (d, J = 7.8 Hz, 2H), 1.96 – 1.91 (m, 3H), 1.32 (t, J = 7.1 Hz, 3H).

*(E)-4-chloro-2-methylbut-2-en-1-ol (**3b**)*: In a flame dried 50 mL two-neck round-bottom flask with nitrogen inlet, **2b** (0.5 g, 3.08 mmol) was dissolved in 10 mL dry dichloromethane and cooled to −40 °C using an acetone/dry ice bath. 1M DIBAL-H in hexane (6.2 mL, 6.17 mmol) was added in dropwise. The mixture was stirred until TLC showed full conversion of starting material for 30 min. The reaction temperature was raised to 0 °C and quenched with saturated aqueous solution of Rochelle's salt (sodium potassium tartrate, 10 ml) and allowed to stir for 20 min at 22 °C. Different layers were separated, and the aqueous layer was washed with dichloromethane (15 mL × 3). The combined organic layers were washed with brine (1 × 30 mL), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The product was purified via silica gel chromatography and mobile phase, hexane and ethylacetate (4:1). The pure 0.24 g colorless liquid of **3b** was isolated with 67% yield.[3]

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.75 (t, J = 7.9 Hz, 1H), 4.15 (d, J = 8.0 Hz, 2H), 4.07 (d, J = 0.5 Hz, 2H), 1.87 (s, 1H), 1.75 (s, 3H).

*(E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (**4b**)*. The pure **3b** was converted to **4b** using general method for pyrophosphorylation described above.

white solid (132 mg, 43% yield). ^{1}H NMR (400 MHz, D_2O) δ 5.60 (t, J = 6.7 Hz, 1H), 4.48 (t, J = 6.9 Hz, 2H), 3.95 (s, 2H), 1.64 (s, 3H). ^{31}P NMR (162 MHz, D_2O) δ –9.58 (d, J = 21.0 Hz), –10.71 (d, J = 21.0 Hz).^{13}C NMR (100 MHz, D_2O) δ 139.6, 120.5 (d, J = 7.5 Hz), 66.3, 62.3 (d, J = 5.1 Hz), 13.0. HRMS (TOF) *m/z* $[M - H]^-$ Calcd for $C_5H_{11}O_8P_2$ 260.9935, found 260.9940.

Expression, overproduction, and purification of recombinant enzymes. The *priB*, *fgaPT2*, and c*dpNPT E. coli* codon-optimized nucleic acid sequences were synthesized (Twist Biosciences) with proper restriction sites. The corresponding fragments were cloned into the *E. coli* expression vector pET28a, transformed into *E. coli* 5α cells and confirmed via sequencing. The validated expression plasmid (pSE_PriB, pSE_FgaPT2 and pSE_CdpNPT) were subsequently transformed into *E. coli* BL21 (DE3) competent cells. All studies employed the corresponding *N*-terminal-His_6 fusion proteins. For protein production, 1L of LB broth (Becton, Dickinson and Company) supplemented with kanamycin (75 μg mL^{-1}) was inoculated with 0.1% (v/v) of the corresponding overnight plasmid-*E. coli* BL21 (DE3) seed culture and grown at 37 °C with shaking (225 rpm). Cultures were induced at OD_{600} of ~0.6–0.8 with isopropyl-*β*-D-thiogalactopyranoside (IPTG, 0.5 mM final concentration) and allowed to grow for an additional 16 h at 20 °C. Cells were harvested by centrifugation and stored in lysis buffer (10 mM imidazole, 50 mM sodium monobasic phosphate, 300 mM NaCl, pH 8.0) at −80 °C until used. All subsequent steps were carried out on 4 °C. Cells were allowed to thaw and were subsequently lysed by sonication (Fisherbrand™ Model 705 Sonic Dismembrator with a microtip, 700W, 2 × 20 sec pulses, 15 sec between pulses). Insoluble debris was removed by centrifugation at 20,000 ×g for 1 h. The supernatant was collected and filtered using 0.22 μm filters and the desired N-His_6-PriB/FgaPT2/CdpNPT fusion protein was purified via Thermo Scientific™ HisPur Ni-NTA Resin (Ni-NTA) affinity chromatography using standard protocols and increasing amount of imidazole in elution buffer (50 mM sodium monobasic phosphate, 300 mM NaCl, pH 8.0). Buffer exchange of each sample was performed using a PD-10 column (GE Healthcare) eluted with 50 mM Tris, 100 mM NaCl, pH 8.0 to yield 10, 8 and 9 mg L^{-1} of each of PriB, FgaPT2 and CdpNPT, respectively. Fractions were collected and concentrated using Amicon Ultra Centrifuge columns 30,000 MWCO (EMD Millipore) and stored in 50 mM Tris, 100 mM NaCl, 10% glycerol pH 8.0 at −80 °C after flash freezing in liquid nitrogen. Protein concentrations were determined by Bradford assay using bovine serum albumin as a standard. Purity and presence of proteins were confirmed by SDS-PAGE gel electrophoresis. The *N*-His_6 fusion proteins (referred to as PriB, FgaPT2 and CdpNPT) were used for all studies.

***In vitro* enzymatic assays.** The initial screening assays were conducted in a total volume of 25 μL containing Tris 50 mM with (FgaPT2 and CdpNPT) and without (PriB) 10 mM $CaCl_2$ (pH 8.0) and a final concentration of 0.4 mM of one of the cyclic dipeptides **7**–**9** or daptomycin **10**, 1.6 mM pyrophosphate substrate, and 33.6 μM of either PriB, FgaPT2 or CdpNPT. After preincubation of the mixture at 37 °C for 30 min, the reactions were initiated by the addition of enzyme and allowed to proceed for 16 h at 37 °C. The reactions were quenched by the addition of 1× MeOH and mixing followed by centrifugation (22,000 rpm, 25 min) to remove precipitated protein. Negative controls were carried out similar to the above-mentioned conditions with the exception of the addition of enzyme. The supernatants were analyzed by UHPLC and RP-HPLC using HPLC methods A, B, C, D and H to calculate conversion rate based on the area of substrate and the product peaks and to confirm masses of the products. When applicable, subsequent product mass analyses were typically accomplished via direct LC-MS analysis method H (LC–QTOF–MS, and/or HR–QTOF–MS (Supplementary Mass Spectra).

Temperature and time optimizations assays were conducted for **7** and **10** at different temperatures (30 °C and 37 °C) and time intervals (2, 5.5, 9 and 16 h).

Determination of kinetics parameters. Reaction mixtures consisted of 50 mM Tris (pH 8.0) containing 10 mM $CaCl_2$ and almost-saturating **4b** (1.6 mM) with variable **7** (2×10^{-2} – 1.6 mM). Reactions were pre-heated to 37 °C in the absence of substrate and initiated by substrate addition. The reactions were performed at 37 °C with 33.6 μM CdpNPT for 960 min and analyzed under initial velocity conditions. Products formation were determined using HPLC with HPLC method C described above. Each data point represents a minimum of three replicate end-point assays; kinetic constants were obtained by nonlinear regression fit to the Michaelis-Menten equation using GraphPad Prism version 9.1.2.

Preparation of cyclo-(L-Trp$_1$-L-Trp$_2$) analogs 11a–d. In a 50 mL centrifuge tube, 39 mg of cyclo-(L-Trp$_1$-L-Trp$_2$) **7** (2.3 mM final concentration) and 26 mg of pyrophosphate **4b** (1.8 mM final concentration) were dissolved in 46 mL Tris 50 mM/10 mM $CaCl_2$ (pH 8.0) followed by the addition of 47.8 μM CdpNPT. Reactions were incubated at 37 °C for 16 h. To calculate the conversion percentage of large-scale reaction, a 100 μL sample from reaction mixture was taken and quenched by adding 100 μL methanol and centrifuged at 20,000 ×g for 15 min and analyzed using HPLC methods A and B and comparing the integrations of the HPLC peaks corresponding to product and starting material **7**. Crude reaction mixtures were subsequently redissolved in DMSO and purified via semi-preparative HPLC method E. Purity was confirmed via HPLC using method C and HR-ESI-MS was determined using HPLC method H. Finally, the compounds were structurally elucidated by 1- and 2D NMR and HR-ESI-MS (**Figures S9**–**S12**, **Tables S1**–**S3**, Supplementary Mass and NMR spectra).

*Cyclo-(6-C-((E)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **(11a)**.* For structural elucidation, purified cyclo-(L-Trp$_1$-L-Trp$_2$) analogs were subjected to HR-ESI-MS (**Table S1**, Supplementary Mass Spectra) and 1D and 2D NMR spectroscopy to determine regiospecificity (**Figure S9**, **Table S2**, Supplementary NMR Spectra). For **11a**, the (+)-HR-ESI-MS $[M + H]^+$ *m/z* 457.2246 indicated a molecular formula of $C_{27}H_{28}N_4O_3$ (Supplementary Mass Spectra). The MS spectrum shows that **11a** has an additional $\Delta m/z = 84$ compared to **7**, consistent with an additional –C_5H_8O– group. The ^{1}H, ^{13}C and HSQC NMR spectroscopic data revealed 27 signals attributable to 1 methyl, 4 methylene, 12 methine, and 10 non-protonated carbons. Assessments of ^{1}H, ^{13}C NMR data implied that the additional 1 methyl $\delta_C = 13.6$ (C-5"), 2 methylene [$\delta_C = 66.2$ (C-4"), $\delta_C = 33.5$ (C-1") ppm], 1 methine $\delta_C = 123.2$ (C-2"), 1 quaternary $\delta_C = 135.1$ (C-3") carbons were reflected which were consistent with proton chemical shifts at $\delta_H = 1.68$ (H-5"), 3.87 – 3.80 (H-4"), 5.57 (H-2"), 3.40 (H-1") ppm and also 1 triplet found at $\delta_H = 4.88$ ppm of OH (H-6") in the ^{1}H NMR spectrum. ^{1}H-^{1}H COSY correlations between H-1"/H-2", H-4"/H-6" as well as the ^{1}H-^{13}C HMBC correlations (H-5"/C-2", C-3", C-4"; H-4"/C-3", C-2", C-5"; H-1"/C-3", C-2"; H-6"/C-4", C-3") indicated the presence of a 4-hydroxy-3-methylbut-2-ene fragment and the presence of a normal (C-1") reaction (**Figure S9**, **Table S2**). The positions of the five carbons were deduced by remaining COSY and HMBC correlations of **11a**. The COSY correlations between H-4 ($\delta_H = 7.26$ ppm)/H-5 ($\delta_H = 6.82$ ppm) and H-1 ($\delta_H = 10.69$ ppm)/H-2 ($\delta_H = 6.56$ ppm) as well as the HMBC correlations between H-5/C-1", C-7 $\delta_C = 110.4$ ppm); H-7 ($\delta_H = 7.06$ ppm)/C-1", C-5 ($\delta_C = 119.6$ ppm), H-2"/C-6 ($\delta_C = 133.8$ ppm) and H-1"/C-5, C-7 revealed the regiospecificity to be at the C-6 of the indole ring. The configurational assignments of C-2"/C-3" was verified as *E* by the NOE spectral correlation (Supplementary NMR Spectra). Thus, cyclo-(6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11a** was established (**Figure S9**).

Cyclo-(6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11a**: white solid (6 mg, isolation 7% yield). (+)-HR–ESI–MS *m/z* calcd for $C_{27}H_{29}N_4O_3$ $[M + H]^+$ 457.2234, observed 457.2246; calcd for $C_{27}H_{27}N_4O_2$ $[M – H_2O + H]^+$ 439.2129, observed 439.2139.

*Cyclo-(5-C-((E)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **(11b)**.* The molecular formula of cyclo-(5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11b** was assigned as $C_{27}H_{28}N_4O_3$ based on HRMS analysis and its ^{13}C NMR data (**Figure S10**, **Table S2**, Supplementary Mass Spectra and Supplementary NMR spectra). These data suggested that **11b** possessed one −C_5H_8O− group beyond that of cyclo-(L-Trp$_1$-L-Trp$_2$) **7**. The ^{13}C and DEPT NMR spectroscopic data complemented with HSQC data revealed 27 signals attributable to 1 methyl, 4 methylene, 12 methine, and 10 non-protonated carbons. Assessments of ^{1}H, ^{13}C NMR data implied that the additional 1 methyl $\delta_C = 13.7$ (C-5"), 2 methylene [$\delta_C = 66.4$ (C-4") and $\delta_C = 33.6$ (C-1") ppm], 1 methine $\delta_C = 123.9$ (C-2"), 1 quaternary $\delta_C = 134.9$ (C-3") carbons and in the 5-*C*-substituted indole of Trp$_1$, consistent with proton chemical shifts at δ_H 5.57 (H-2"), 3.83 (H-4"), 3.39 (H-1"), 1.69 (H-5") and an additional triplet found at 4.74 (H-6") of OH in the ^{1}H NMR spectrum. The positions of the five carbons were deduced by HMBC correlations of H-1"/C-2", C-3", C-4 ($\delta_C = 117.5$), C-5 ($\delta_C = 130.8$), C-6 ($\delta_C = 121.7$) and H-2"/C-1", C-5", C-4", C-6, together with the ^{1}H−^{1}H COSY correlations of H-1"/H-2" and H-4"/ H-6" (OH). The configurational assignments of C-2"/C-3" was *E* as verified by the NOE spectral correlation. Thus, cyclo-(5-*C*-((*E)*-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11b** was established.

(3S,6S)-3-((1H-indol-3-yl)methyl)-6-((5-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-1H-indol-3-yl)methyl)piperazine-2,5-dione or cyclo-(5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11b**: white solid (10 mg, isolation 11% yield). (+)-HR–ESI–MS *m/z* calcd for $C_{27}H_{29}N_4O_3$ $[M + H]^+$ 457.2234, observed 457.2251; calcd for $C_{27}H_{27}N_4O_2$ $[M – H_2O + H]^+$ 439.2129, observed 439.2144.

*3-C-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b] indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **(11c)**.* The molecular formula of reverse modified compound **11c** was assigned as $C_{27}H_{28}N_4O_3$ based on HRMS analysis and its ^{13}C NMR data (**Figure S11**, **Table S3**, Supplementary Mass Spectra and Supplementary NMR Spectra). These data suggested that **11c** possess one −C_5H_8O− group beyond that of cyclo-(L-Trp$_1$-L-Trp$_2$) **7**. The ^{13}C and DEPT NMR spectroscopic data complemented with HSQC data revealed 27 signals attributable to 1 methyl, 4 methylene, 13 methine, and 9 non-protonated carbons. Assessments of ^{1}H, ^{13}C NMR data implied that the additional 1 methyl $\delta_C = 16.4$ (C-5"), 2 methylene $\delta_C = 115.4$ (C-1"), $\delta_C = 65.4$ (C-4") ppm, 1 methine $\delta_C = 140.9$ (C-2"), 1 quaternary $\delta_C = 45.1$ (C-3") carbons were reflected which were consistent with proton chemical shifts at δ_H 5.07–4.94 (H-1"), 5.83 (H-2"), 3.31, 3.24 (H-4"), 0.59 (H-5") and additional triplet found at 4.56 of OH in the ^{1}H NMR spectrum. The positions of the five carbons were deduced by HMBC correlations

of H-1"/C-2", C-3" and H-2"/C-1", C-5", C-4", C-3 (δ_C = 59.8), together with the ^{1}H–^{1}H COSY correlations of H-1"/H-2" and H-4"/H-6" (OH proton). The hexahydropyrrolo[2,3-b] indole ring was confirmed by HMBC correlation of H-2 (δ_H = 5.31)/C-7a (δ_C = 151.2), C-3a (δ_C = 128.8), C-8 (δ_C = 37.9), C-9 (δ_C = 57.9), C-11 (δ_C = 164.7), C-3" and the configurational assignments of the compound at C-2 and C-3 were verified by the NOE spectral correlation (**Figure S11**, **Table S3**) and the fact that the starting dipeptide is assigned as L.

((3S,5aR,10bR,11aS)-3-((1H-indol-3-yl)methyl)-10b-(1-hydroxy-2-methylbut-3-en-2-yl)-2,3,6,10b,11,11a-hexahydro-4H-pyrazino[1',2':1,5]pyrrolo[2,3-b]indole-1,4(5aH)-dione) **11c**: white solid (4 mg, 4% isolation yield). (+)-HR–ESI–MS *m/z* calcd for $C_{27}H_{29}N_4O_3$ $[M + H]^+$ 457.2234, observed 457.2196.

*N-formyl-3-C-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) (**11d**)*. The molecular formula of **11d** was assigned as $C_{28}H_{28}N_4O_4$ based on HRMS analysis and its ^{13}C NMR data (**Figure S12**, **Table S3**, Supplementary Mass Spectra and Supplementary NMR Spectra). These data suggested that **11d** possessed one $-C_6H_8O_2-$ group beyond that of cyclo-(L-Trp$_1$-L -Trp$_2$) **7**. The ^{13}C and DEPT NMR spectroscopic data complemented with HSQC data revealed 28 signals attributable to 1 methyl, 4 methylene, 13 methine, and 10 non-protonated carbons. Assessments of ^{1}H, ^{13}C NMR data implied that the additional 1 methyl δ_C = 16.6 (C-5"), 2 methylene δ_C = 115.9 (C-1"), δ_C = 65.4 (C-4") ppm, 1 methine δ_C = 139.9 (C-2") ppm, 1 quaternary δ_C = 44.7 (C-3") ppm carbons were reflected which were consistent with proton chemical shifts at δ_H 5.10 – 5.02 (H-1"), 5.86 (H-2"), 3.27/3.13 (H-4"), 0.62 (H-5") and additional triplet found at 4.84 of OH in the ^{1}H NMR spectrum. Along with this 1 additional carbonyl (counted as methine group) δ_C = 161.9 (1-OCH), δ_H = 8.91 (1-OCH) ppm The positions of the five carbons were deduced by HMBC correlations of H-1"/C-2", C-3", C-4, C-5, C-6 and H-2"/C-1", C-5", C-4" together with the ^{1}H–^{1}H COSY correlations of H-1"/H-2" and H-4"/H-6" (OH proton). The pyrrolo[2,3-b] indole ring was confirmed by HMBC correlation of H-2 (δ_H = 6.15)/C-7a (δ_C = 141.4), C-3a (δ_C = 132.7), C-8 (δ_C = 37.5), C-9 (δ_C = 57.6), C-11 (δ_C = 165.8), C-3", 1-O=C. The configurational assignments of C-3 were verified by the NOE spectral correlation (**Figure S12**, **Table S3**) and the fact that the starting dipeptide is assigned as L.

(3S,5aR,10bR,11aS)-3-((1H-indol-3-yl)methyl)-10b-(1-hydroxy-2-methylbut-3-en-2-yl)-1,4-dioxo-1,2,3,4,5a,10b,11,11a-octahydro-6H-pyrazino[1',2':1,5]pyrrolo[2,3-b]indole-6-carbaldehyde **11d**: white solid (4 mg, 4% isolation yield). (+)-HR–ESI–MS *m/z* calcd for $C_{28}H_{29}N_4O_4$ $[M + H]^+$ 485.2183, observed 485.2138.

Preparation of 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$ daptomycin 12. In a 50 mL centrifuge tube, 65 mg of daptomycin **10** (0.8 mM final concentration) and 32.9 mg of pyrophosphate **4b** (2 mM final concentration) were dissolved in 50 mL Tris 50mM/10 mM $CaCl_2$ (pH 8.0) and to the clear solution 32 μM CdpNPT and incubated at 37 °C for 16 h. To calculate the conversion percentage, a 100 μL sample from reaction mixture was taken and quenched by adding 100 μL methanol and centrifuged at 20,000 × g for 15 min and analyzed using HPLC methods A and B and comparing the integrations of the HPLC peaks corresponding to product and starting material **10**. Crude reaction mixtures were subsequently redissolved in DMSO and purified via semi-preparative HPLC method F and the semi-purified fraction was purified using HPLC method G. Purity was confirmed using HPLC method D and HR-ESI-MS was determined using HPLC method H. Finally, the compounds were structurally elucidated by 1- and 2D NMR and HR-ESI-MS (**Figure S12**, **Table S4**, Supplementary Mass and NMR Spectra).

*Structural Elucidation of 5-C-((E)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$ daptomycin (**12**)*. The (+)-HR-ESI-MS $[M + H]^+$ *m/z* 1704.7761 indicated a molecular formula of $C_{77}H_{109}N_{17}O_{27}$ and a $\Delta m/z$ = 84 compared to **10**, consistent with an additional $-C_5H_8O-$ group. The ^{1}H, ^{13}C and HSQC NMR spectroscopic data revealed 77 signals corresponding to 5 methyl, 23 methylene, 22 methine, and 27 non-protonated carbons (**Table S4**). NMR data of **12** were in full agreement with that of **10** except of additional one methyl δ_C = 13.7 (C-5"), two methylene δ_C = 33.6 (C-1") and δ_C = 66.4 (C-4"), one methine, δ_C = 123.9 (C-2"), and one non-protonated δ_C = 135.0 (C-3") carbon. COSY, including long-range correlations appear between H-1" (δ_H = 3.39)/H-2" (δ_H = 5.56), H-2"/H-4" (δ_H = 3.81) and H-2"/H-5" (δ_H = 1.69) (**Figure 4b**). Indeed, four and five bond COSY correlations have been reported in allyl compounds.[4] Additionally, HMBC correlations [(**Figure 4b**) (H-5"/C-2"; H-4"/C-3", C-2" and H-1"/C-3", C-2")] confirmed the presence of a 4-hydroxy-3-methyl-2-butene group, indicating the presence of a normal (C-1") not reverse (C-3"), hydroxyprenyl group (**Figure 4c**, **Figure S13**, **Table S4**). The COSY correlations between H-6 (δ_H = 6.87)/H-7 (δ_H = 7.22) and H-1 (δ_H = 10.63)/H-2 (δ_H = 7.09) as well as the HMBC correlations between H-4 (δ_H = 7.32)/C-1", C-6 (δ_C = 121.7); H-6/C-1", C-4 (δ_C = 117.4), H-2"/C-5 (δ_C = 130.8) and H-1"/C-4, C-6 (**Figure 4b**) revealed the regiospecificity to be at the C-5 of the indole Trp moiety (**Figure 4c**). The configurational assignments between C-2" and C-3" were verified by the strong NOE effect between H-2"/H-4" and deduced as *E* (**Figure 4b**, **Figure S13**).

5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$ daptomycin (**12**). white solid (10 mg, 15% isolation yield). (+)-HR–ESI–MS *m/z* calcd for $C_{77}H_{110}N_{17}O_{27}$ $[M + H]^+$ 1704.7752, observed 1704.7761; calcd for $C_{77}H_{111}N_{17}O_{27}$ $[M + 2H]^{2+}$ 852.8912, observed *m/z*: 852.8931.

II-Supplementary Figures

5′-
ATGGGTGGTCCGATGTCTGGTTTCCACTCTGGTGAAGCGCTGCTGGGTGACCTGGCGACCGGTCAGCTGA
CCCGTCTGTGCGAAGTTGCGGGTCTGACCGAAGCGGACACCGCGGCGTACACCGGTGTTCTGATCGAATC
TCTGGGTACCTCTGCGGGTCGTCCGCTGTCTCTGCCGCCGCCGTCTCGTACCTTCCTGTCTGACGACCAC
ACCCCGGTTGAATTCTCTCTGGCGTTCCTGCCGGGTCGTGCGCCGCACCTGCGTGTTCTGGTTGAACCGG
GTTGCTCTTCTGGTGACGACCTGGCGGAAAACGGTCGTGCGGGTCTGCGTGCGGTTCACACCATGGCGGA
CCGTTGGGGTTTCTCTACCGAACAGCTGGACCGTCTGGAAGACCTGTTCTTCCCGTCTTCTCCGGAAGGT
CCGCTGGCGCTGTGGTGCGCGCTGGAACTGCGTTCTGGTGGTGTTCCGGGTGTTAAAGTTTACCTGAACC
CGGCGGCGAACGGTGCGGACCGTGCGGCGGAAACCGTTCGTGAAGCGCTGGCGCGTCTGGGTCACCTGCA
GGCGTTCGACGCGCTGCCGCGTGCGGACGGTTTCCCGTTCCTGGCGCTGGACCTGGGTGACTGGGACGCG
CCGCGTGTTAAAATCTACCTGAAACACCTGGGTATGTCTGCGGCGGACGCGGGTTCTCTGCCGCGTATGT
CTCCGGCGCCGTCTCGTGAACAGCTGGAAGAATTCTTCCGTACCGCGGGTGACCTGCCGGCGCCGGGTGA
CCCGGGTCCGACCGAAGACACCGGTCGTCTGGCGGGTCGTCCGGCGCTGACCTGCCACTCTTTCACCGAA
ACCGCGACCGGTCGTCCGTCTGGTTACACCCTGCACGTTCCGGTTCGTGACTACGTTCGTCACGACGGTG
AAGCGCGTGACCGTGCGGTTGCGGTTCTGCGTGAACACGACATGGACTCTGCGGCGCTGGACCGTGCGCT
GGCGGCGGTTTCTCCGCGTCCGCTGTCTGACGGTGTTGGTCTGATCGCGTACCTGGCGCTGGTTCACCAG
CGTGGTCGTCCGACCCGTGTTACCGTTTACGTTTCTTCTGAAGCGTACGAAGTTCGTCCGCCGCGTGAAA
CCGTTCCGACCCGTGACCGTGCGCGTGCGCGTCTGTAA-3′

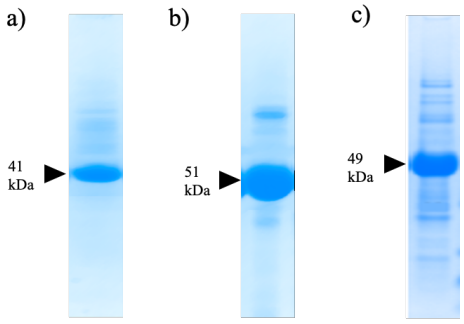
Supplementary Figure S1. Nucleotide sequence of the codon optimized synthesized gene for PriB.

5′-
ATGAAGGCTGCGAATGCCAGCAGCGCAGAAGCATATCGTGTTCTTTCACGTGCTTTTCGGTTTGACAACGAGGA
CCAAAAGCTGTGGTGGCATTCAACTGCCCCTATGTTCGCTAAAATGCTGGAGACTGCCAACTACACTACGCCTT
GCCAATACCAATATTTGATCACCTACAAGGAGTGTGTTATCCCAAGCCTTGGATGCTACCCGACAAATAGTGCT
CCACGGTGGTTAAGCATTCTGACGCGGTACGGAACTCCCTTTGAGTTGTCTCTGAACTGTTCCAATTCGATTGT
GCGTTACACGTTTGAACCGATCAACCAACACACAGGCACTGACAAGGACCCATTTAACACGCATGCTATTTGGG
AGTCGTTACAGCATCTGCTTCCACTGGAAAAGTCTATCGACCTTGAGTGGTTCAGACACTTTAAGCACGATCTG
ACGCTGAACTCTGAAGAGTCAGCTTTCTTAGCACACAATGACAGACTGGTCGGCGGAACGATCCGTACCCAAAA
CAAACTGGCTCTTGACCTGAAAGACGGTCGCTTCGCTTTAAAGACCTATATCTACCCGGCCTTGAAGGCTGTTG
TCACTGGCAAGACTATCCACGAATTGGTGTTTGGTAGTGTTCGTCGCTTGGCAGTTCGCGAGCCGCGCATCTTA
CCTCCGCTTAACATGCTGGAGGAGTACATTAGATCGCGCGGTTCAAAGTCCACCGCTTCACCGAGATTAGTCTC
GTGTGACTTGACATCACCTGCCAAGAGTCGTATCAAAATTTATTTATTGGAACAAATGGTTTCATTAGAGGCGA
TGGAAGATCTTTGGACCCTTGGGGGTCGTCGTCGTGACGCCTCTACTCTTGAAGGGTTGTCGCTTGTCCGTGAA
CTTTGGGACCTGATCCAACTGTCGCCGGGATTAAAATCCTACCCTGCGCCCTATCTGCCCCTGGGGGTAATACC
TGATGAACGGTTGCCATTAATGGCTAATTTTACACTTCACCAAAATGATCCTGTCCCAGAGCCTCAAGTTTACT
TTACCACATTTGGTATGAACGACATGGCTGTAGCCGATGCTTTAACAACGTTTTTCGAGAGACGTGGCTGGTCC
GAGATGGCCCGTACTTACGAAACCACGCTTAAGTCGTATTACCCGCATGCGGATCACGACAAACTTAATTATTT
ACATGCATATATCTCCTTTTCCTACCGGGATCGTACTCCATATTTGTCAGTTTACTTACAATCATTCGAAACAG
GCGATTGGGCGGTAAAGTGTCAAGATGCGGCGTGCCAGCCCACCAGTTTGCCCCCTGACTTGTCGAAGACGGGG
GTTTATTATAGCGGCCTGCATTGA-3′

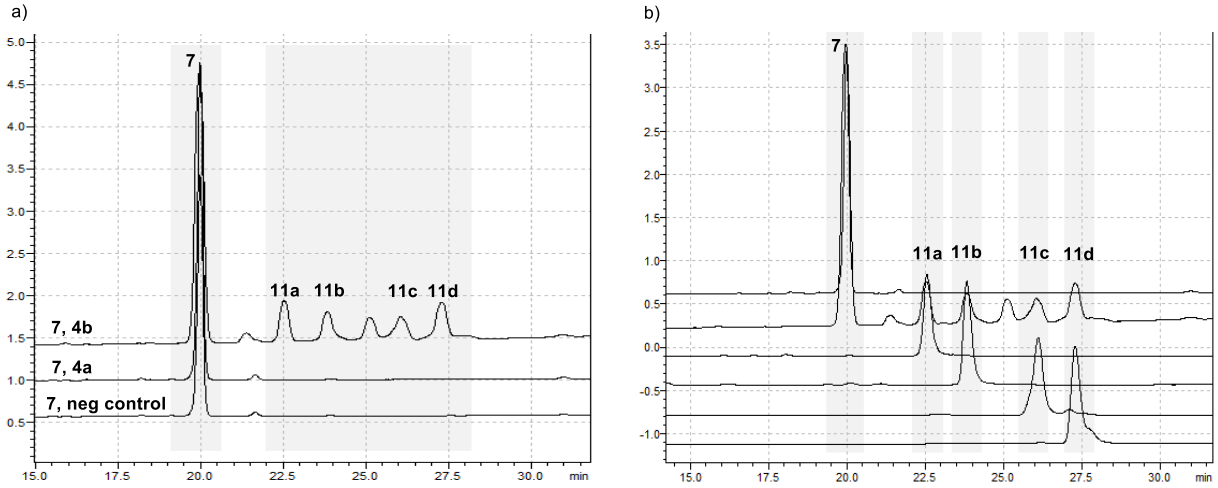
Supplementary Figure S2. Nucleotide sequence of the codon optimized synthesized gene for FgaPT2.

5′-
ATGGACGGTGAAATGACCGCATCGCCGCCGGACATCTCAGCTTGTGATACCTCAGCAGTTGACGAACAGA
CGGGCCAATCGGGCCAGAGCCAGGCGCCGATTCCGAAAGATATCGCCTATCATACCCTGACGAAAGCACT
GCTGTTTCCGGATATTGACCAGTACCAACACTGGCATCACGTTGCGCCGATGCTGGCCAAAATGCTGGTC
GATGGCAAATATAGCATCCATCAGCAATATGAATACCTGTGCCTGTTCGCACAGCTGGTCGCTCCGGTGC
TGGGTCCGTATCCGTCACCGGGTCGTGACGTGTACCGCTGTACCCTGGGCGGTAACATGACGGTTGAACT
GTCGCAGAATTTTCAACGTAGCGGCTCTACCACGCGCATTGCTTTCGAACCGGTGCGTTACCAGGCGTCT
GTTGGTCATGATCGTTTTAACCGCACCAGTGTGAATGCCTTTTTCTCCCAGCTGCAACTGCTGGTTAAAT
CAGTCAACATCGAACTGCATCACCTGCTGTCGGAACACCTGACCCTGACGGCGAAAGATGAACGTAACCT
GAATGAAGAACAGCTGACCAAATATCTGACGAATTTTCAGGTCAAAACCCAATACGTGGTTGCCCTGGAC
CTGCGCAAAACGGGCATTGTGGCAAAAGAATATTTCTTTCCGGGTATCAAATGCGCAGCAACCGGTCAGA
CGGGTTCAAACGCGTGTTTCGGCGCAATTCGTGCTGTTGATAAAGACGGTCATCTGGATAGCCTGTGCCA
ACTGATTGAAGCTCACTTTCAGCAATCTAAAATCGATGACGCCTTCCTGTGCTGTGATCTGGTCGACCCG
GCCCATACCCGCTTTAAAGTCTATATTGCAGACCCGCTGGTGACCCTGGCACGTGCAGAAGAACACTGGA
CCCTGGGCGGTCGCCTGACGGATGAAGACGCAGCTGTGGGCCTGGAAATTATCCGTGGTCTGTGGAGTGA
ACTGGGCATTATCCAGGGTCCGCTGGAACCGTCCGCAATGATGGAAAAAGGCCTGCTGCCGATTATGCTG
AATTATGAAATGAAAGCGGGTCAGCGCCTGCCGAAACCGAAACTGTACATGCCGCTGACCGGCATCCCGG
AAACGAAAATTGCGCGTATCATGACCGCCTTTTTCCAGCGCCATGATATGCCGGAACAAGCTGAAGTTTT
CATGGAAAACCTGCAGGCGTATTACGAAGGTAAAAATCTGGAAGAAGCAACCCGCTATCAAGCTTGGCTG
TCGTTCGCTTACACCAAAGAAAAAGGTCCGTATCTGAGCATTTATTACTTCTGGCCGGAATAA-3′

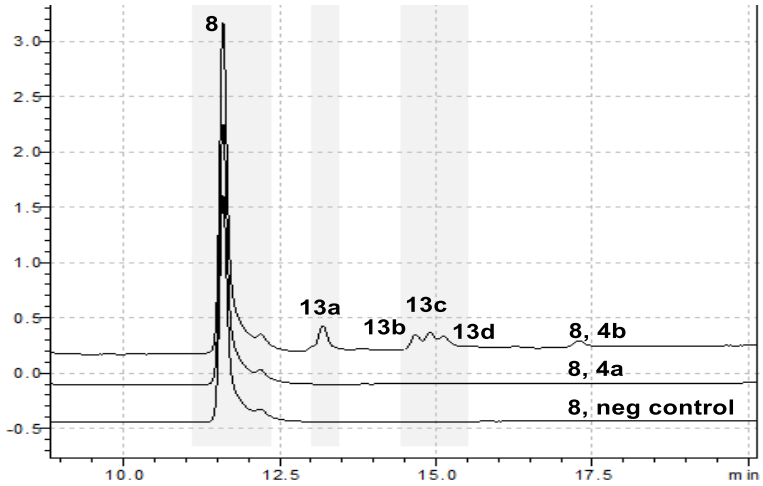
Supplementary Figure S3. Nucleotide sequence of the codon optimized synthesized gene for CdpNPT.



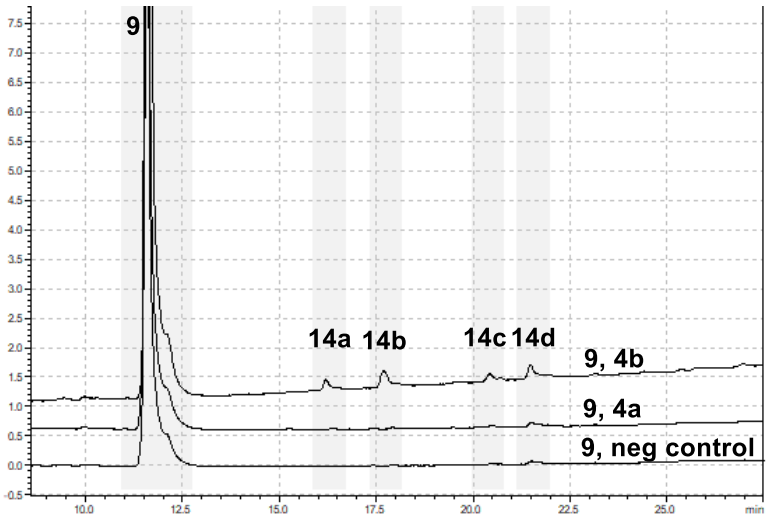
Supplementary Figure S4. SDS-PAGE of overproduced and purified a) PriB, b) FgaPT2, and c) CdpNPT proteins.



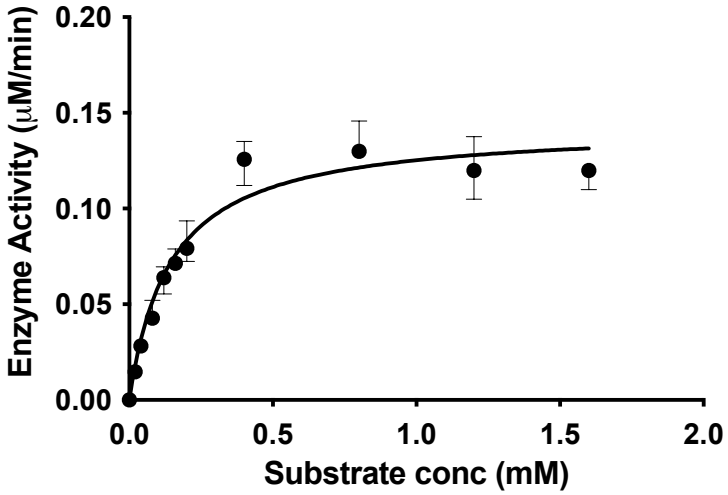
Supplementary Figure S5. a) HPLC chromatograms of reaction mixtures containing cyclo-(L-Trp-L-Trp) **7** in Tris 50 mm/$CaCl_2$ 10 mm (pH 8.0) incubated for 16 h at 37 ºC in the presence of each of **4a** and **4b**. b) HPLC chromatograms of purified products **11a**–**11d** with reaction mixture and **7**. One peak was not isolated due to instability issues.



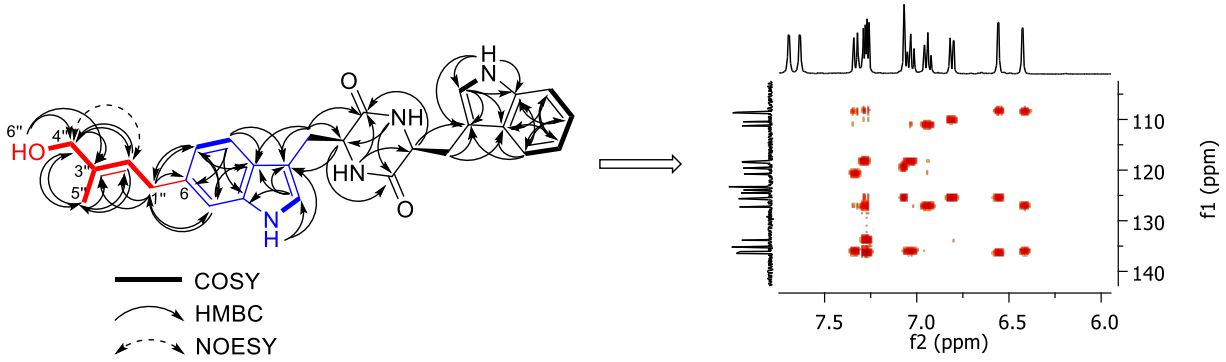
Supplementary Figure S6. HPLC chromatograms of reaction mixtures containing cyclo-(L-Trp-L-Tyr) **8** in Tris 50 mm/$CaCl_2$ 10 mm (pH 8.0) incubated for 16 h at 37 ºC in the presence of each of **4a** and **4b**.



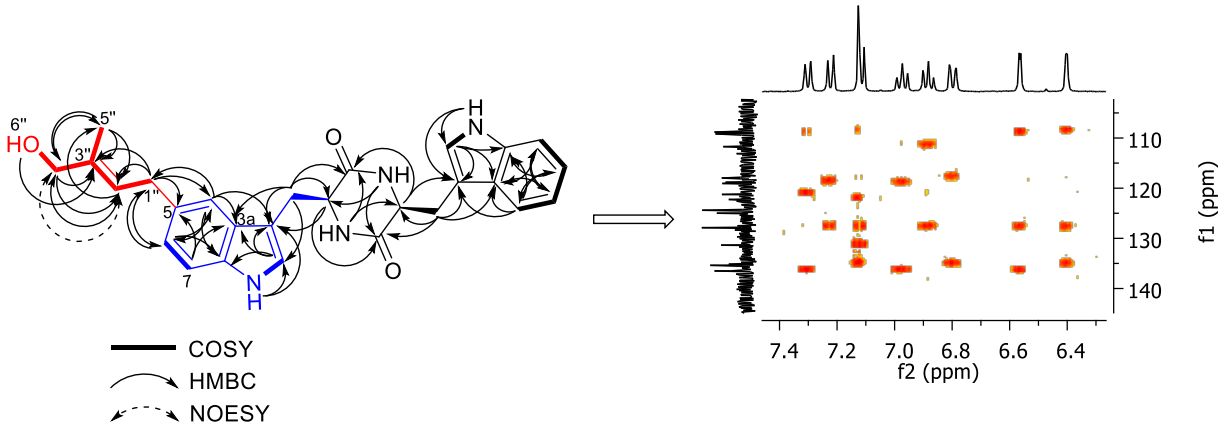
Supplementary Figure S7. HPLC chromatograms of reaction mixtures containing cyclo-(L-Trp- Gly) **9** in Tris 50 mm, $CaCl_2$ 10 mm (pH 8.0) incubated for 16 h at 37 °C in the presence of each of **4a** and **4b**.



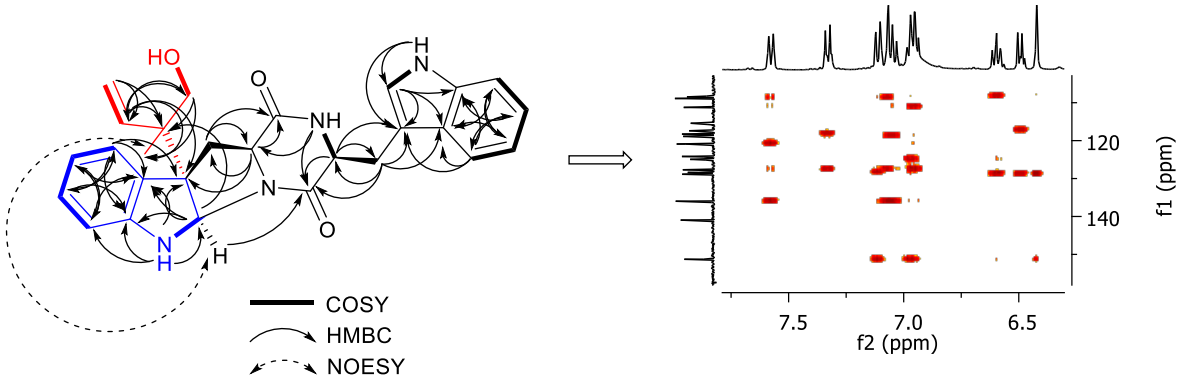
Supplementary Figure S8. Steady state kinetics of recombinant CdpNPT in the presence of **4b** as donor and cyclo-(L-Trp-L-Trp) as acceptor **7** as acceptor. Assays consisted of 50 mM Tris supplemented with 10 mM $CaCl_2$ (pH 8.0), almost-saturating **4b** (1.6 mM) with variable **7** (2×10^{-2} – 1.6 mM) and 33.6 μM. Reactions were incubated at 37 °C for 960 min. Total conversion of products were combined.



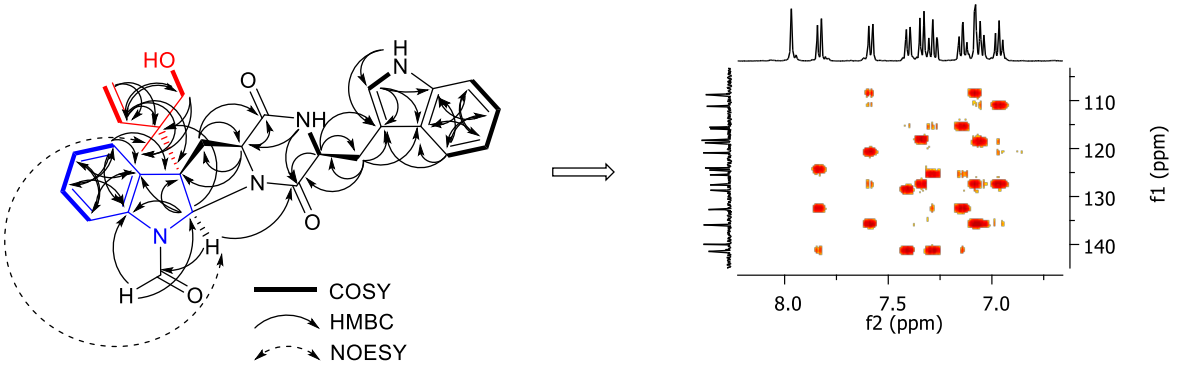
Supplementary Figure S9. ^{1}H–^{1}H COSY (—) and ^{1}H–^{13}C HMBC (⌒) ^{1}H–^{1}H NOSEY (⌒) correlations of cyclo-(6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) (d_6–DMSO) **11a**. Expanded HMBC spectrum of the region 6.0 – 7.7 with key correlations is shown. Full spectrum is presented in NMR spectra below. Long-rang ^{1}H-^{1}H COSY correlations between H-2"/H-4", H-5" are shown.



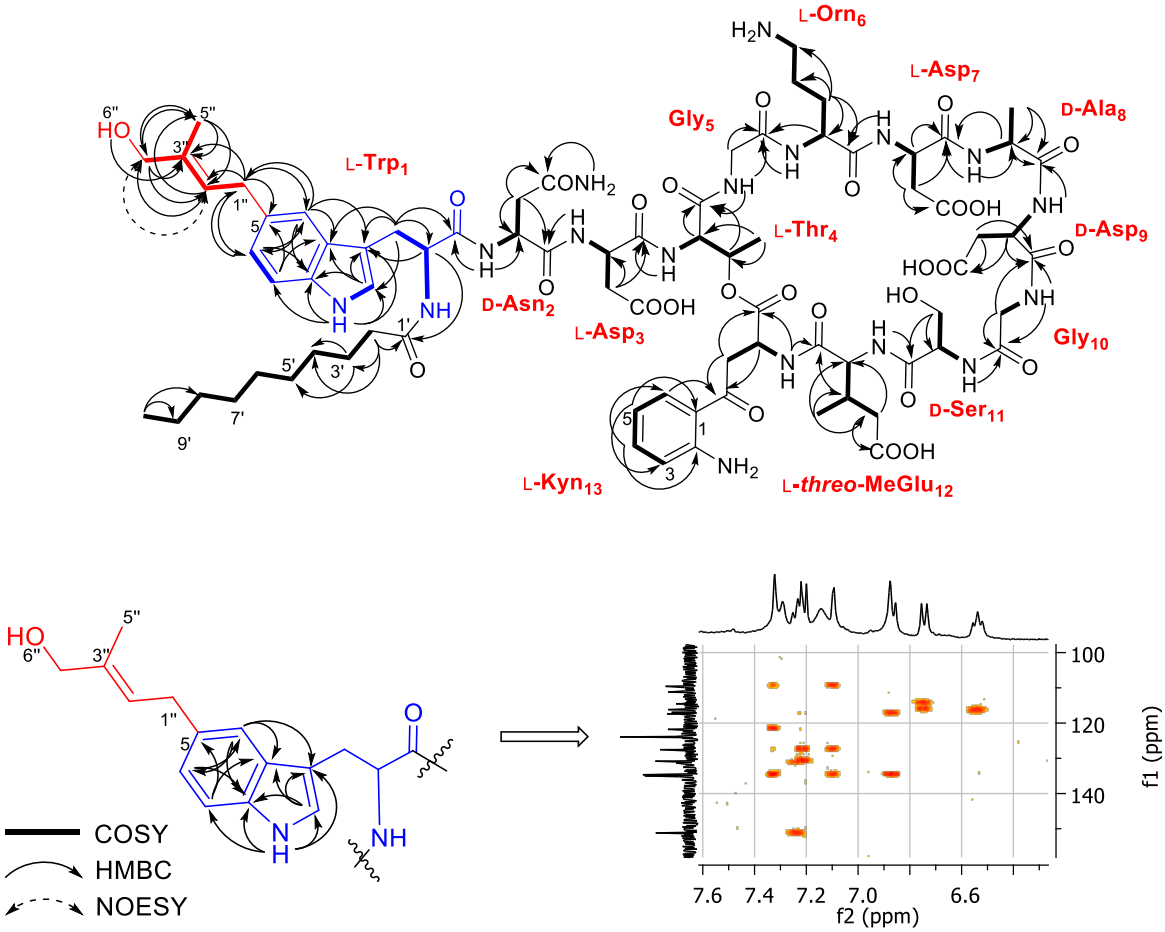
Supplementary Figure S10. ^{1}H–^{1}H COSY (—),^{1}H–^{13}C HMBC (⌒), and ^{1}H–^{1}H NOSEY (⌒) correlations of cyclo-(5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) (d_6–DMSO) **11b**. Expanded HMBC spectrum of the region 6.3 – 7.4 with key correlations is shown. Full spectrum is presented in NMR spectra below. Long-rang ^{1}H-^{1}H COSY correlations between H-2"/H-4", H-5" are shown.



Supplementary Figure S11. ^{1}H–^{1}H COSY (—), ^{1}H–^{13}C HMBC (⌒), and ^{1}H–^{1}H NOSEY (⌒) correlations of 3-*C*-(4-hydroxy)-reverse prenylated derivative **11c** (*d*$_6$–DMSO). Expanded HMBC spectrum of the region 6.4 – 7.7 with key correlations is shown. Full spectrum is presented in NMR spectra below.



Supplementary Figure S12. ^{1}H–^{1}H COSY (—), ^{1}H–^{13}C HMBC (⌒) and ^{1}H–^{1}H NOSEY (⌒) correlations of 3-*C*-(4-hydroxy)-reverse prenylated derivative **11d** (*d*$_6$–DMSO). Expanded HMBC spectrum of the region 6.5 – 8.1 with key correlations is shown. Full spectrum is presented in NMR spectra below.



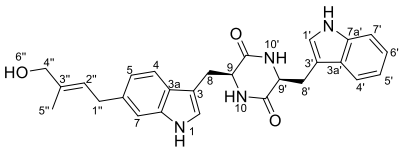
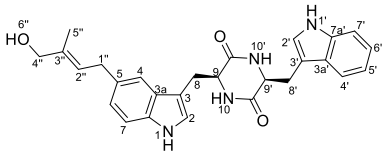
Supplementary Figure S13. ^{1}H–^{1}H COSY (▬), ^{1}H–^{13}C HMBC (⌒) and ^{1}H–^{1}H NOSEY (⌒) correlations of 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$ daptomycin **12**. Only correlations within the ring is shown in the lower figure. Expanded HMBC spectrum of the region 6.3 – 7.6 with key correlations is shown. Full spectrum is presented in NMR spectra below. Long-rang ^{1}H-^{1}H COSY correlations are shown between H-2"/H-4", H-5".

III-Supplementary Tables

Supplementary Table S1. Summary of molecular formula, calculated and observed high-resolution mass spectrpmetry data of hydroxy-bearing allyl modified peptides generated in study.

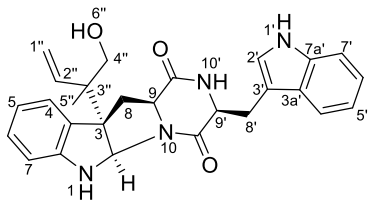
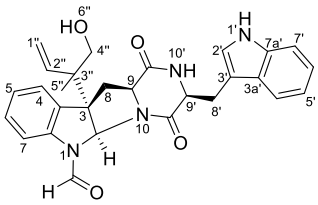
Compound	Molecular Formula	Ionization	Calculated Mass (*m/z*)	Observed Mass (*m/z*)
11a	$C_{27}H_{28}N_4O_3$	$[M + H]^+$	457.2234	457.2246
		$[M - H_2O + H]^+$	439.2129	439.2139
11b	$C_{27}H_{28}N_4O_3$	$[M + H]^+$	457.2234	457.2251
		$[M - H_2O + H]^+$	439.2129	439.2144
11c	$C_{27}H_{28}N_4O_3$	$[M + H]^+$	457.2234	457.2196
		$[2M + H]^+$	913.4396	913.4307
11d	$C_{28}H_{28}N_4O_4$	$[M + H]^+$	485.2183	485.2138
13a	$C_{25}H_{27}N_3O_4$	$[M + H]^+$	434.2074	434.2095
13b	$C_{25}H_{27}N_3O_4$	$[M + H]^+$	434.2074	434.2097
		$[M - H_2O + H]^+$	416.1969	416.1992
13c	$C_{25}H_{27}N_3O_4$	$[M + H]^+$	434.2074	434.2094
		$[M - H_2O + H]^+$	416.1969	416.1991
13d	$C_{25}H_{27}N_3O_4$	$[M + H]^+$	434.2074	434.2099
		$[M - H_2O + H]^+$	416.1969	416.1994
14a	$C_{18}H_{21}N_3O_3$	$[M + H]^+$	328.1656	328.1662
14b	$C_{18}H_{21}N_3O_3$	$[M + H]^+$	328.1656	328.1662
		$[M - H_2O + H]^+$	310.1550	310.1556
14c	$C_{18}H_{21}N_3O_3$	$[M + H]^+$	328.1656	328.1660
		$[M - H_2O + H]^+$	310.1550	310.1557
14d	$C_{18}H_{21}N_3O_3$	$[M + H]^+$	328.1656	328.1681
		$[M - H_2O + H]^+$	310.1550	310.1558
12	$C_{77}H_{109}N_{17}O_{27}$	$[M + H]^+$	1704.7752	1704.7761
		$[M + 2H]^{2+}$	852.8912	852.8931

Supplementary Table S2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of 6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-cyclo-(L-Trp-L-Trp) **11a** and 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-cyclo-(L-Trp-L-Trp) **11b** (d_6–DMSO).

Residue	Position	 **(11a, C-6)**[a]		 **(11b, C-5)**[a]	
		δ_C, type	**δ_H, mult (*J* in Hz)**	**δ_C, type**	**δ_H, mult (*J* in Hz)**
L-Trp$_1$					
	1		10.69, s		10.69, s
	2	123.9, CH	6.56, d (2.1)	124.5 CH	6.40, d (2.1)
	3	108.5, C		108.3, C	
	3a	125.6, C		127.4, C	
	4	118.5, CH	7.26, dd (8.1, 4.3)	117.5, CH	7.19, s
	5	119.6, CH	6.82, dd (8.2, 1.3)	130.8, C	
	6	133.8, C		121.7, CH	6.87, d (7.4)
	7	110.4, CH	7.06, s	111.2, CH	7.29, d (7.8)
	7a	136.4, C		134.6, C	
	1"	33.5, CH_2	3.40, d (7.3)	33.6, CH_2	3.39, d (7.6)
	2"	123.2, CH	5.57, td (7.4, 1.2)	123.9, CH	5.57, t (7.3, 1.1)
	3"	135.1, C		134.9, C	
	4"	66.2, CH_2	3.87–3.80, m	66.4, CH_2	3.83, d (5.2)
	5"	13.6, CH_3	1.68, s	13.7, CH_3	1.69, s
	6"-OH		4.88, t (5.5)		4.74, t (5.6)
	8	30.0, CH_2	2.79–2.63, m 2.26, dd (14.2, 6.4)	30.1, CH_2	2.66, dd (14.4, 4.0) 2.04, dd (14.3, 6.5)
	9	55.1, CH	3.87–3.80, m	55.0, CH	3.83–3.80 m
	10		7.63, d (2.5)		7.73, d (2.4)
	C=O	166.7[b], C		166.7[b], C	
L-Trp$_2$					
	1'		10.80, s		10.83, s
	2'	124.5, CH	6.42, d (1.9)	124.5, CH	6.63, d (2.1)
	3'	108.6, C		108.7, C	
	3a'	127.2, C		127.4, C	
	4'	118.4, CH	7.34, d (7.9)	118.9, CH	7.37, d (7.8)
	5'	118.3, CH	6.95, t (7.4)	118.3, CH	6.96, t (7.4)
	6'	120.7, CH	7.05, t (7.5)	120.7, CH	7.05, t (8.1)
	7'	111.2, CH	7.28, d (7.2)	111.1, CH	7.19, d (8.1)
	7a'	136.0, C		136.0, C	
	8'	30.1, CH_2	2.70–2.63, m 2.06, dd (14.2, 7.0)	29.9, CH_2	2.77, dd (14.2, 4.1) 2.30, dd (14.3, 6.5)
	9'	55.3, CH	3.87–3.80, m	55.3, CH	3.90–3.86 m
	10'		7.69, d (2.4)		7.66, d (2.4)
	C=O	166.7[b], C		166.7[b], C	

Assignments supported by 2D COSY, HSQC, HMBC experiments and by comparison with related compounds from literatures. [a]see Supplementary NMR Spectral Data. [b]interchangeable assignments due to overlapped signals.

Supplementary Table S3. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of **11c** and **11d** (d_6–DMSO).

Residue	Position	 **11c**[a]		 **11d**[a]	
		δ_C, type	**δ_H, mult (*J* in Hz)**	**δ_C, type**	**δ_H, mult (*J* in Hz)**
L-Trp$_1$					
	1		6.42, s		
	2	76.5 CH	5.31, d (2.1)	76.7, CH	6.15, s
	3	59.8, C		58.8, C	
	3a	128.8, C		132.7, C	
	4	124.9, CH	7.12, d (7.2)	125.4, CH	7.41, d (7.2)
	5	117.3, CH	6.60, t (6.9)	124.4, CH	7.15, t (7.6)
	6	128.3, CH	6.98–6.96, m	128.6, CH	7.30, t (7.6)
	7	108.4, CH	6.50, d (7.4)	115.5, CH	7.83, d (7.8)
	7a	151.2, C		141.4, C	
	1"	115.4, CH_2	5.07–4.94, m	115.9, CH_2	5.10–5.02, m
	2"	140.9, CH	5.83, dd (17.5, 11.0)	139.9, CH	5.86, dd (17.5, 11.0)
	3"	45.1, C		44.7, C	
	4"	65.4, CH_2	3.31, bs 3.24, bs	65.4, CH_2	3.27, d (4.3) 3.13, d (4.6)
	5"	16.4, CH_3	0.59, s	16.6, CH_3	0.62, s
	6"-OH		4.56, bs		4.84, t (4.7)
	1-C=O			161.9, C	
	1-OCH				8.91, s
	8	37.9, CH_2	2.27, dd (12.6, 5.7) 1.86, dd (12.1, 1.0)	37.5, CH_2	2.39, dd (12.7, 5.7) 1.89, dd (12.3)
	9	57.9, CH	3.67, dd (11.5, 5.7)	57.6, CH	3.71, dd (11.0, 6.3)
	10				
	C=O	164.7, C		165.8, C	
L-Trp$_2$					
	1'		10.89, s		10.90, s
	2'	124.1, CH	7.07, d (2.1)	124.0, CH	7.08, d (2.4)
	3'	108.8, C		108.7, C	
	3a'	127.6, C		127.5, C	
	4'	118.8, CH	7.58, d (8.1)	118.7, CH	7.59, d (7.9)
	5'	118.2, CH	6.96–6.94, m	118.4, CH	6.98, t (7.4)
	6'	120.7, CH	7.04, t (8.0)	120.8, CH	7.07, t (7.5)
	7'	111.2, CH	7.34, d (8.1)	111.2, CH	7.34, d (7.1)
	7a'	135.9, C		135.9, C	
	8'	29.9, CH_2	3.18–3.14, m	26.2, CH_2	3.19–3.11, m
	9'	55.3, CH	4.32, t (4.5)	55.3, CH	4.43, t (3.8)
	10'		7.87, s		7.96, s
	C=O	167.7, C		167.0, C	

Assignments supported by 2D COSY, HSQC, HMBC experiments and by comparison with related compounds from literatures. [a]see Supplementary NMR Spectral Data.

Supplementary Table S4. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of daptomycin and 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp_1 daptomycin **12**.

Residue	Position	daptomycin (10)[a]		5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp_1 daptomycin 12[b]	
		δ_C, type	**δ_H, mult (*J* in Hz)**	**δ_C, type**	**δ_H, mult (*J* in Hz)**
L-Trp_1	NH		7.98, d (6.4)		8.00, d (6.2)
	α	53.9, CH	4.46–4.39, m	54.2, CH	4.39–4.33, m
	β	27.4, CH_2	2.91–2.85, m	27.2, CH_2	2.89, dd (9.3, 5.0)
			3.07, dd (14.8,4.4)		3.03, dd (13.8, 4.8)
	1		10.76, br s		10.63, br s
	2	123.7, CH	7.14, br s	123.8, CH	7.09, d (1.6)
	3	110.0, C		109.5, C	
	3a	127.3, C		127.5, C	
	4	118.1, CH	7.57, d (7.9)	117.4, CH	7.32, s
	5	118.4, CH	6.95, t (7.8)	130.8, C	
	6	120.7, CH	7.03, t (7.6)	121.7, CH	6.87, d (8.0)
	7	111.2, CH	7.14, d (8.0)	111.2, CH	7.22, d (8.0)
	7a	136.0, C		134.6, C	
	C=O	172.8[b], C		172.5[b], C	
	1'	173.9[c], C		173.9[c], C	
	2'	35.1, CH_2	2.02, t (7.2)	35.1, CH_2	2.01, t (7.0)
	3'	25.0, CH_2	1.35–1.31, m	25.0, CH_2	1.37–1.33, m
	4'	28.9, CH_2	1.25–1.19, m	29.0, CH_2	1.22–1.18, m
	5'	28.8, CH_2	1.25–1.19, m	28.9, CH_2	1.22–1.18, m
	6'	28.7, CH_2	1.25–1.19, m	28.7, CH_2	1.22–1.18, m
	7'	28.6, CH_2	1.25–1.19, m	28.6, CH_2	1.22–1.18, m
	8'	31.3, CH_2	1.14–1.07, m	31.3, CH_2	1.22–1.18, m
	9'	22.1, CH_2	1.14–1.07, m	22.1, CH_2	1.22–1.18, m
	10'	14.0, CH_3	0.84, t, (7.2)	14.0, CH_3	0.83, t (7.0)
	1"			33.6, CH_2	3.39, d (4.0)
	2"			123.9, CH	5.56, t (7.0)
	3"			135.0, C	
	4"			66.4, CH_2	3.81, bs
	5"			13.7, CH_3	1.69, t (7.2)
	6"-OH				4.39, bs
D-Asn_2					
	NH		8.32, br s		8.37, br s
	α	50.2, CH	4.46–4.39, m	49.8, CH	4.57–4.54, m
	β	38.4, CH_2	2.37–2.30, m	37.1, CH_2	2.37–2.30, m
			2.48–2.43, m		2.45–240, m
	C=O	174.0, C		173.8, C	
	$\underline{CO}NH_2$	171.5, C		171.6, C	
	$CONH_2$		7.30, br s		7.29, br s
			6.87, s		6.87, s
L-Asp_3					
	NH		8.13–8.04, br m		8.00, d (6.8)
	α	49.7, CH	4.60–4.53, m	54.1, CH	4.40–4.34, m
	β	37.3, CH_2	2.37–2.30, m	38.5, CH_2	2.37–2.30, m
			2.91–2.85, m		2.89–2.78, m
	C=O	170.8[d], C		170.7[d], C	
L -Thr_4	$\underline{CO}OH$	171.4, C		171.5, C	
	NH		7.91, d (8.0)		7.93, d (6.8)
	α	56.0, CH	4.28–4.25, m	55.6, CH	4.40–4.34, m
	β	70.1, CH	5.28, s	70.2, CH	5.19, br s
	γ	15.9, CH_3	1.09, d (6.4)	15.6, CH_3	1.09, d (6.0)
Gly_5	C=O	169.0, C		168.7, C	
	NH		8.13–8.04, br m		8.13–8.07, br m
	α	42.1, CH_2	3.80, br s	42.2, CH_2	3.81, s
L-Orn_6	C=O	169.1, C		168.5, C	

	NH		8.32, br s		8.27, d (3.6)
	α	52.9, CH	4.16–4.13, m	52.6, CH	4.20–4.16, m
	β	28.0, CH_2	1.71–1.65, m	28.1, CH_2	1.70–1.66, m
	γ	23.3, CH_2	1.62–1.55, m	23.5, CH_2	1.62–1.58, m
	δ	38.2, CH_2	2.79–2.75, m	38.5, CH_2	2.83–2.78, m
	NH_2		7.28, br s		7.28, br s
L-Asp_7	C=O	171.3, C		171.5, C	
	NH		8.48, s		8.42, bs
	α	50.2, CH	4.60–4.53, m	49.8, CH	4.63–4.54, m
	β	36.9, CH_2	2.37–2.31, m	36.9, CH_2	2.37–2.30, m
			2.62–2.58, m		2.66–2.59, m
	C=O	171.5, C		171.5, C	
D-Ala_8	COOH	171.8, C		171.9, C	
	NH		7.98, d (6.4)		7.97, d (4.1)
	α	48.8, CH	4.16–4.13, m	48.6, CH	4.20–4.16, m
	β	17.3, CH_3	1.25–1.19, m	17.6, CH_3	1.22–1.15, m
L-Asp_9	C=O	172.8[c], C		172.5[c], C	
	NH		8.48, s		8.42, br s
	α	50.3, CH	4.50, m	50.5, CH	4.54–4.45, m
	β	36.9, CH_2	2.62–2.58, m	36.0, CH_2	2.66–2.59, m
			2.44, s		2.45–2.40, m
	C=O	171.4, C		171.4, C	
Gly_{10}	COOH	173.8, C		173.7, C	
	NH		8.13–8.04, m		8.22, br s
	α	42.1, CH_2	3.80, br s	42.1, CH_2	3.81, br s
D-Ser_{11}	C=O	170.4, C		170.4, C	
	NH		8.13–8.04, br m		8.13–8.04, br m
	α	56.3, CH	4.28–4.25, m	55.8, CH	4.40–4.34, m
	β	61.3, CH_2	3.66, s	61.4, CH_2	3.61, s
L-threo-$MeGlu_{12}$	C=O	170.7, C		170.5, C	
	NH		8.32, s		8.32, br s
	α	56.0, CH	4.45–4.40, m	55.6, CH	4.50–4.45, m
	β	37.3, CH	2.37–2.31, m	37.1, CH	2.37–2.30, m
	γ1	37.7, CH_2	2.44–2.43, m	38.0, CH_2	2.45–2.40, m
			2.32–2.31, m		2.37–2.30, m
	γ2	14.7, CH_3	0.88, d (6.4)	14.5, CH_3	0.86, d (6.8)
	C=O	171.4, C		171.9, C	
L-Kyn_{13}	COOH	172.6, C		172.9, C	
	NH		8.32, s		8.32, br s
	α	48.9, CH	4.70, m	48.6, CH	4.72, m
	β	42.1, CH_2	3.66, br m	41.9, CH_2	3.61, br m
			3.58, m		
	γ	197.4, C		197.5, C	
	1	116.2, C		116.1, C	
	2	151.1, C		151.1, C	
	2-NH_2		7.16, br s		7.14, br s
	3	116.9, CH	6.75, d (8.2)	116.9, CH	6.75, d (8.2)
	4	134.3, CH	7.24, t (7.2)	134.4, CH	7.25 t (7.6)
	5	114.4, CH	6.54, t (6.8)	114.5, CH	6.56, t (7.3)
	6	131.2, CH	7.72, d (6.0)	131.3, CH	7.75, d (6.8)
	C=O	171.8[d], C		171.9[d], C	

Assignments supported by 2D COSY, HSQC, HMBC experiments and by comparison with related compounds from literatures. [a]The daptomycin referral data was accessed by our recent report [b]see Supplementary NMR Spectral Data. [c-d]interchangeable assignments due to overlapped signals and/or the lack of observed HMBC correlations.

IV-Supplementary References

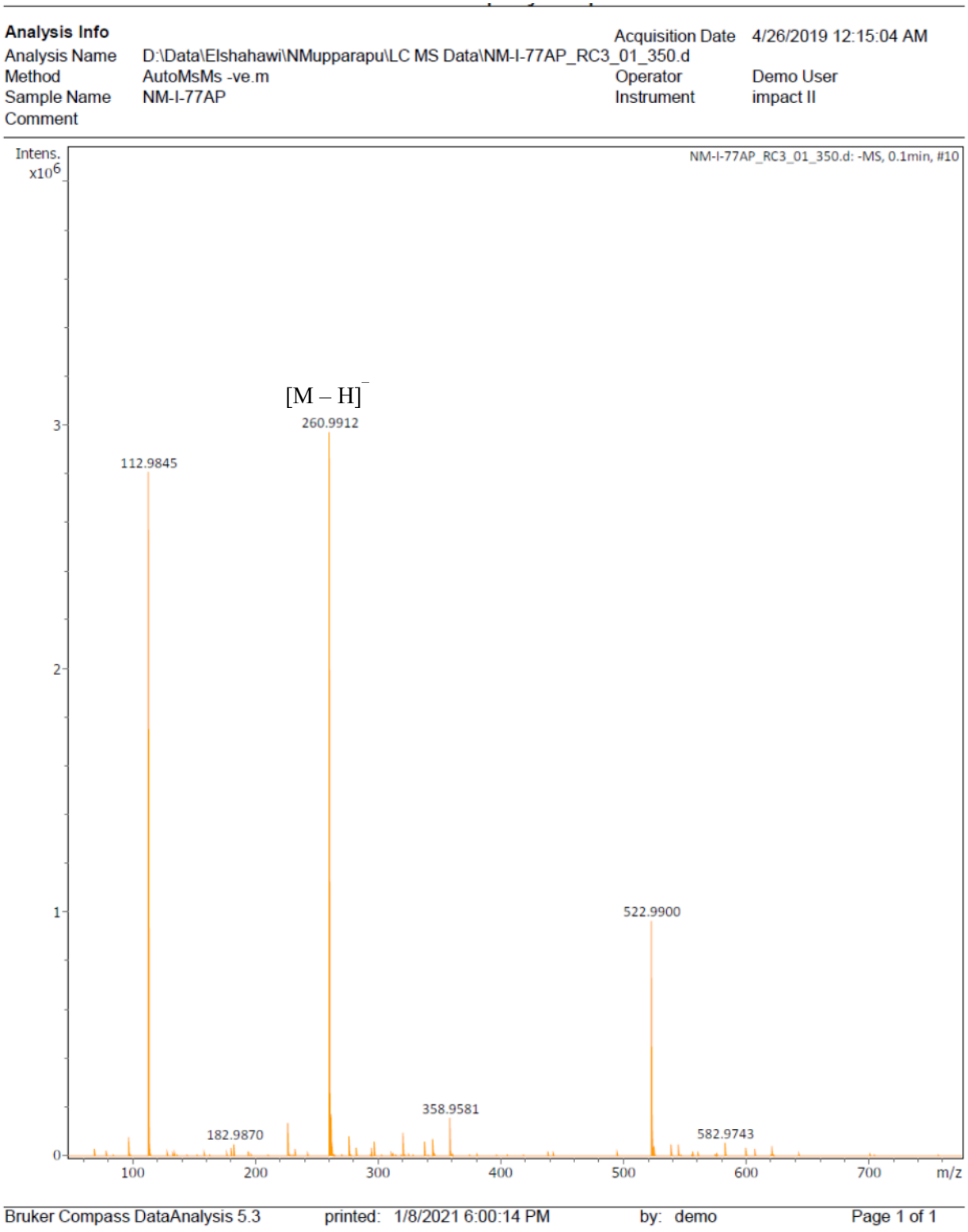
[1] K. Heckenbichler, A. Schweiger, L. A. Brandner, A. Binter, M. Toplak, P. Macheroux, K. Gruber, R. Breinbauer, *Angew. Chem. Int. Ed Engl.* **2018**, *57*, 7240–7244.

[2] I. Span, K. Wang, W. Wang, J. Jauch, W. Eisenreich, A. Bacher, E. Oldfield, M. Groll, *Angew. Chem. Int. Ed Engl.* **2013**, *52*, 2118–2121.

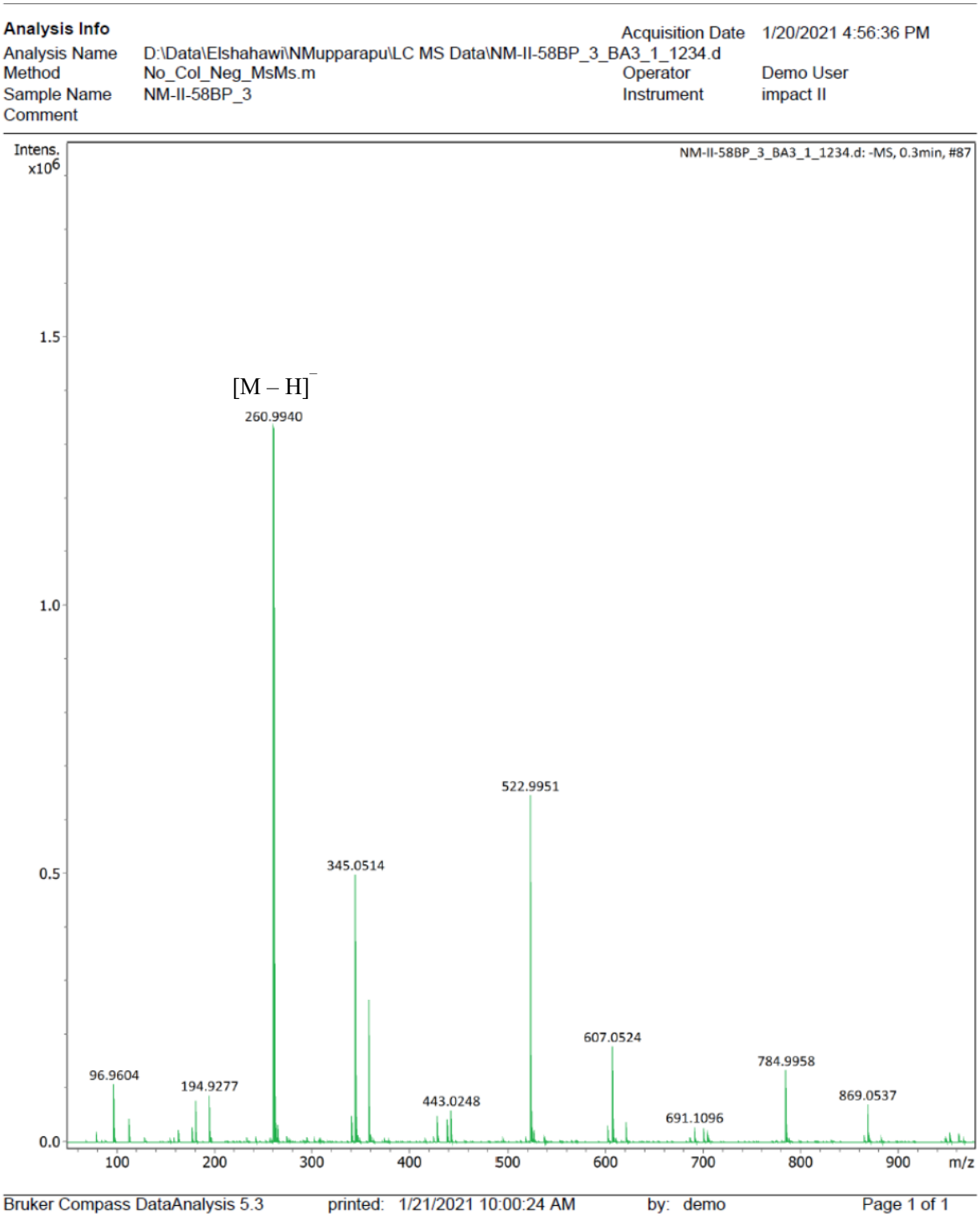
[3] S. P. Bew, G. D. Hiatt-Gipson, G. P. Mills, C. E. Reeves, *Beilstein J. Org. Chem.* **2016**, *12*, 1081–1095.

[4] D. C. Burns, E. P. Mazzola, W. F. Reynolds, *Nat. Prod. Rep.* **2019**, *36*, 919–933.

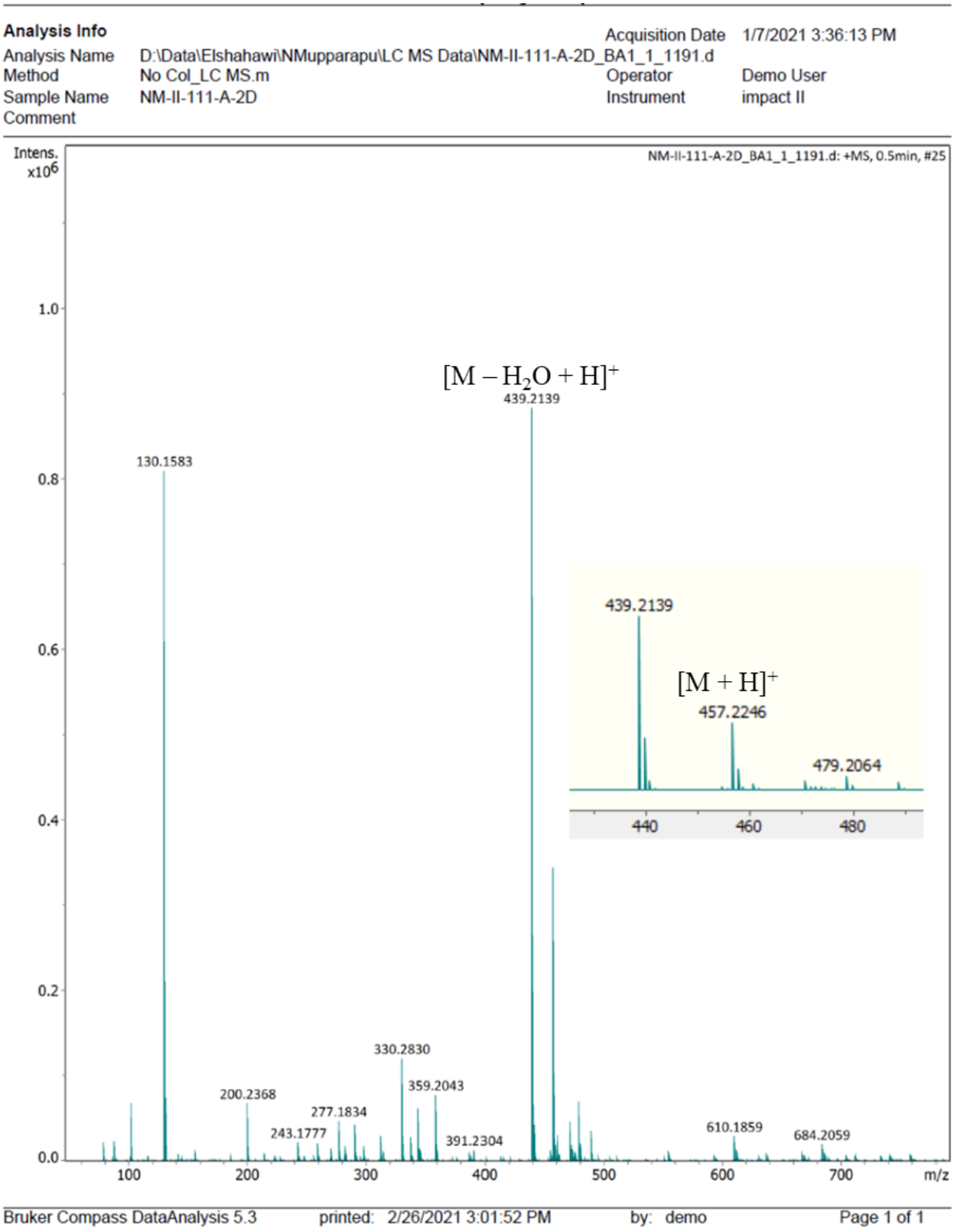
V-Supplementary Mass Spectra



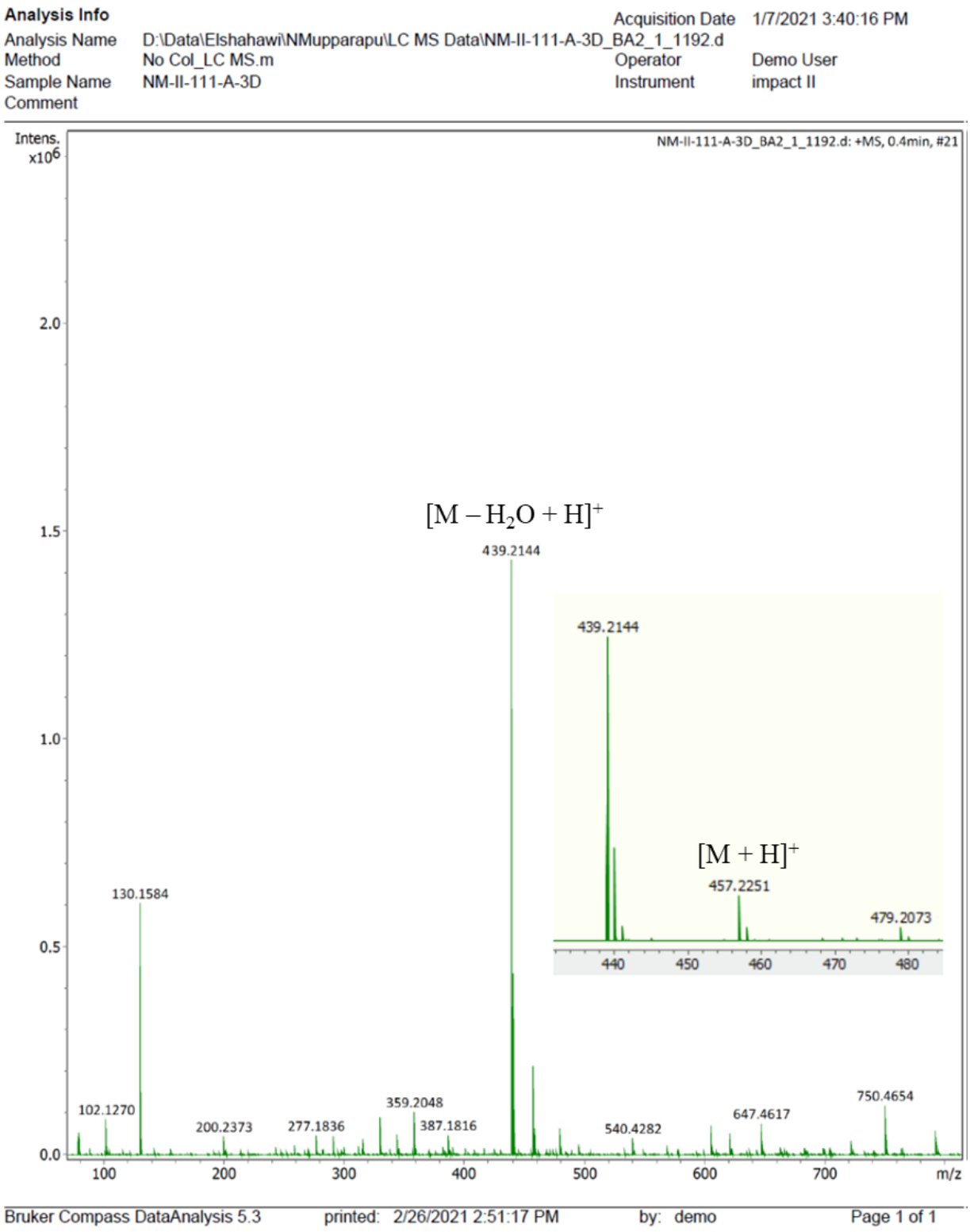
LC-HR-ESI-MS data of (*E*)-4-hydroxy-2-methylbut-2-en-1-yl diphosphate **4a**



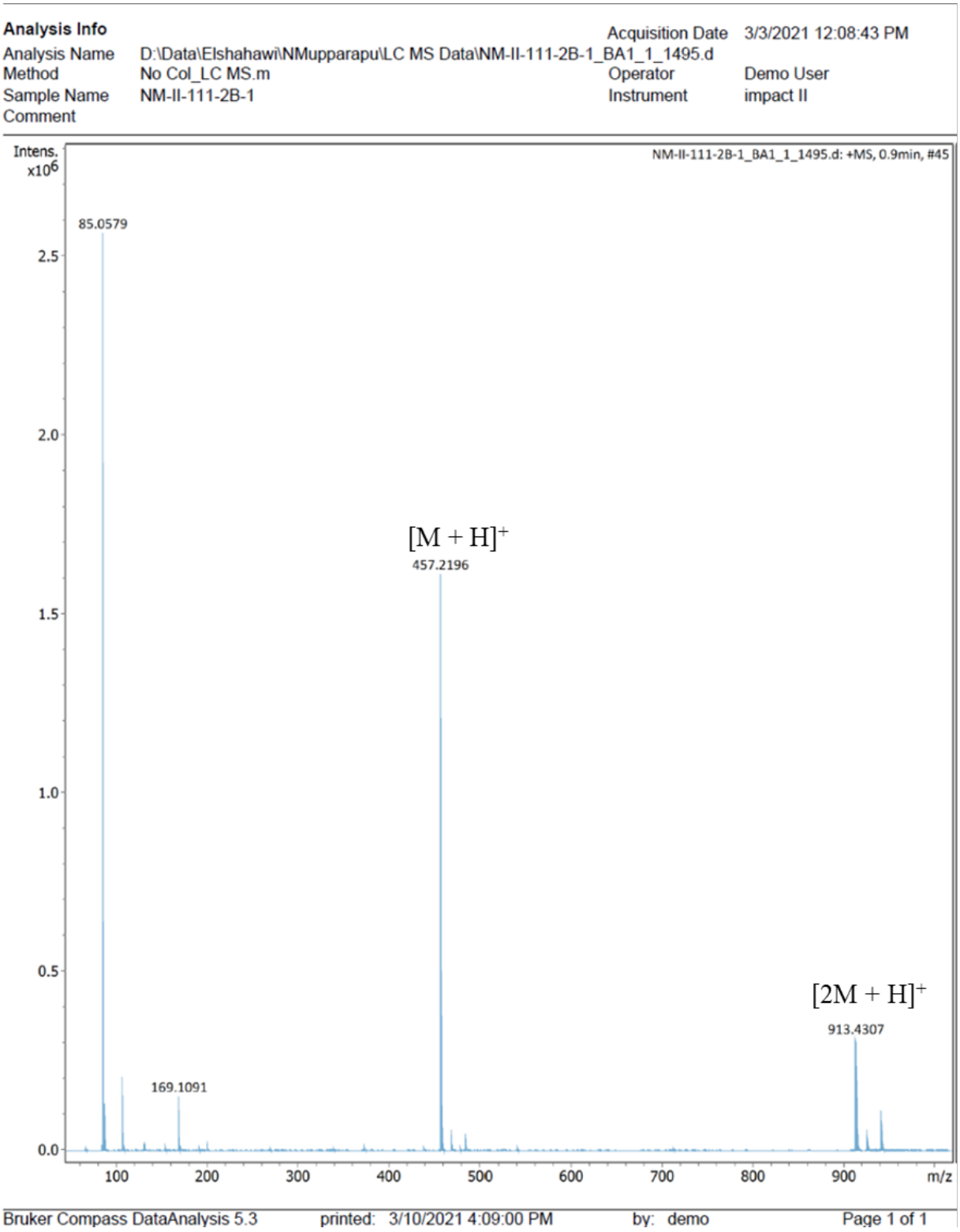
LC-HR-ESI-MS data of (*E*)-4-hydroxy-3-methyl-2-butene-diphosphate 4**b**



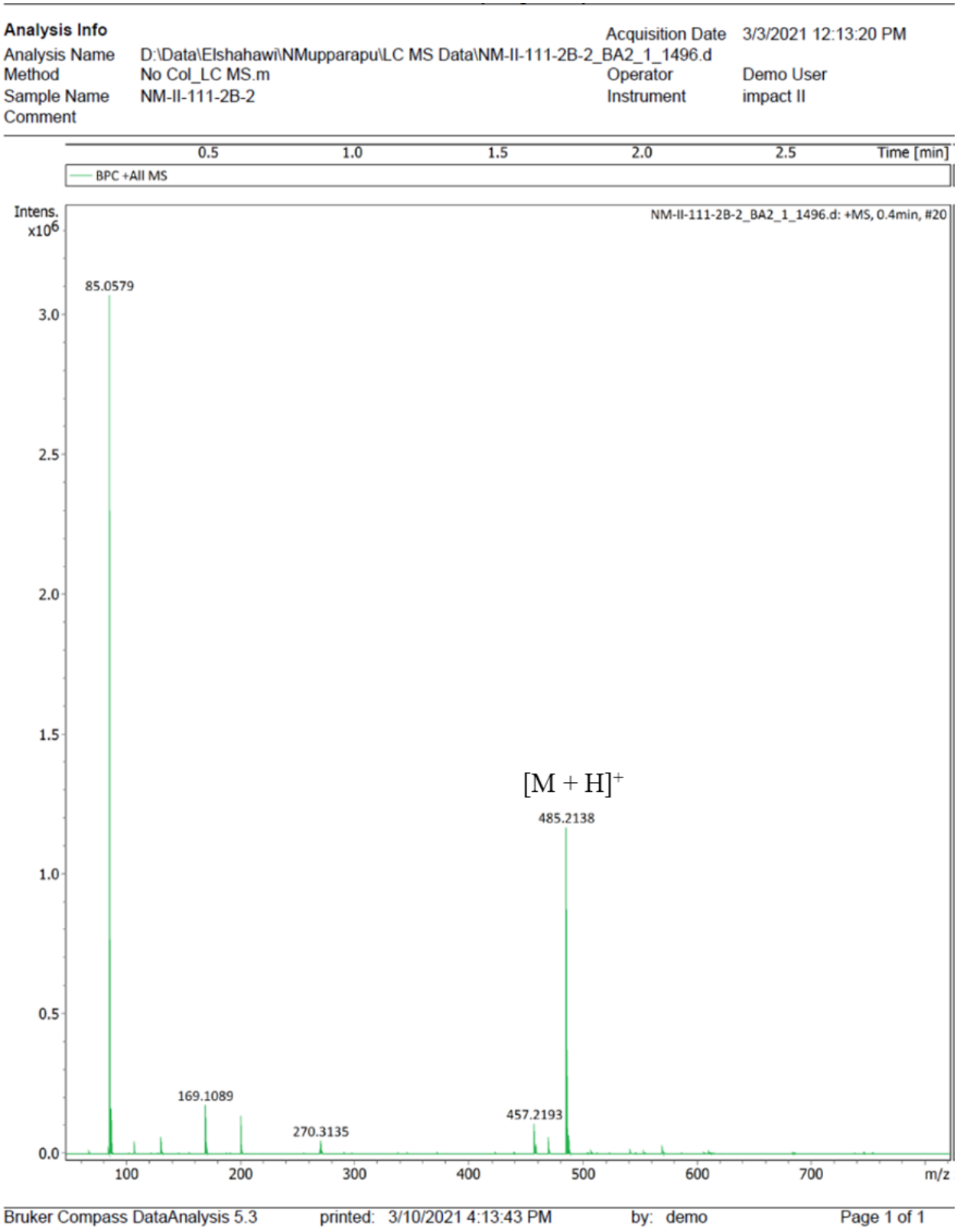
Mass spectrum of cyclo-(6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11a**.



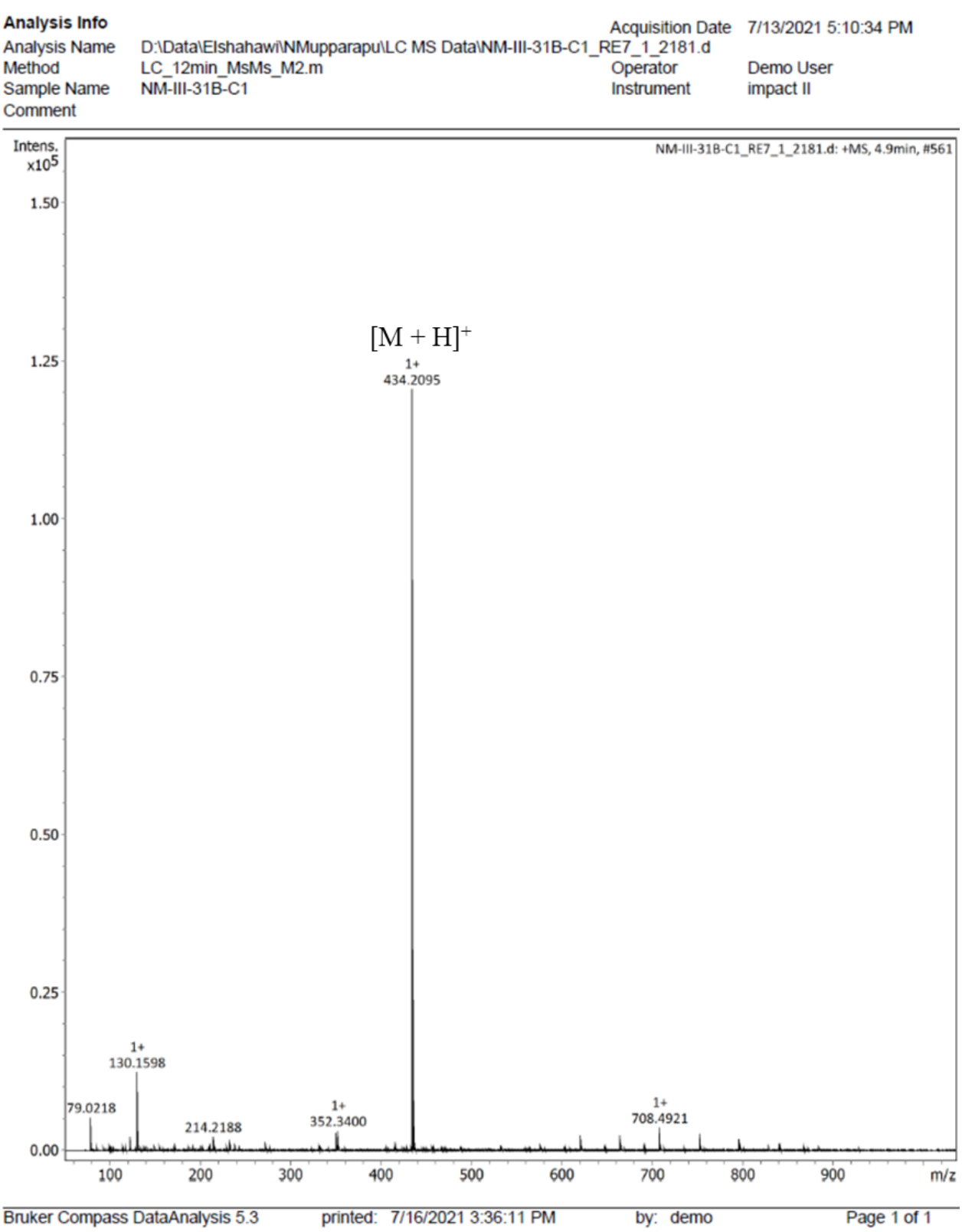
Mass spectrum of cyclo-(5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11b**.



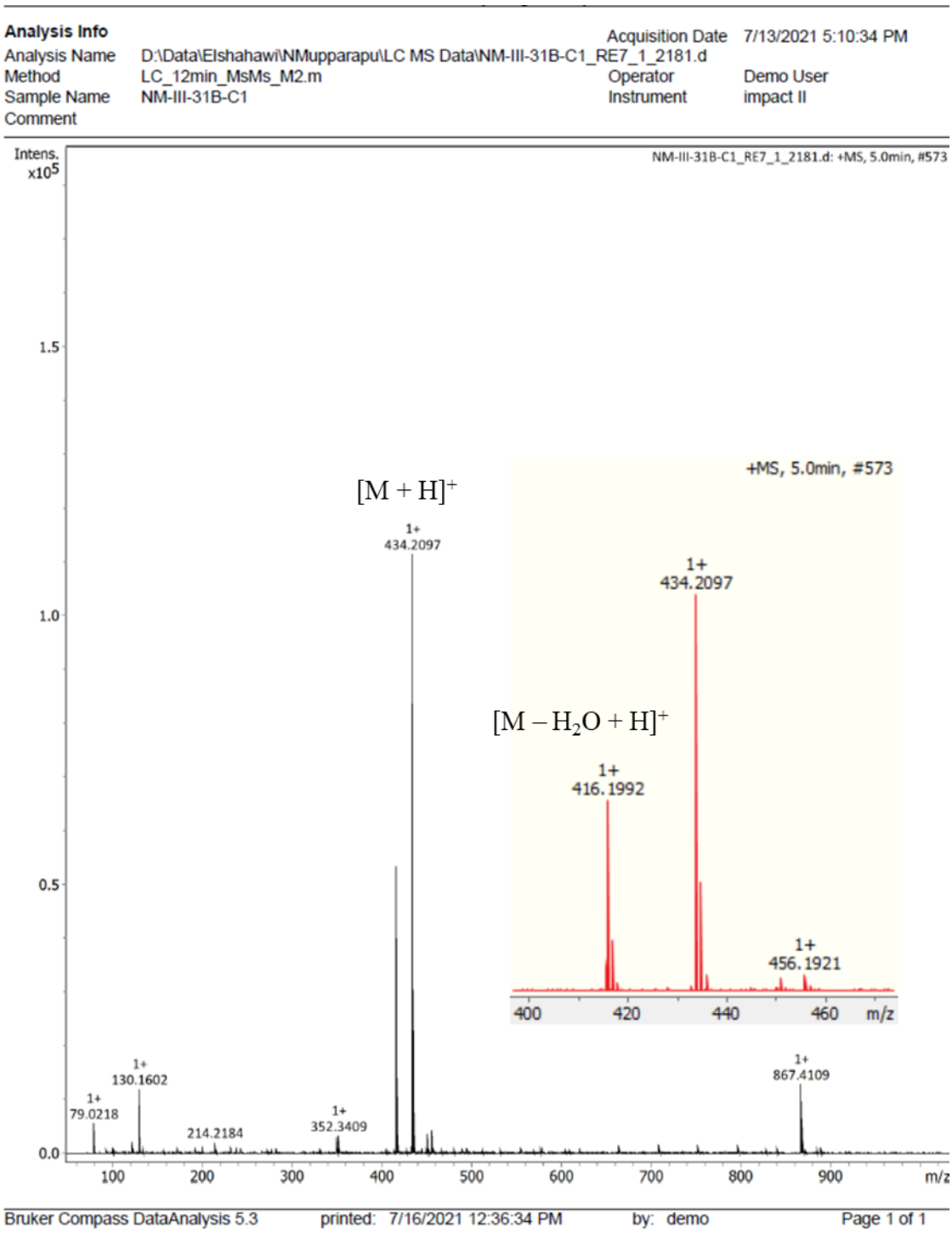
Mass spectrum of 3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11c**.



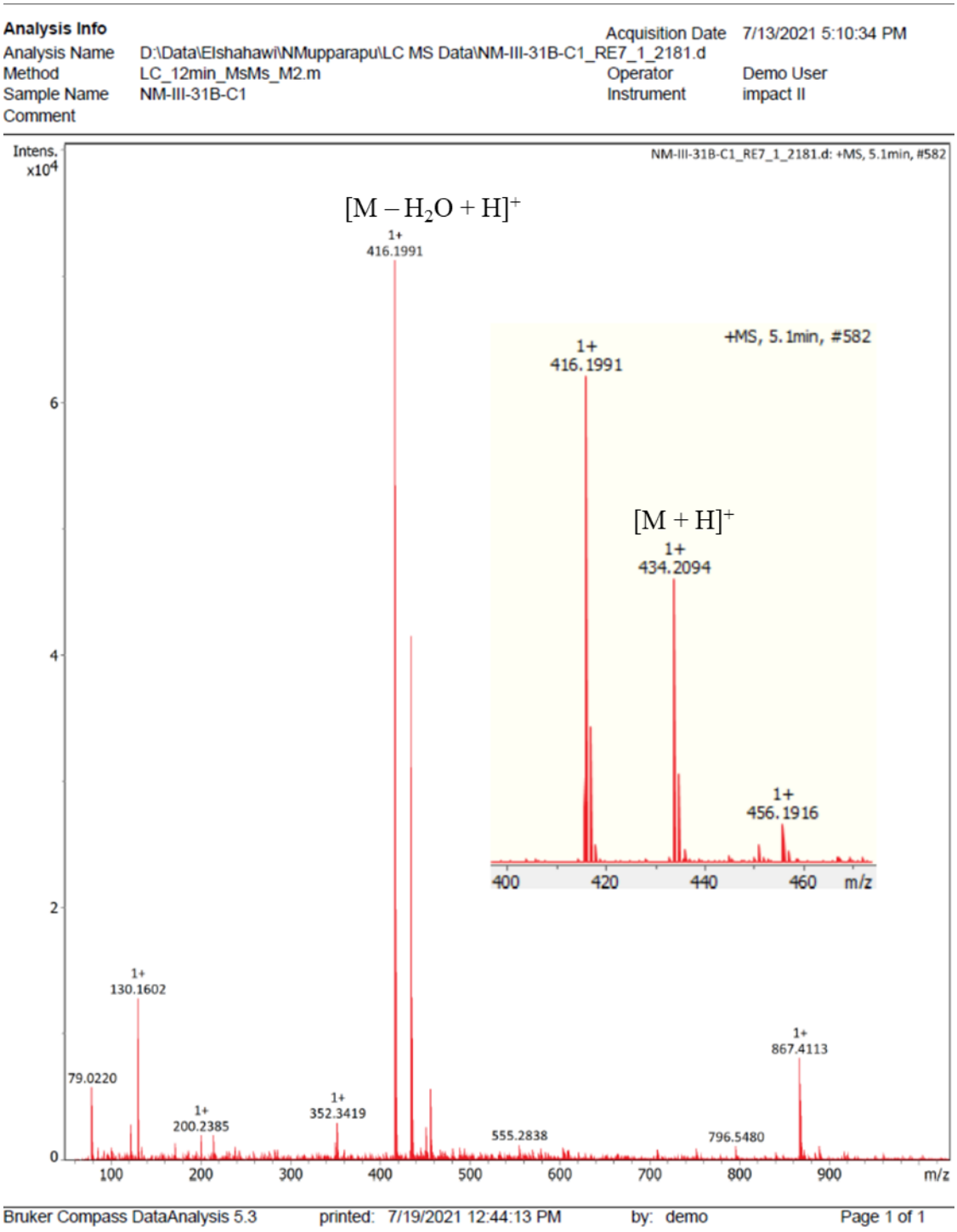
Mass spectrum of *N*-formyl-3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11d**.



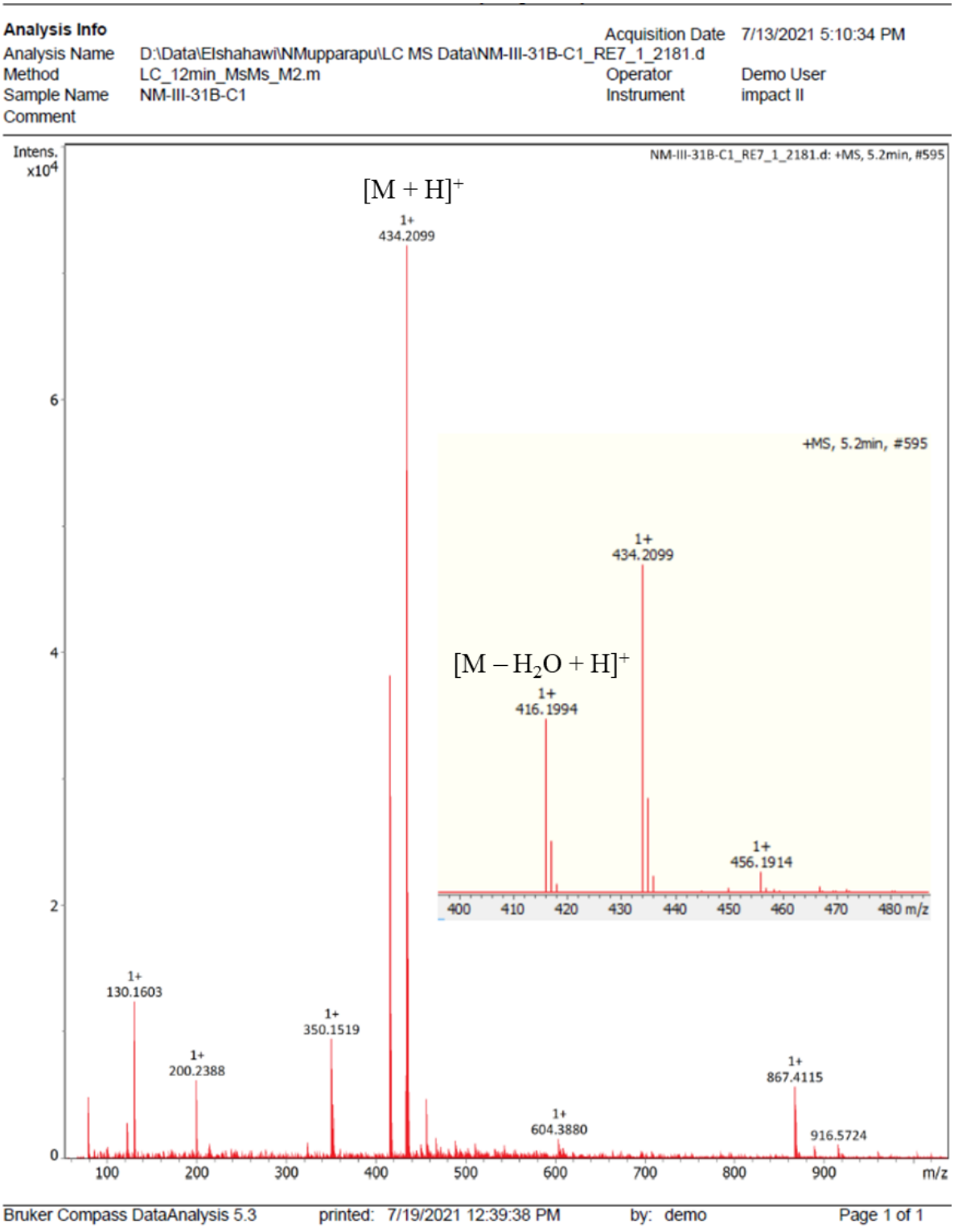
Mass spectrum of hydroxy-bearing allyl modified cyclo-(L-Trp-L-Tyr) **13a**.



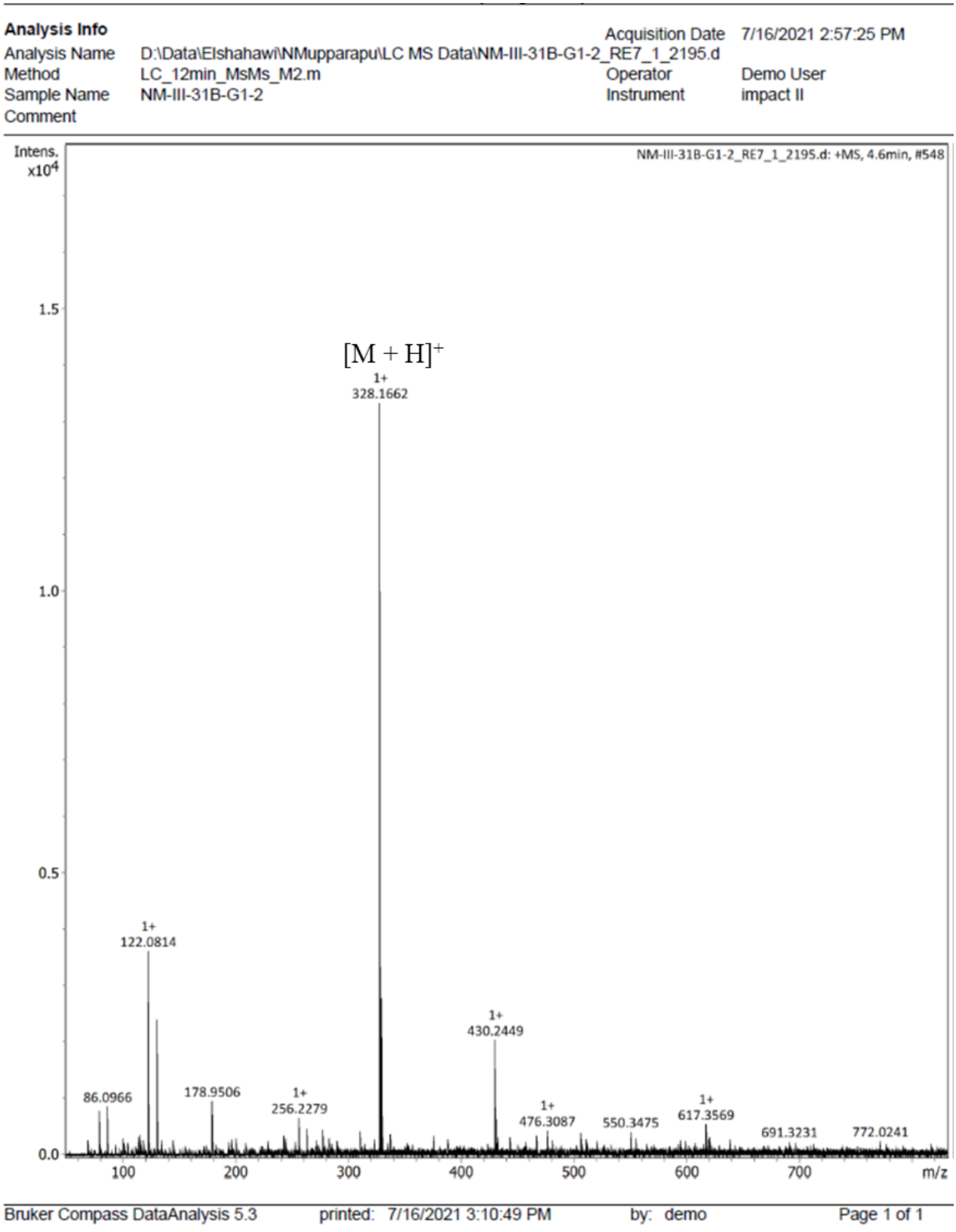
Mass spectrum of hydroxy-bearing allyl modified cyclo-(L-Trp-L-Tyr) **13b**.



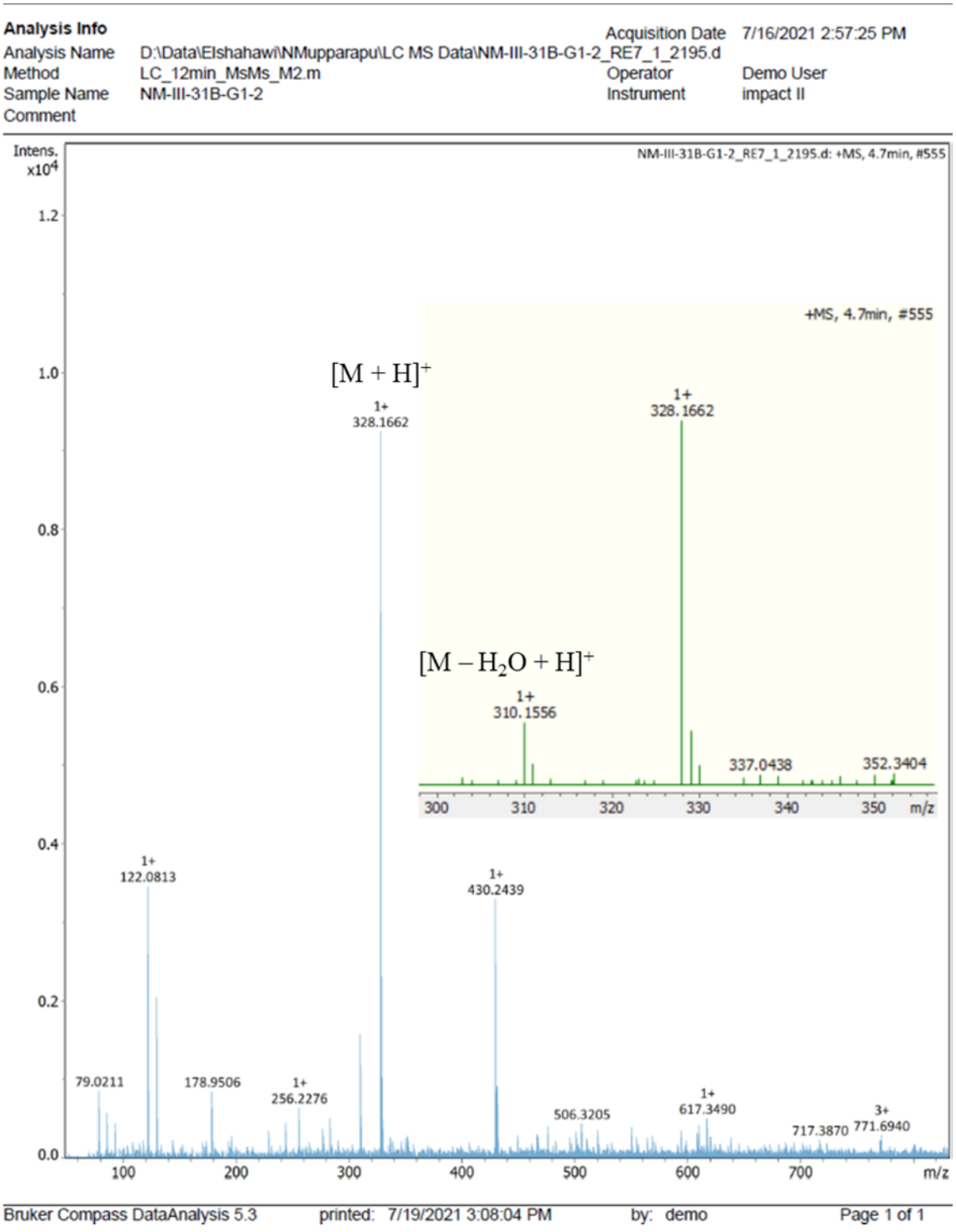
Mass spectrum of hydroxy-bearing allyl modified cyclo-(L-Trp-L-Tyr) **13c**.



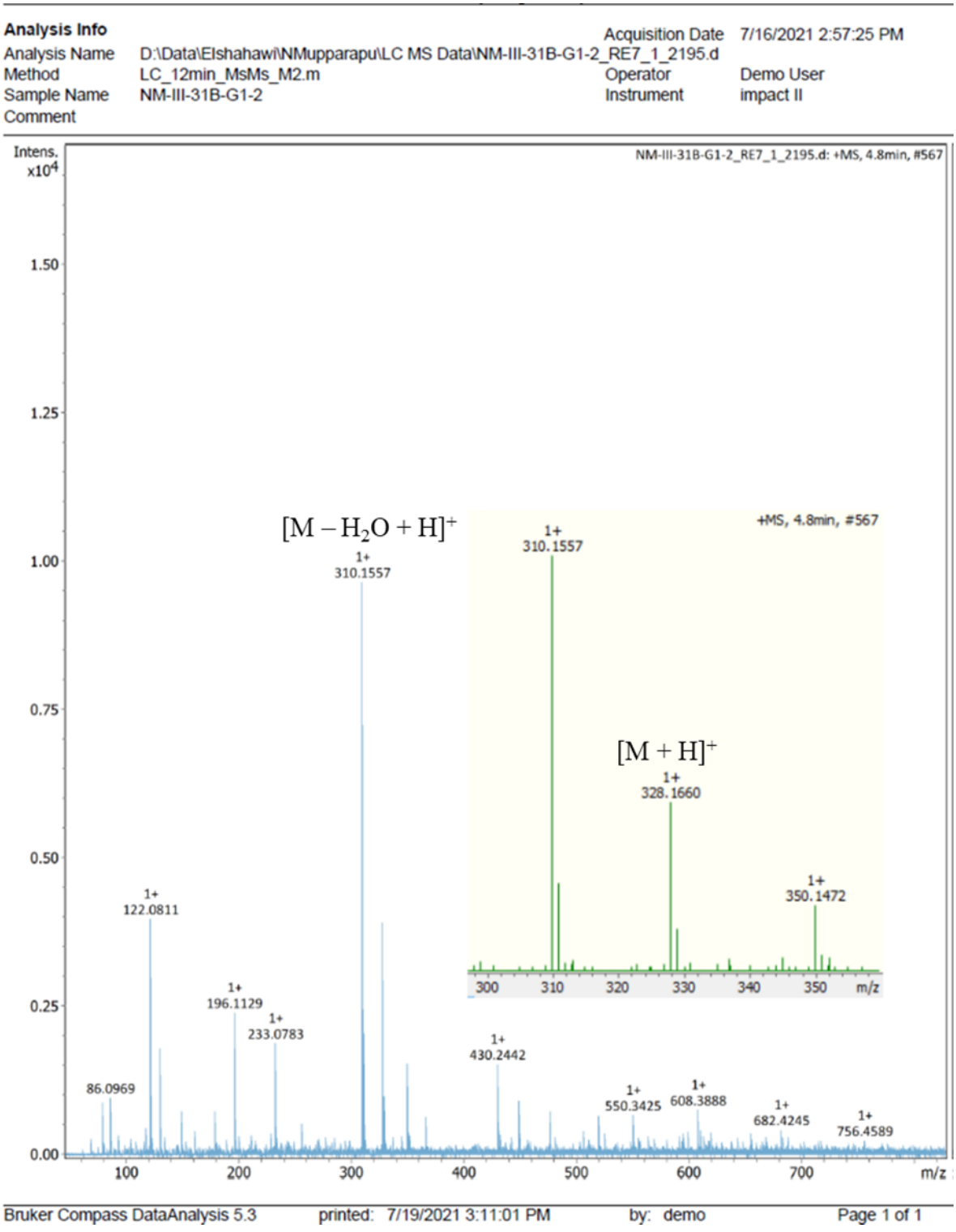
Mass spectrum of hydroxy-bearing allyl modified cyclo-(L-Trp-L-Tyr) **13d**.



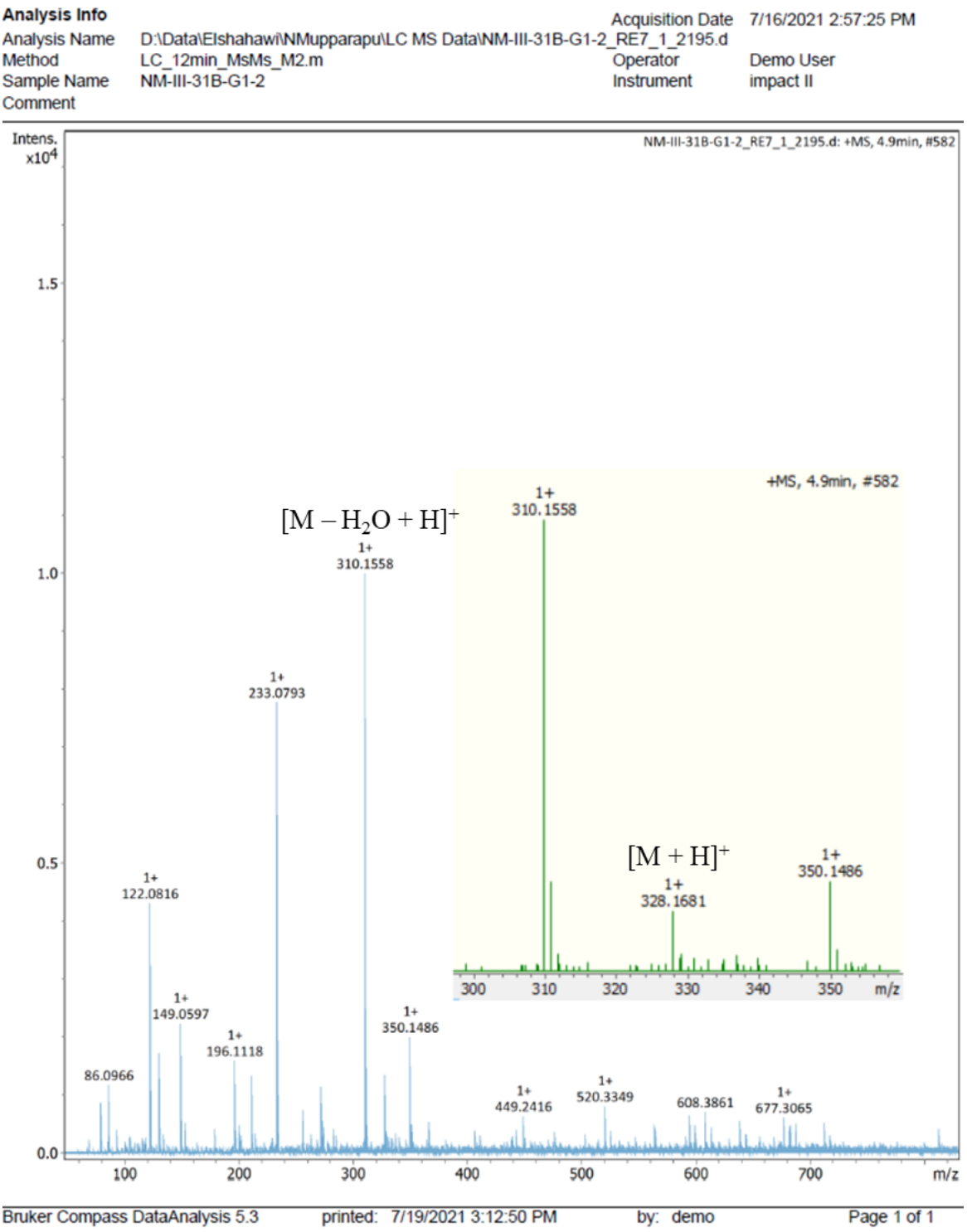
Mass spectrum of hydroxy-bearing allyl-modified cyclo-(L-Trp-Gly) **14a**.



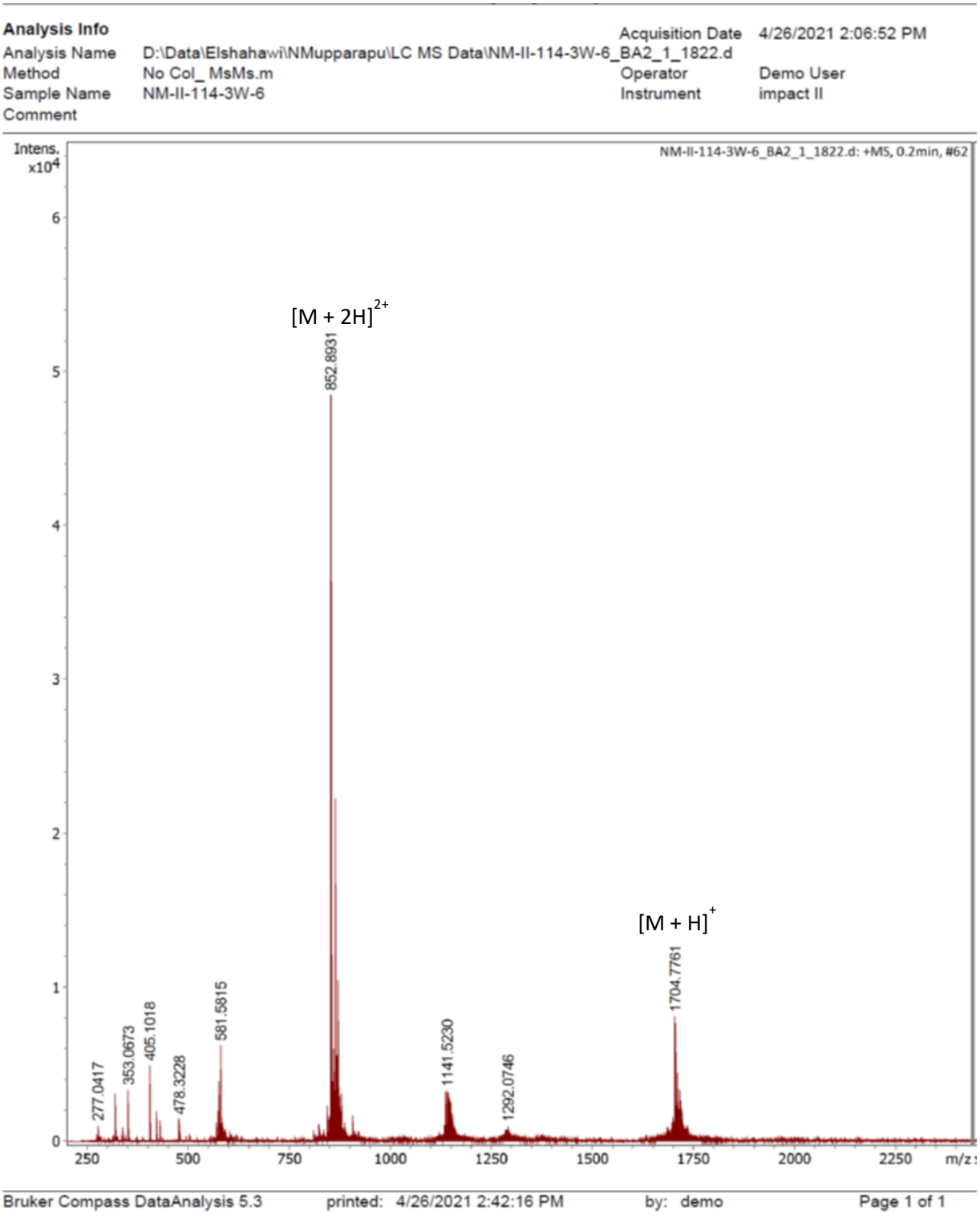
Mass spectrum of hydroxy-bearing allyl-modified cyclo-(L-Trp-Gly) **14b**.



Mass spectrum of hydroxy-bearing allyl-modified cyclo-(L-Trp-Gly) **14c**.

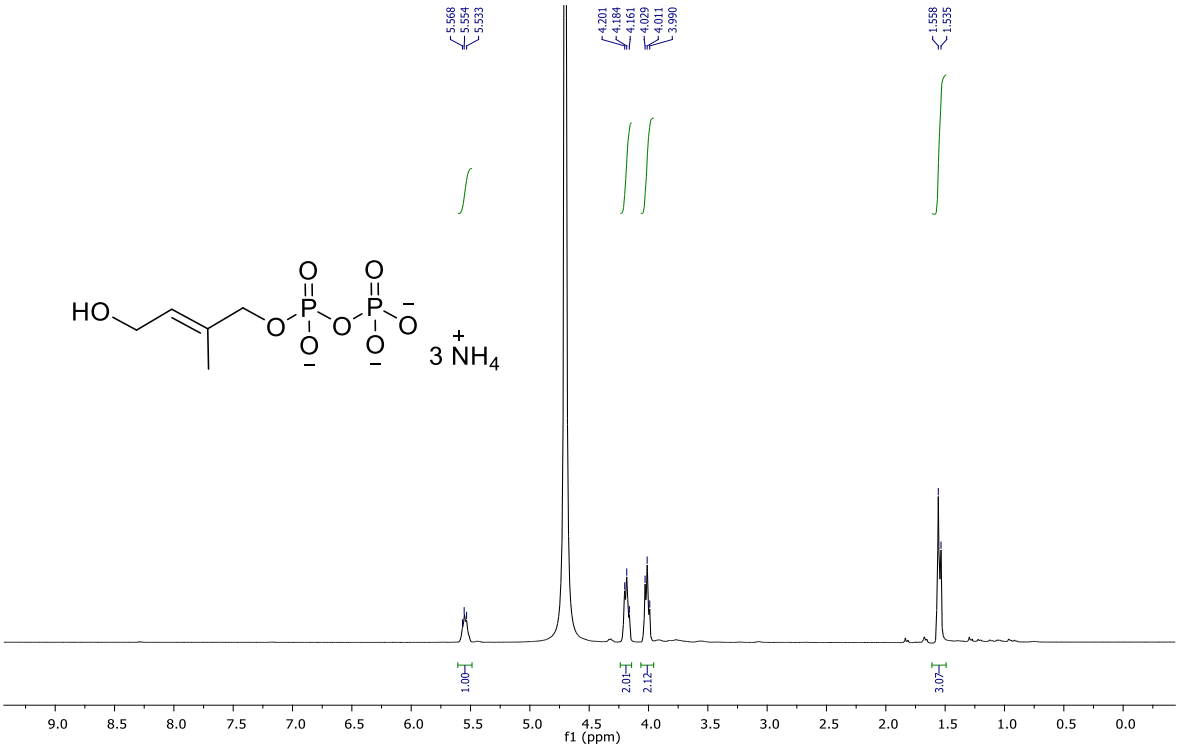


Mass spectrum of hydroxy-bearing allyl-modified cyclo-(L-Trp-Gly) **14d**.

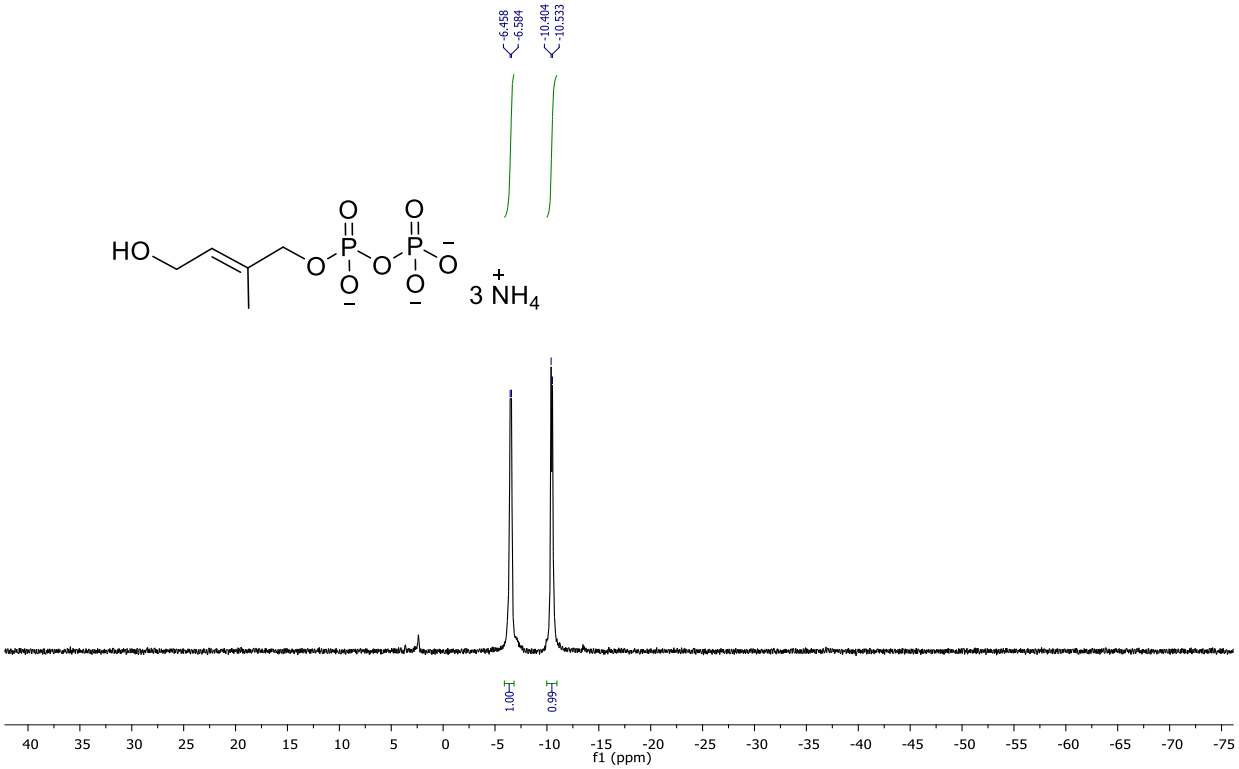


LC-LR-ESI-MS data of 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$ daptomycin **12**.

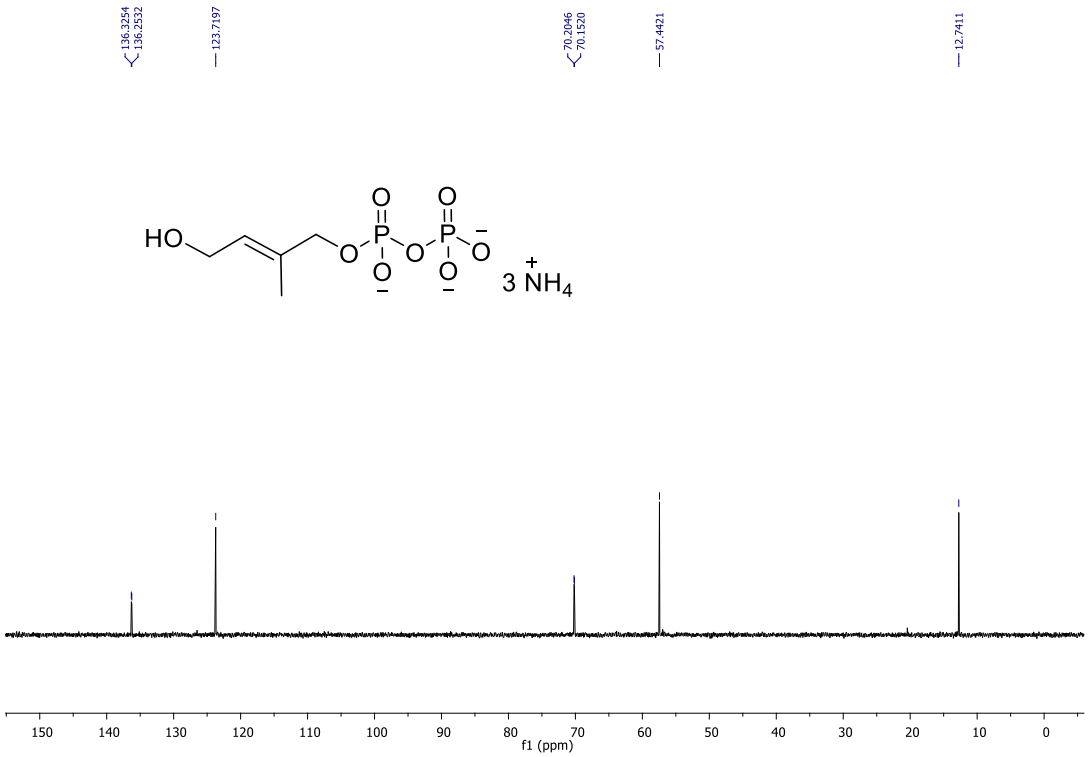
VI-Supplementary NMR Spectra



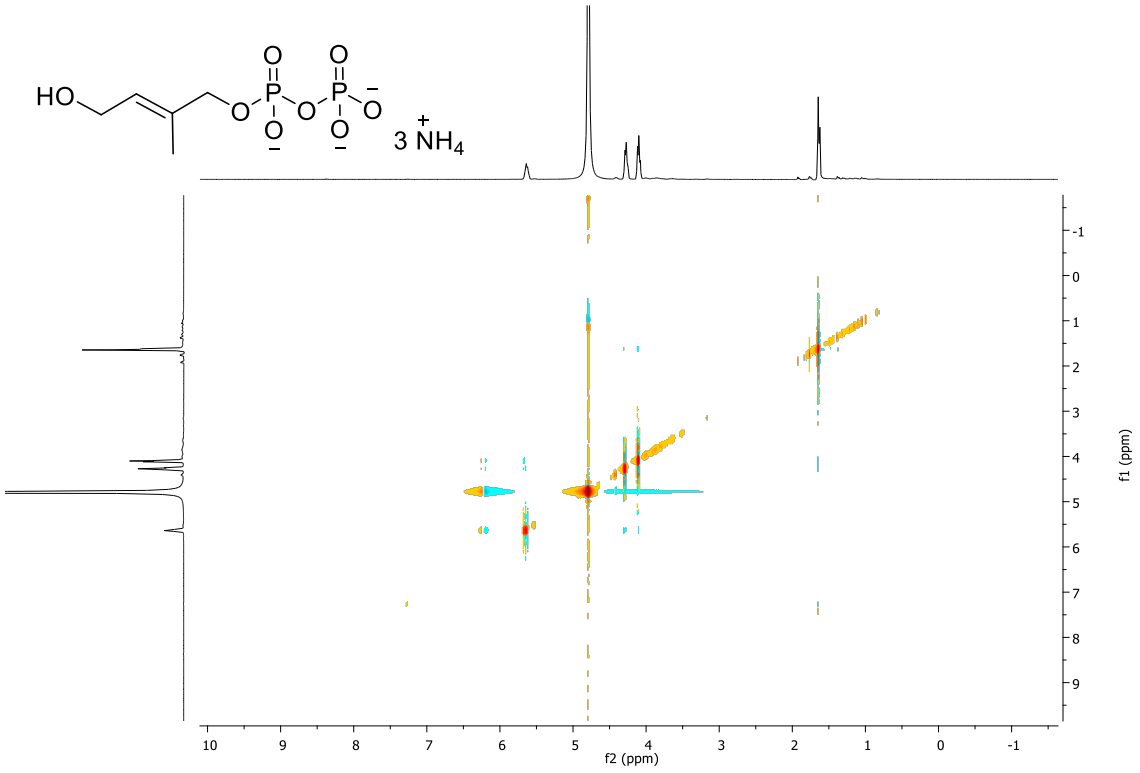
^{1}H NMR (D_2O, 400 MHz) of (*E*)-4-hydroxy-2-methylbut-2-en-1-yl diphosphate **4a**.



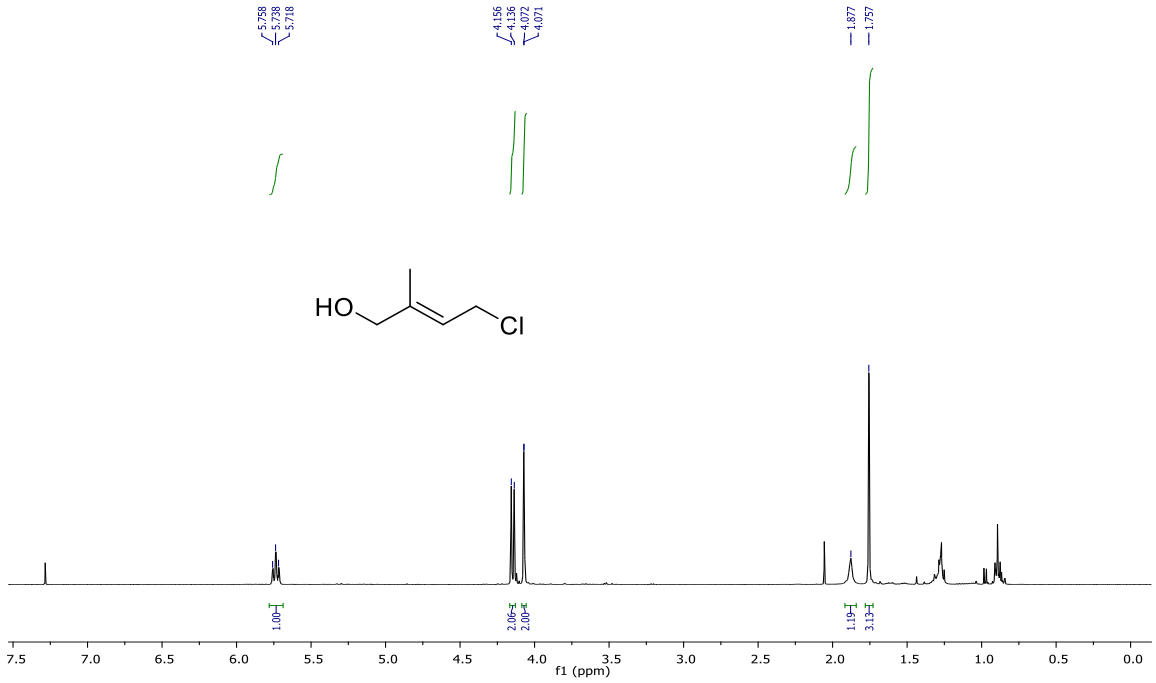
^{31}P NMR (D_2O, 164 MHz) of (*E*)-4-hydroxy-2-methylbut-2-en-1-yl diphosphate **4a**.



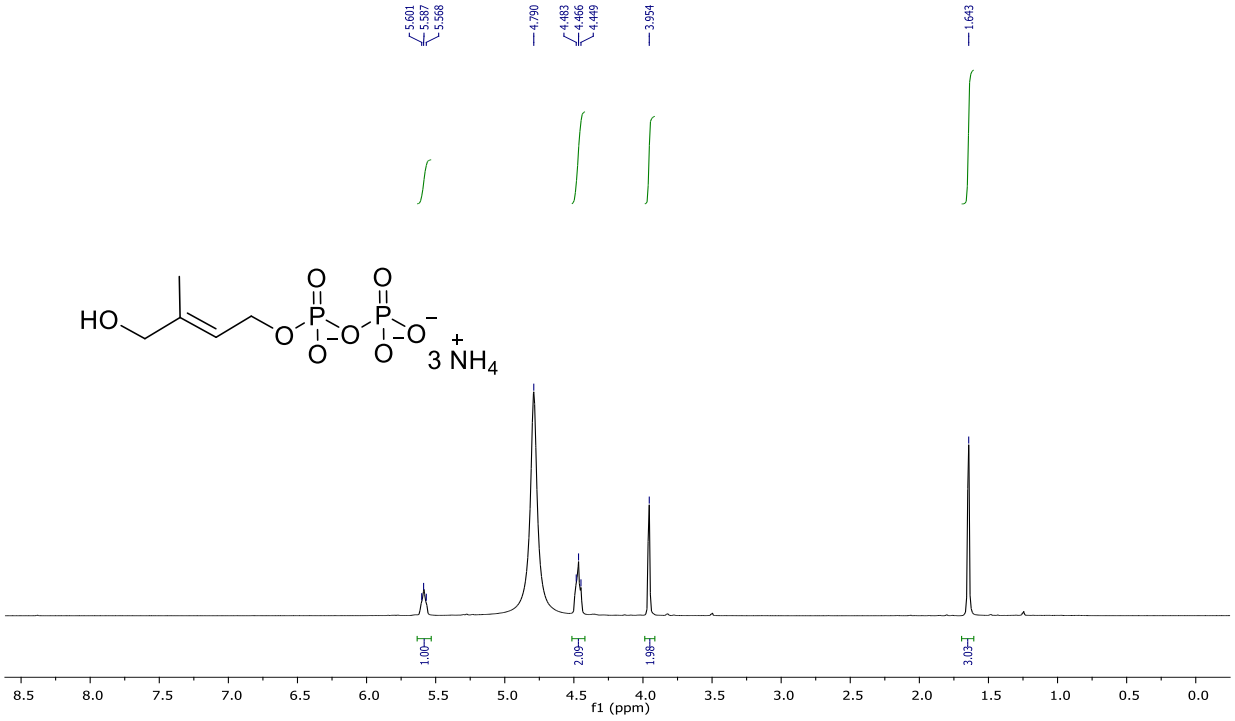
^{13}C NMR (D_2O, 100 MHz) of (*E*)-4-hydroxy-2-methylbut-2-en-1-yl diphosphate **4a**.



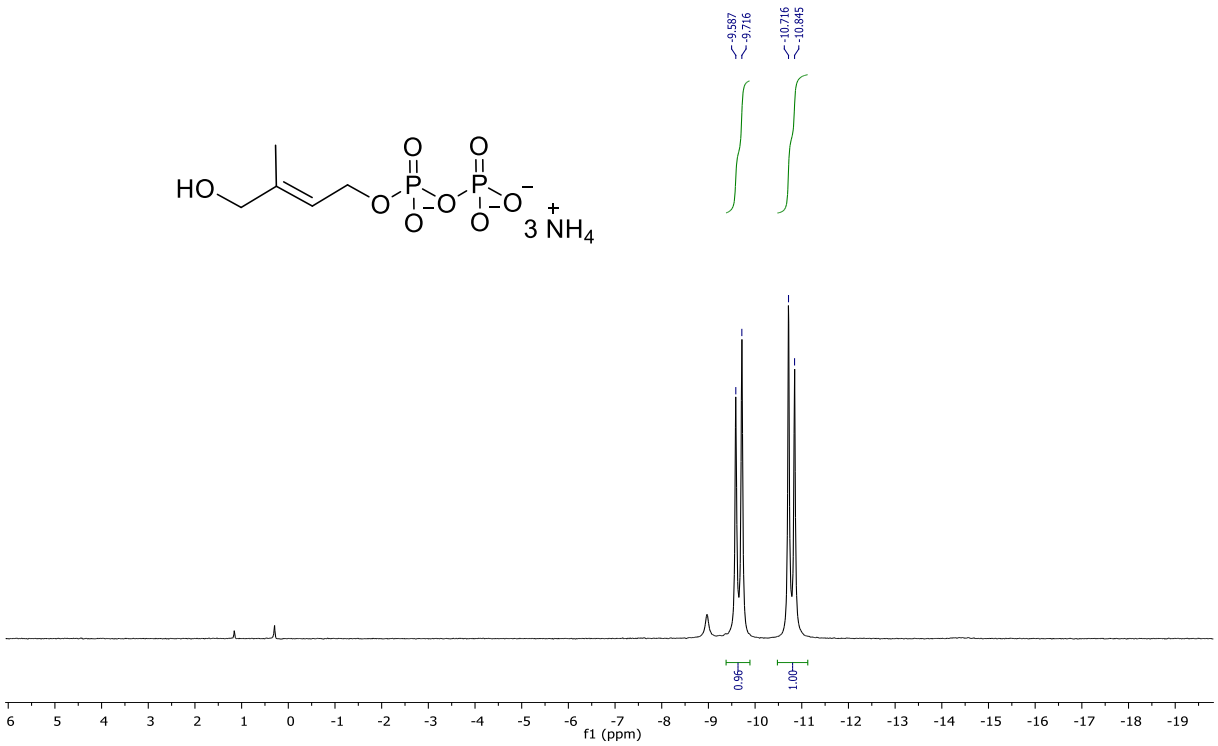
NOESY (D_2O, 400 MHz) of (*E*)-4-hydroxy-2-methylbut-2-en-1-yl diphosphate **4a**.



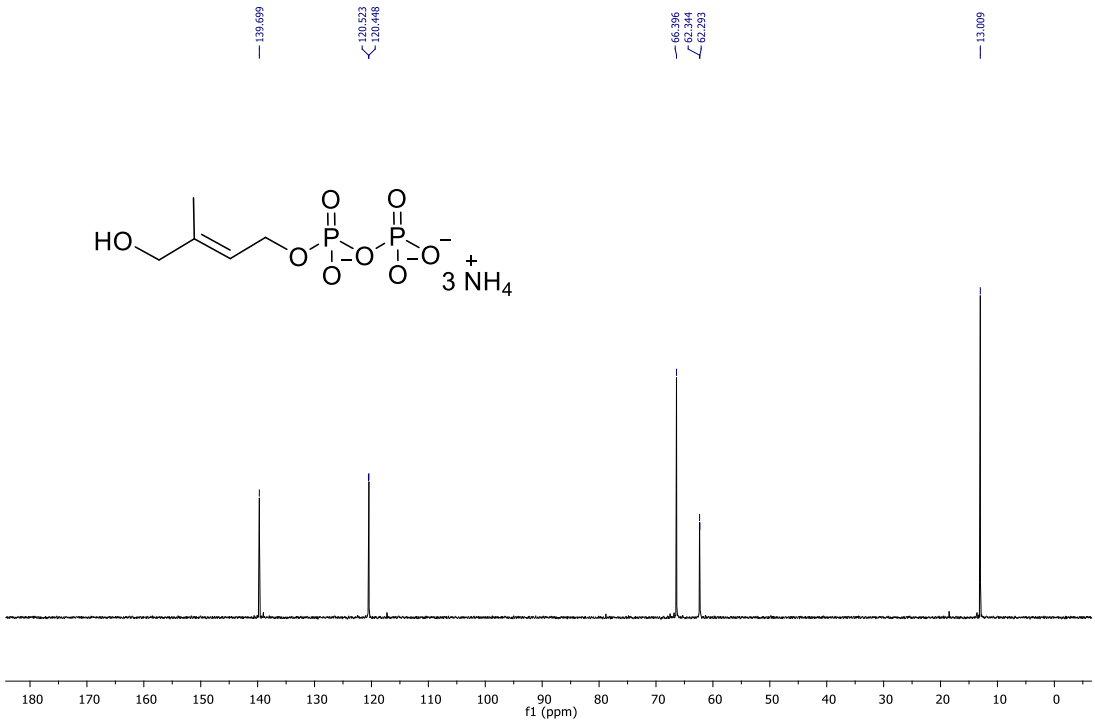
^{1}H NMR ($CDCl_3$, 400 MHz) of (*E*)-4-chloro-2-methylbut-2-en-1-ol **3b**.



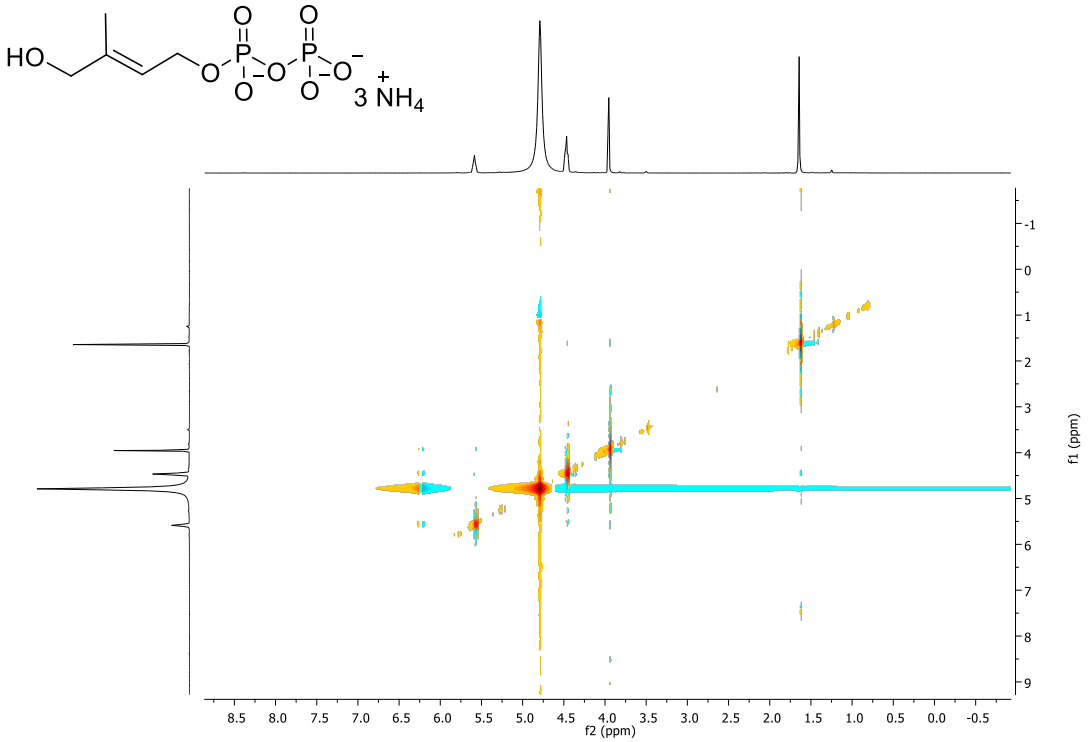
^{1}H NMR (D_2O, 400 MHz) of (*E*)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate **4b**.



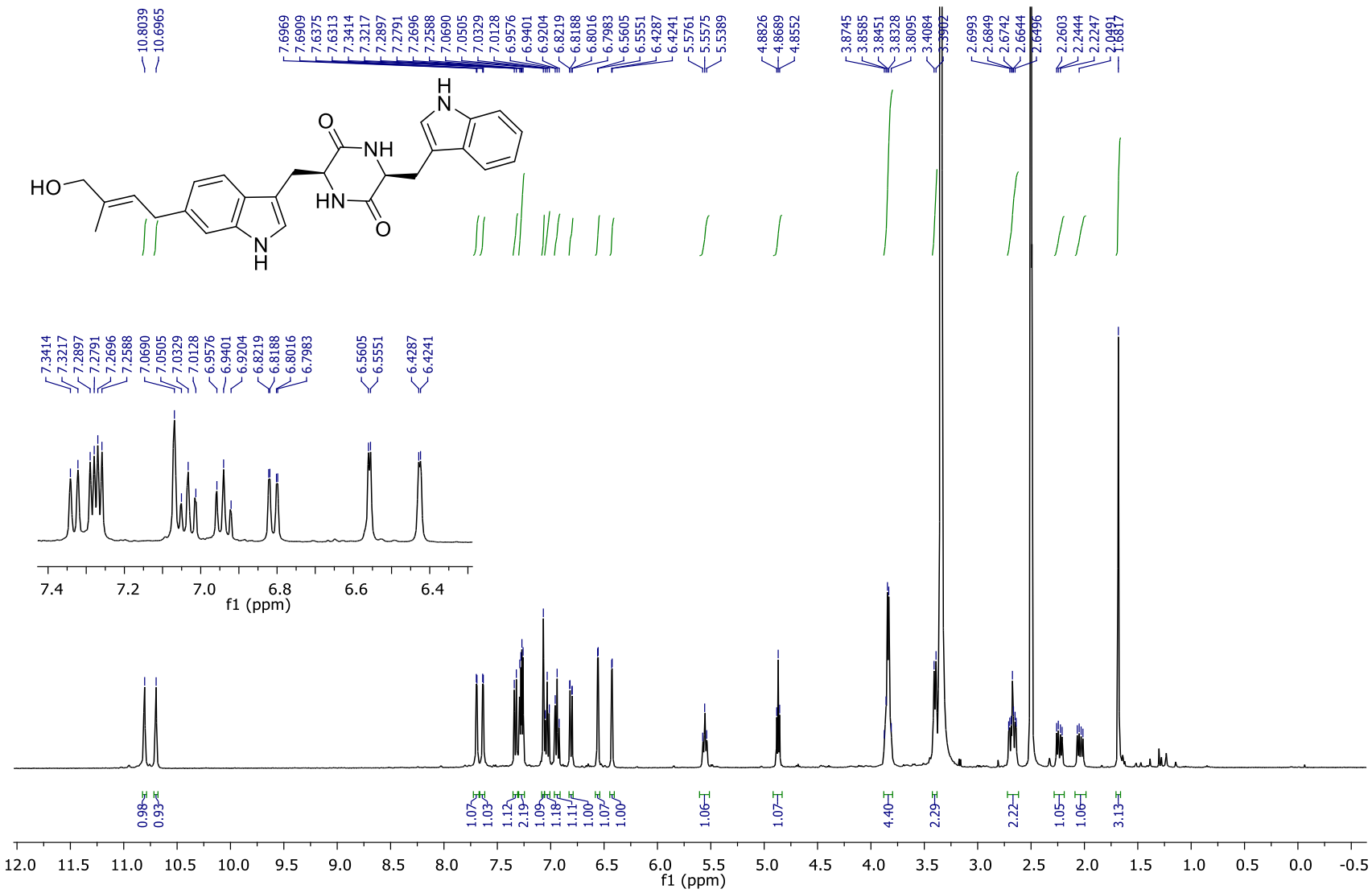
^{31}P NMR (D_2O, 164 MHz) of (*E*)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate **4b**.



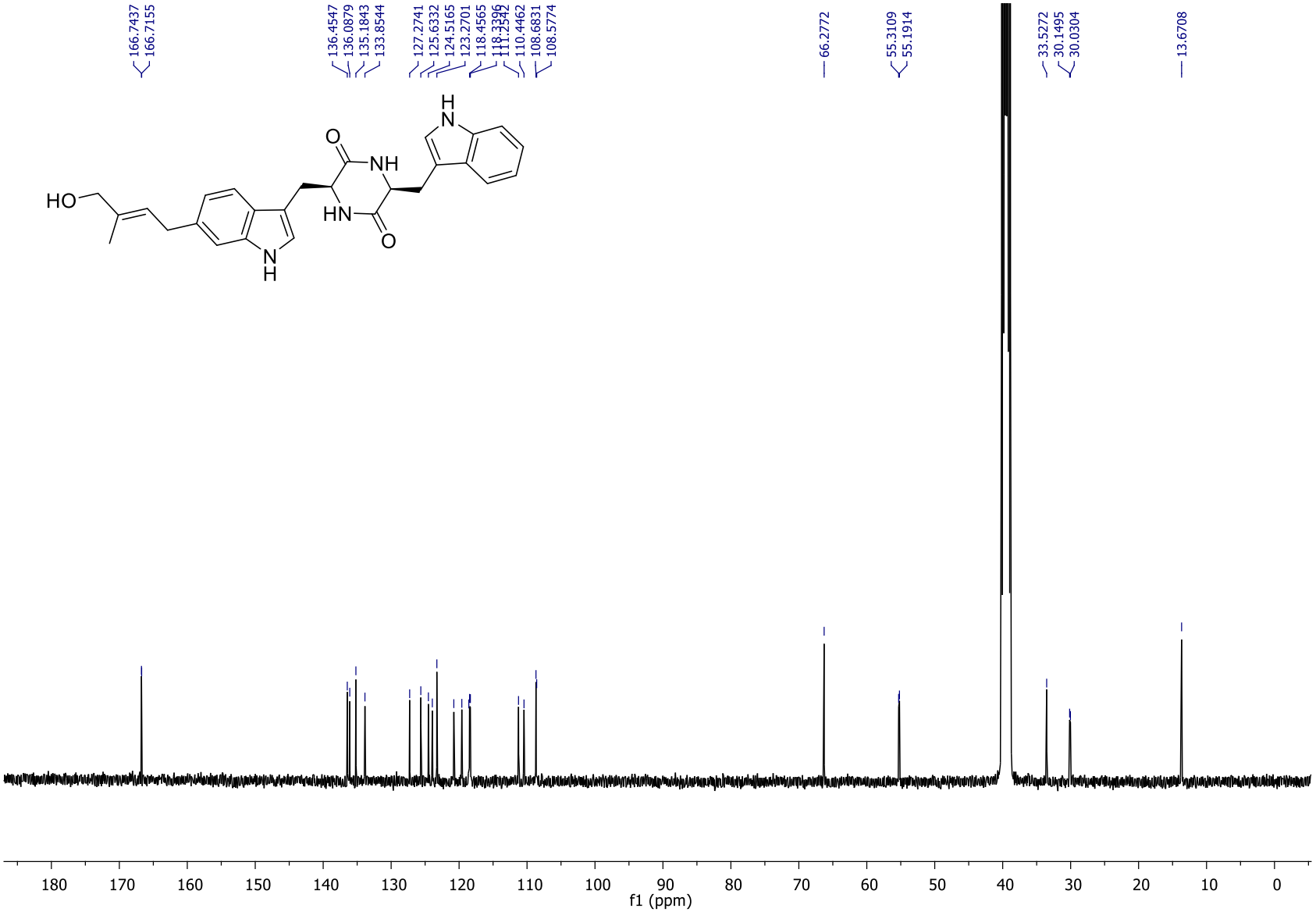
^{13}C NMR (D_2O, 100 MHz) of (*E*)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate **4b**.



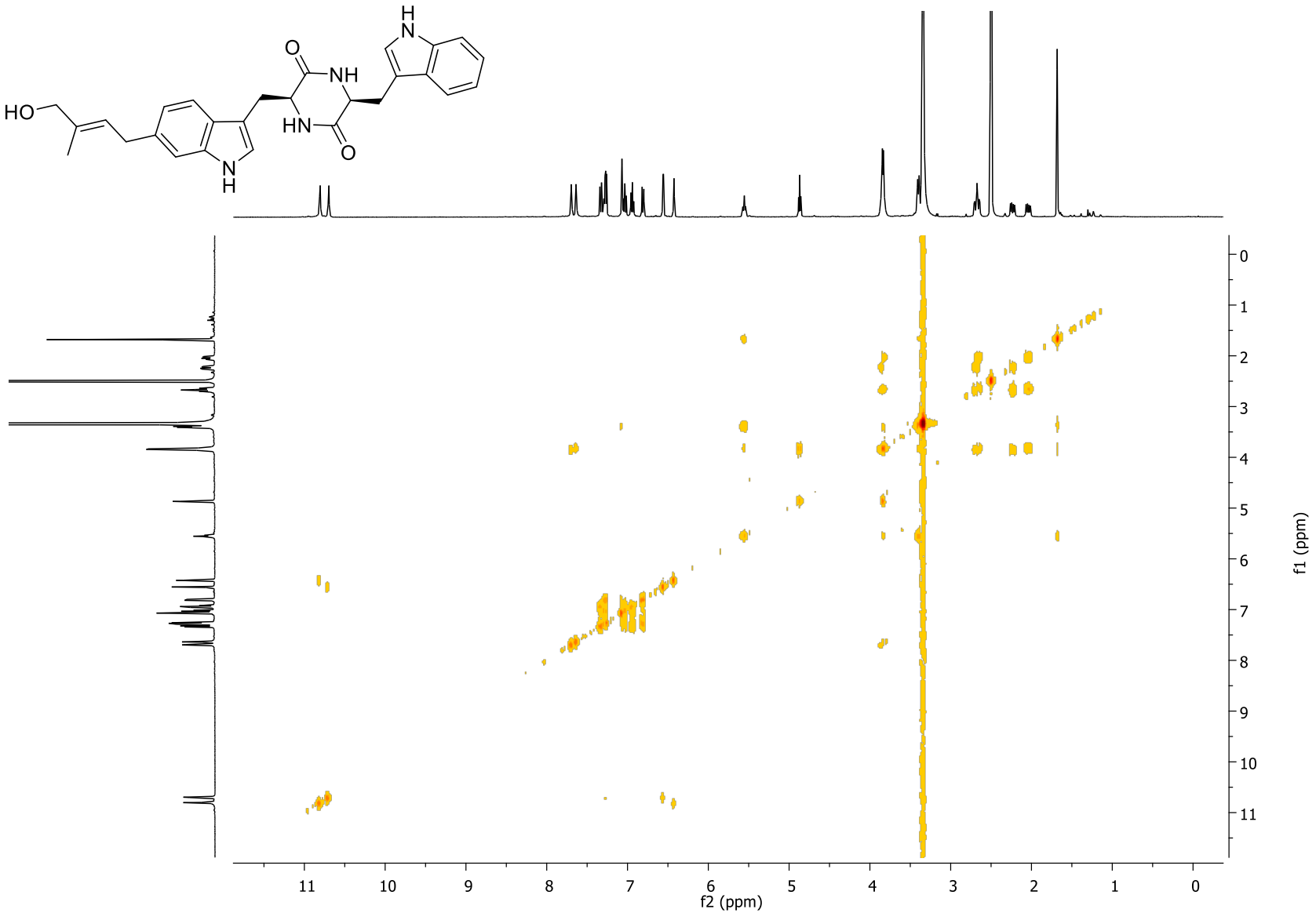
NOESY (D_2O, 400 MHz) of (*E*)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate **4b**.



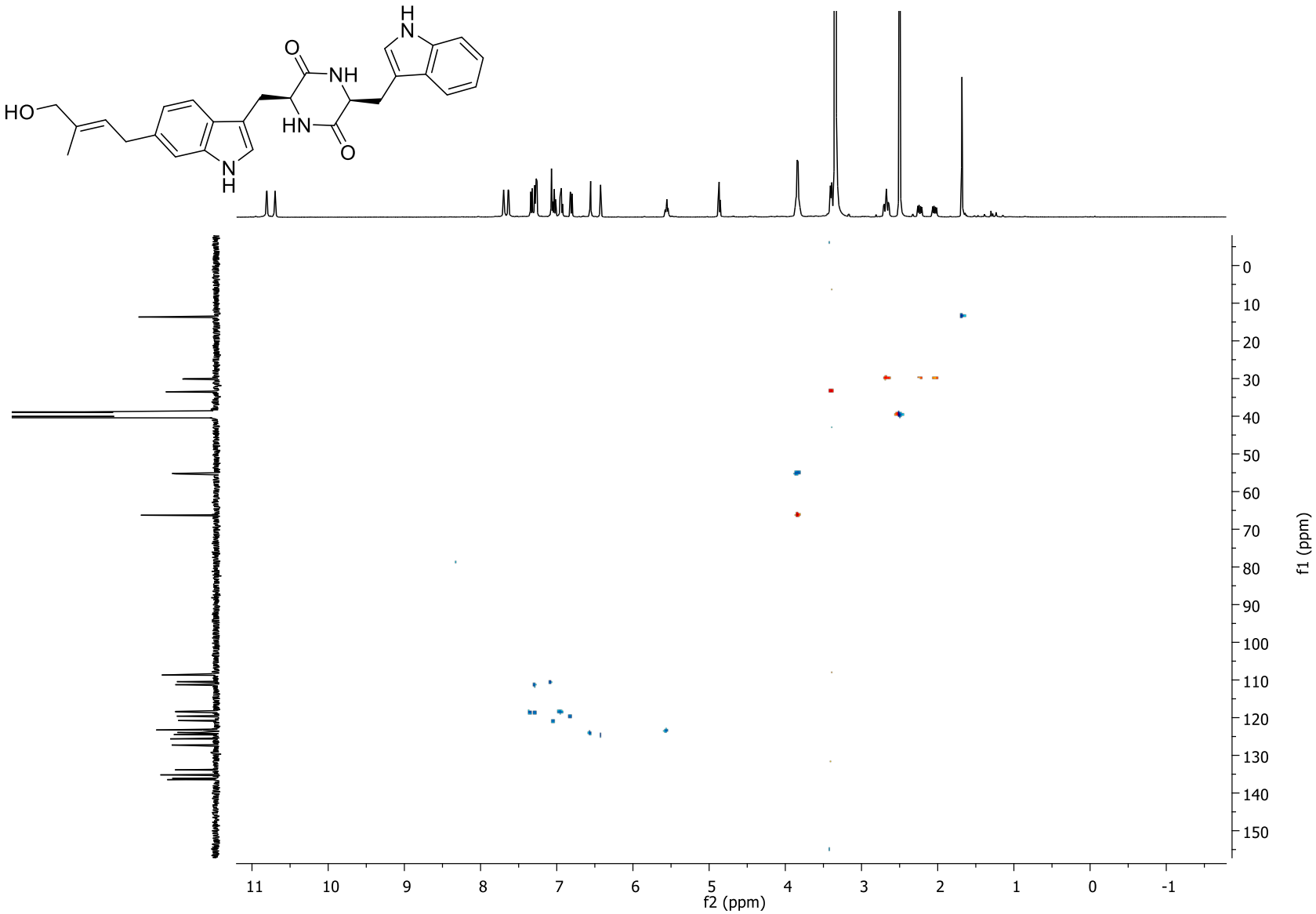
^{1}H NMR spectrum (d_6–DMSO, 400 MHz) of cyclo-(6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11a**.



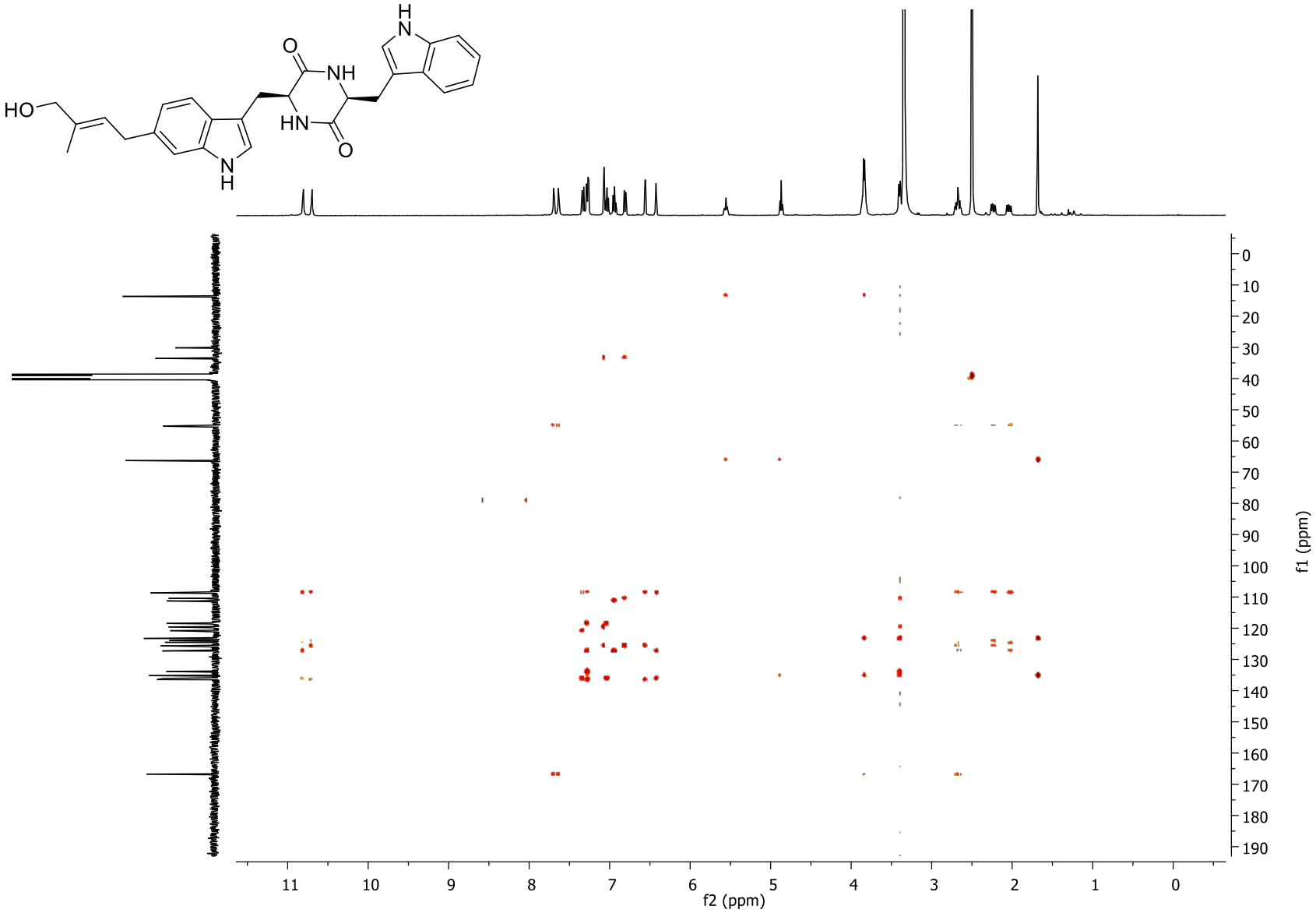
^{13}C NMR spectrum (d_6–DMSO, 100 MHz) of cyclo-(6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11a**.



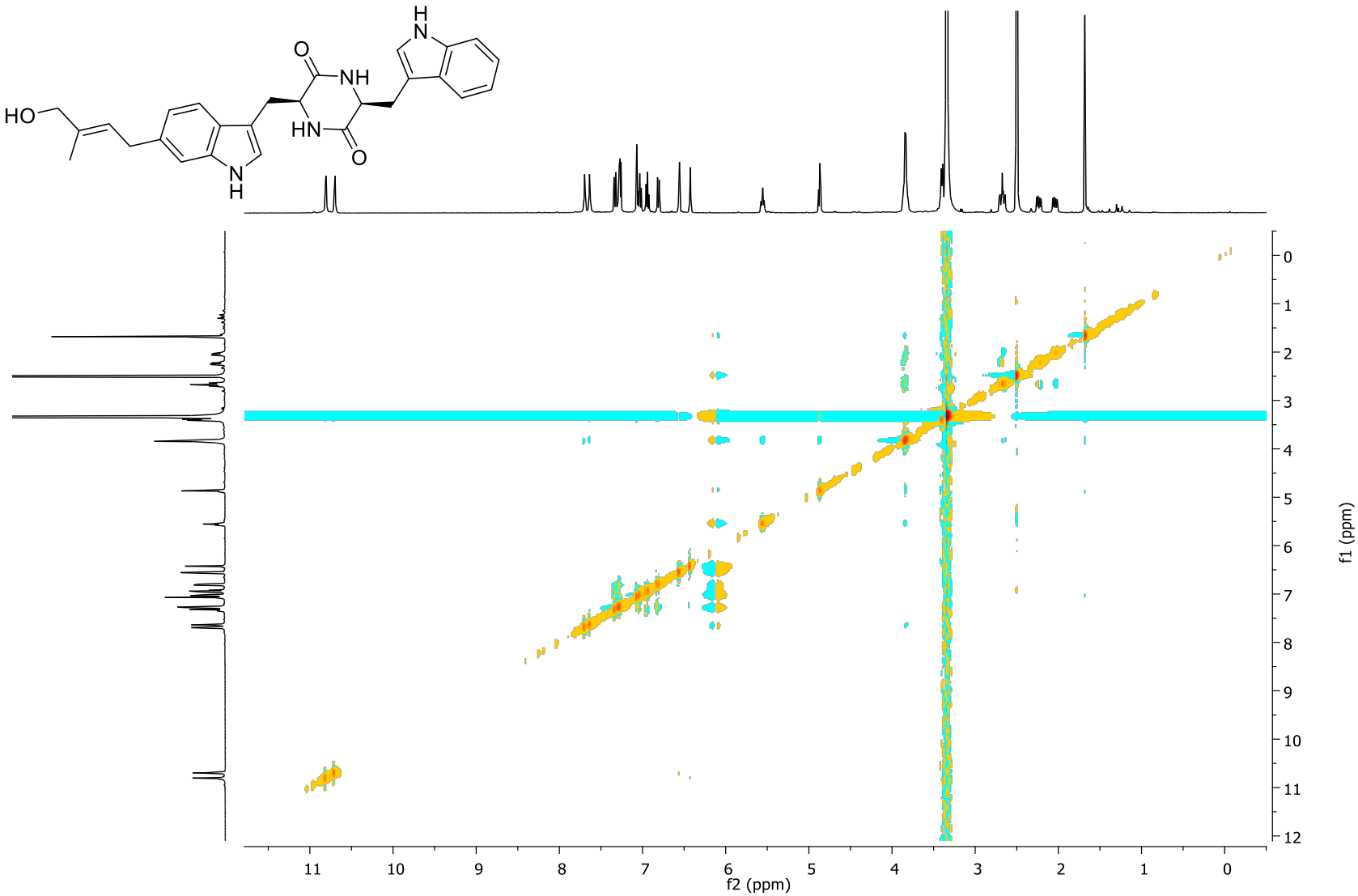
^{1}H-^{1}H COSY NMR spectrum (d_6–DMSO, 400 MHz) of cyclo-(6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11a**.



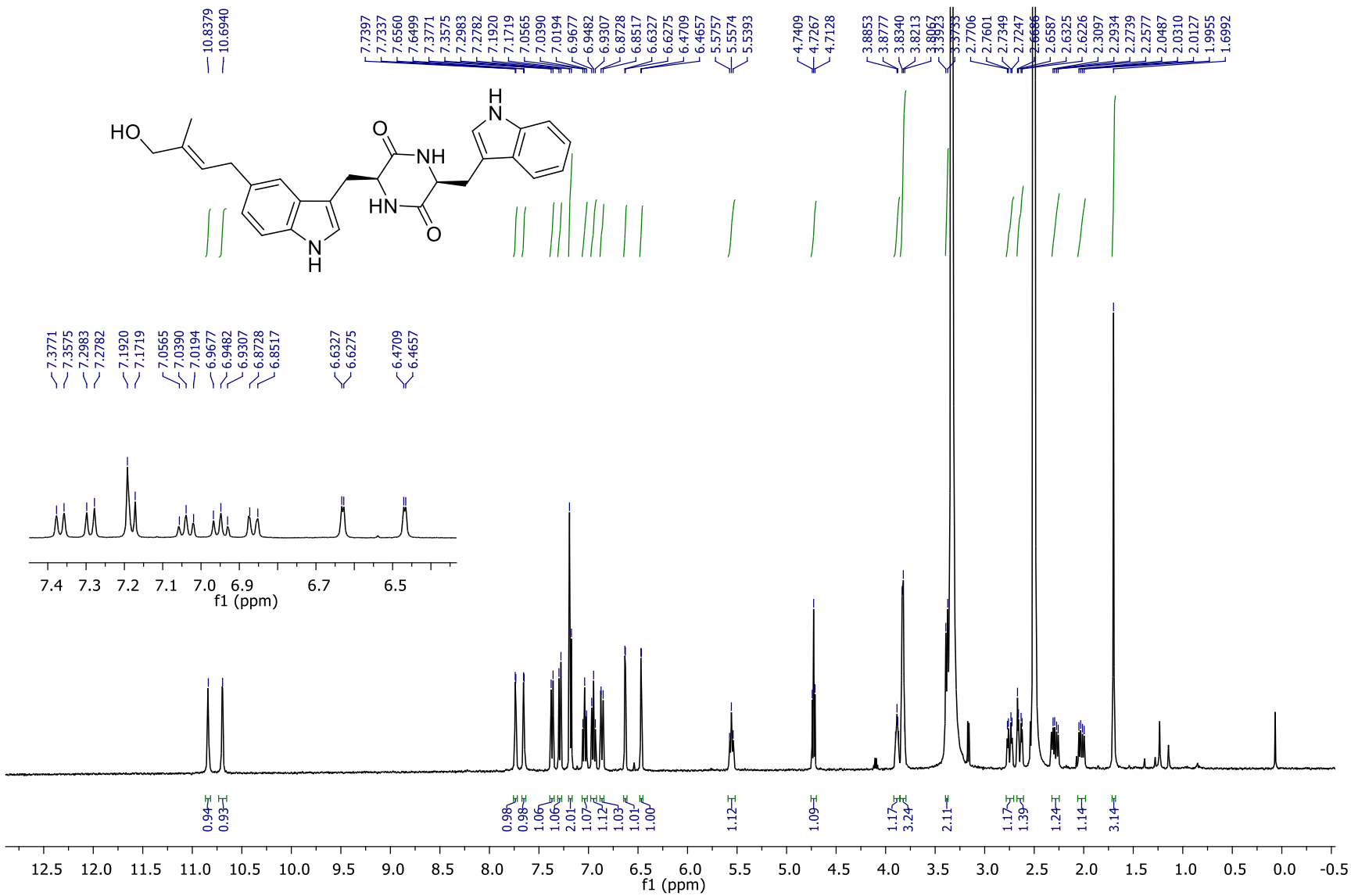
^{1}H -^{13}C HSQC spectrum (*d*$_6$–DMSO, 400 MHz) of cyclo-(6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11a**.



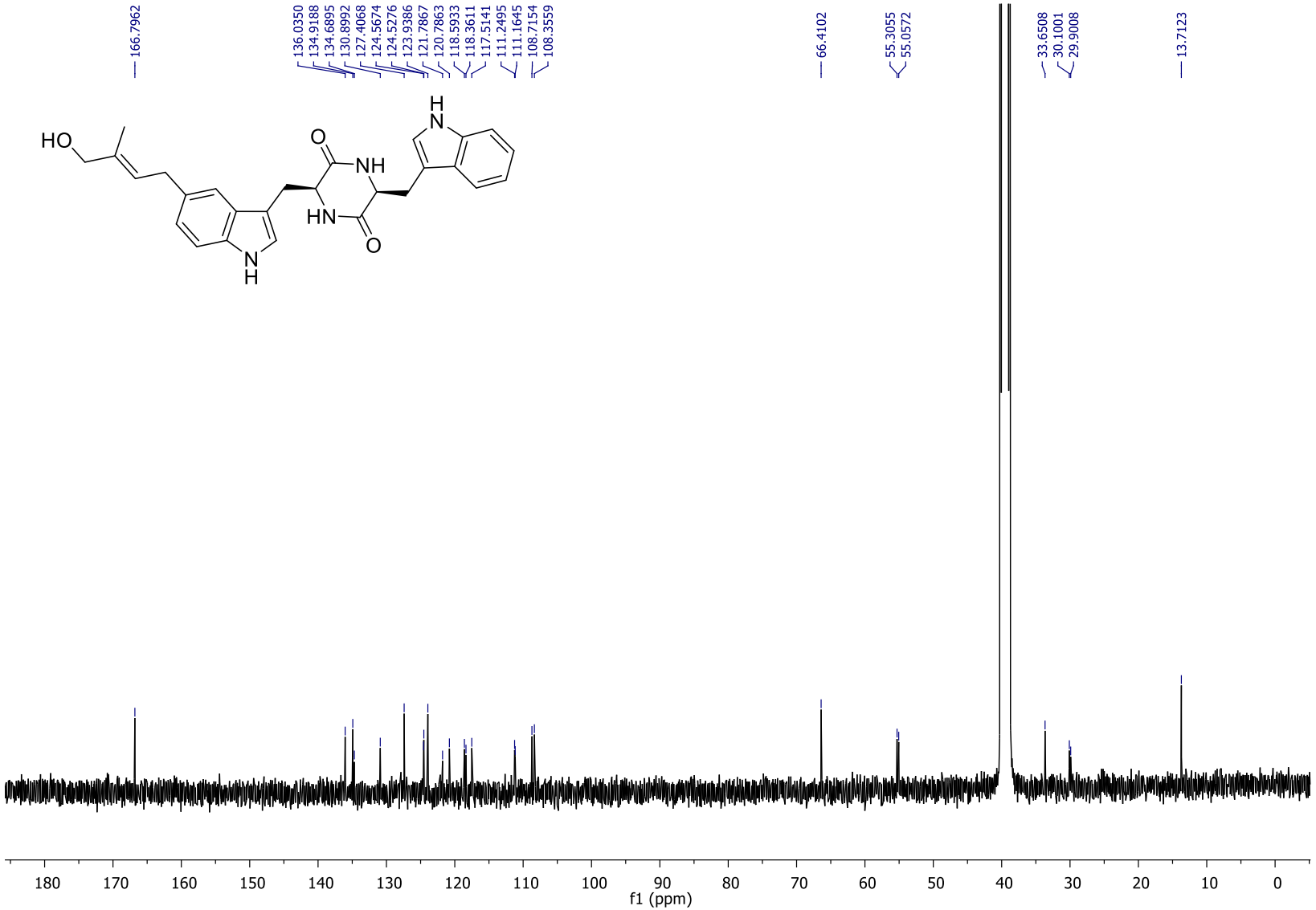
^{1}H-^{13}C HMBC NMR spectrum (d_6–DMSO, 400 MHz) of cyclo-(6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11a**.



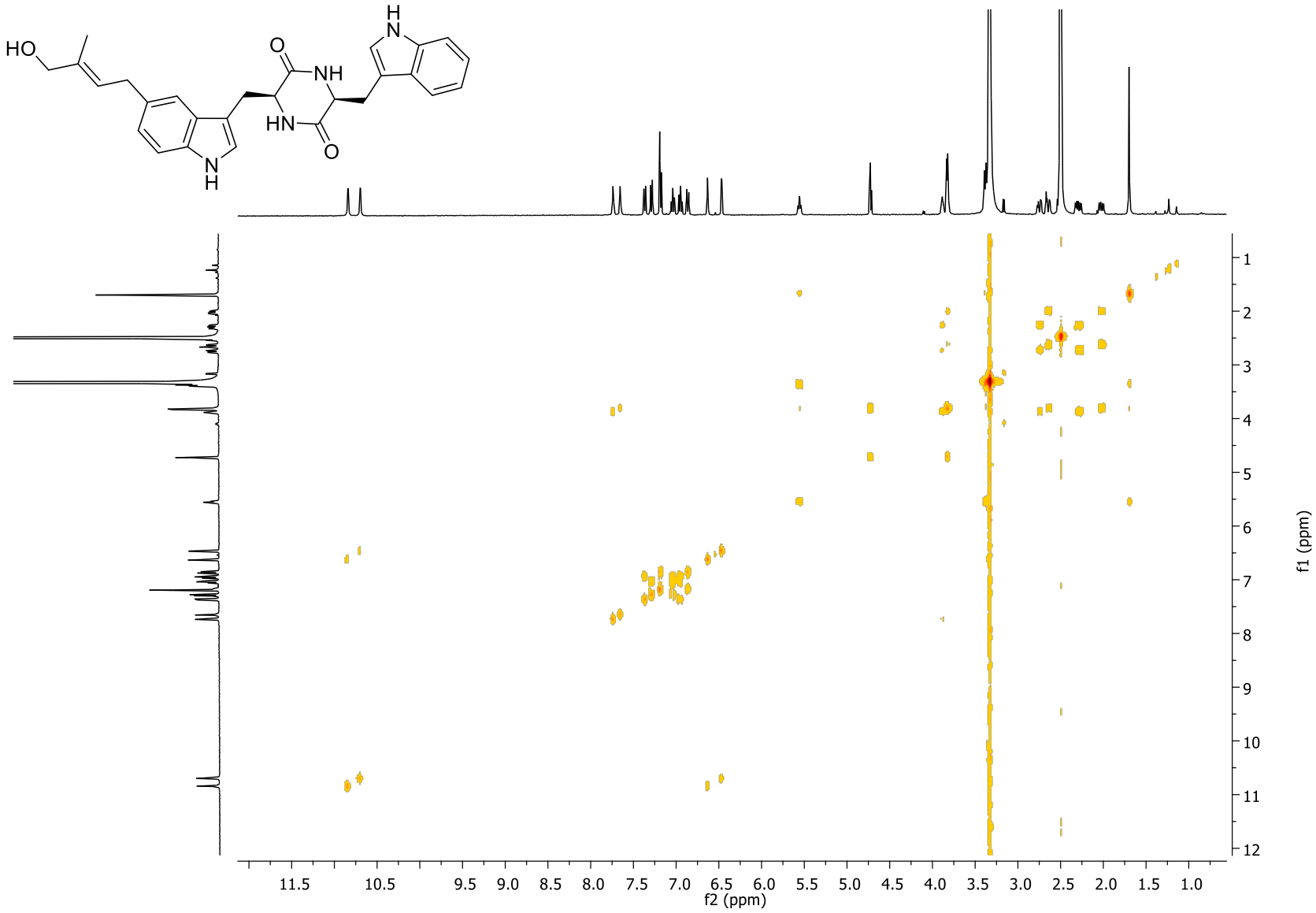
^{1}H-^{1}H NOESY NMR spectrum (*d*$_{6}$–DMSO, 400 MHz) of cyclo-(6-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_{1}$-L-Trp$_{2}$) **11a**.



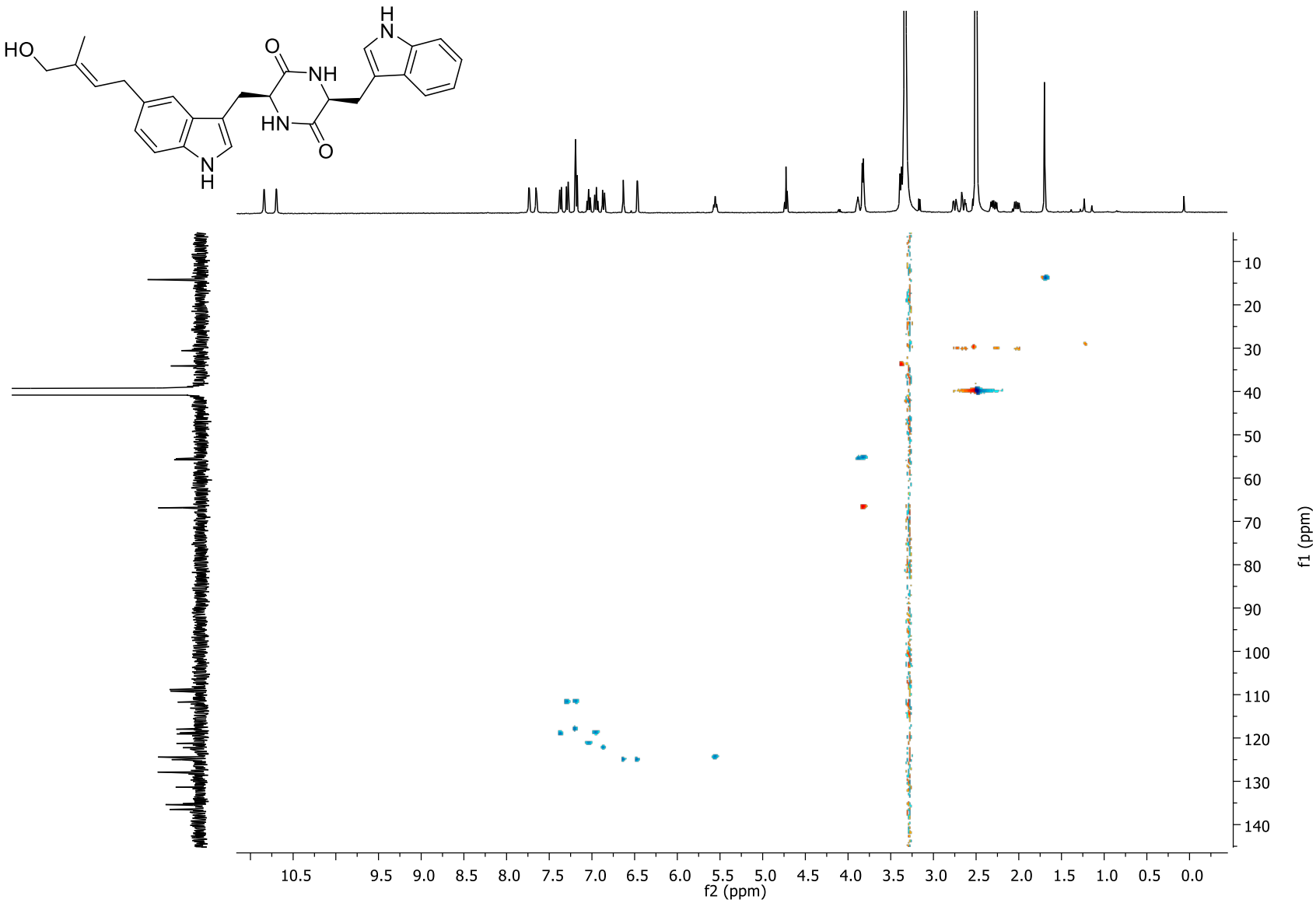
[1]H NMR spectrum (d_6–DMSO, 400 MHz) of cyclo-(5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11b**.



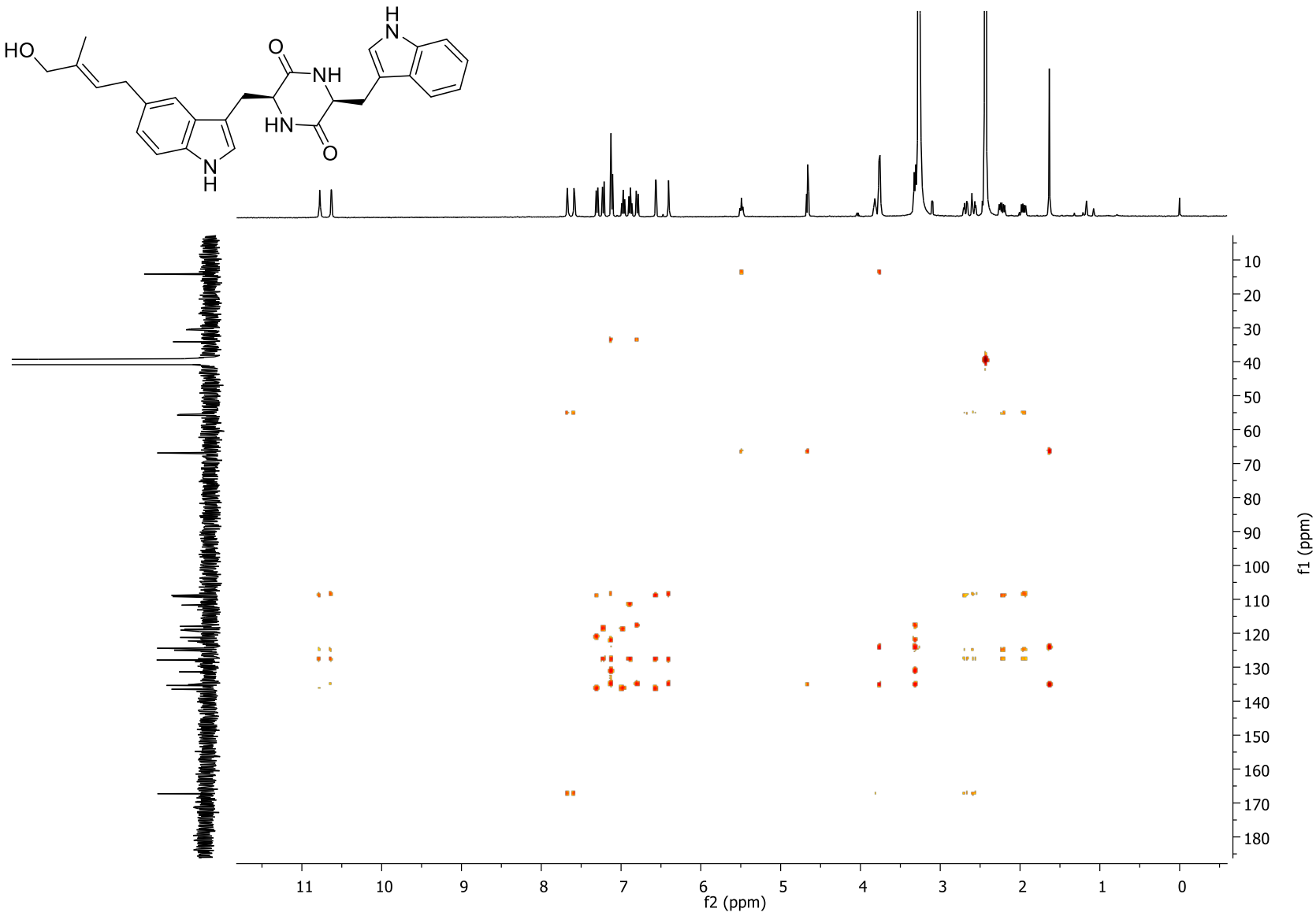
^{13}C NMR spectrum (*d*$_6$–DMSO, 100 MHz) of cyclo-(5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11b**.



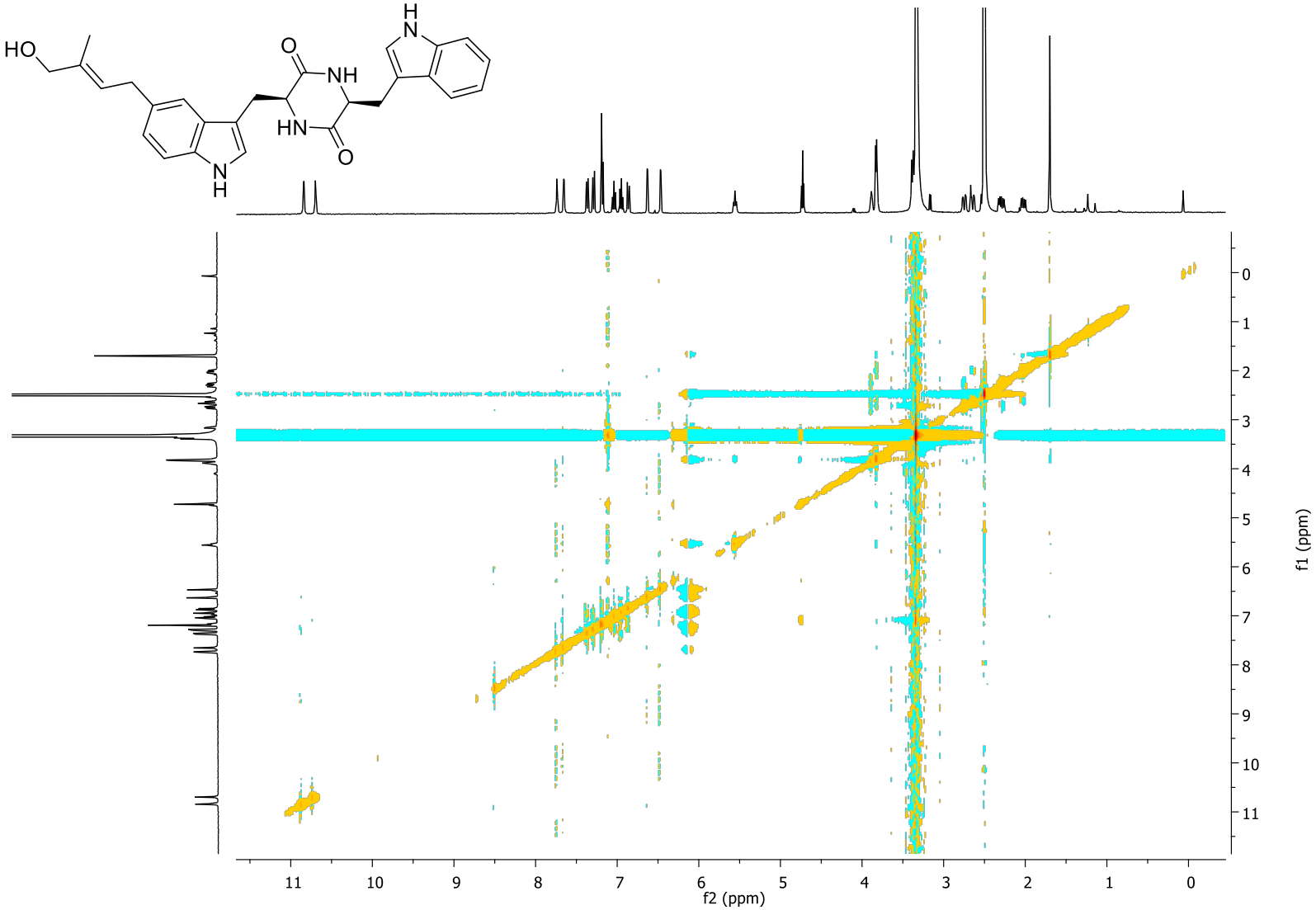
^{1}H-^{1}H COSY NMR spectrum (*d*$_{6}$–DMSO, 400 MHz) of cyclo-(5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_{1}$-L-Trp$_{2}$) **11b**.



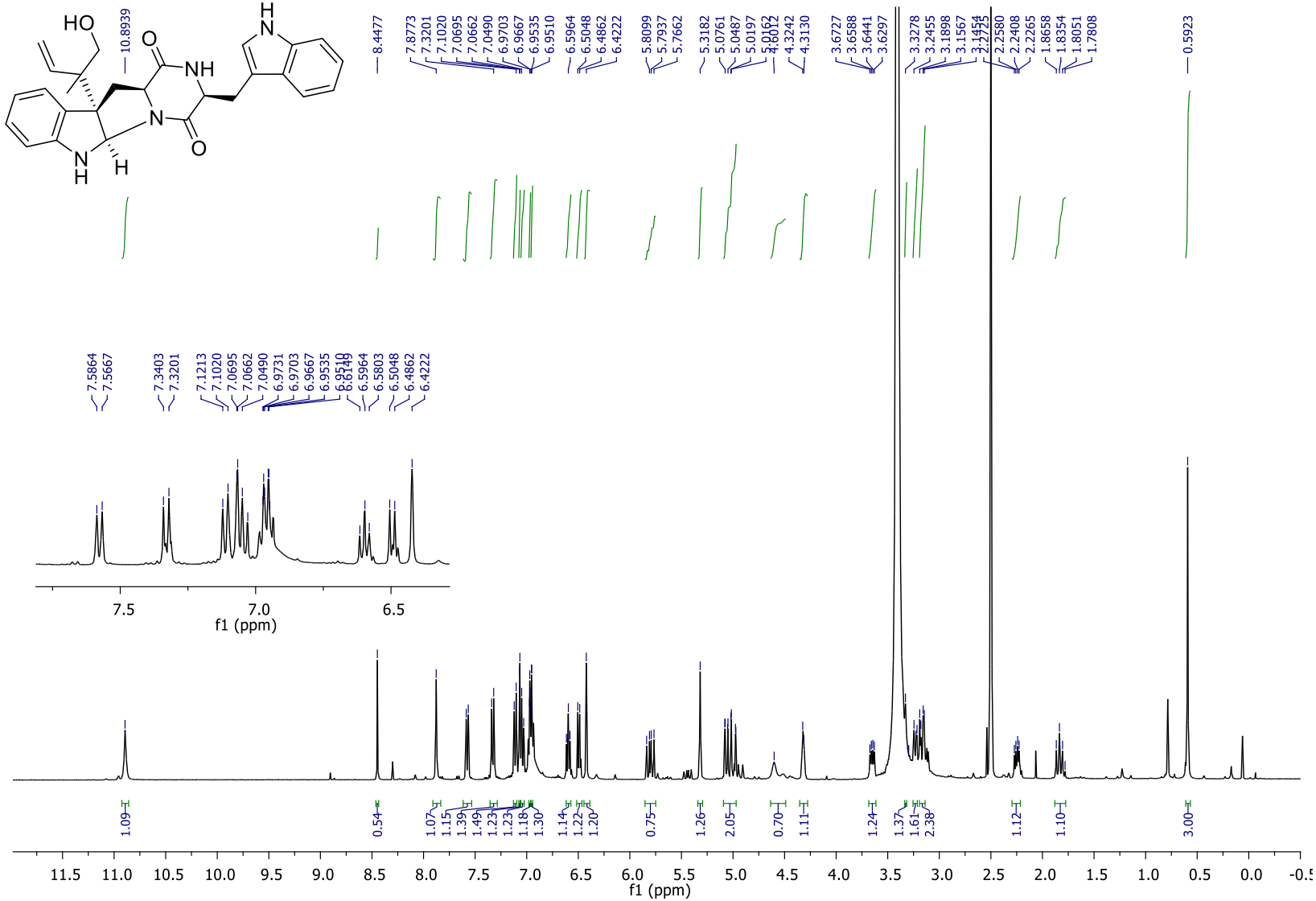
^{1}H -^{13}C HSQC spectrum (*d*$_{6}$–DMSO, 400 MHz) of cyclo-(5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_{1}$-L-Trp$_{2}$) **11b**.



^{1}H-^{13}C HMBC NMR spectrum (d_6–DMSO, 400 MHz) of cyclo-(5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$-L-Trp$_2$) **11b**.

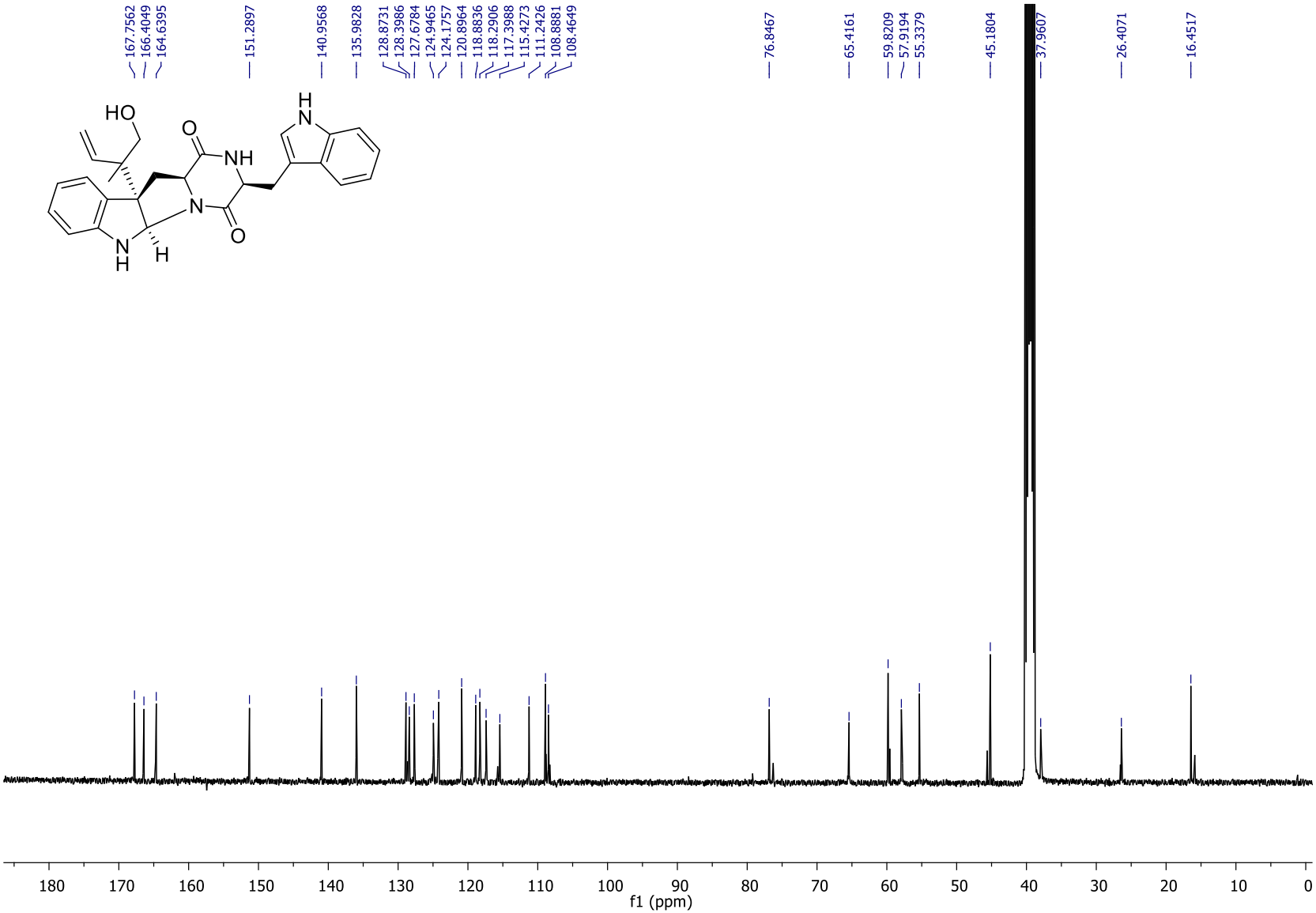


^{1}H-^{1}H NOESY NMR spectrum (*d*$_{6}$–DMSO, 400 MHz) of cyclo-(5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_{1}$-L-Trp$_{2}$) **11b**.

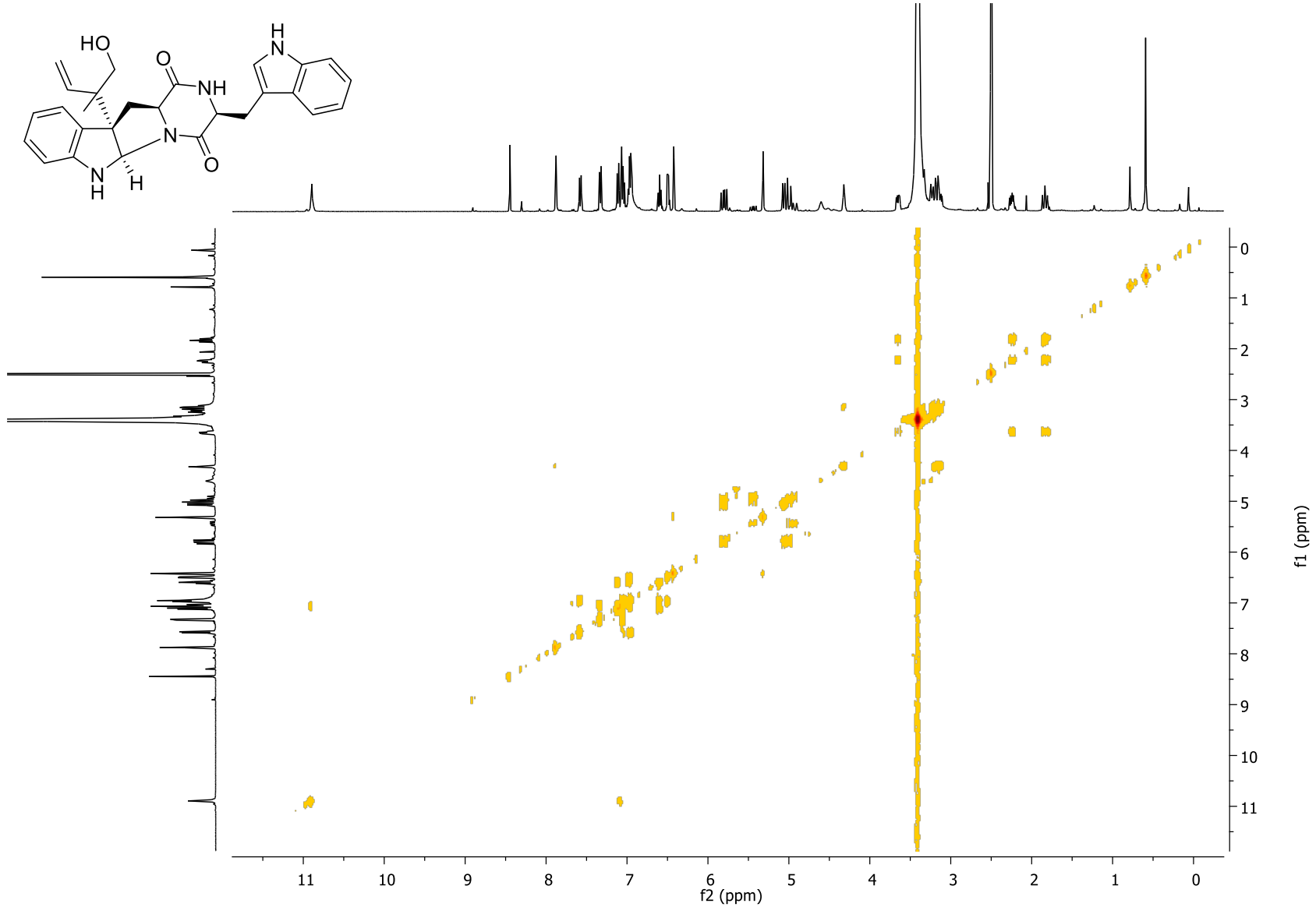


^{1}H NMR spectrum (d_6–DMSO, 400 MHz) of 3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11c**.

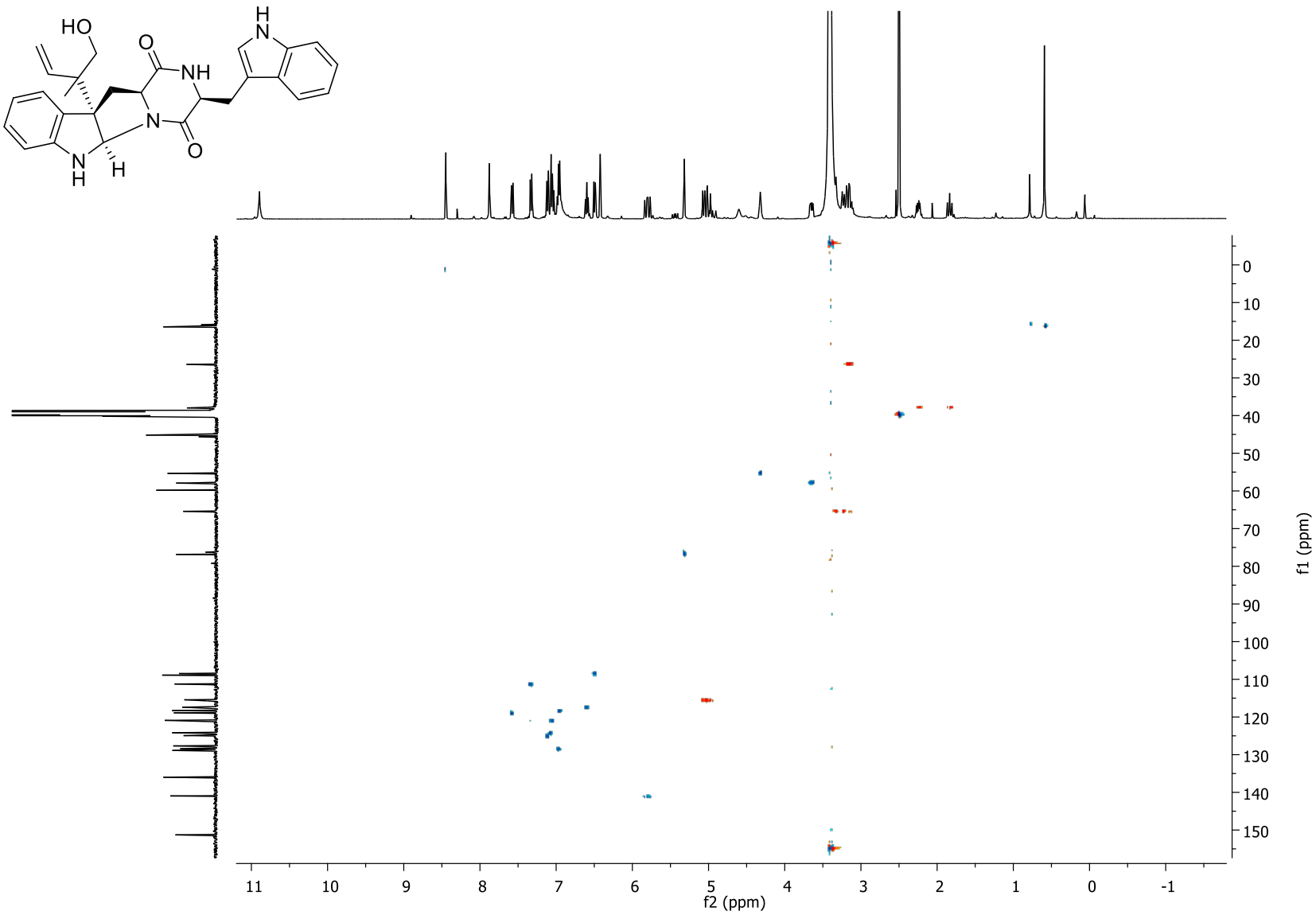
.



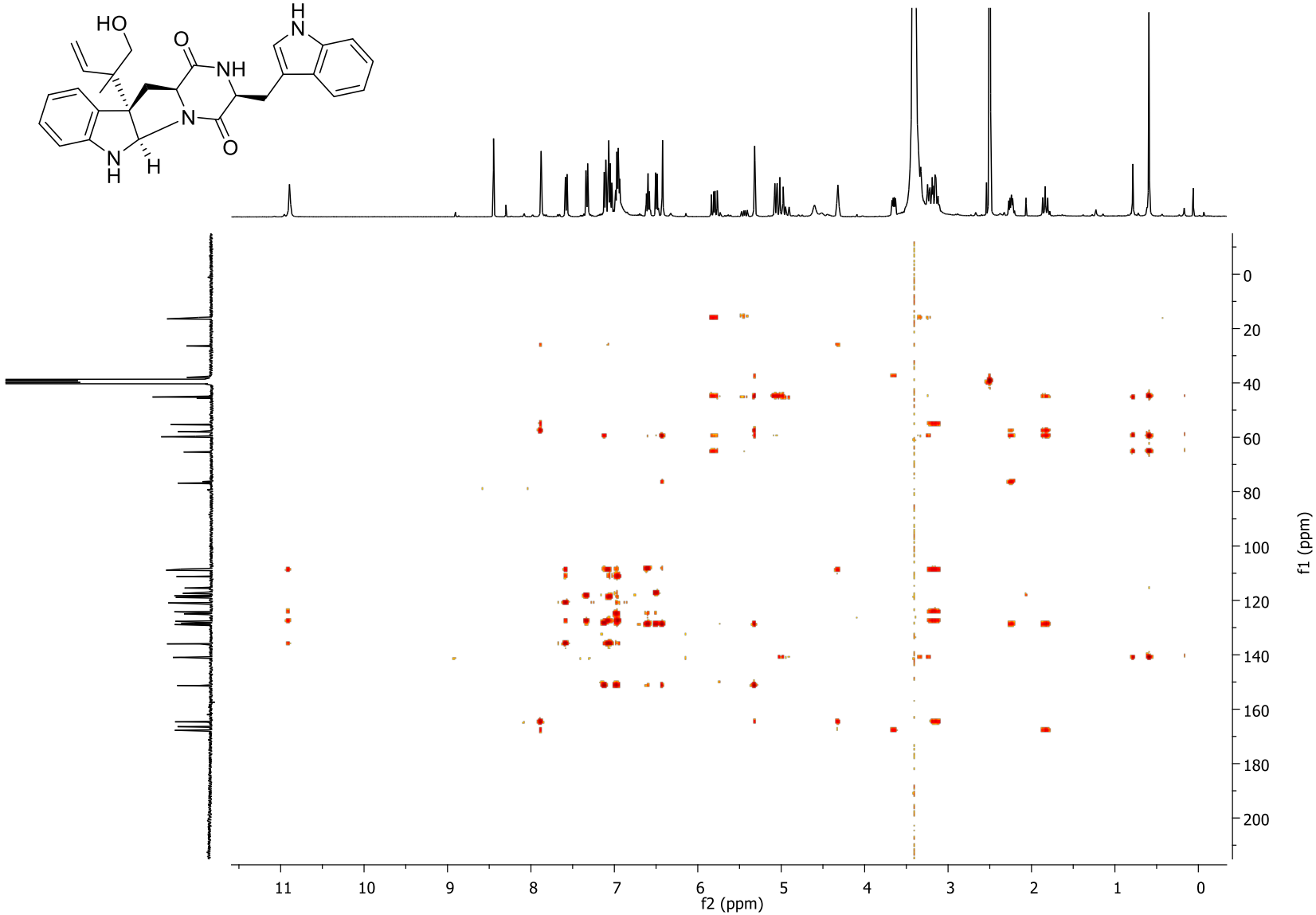
^{13}C NMR spectrum (d_6–DMSO, 100 MHz) of 3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11c**.



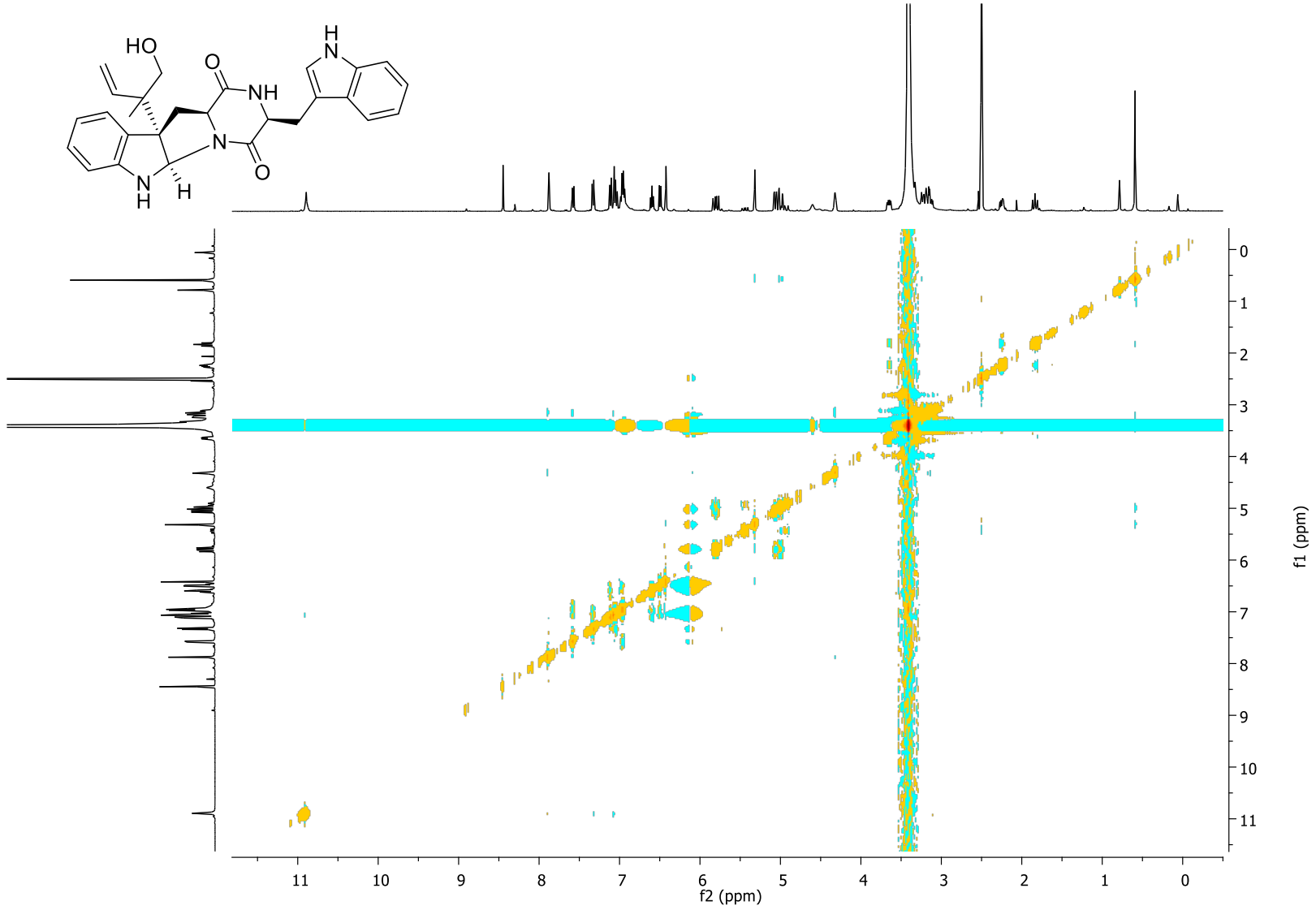
^{1}H-^{1}H COSY NMR spectrum (d_6–DMSO, 400 MHz) of 3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11c**.



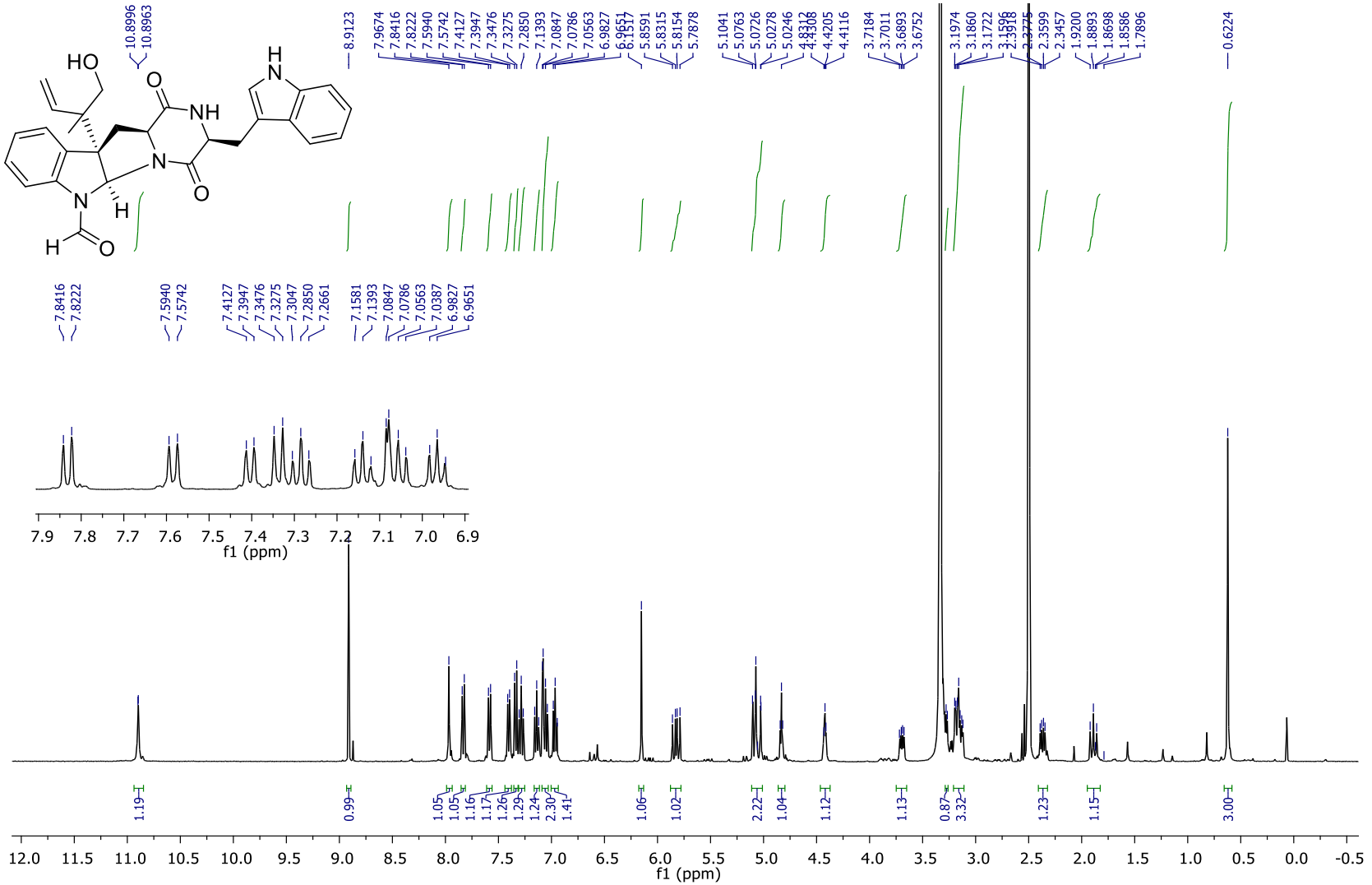
^{1}H -^{13}C HSQC spectrum (*d*$_6$–DMSO, 400 MHz) of 3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11c**.



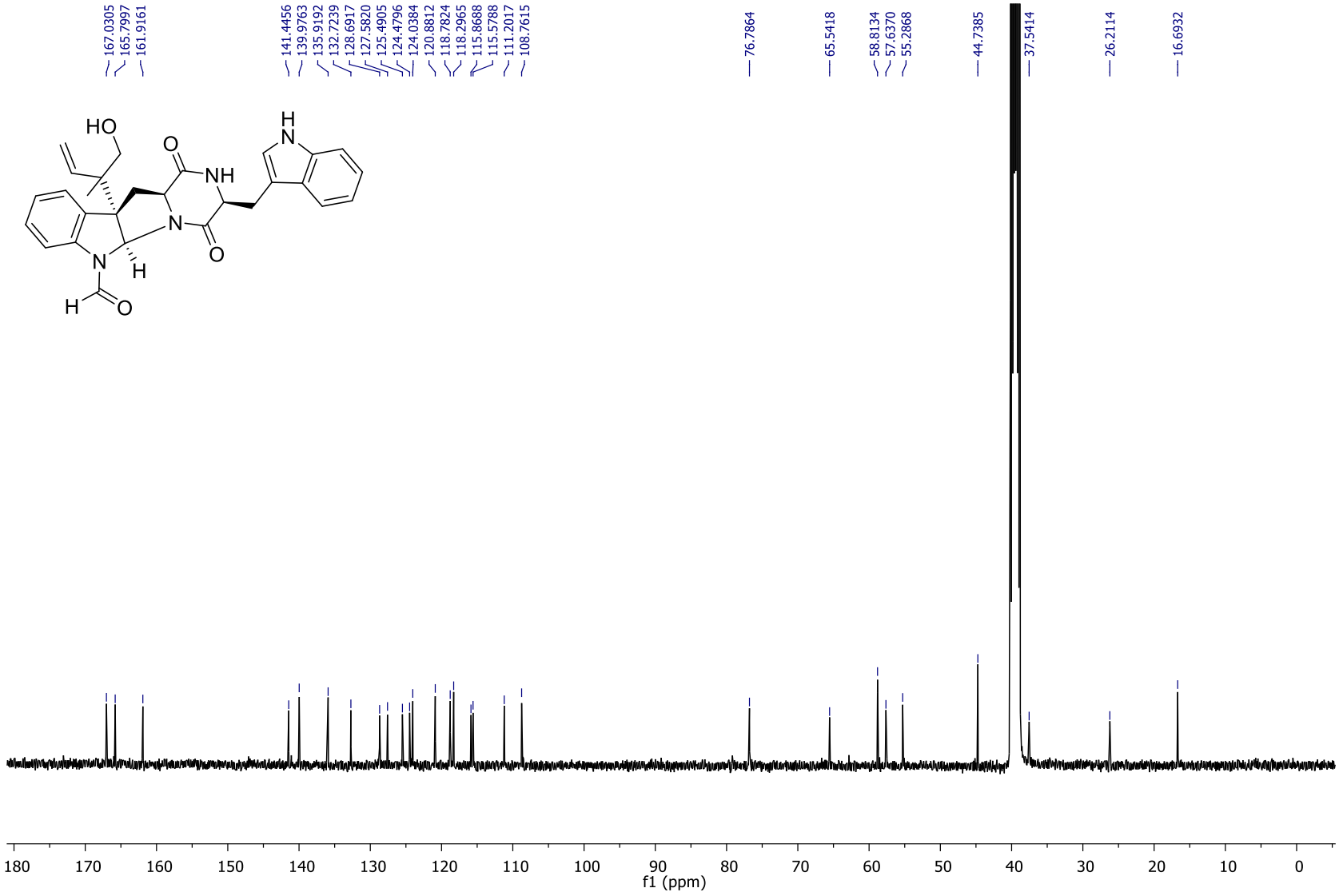
^{1}H-^{13}C HMBC NMR spectrum (*d*$_6$–DMSO, 400 MHz) of 3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11c**.



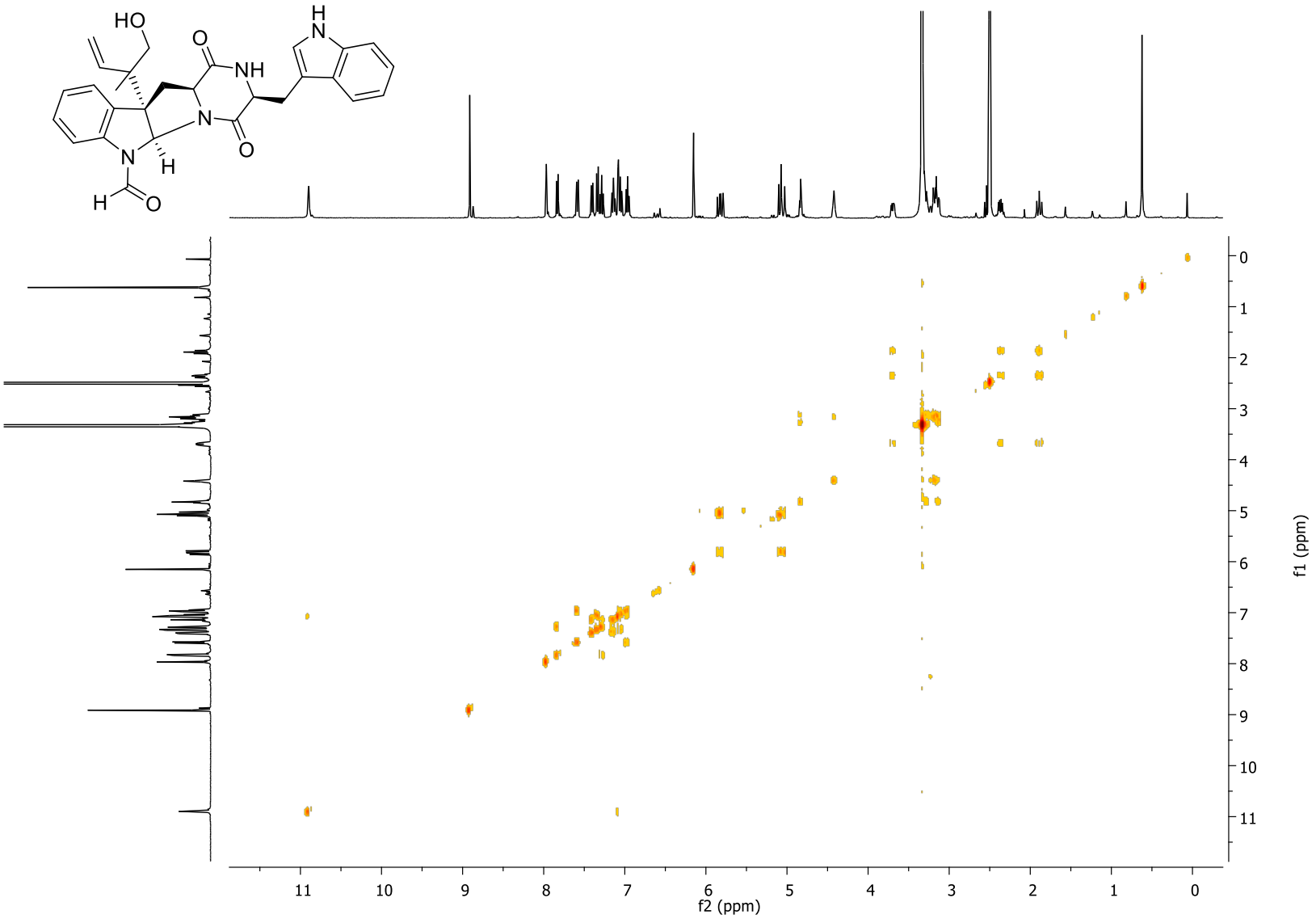
^{1}H-^{1}H NOESY NMR spectrum (*d*$_{6}$–DMSO, 400 MHz) of 3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_{1}$-L-Trp$_{2}$) **11c**.



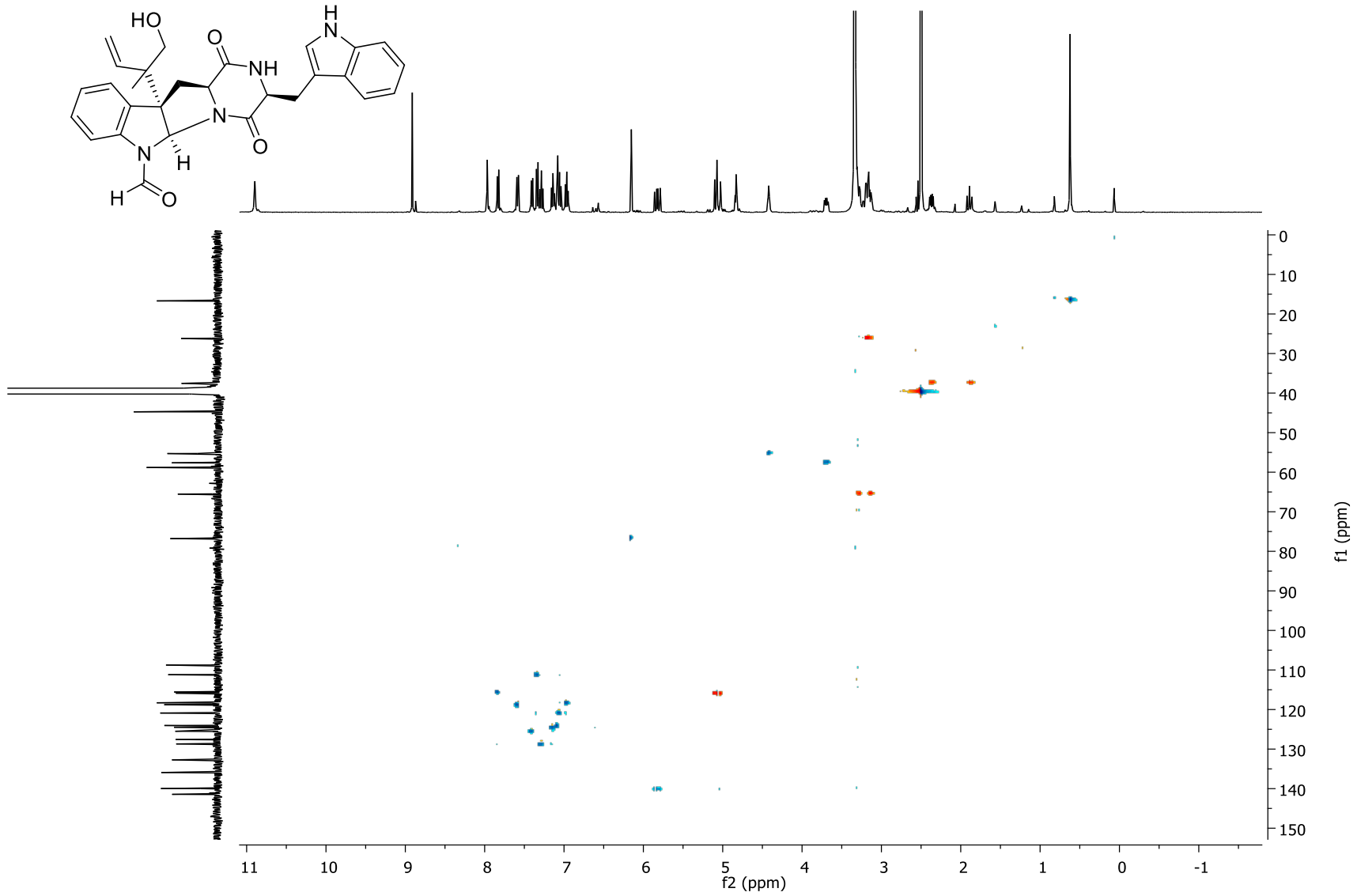
^{1}H NMR spectrum (d_6–DMSO, 400 MHz) of *N*-formyl-3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11d**.



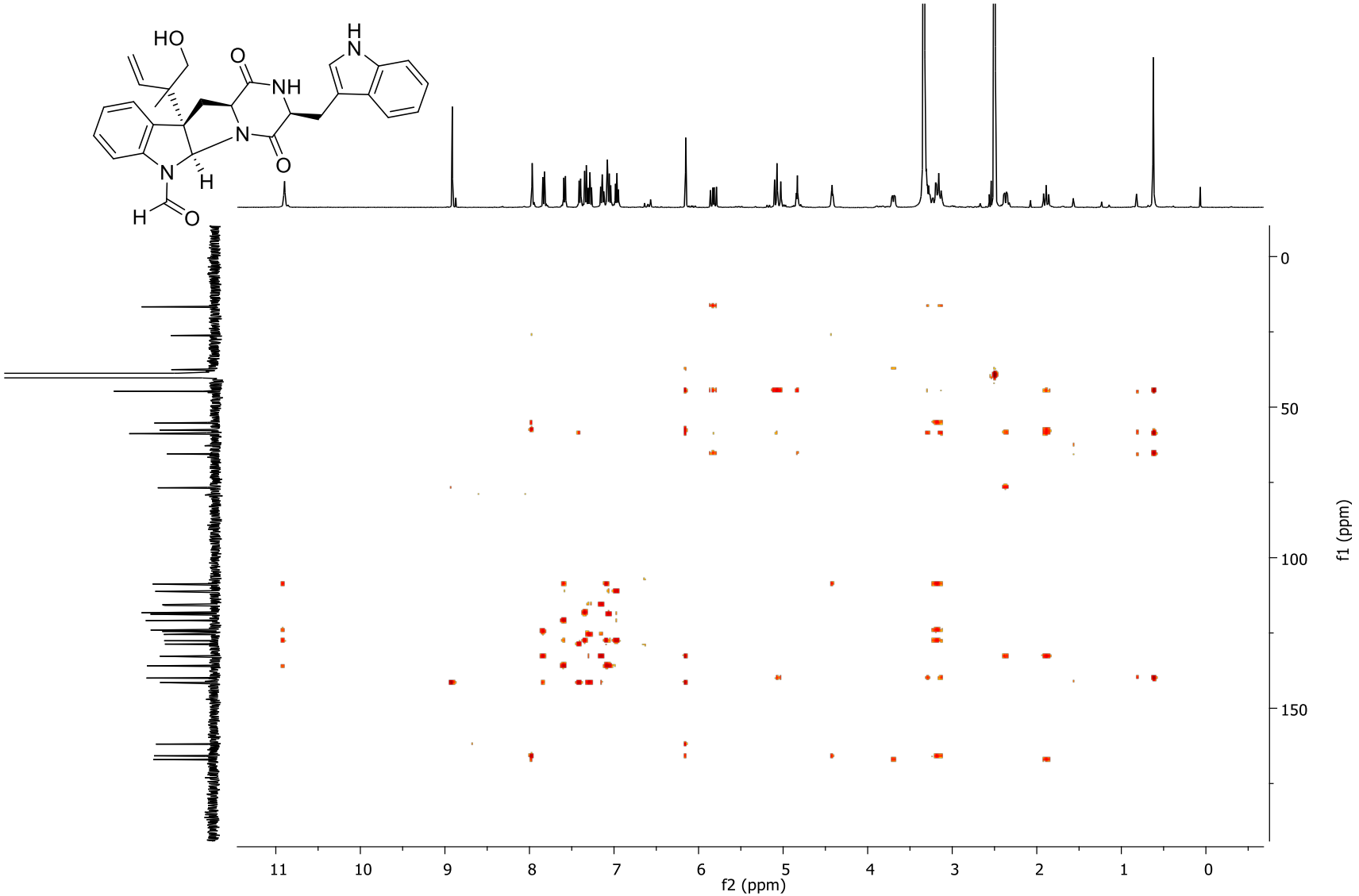
^{13}C NMR spectrum (d_6–DMSO, 100 MHz) of *N*-formyl-3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11d**.



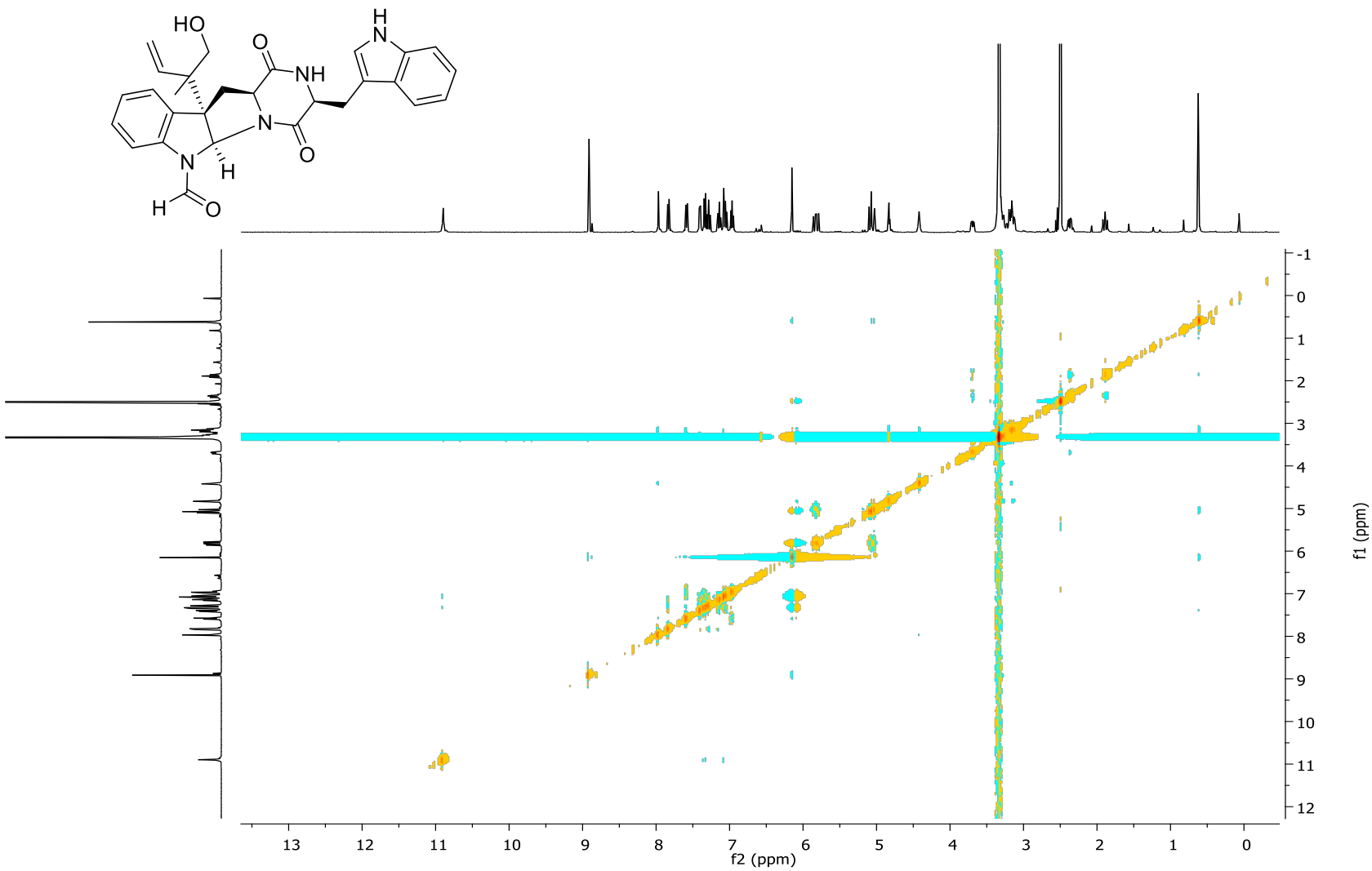
^{1}H-^{1}H COSY NMR spectrum (*d*$_{6}$–DMSO, 400 MHz) of *N*-formyl-3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo(L-Trp$_{1}$-L-Trp$_{2}$) **11d**.



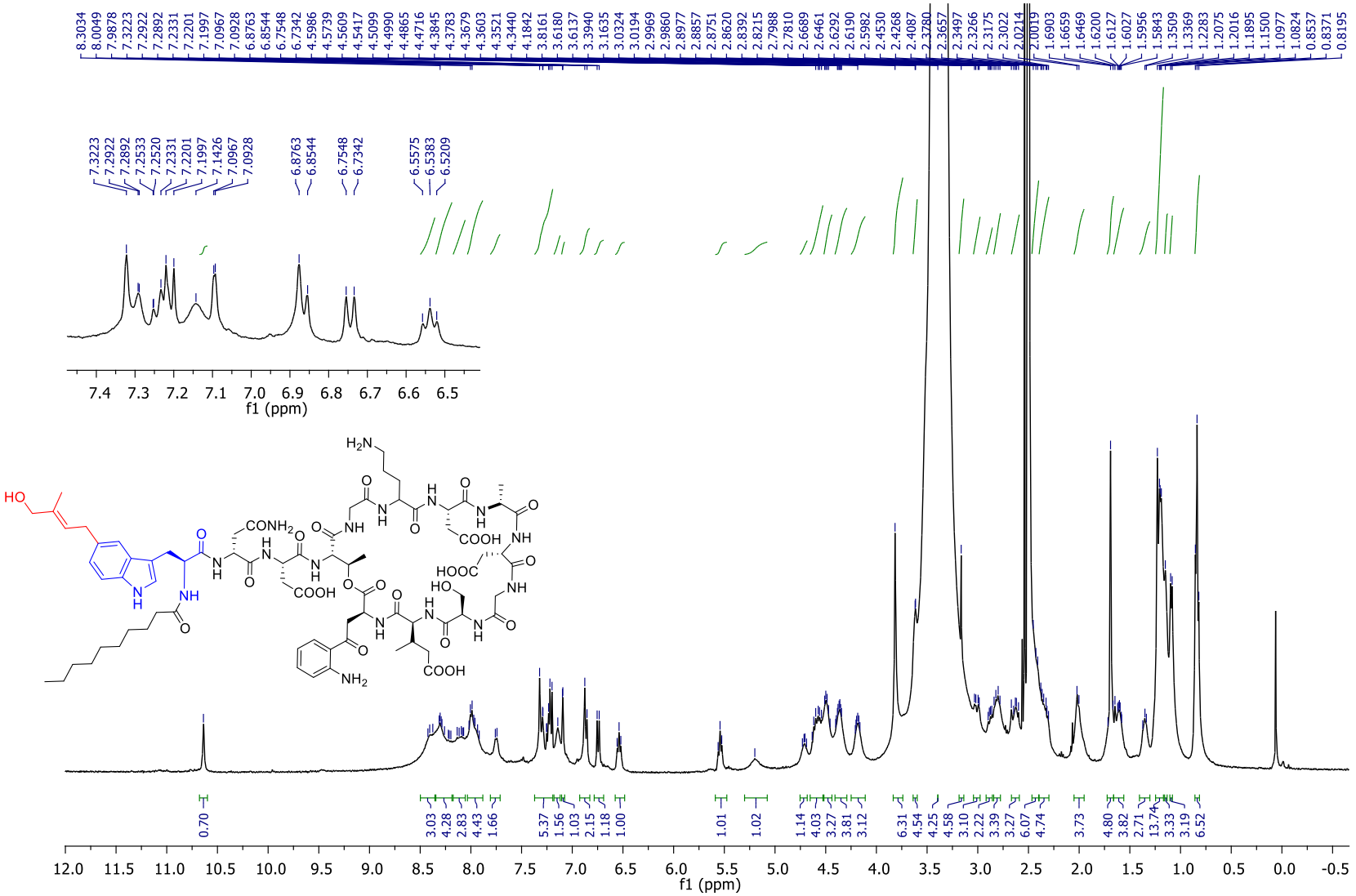
^{1}H -^{13}C HSQC spectrum (d_6–DMSO, 400 MHz) of *N*-formyl-3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11d**.



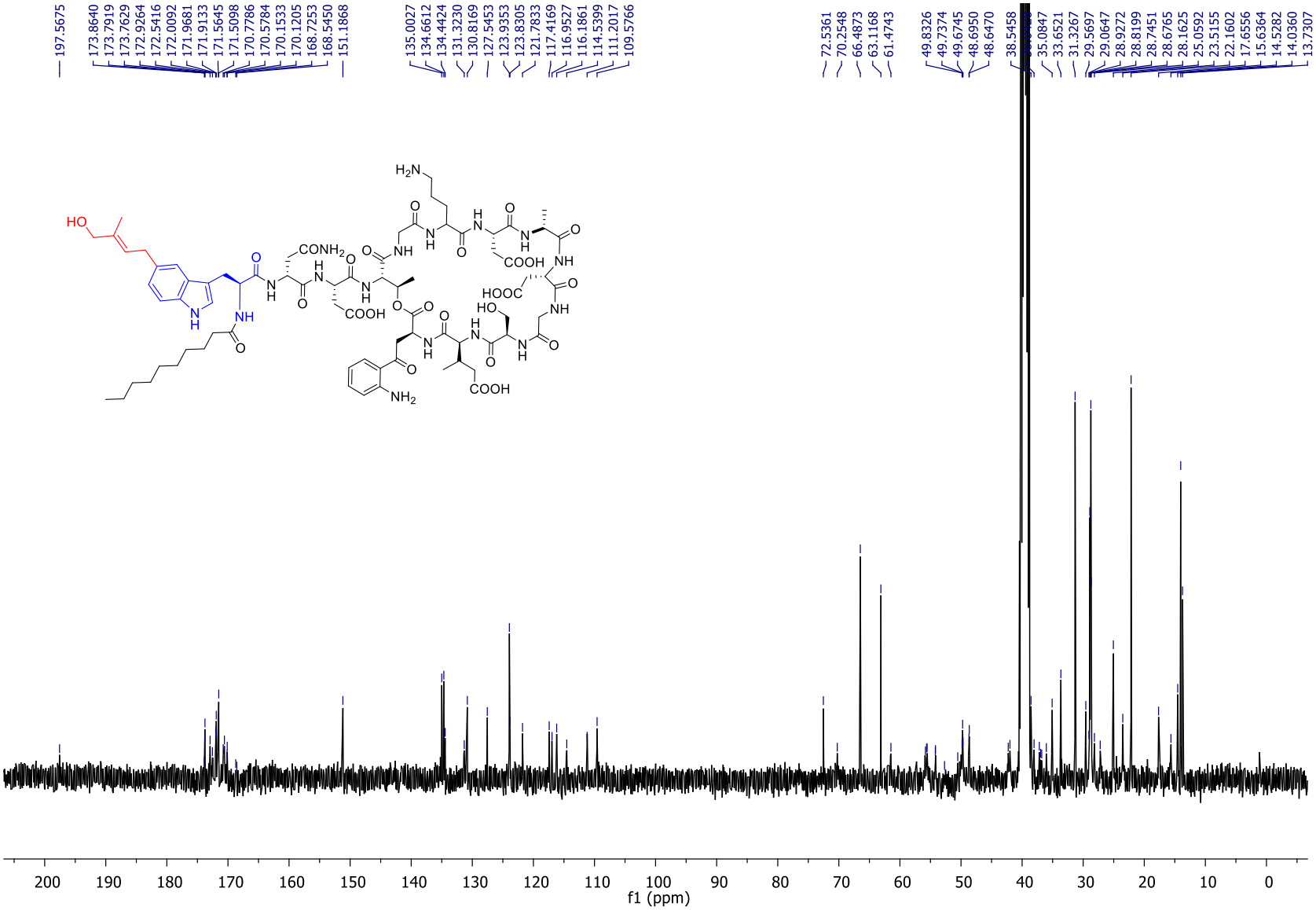
^{1}H-^{13}C HMBC NMR spectrum (d_6–DMSO, 400 MHz) of *N*-formyl-3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11d**.



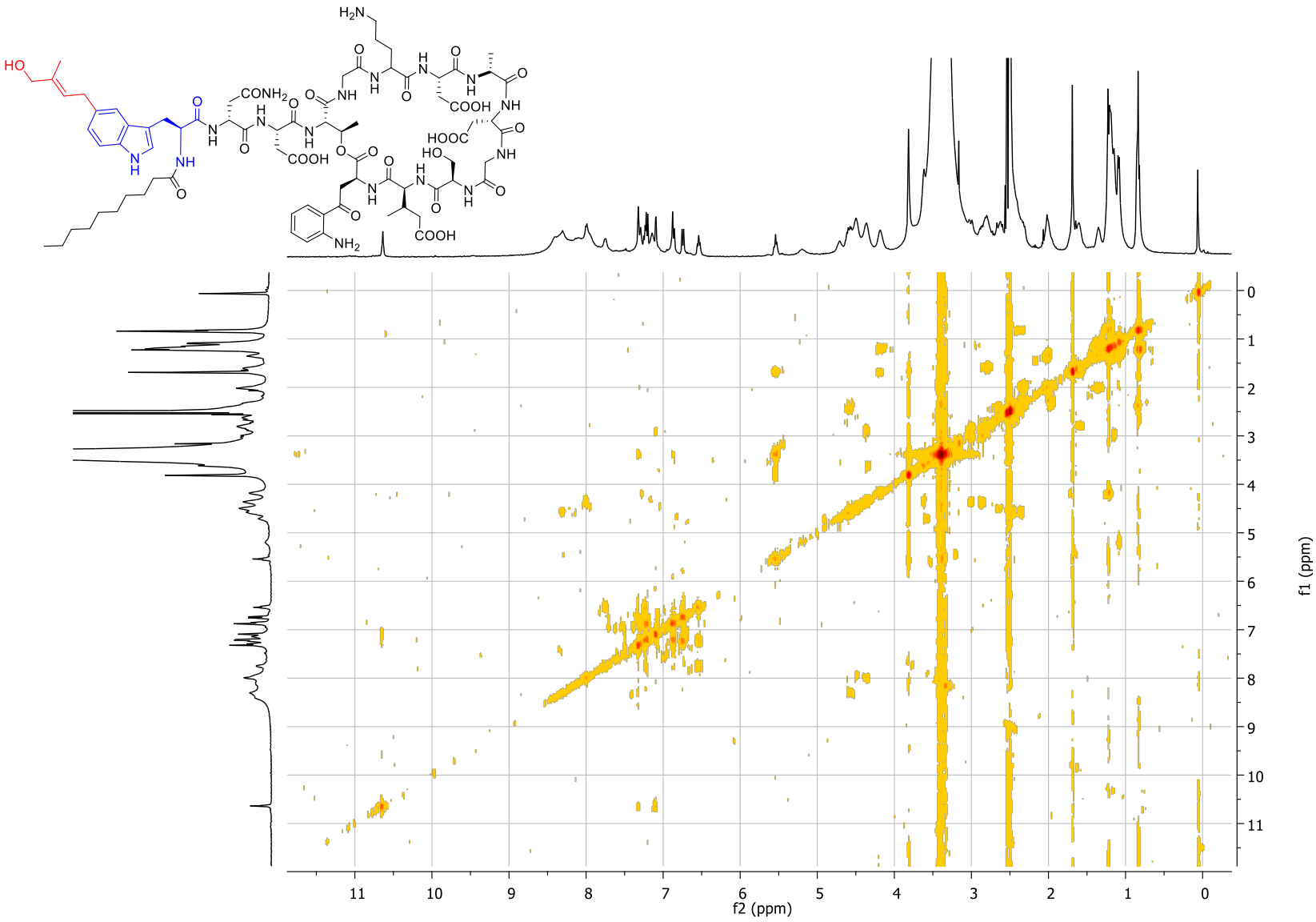
^{1}H-^{1}H NOESY NMR spectrum (d_6–DMSO, 400 MHz) of *N*-formyl-3-*C*-(1-hydroxy-2-methylbut-3-en-2-yl)-pyrrolo[2,3-b]indole derivative of cyclo-(L-Trp$_1$-L-Trp$_2$) **11d**.



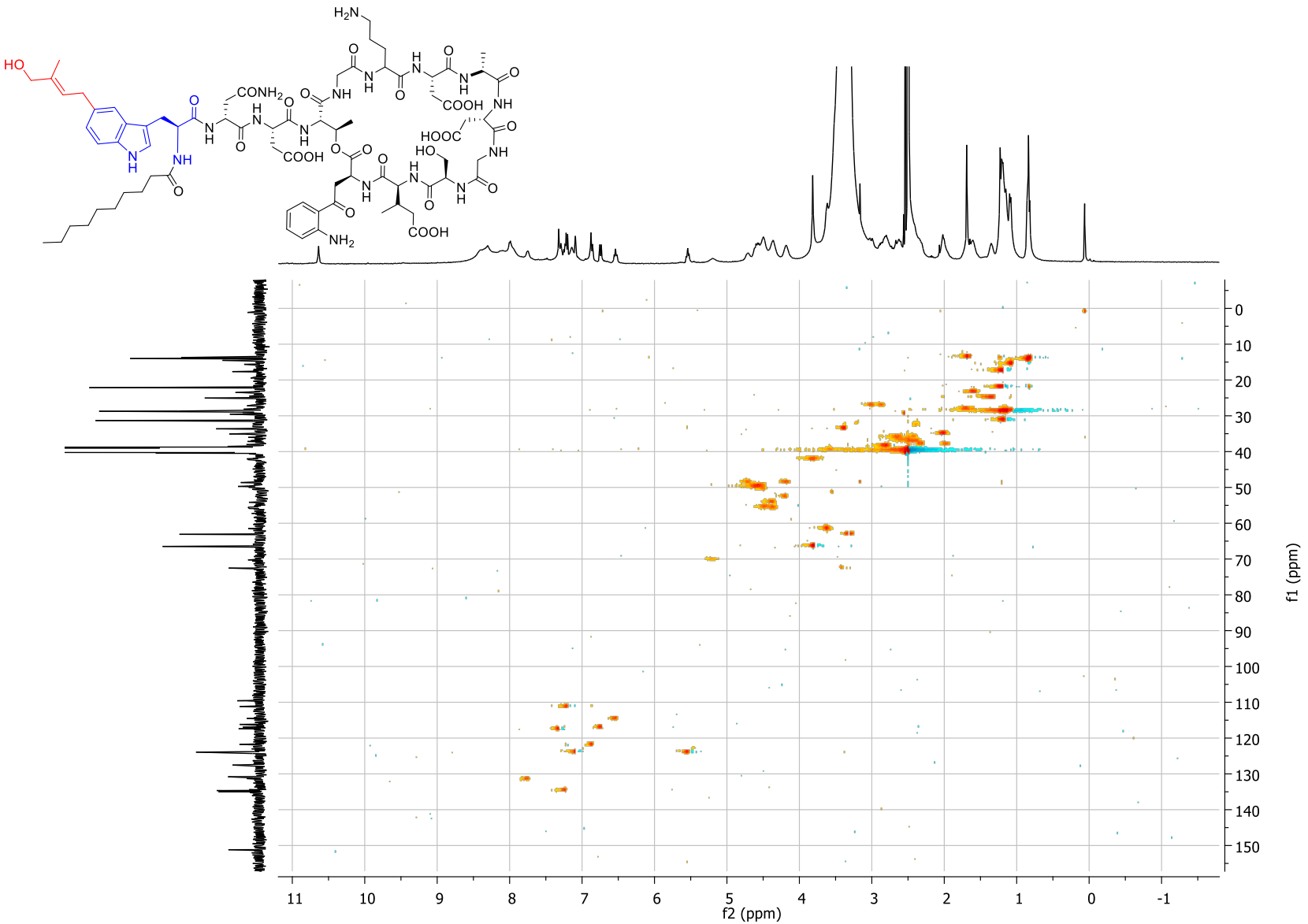
[1]H NMR spectrum (DMSO-*d6*, 400 MHz) of 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp[1] daptomycin **12**.



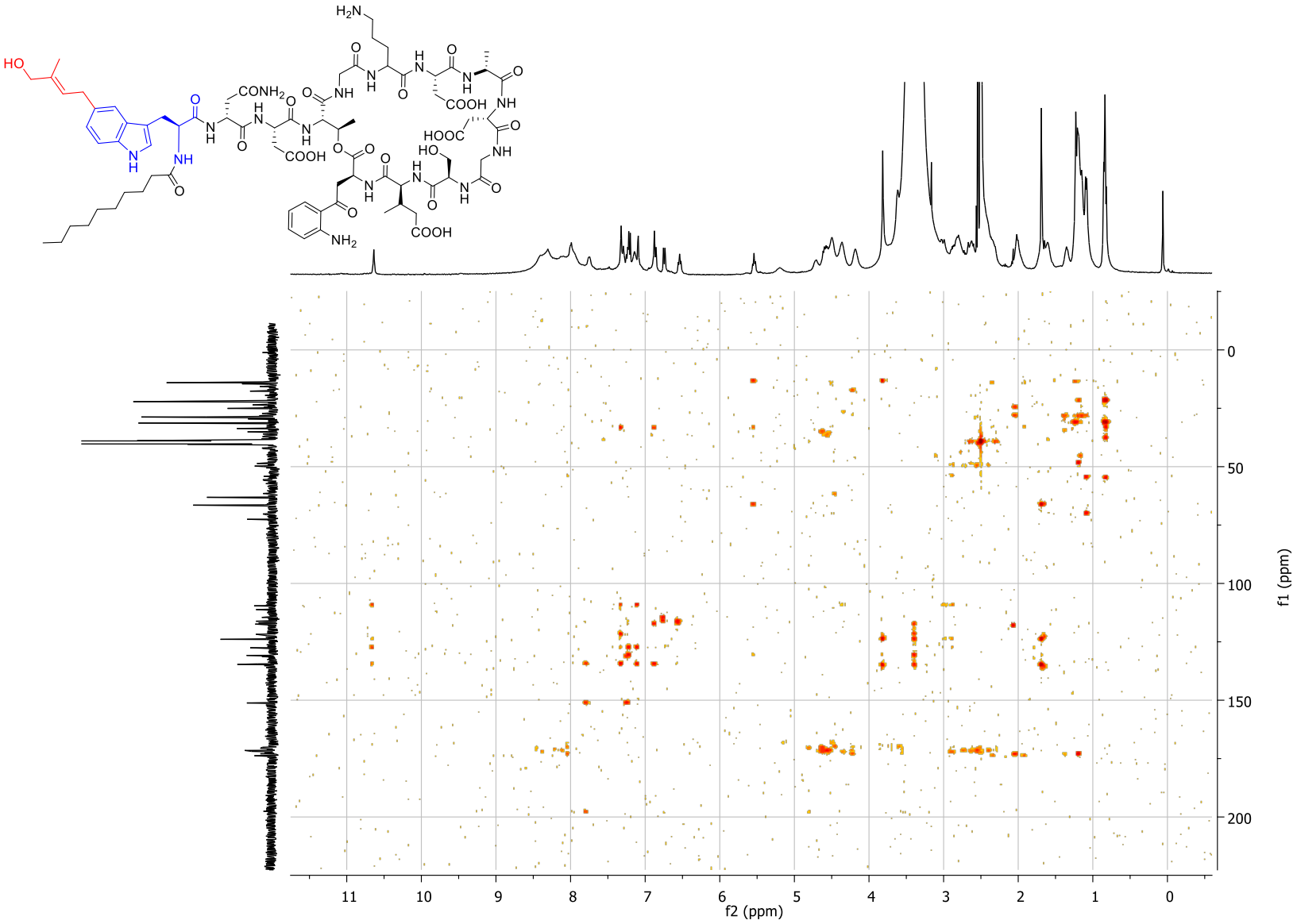
^{13}C NMR spectrum (DMSO-*d6*, 100 MHz) of 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$ daptomycin **12**.



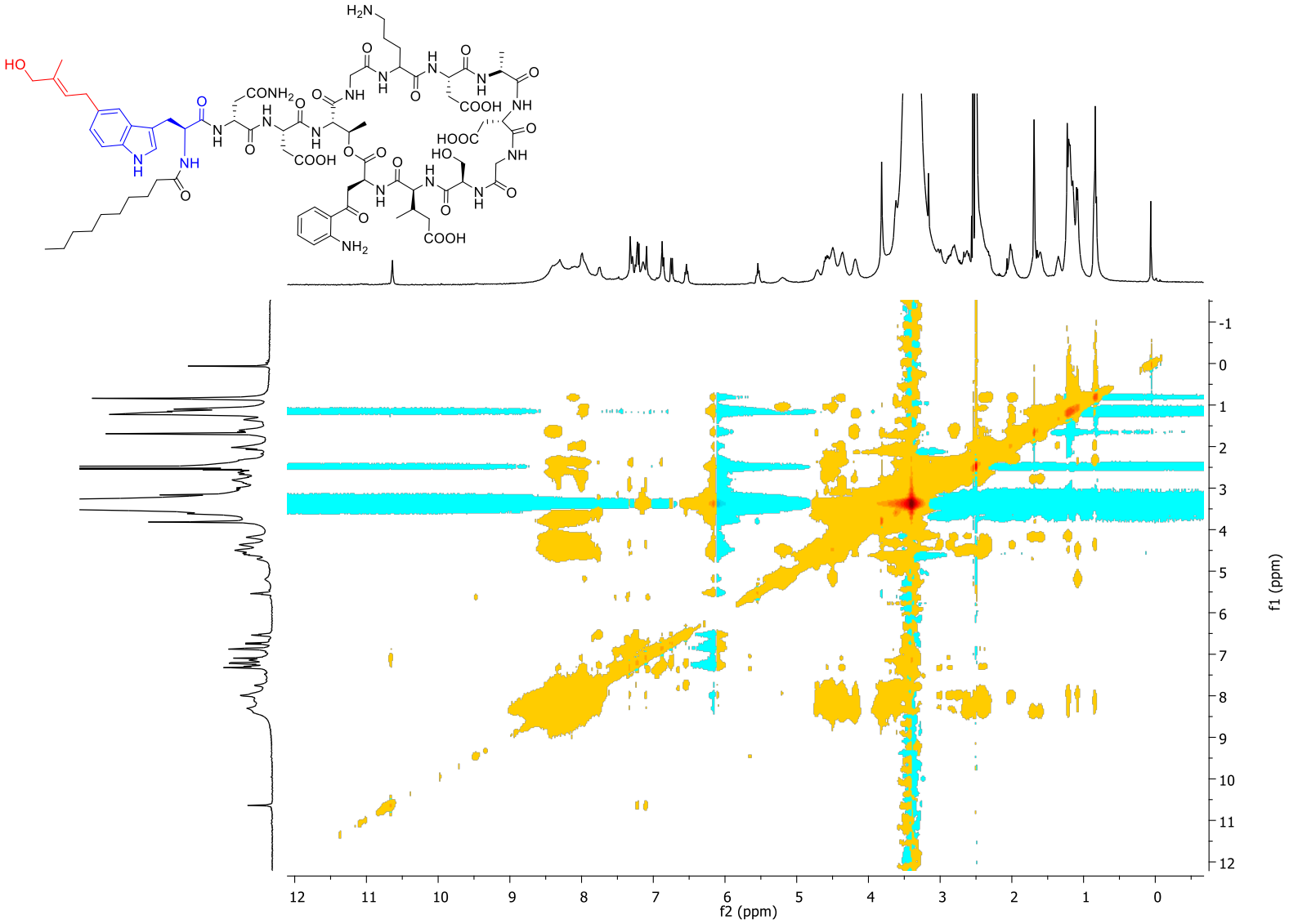
^{1}H-^{1}H COSY NMR spectrum (DMSO-*d6*, 400 MHz) of 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp_1 daptomycin **12**.



^{1}H -^{13}C HSQC spectrum (DMSO-*d6*, 400 MHz) of 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_{1}$ daptomycin **12**.



^{1}H -^{13}C HMBC spectrum (DMSO-*d6*, 400 MHz) of 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_1$ daptomycin **12**.



^{1}H -^{1}H NOESY spectrum (DMSO-*d6*, 400 MHz) of 5-*C*-((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)-L-Trp$_{1}$ daptomycin **12**.