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Supporting Information

for

Biapillarmycin A: a novel alkaloid from Ghana with potent antitrypanosomal activity

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Experimental protocols, phylogenetic data, compound isolation and characterization data (HPLCs, 1D, 2D NMR, IR, UV), tables, and figures

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Supplementary methods

General experimental procedures

1D and 2D NMR analyses were performed on a Bruker Ascend 500 MHz spectrometer (Bruker, Sylvenstein, Germany). This instrument was optimized for ^{1}H observation with ^{13}C and ^{15}N pulsing/decoupling, with 2H lock channels equipped with shielded z-gradients and cooled preamplifiers for ^{1}H and ^{13}C. Solvent signals at δ_H 3.31 and δ_C 49.00 ppm in CD_3OD, were used as references for ^{1}H and ^{13}C chemical shifts. High-resolution mass spectrometry data were obtained using a Thermo Instrument MS system (LTQ XL/LTQ Orbitrap Discovery, UK) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler, and Accela pump). Conditions used in data acquisition were capillary voltage of 45 V, a flow rate of 40–50 arbitrary units of sheath gas, a mass range of 100–2000 amu (maximum resolution of 30,000), a capillary temperature of 320 °C, 4.5 kV of spray voltage, and an auxiliary gas flow rate between 10–20 arbitrary units. The IR spectrum was measured using a Nexus 870 FT-IR, UK. Optical rotation was measured using an Autopol IV automatic polarimeter (Rudolph Research Analytical, USA). Separations on HPLC were performed using a reversed-phase Phenomenex (C-18, 5 µm, 100 Å, 250 × 6.10 mm, L × i.d.) column, coupled to a Waters 1525 series binary pump (France), an in-line degasser, and a column heater. Detection and monitoring were performed using a Waters 2998 photodiode array (PDA) detector (France). All solvents used were of HPLC grade and purchased from VWR, UK.

Soil sample collection site

The Ghanaian *Streptomyces* sp. BNP Pillar 1G 1/10B is a soil-dwelling bacterium that was isolated from soils collected from the Bia National Park (6˚30´0.00″ N and 3˚0´0.00″ W), a highly biodiverse biosphere reserve located in the Western North region of Ghana (Figure S1). The forest, covering approximately 563 km^2, is home to a wide variety of species. To date, the park has been maintained in its natural state with frequent and deliberate eviction of nearby settlements, leading to a significant reduction in anthropogenic activities and hence, making the area a promising environment for the isolation of novel microbial strains.

In this study, rhizosphere soil samples were collected from the most natural areas of the forest at 300 m intervals. The soils were collected by first shoving off the topsoil from plant debris and digging to a depth of about 0.8−1.6 m. Deep-soil samples were scooped into 50 ml sterile Falcon tubes, covered, labelled, and stored for transportation to the University of Ghana laboratory.

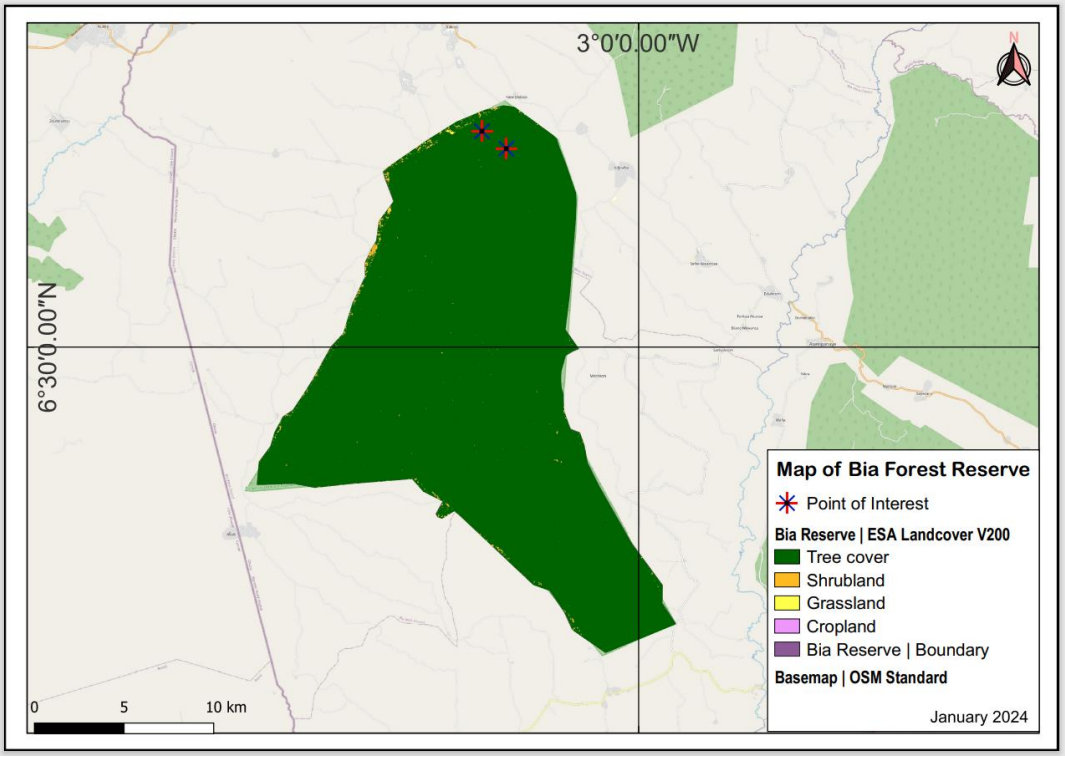

Figure S1: Topographic map of the soil sample collection site in the Bia National Park.

Culture and isolation of the strain *Streptomyces* sp. BNP Pillar 1G 1/10B from soil sediments

Using procedures described previously in the literature [1], the soil sample coded BNP Pillar 1G 1/10B was prepared for bacterial strain isolation by placing 5 g of the soil sample into a new sterile 50 mL Falcon tube. About 10 mL of sterilized Milli-Q water was added, and the Falcon tube was capped under sterile conditions. The sample was subsequently placed in a hot water bath at 55 °C for 1 hour to prevent the growth of fast-growing gram-negative bacteria and fungi. The heated soil sample was allowed to cool, then transferred to a clean bench that had been previously sterilized with 70% ethanol and exposed to ultraviolet (UV) light for an hour. About 10 mL of sterilized Milli-Q water was added to the sample, and dilutions at 10^{-1}, 10^{-2}, and 10^{-3} were prepared. This was done by pipetting 1 mL of the stock 10 mL suspension into a new sterile 50

mL Falcon tube, diluting with 9 mL sterile Milli-Q water, and shaking thoroughly to afford the 10^{-1} dilution. A 1 ml aliquot of the 10^{-1} suspension was used to prepare the subsequent serial dilutions. Pre-sterilized inoculation loops were used to evenly spread about 5 µL of the different concentrations of the soil sample onto pre-modified ISP2 agar plates at pH 5.5, supplemented with 25 µg/mL each of nystatin and nalidixic acid antibiotics. The master plates were parafilmed and incubated for 7 days at 28 °C to allow bacterial growth. After 7 days, the agar plates were removed from the incubator and observed on a clean bench. Single colonies with different phenotypes were re-plated on new agar plates using sterilized inoculation loops in the clean bench to obtain pure strains. Parent or master plates that had fungal contamination or lacked single individual colonies to pick were discarded to prevent contamination of the lot. The plates were sealed with parafilm and placed in the incubator to allow for continued bacterial growth. Pure strains transferred to new agar plates were also parafilmed and incubated for periodic observation. Pure strains were obtained by successive subculturing of all colonies originally subcultured from the parent or master plates. A pure strain of BNP Pillar 1G 1/10B (Figure S2) was obtained through this process and its whole DNA was submitted to the Department of Biomedical Engineering, University of Samsun, Turkey for 16S rRNA gene sequencing.

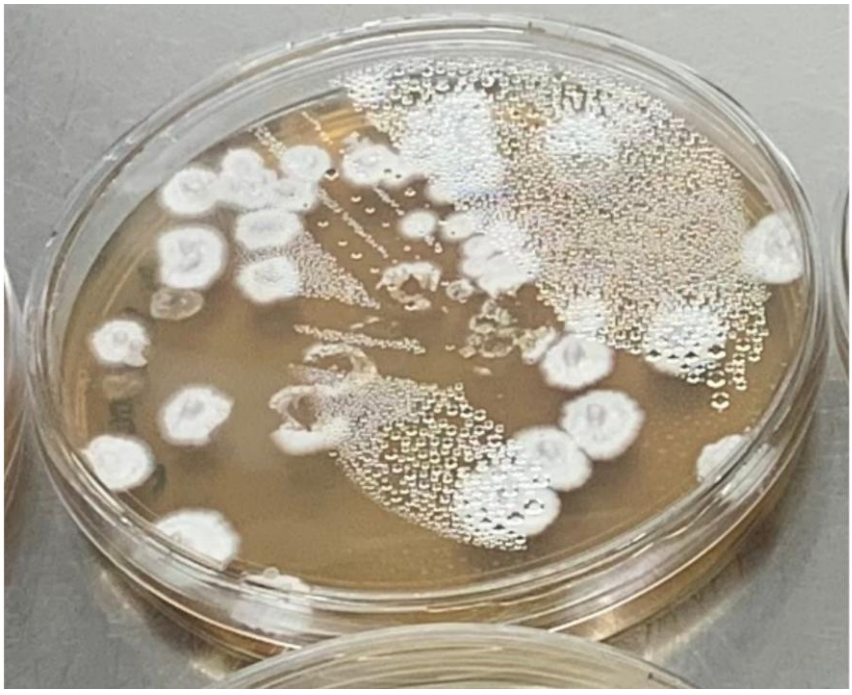

Figure S2: Pure *Streptomyces* sp. BNP-Pillar-1G-1/10B growing on ISP2 agar plates at pH 5.5 and 28 °C incubation temperature.

Phylogenetic analyses of *Streptomyces* sp. BNP Pillar 1G 1/10B from soil sediments

For genomic DNA extraction, strain BNP Pillar 1G 1/10B was cultivated in ISP2 broth at 28 °C for 7 days. Genomic DNA was isolated using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA) following the manufacturer's protocol. The quality and concentration of the extracted DNA were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The 16S rRNA gene was amplified using the universal bacterial primers 27F and 1525R, Lane,1991 **[2]**. The PCR amplicon was subsequently purified and sequenced with an ABI PRISM 3730 XL automated sequencer (Applied Biosystems, Thermo Fisher Scientific, USA by Macrogen Inc., The Netherlands). The nearly complete 16S rRNA gene sequence was compared with type strain sequences available in the EzBioCloud database https://www.ezbiocloud.net, Yoon et al. 2017 [3]. Sequence alignment was performed using ClustalW in MEGA 11 software, which was also used for phylogenetic analysis, Tamura et al. 2021 [4]. The phylogenetic tree (Figure 2) was constructed using the neighbor-joining method and inferred using the Saitou et al. 1987 algorithm [5]. Evolutionary distances were calculated using the Jukes-Cantor model [6]. Topologies of the resultant trees were evaluated by bootstrap analysis Felsentein, 1985 based on 1000 re-samplings [7].

Culture and fermentation of *Streptomyces* sp. BNP Pillar 1G 1/10B

The *Streptomyces* sp. BNP Pillar 1G 1/10B was grown on ISP2 agar plates made up of 10 g of malt extract, 4 g each of yeast extract and D-glucose, and 15 g of agar at pH 5.5 with incubation temperature 28 °C for 72 hours. Subsequently, a small-scale culture was prepared by inoculating 100 mL of a TSBY fermentation media broth composed of 17 g tryptone, 3 g of phytone, 5 g of sodium chloride, and 2.5 g of each disodium phosphate and glucose in distilled water in a 250 mL Erlenmeyer flask plugged with non-absorbent cotton wool and autoclaved with spores of the strain *Streptomyces* sp. BNP Pillar 1G 1/10B. This small-scale culture was incubated for 7 days at 28 °C, 220 rpm, and 25 g/L of autoclaved HP-20 resin added under sterile conditions. For large-scale fermentation cultures, an inoculum was prepared by

placing 50 mL of TSBY fermentation media in a 250 mL Erlenmeyer flask plugged with non-absorbent cotton wool, autoclaving it, and subsequently inoculating this with spores of the strain *Streptomyces* sp. BNP Pillar 1G 1/10B followed by incubation for 3 days at 28 °C with 220 rpm. The inoculum was used to inoculate nine 1 L Erlenmeyer flasks, each containing 200 mL of TSBY media, plugged with non-absorbent cotton wool, and previously sterilized. These flasks were then cultured for 10 days at 28 °C, 220 rpm. Exactly 24 hours prior to the end of the culture period, 50 g/L of autoclaved HP-20 resin was added to each flask under sterile conditions, and the flasks were incubated at the same temperature and shaking conditions.

Extraction and purification procedures

The *Streptomyces* sp. BNP Pillar 1G 1/10B fermentation broth (1.8 L) was subjected to suction filtration using a Büchner funnel equipped with a piece of glass wool to separate the supernatant from the mycelia. The supernatant was extracted with ethyl acetate while the mycelia and HP-20 resin were placed in a 1 L flask and repeatedly extracted with methanol and dichloromethane. To obtain the total crude extract (TCE = 2.84 g), all extracts were combined and concentrated under reduced pressure. The TCE was subjected to a modified Kupchan's solvent partitioning process [8], which afforded the four fractions FH (0.65 g), FD (0.48 g), FM (0.32 g), and WB (1.08 g) in increasing order of polarity. Therefore, the WB fraction was subjected to column chromatography by gravity using Sephadex LH-20 as the stationary phase and an isocratic elution methanol:acetonitrile (1:1) to obtain five sub-fractions that were labelled WB-SF1-SF5. Microchemical screening with Ninhydrin reagent on pre-run TLC plates, in addition to ^{1}H-NMR data, showed that the compound of interest was concentrated in the WB-SF3 (0.15 g).

Initial injection of the crude BNP-Pillar-1G-1/10B-WB-SF3 fraction on reverse-phase HPLC produced a profile that did not efficiently separate the compound and its many derivatives. Therefore, the compound biapillarmycin A (**1**) was isolated from the BNP-Pillar-1G-1/10B-WB-SF3 fraction by systematic gradient normal-phase gravity column chromatography equipped with UV-detection. Selective gradient elution was achieved using hexane, ethyl acetate, methanol, and ammonia in various strategic combinations. The solvent mixture deployed to elute compound **1** was ethyl acetate (10%): methanol (90%) (200 mL). To ensure the absolute purity of the compound **1**, the normal-phase eluted fraction was profiled on a reversed-phase semi-preparative

HPLC system equipped with a PDA detector (200–800 nm). Analysis of the UV profile confirmed that the compound of interest was indeed contained in the WB-SF3-C7 fraction. Subsequent repeated HPLC (Figure S3) to obtain enough quantities of the compound **1** involved the use of Phenomenex Luna C18 column (C18 250 × 10 mm, L × i.d.), Gradients of Solvent A: (80% H_2O: 20% MeOH) and Solvent B: (100% MeCN) (100% A to 100% B in 30 min and hold for 30 min) were used as eluents with column flow rates set at 1.5 ml/min to afford compound **1** (3.8 mg, t_R = 14.36 min). Compound **1** was obtained as a pale white substance, colourless when in solution of methanol and/or dichloromethane.

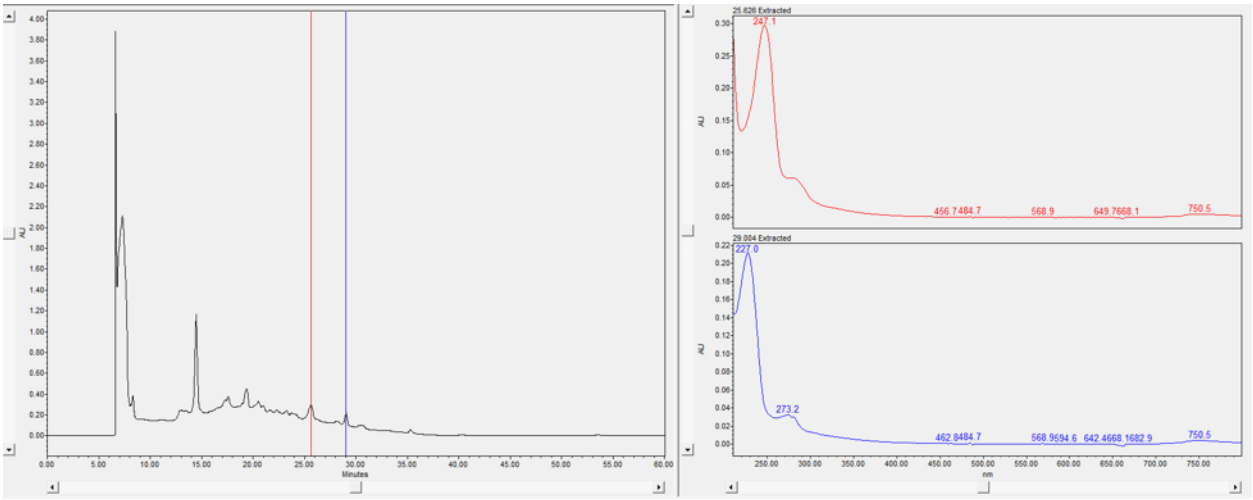

Figure S3: Reverse phase HPLC chromatogram shows the biapillarmycins undergoing separation.

Physical data for compound 1

Pale white substance, colourless when in solution of methanol and/or dichloromethane.; HR-ESI-PDA-MSMS (*m/z*): 286.1438 $[M + H]^+$ (calculated for $C_{17}H_{20}NO_3^+$, 286.1438); ^1H, ^{13}C, HSQC, COSY, HMBC, HSQC-TOCSY and NOESY NMR data are shown in Table 1 and Figures S4–S19.

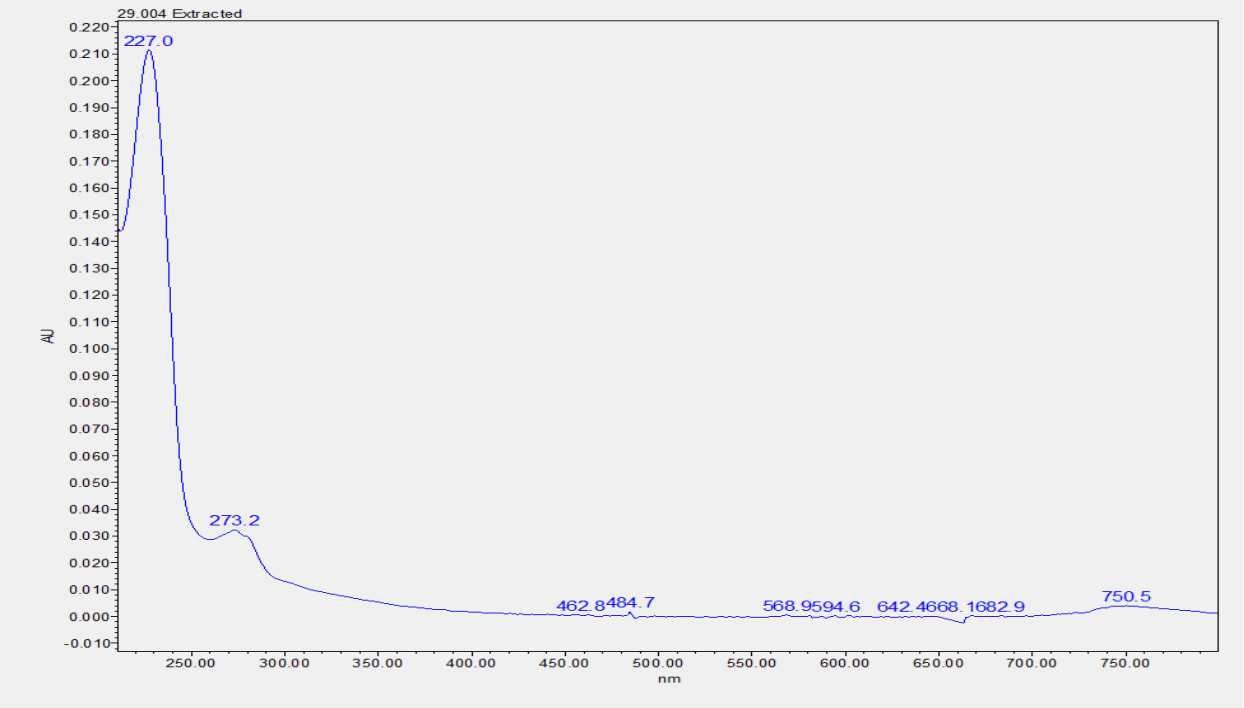

Figure S4: Full UV–visible spectrum of biapillarmycin A (**1**).

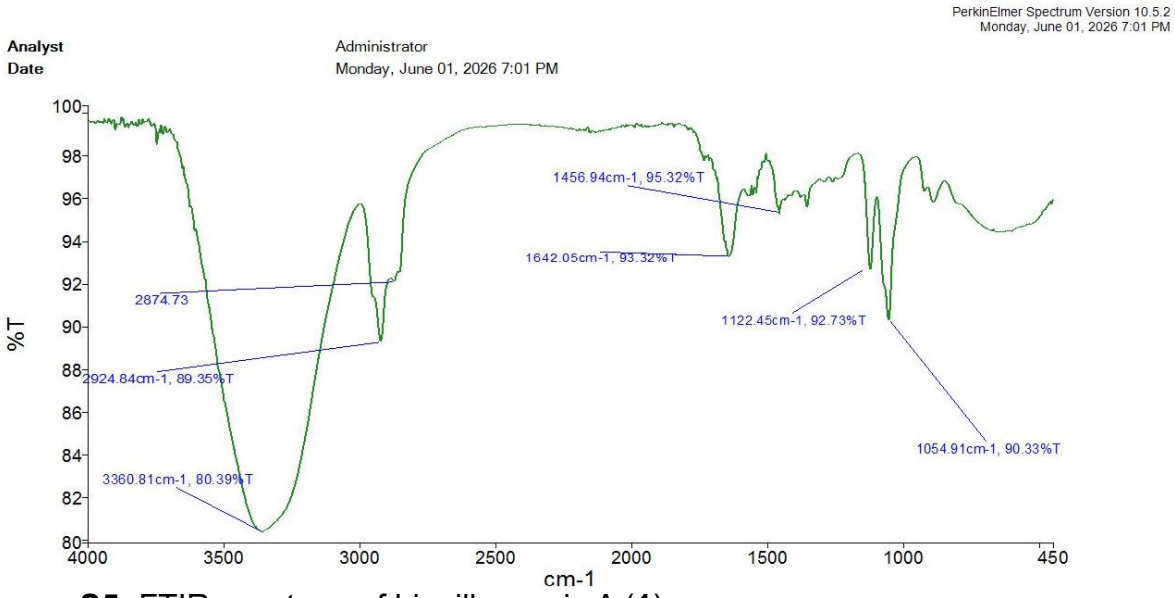

Figure S5: FTIR spectrum of biapillarmycin A (**1**).

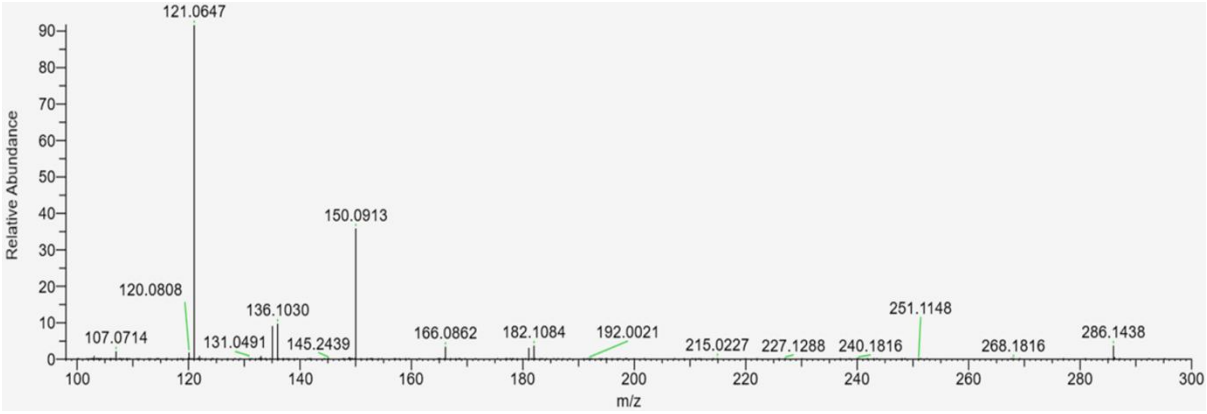

Figure S6: HRMS data of biapillarmycin A (**1**).

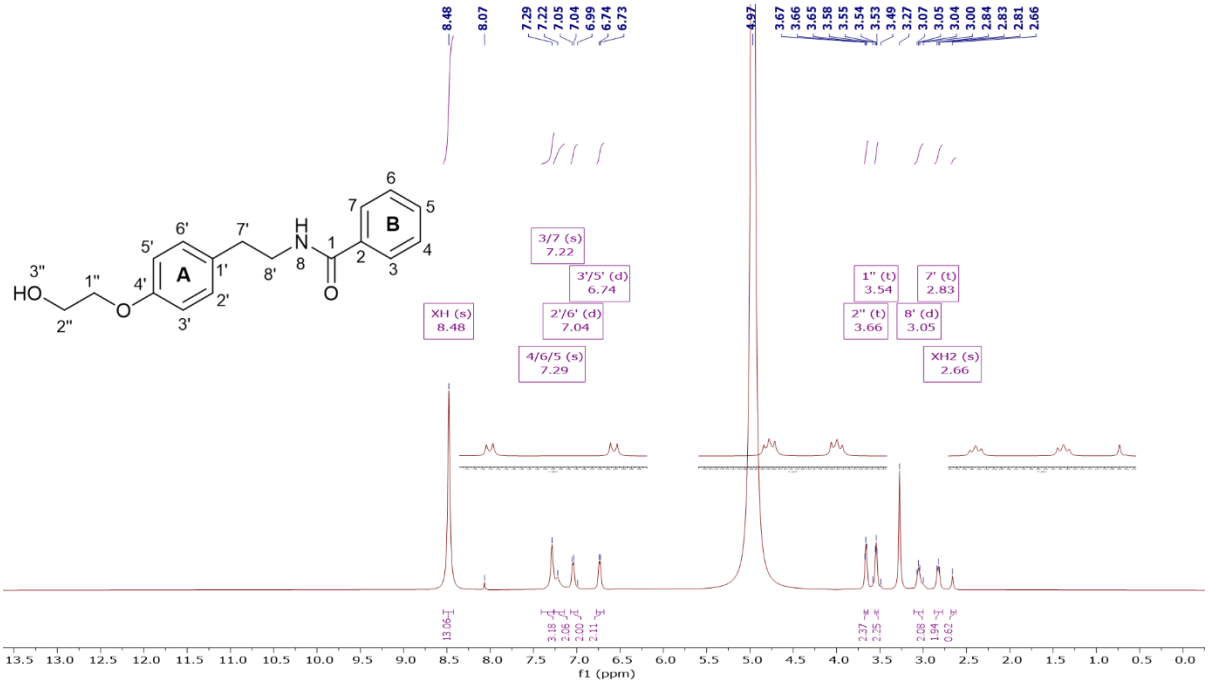

Figure S7: 500 MHz ^{1}H NMR spectrum of compound **1** in CD_3OD.

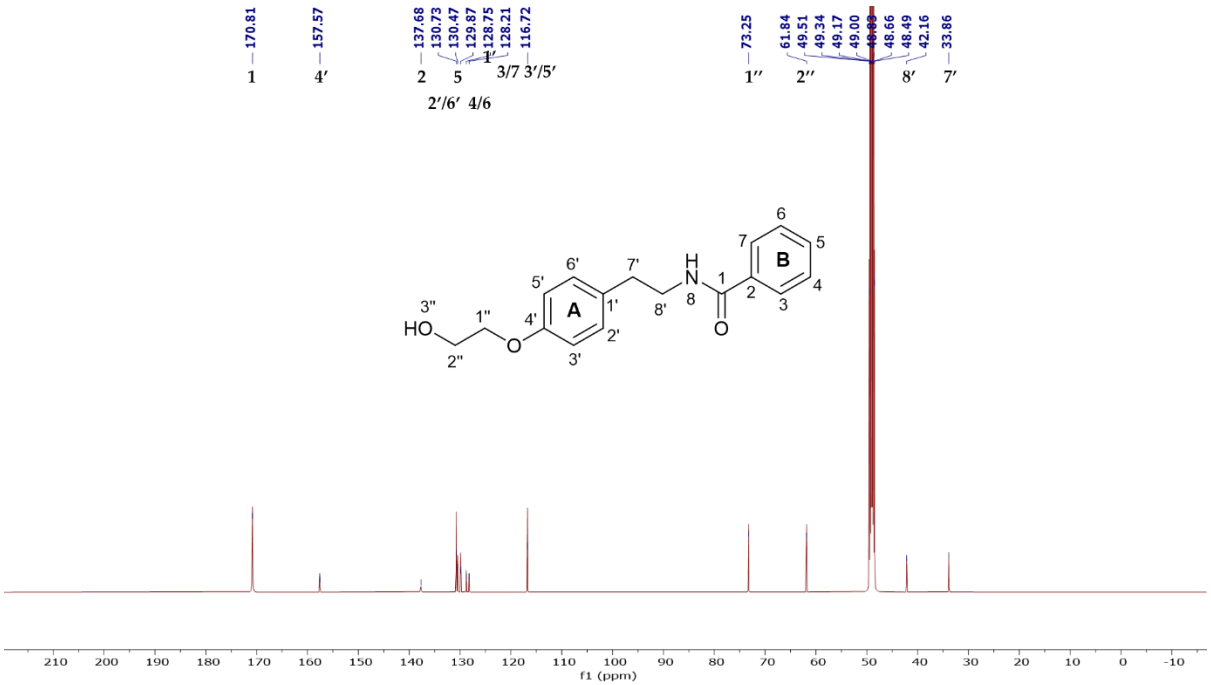

Figure S8: 500 MHz ^{13}C NMR spectrum of compound **1** in CD_3OD.

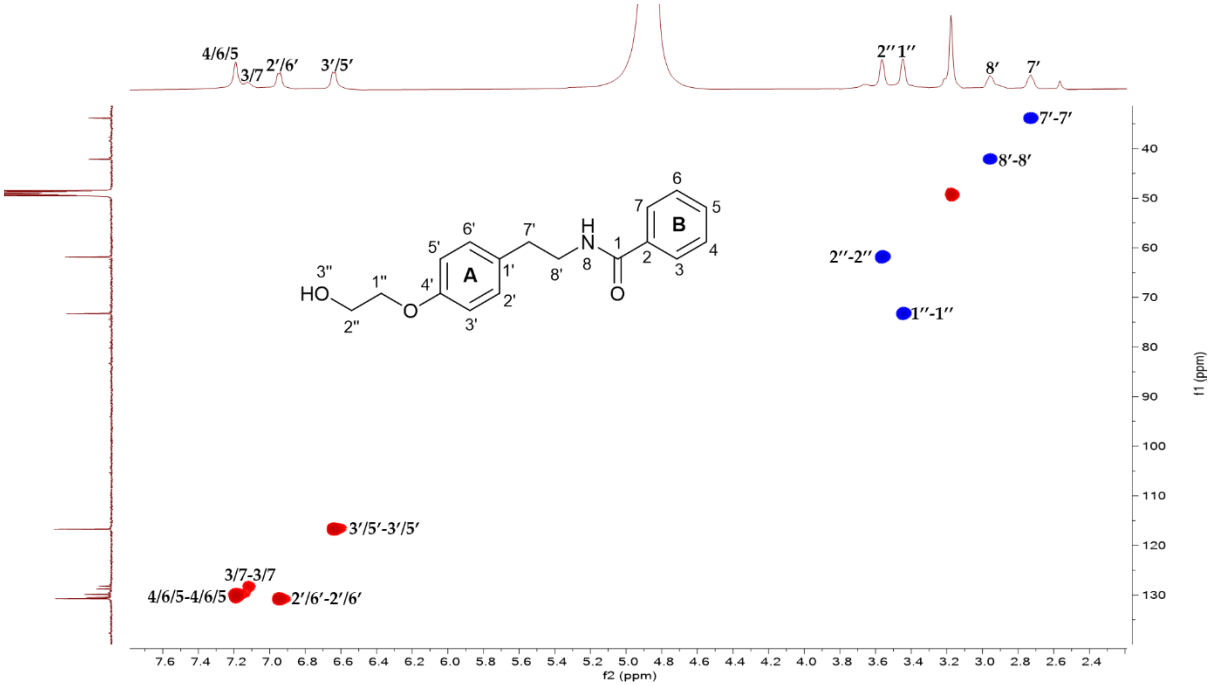

Figure S9: 500 MHz HSQC NMR spectrum of compound **1** in CD_3OD.

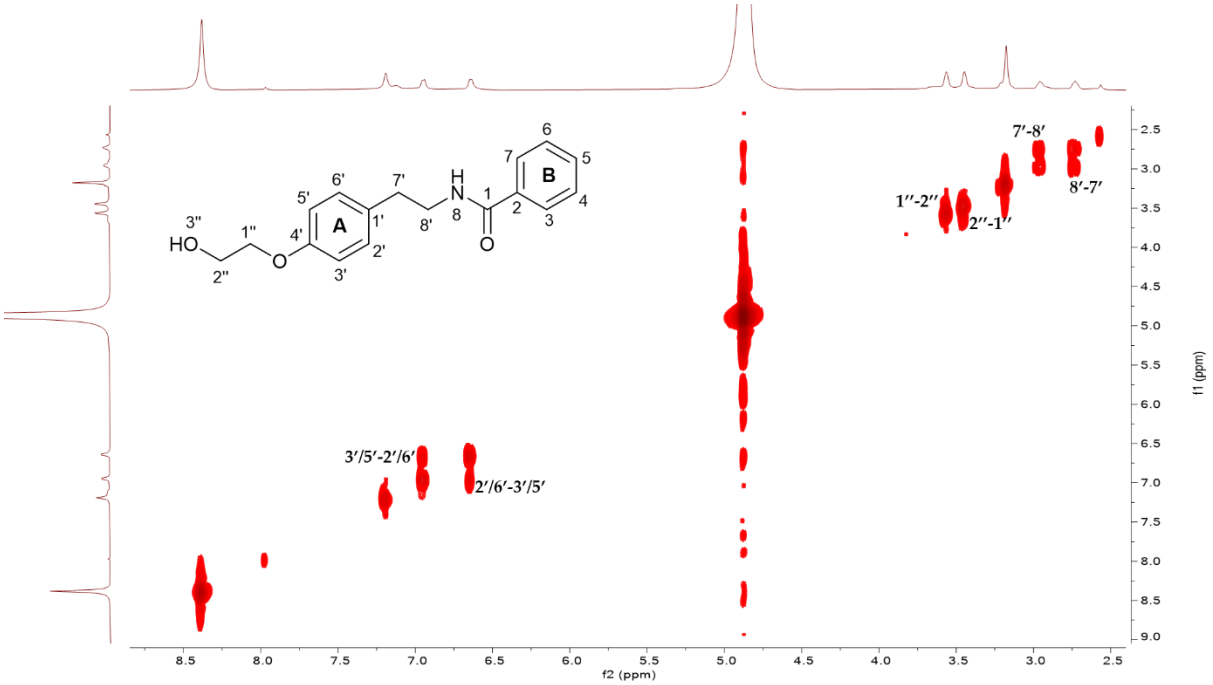

Figure S10: 500 MHz COSY NMR spectrum of compound **1** in CD_3OD.

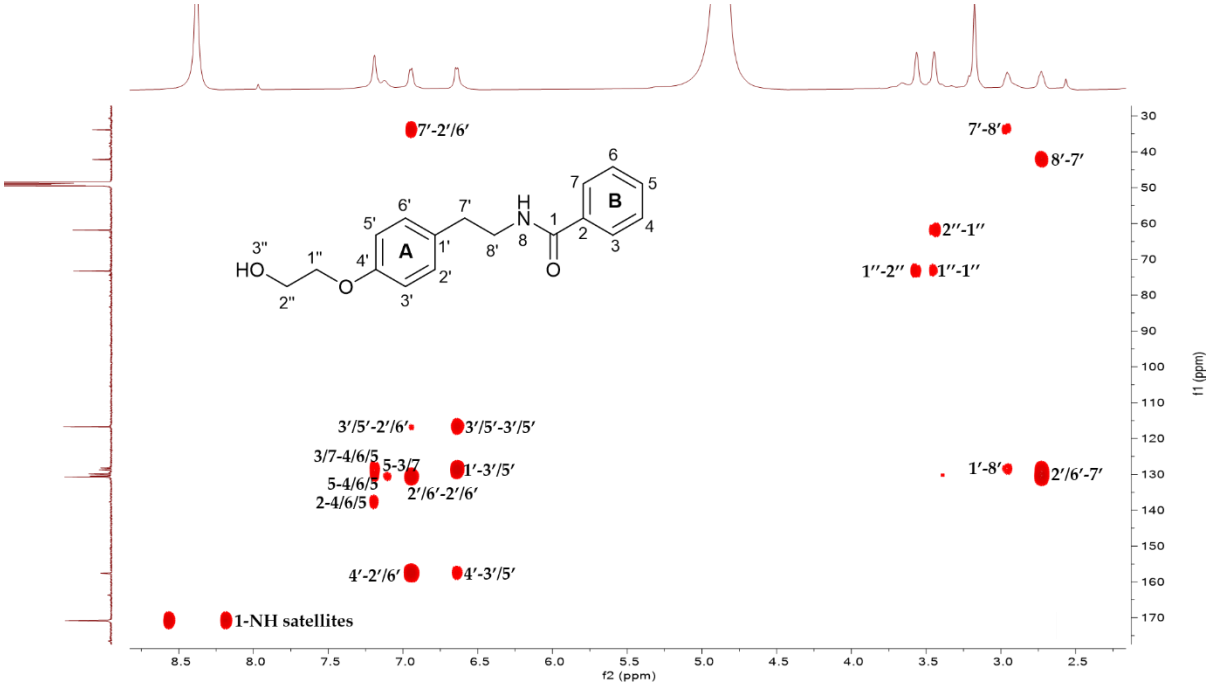

Figure S11: 500 MHz HMBC NMR spectrum of compound **1** in CD_3OD.

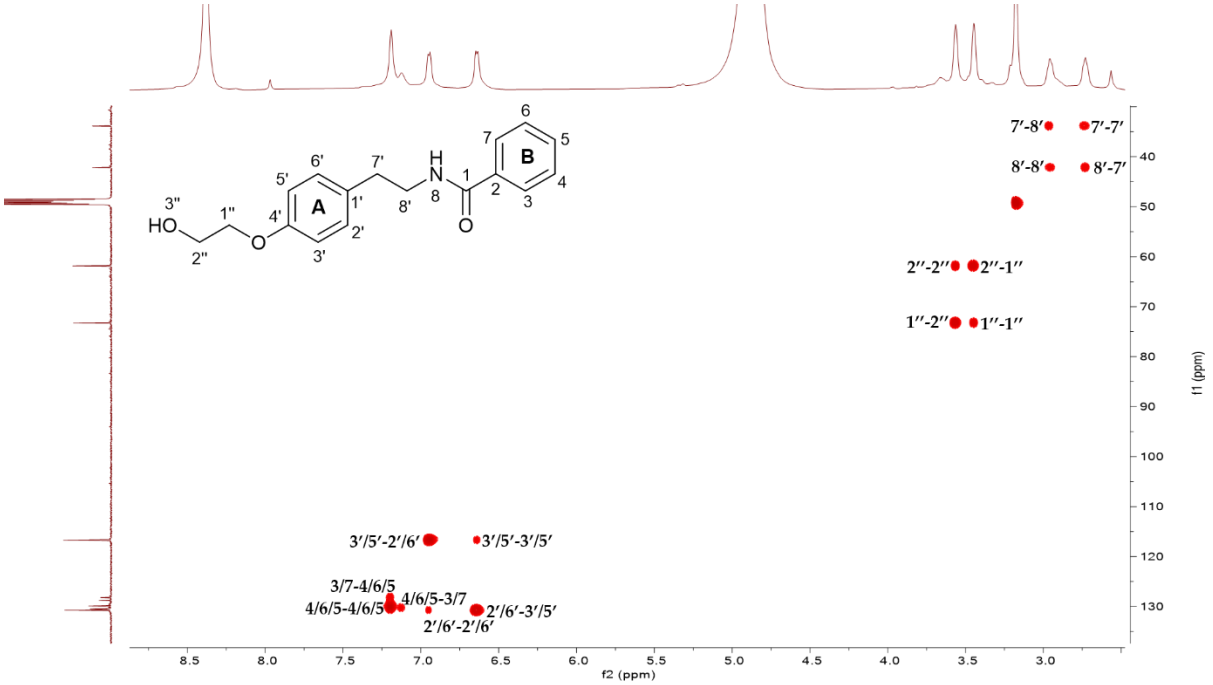

Figure S12: 500 MHz gHSQC-TOCSY NMR spectrum of compound **1** in CD_3OD.

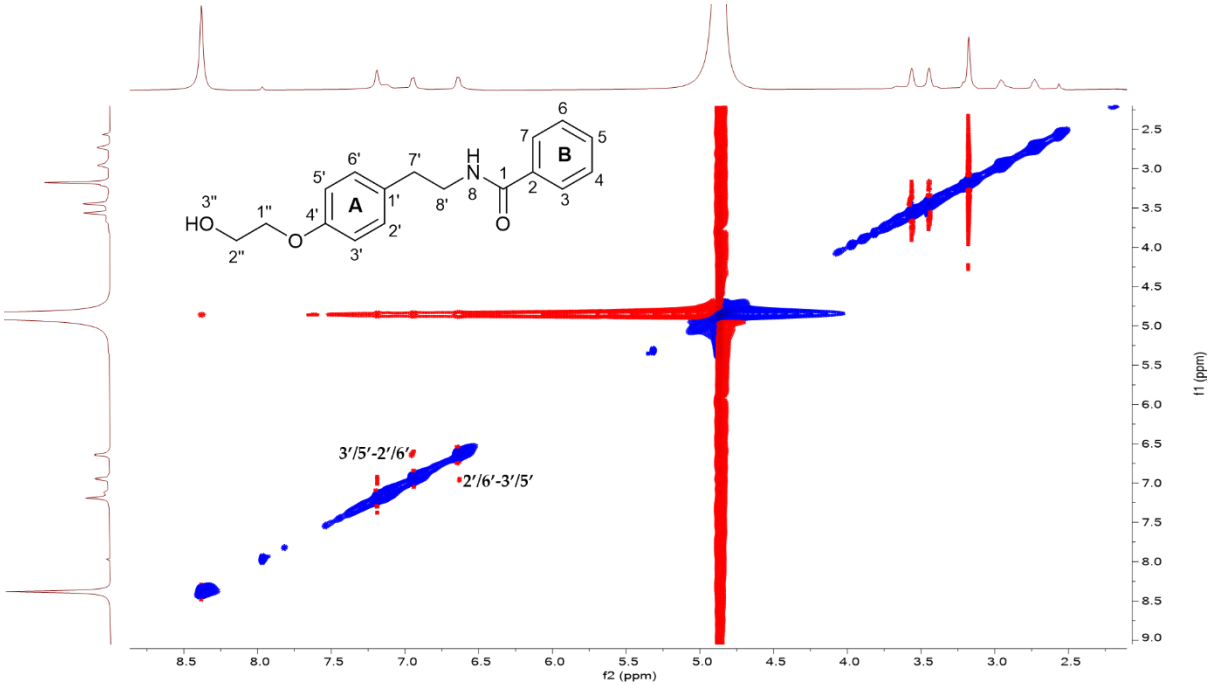

Figure S13: 500 MHz ROESY NMR spectrum of compound **1** in CD_3OD.

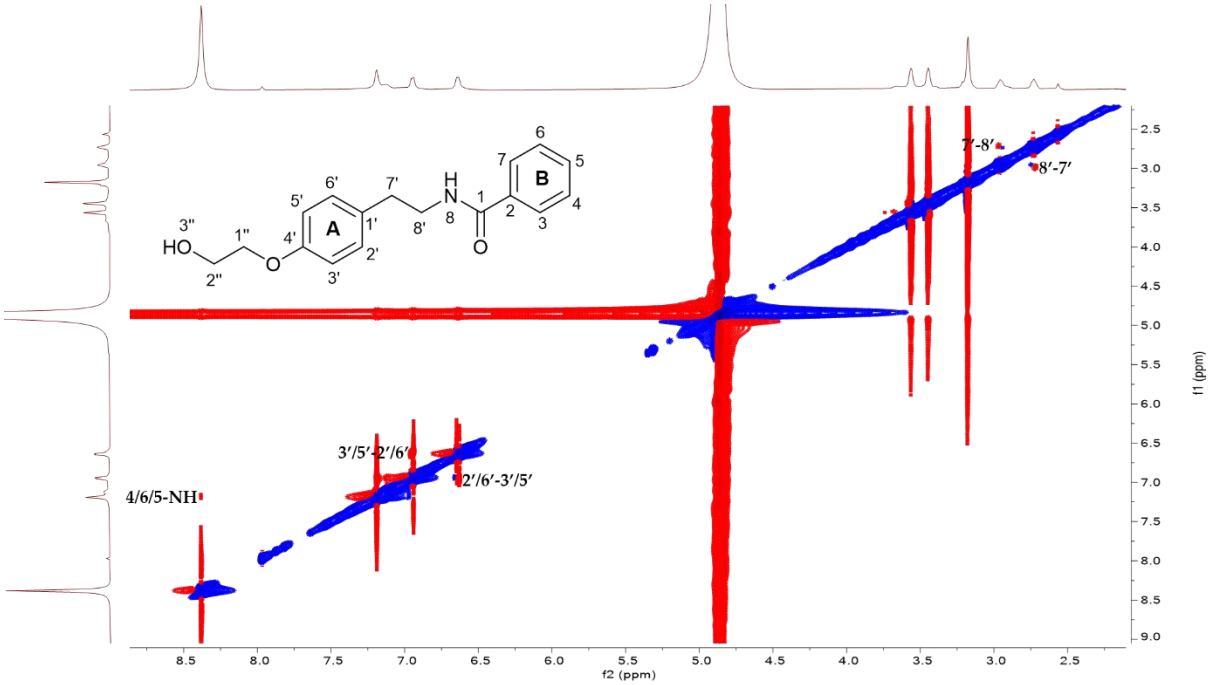

Figure S14: 500 MHz ROESY NMR spectrum of compound **1** in CD_3OD.

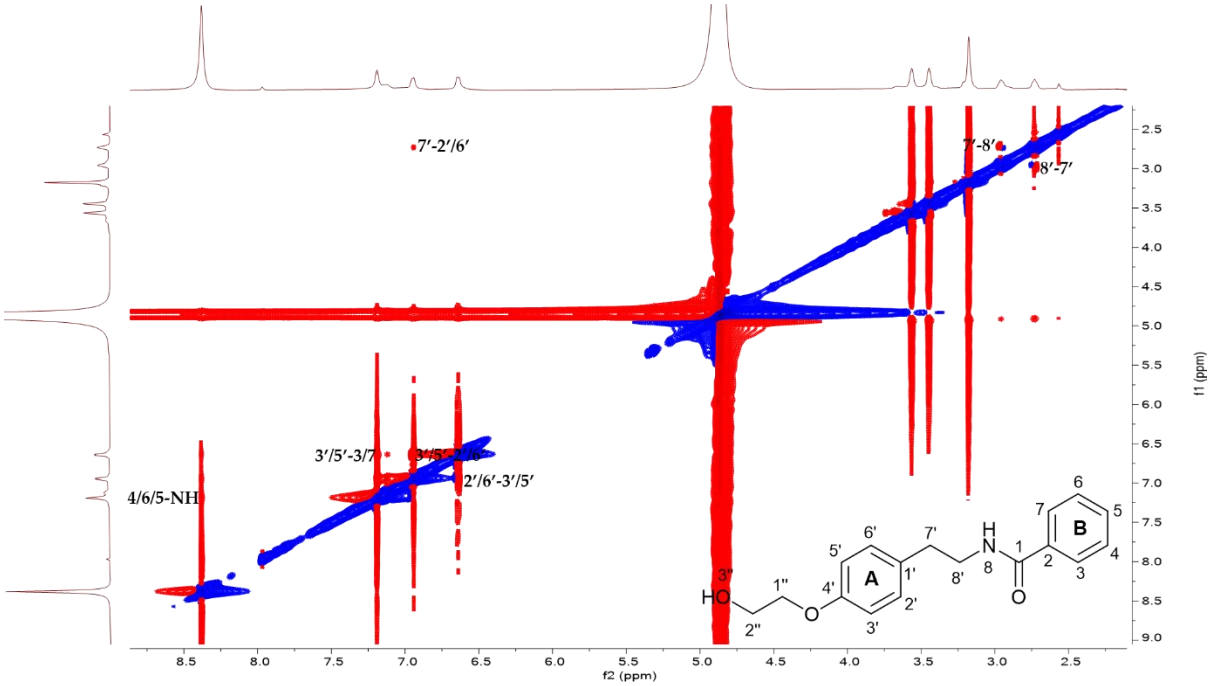

Figure S15: 500 MHz ROESY NMR spectrum of compound **1** in CD_3OD.

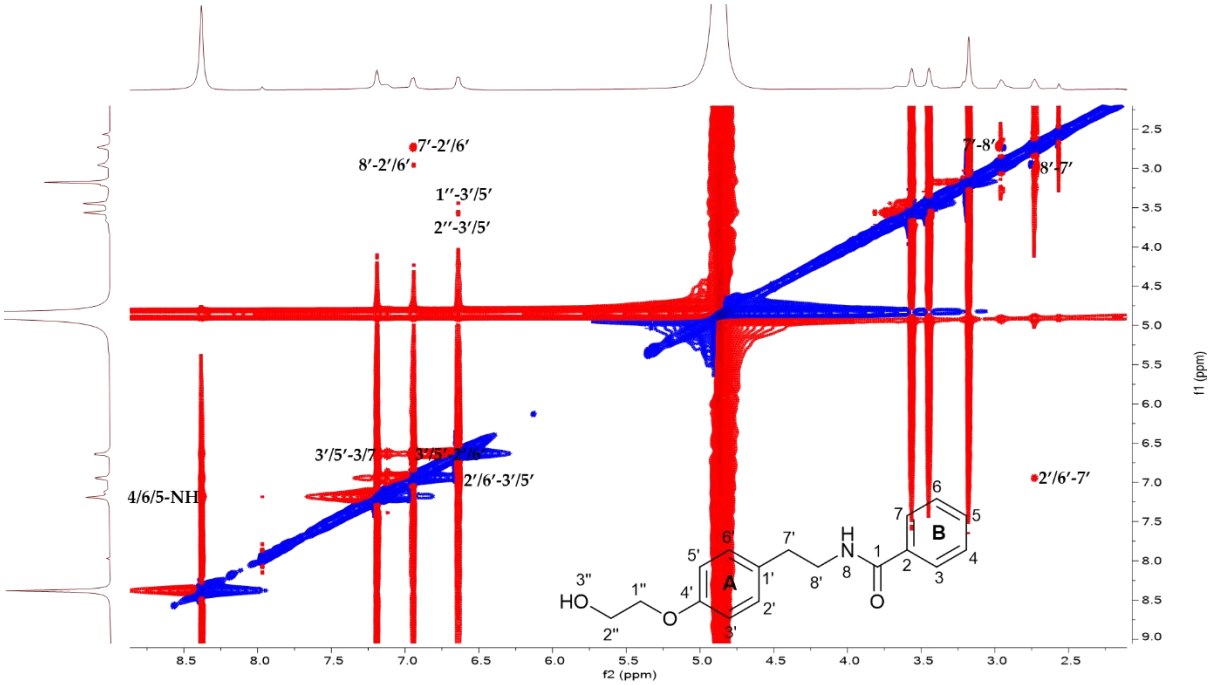

Figure S16: 500 MHz ROESY NMR spectrum of compound **1** in CD_3OD.

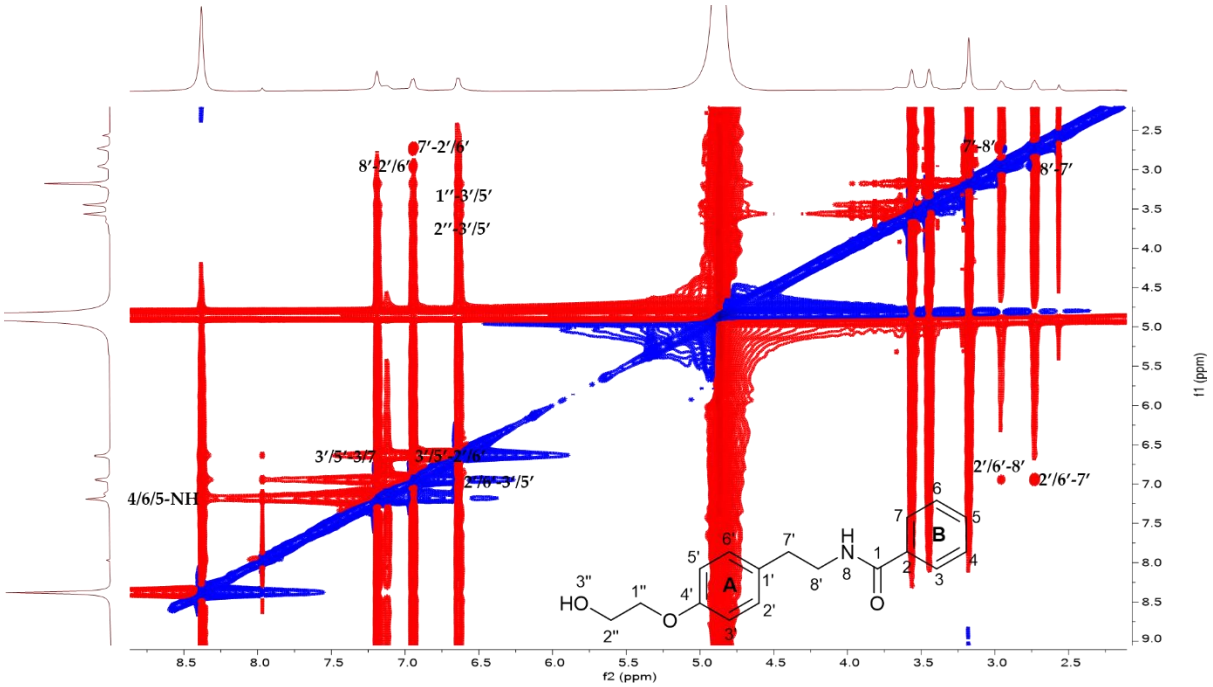

Figure S17: 500 MHz ROESY NMR spectrum of compound **1** in CD_3OD.

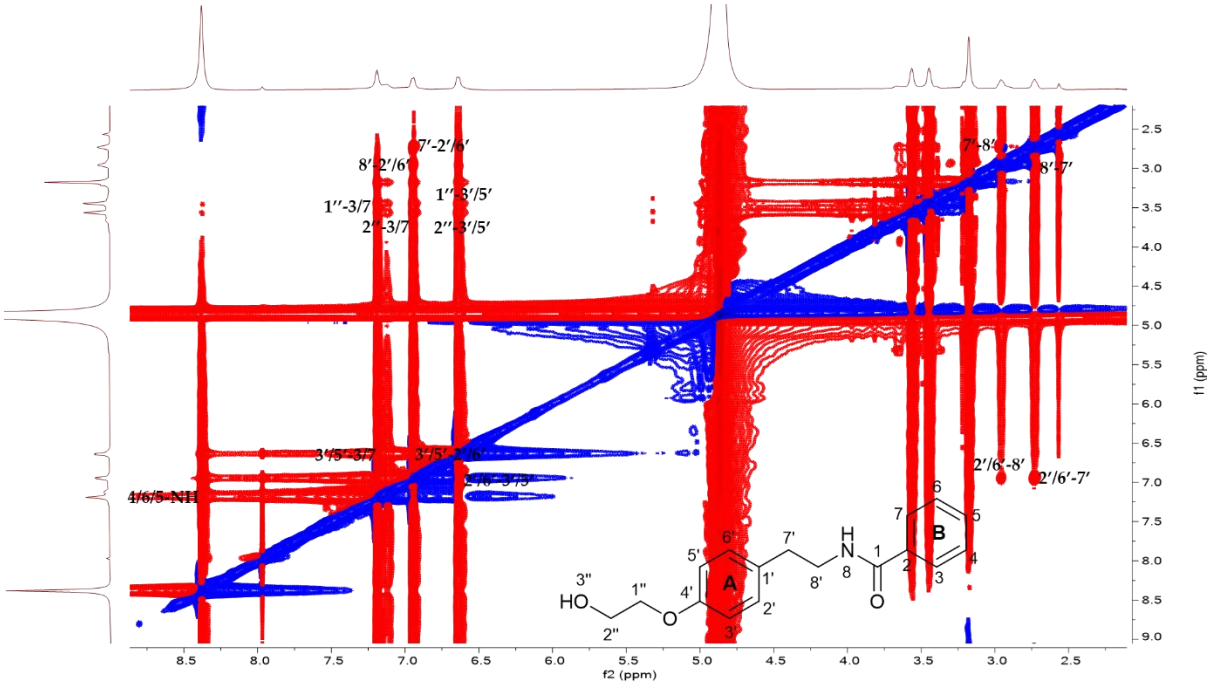

Figure S18: 500 MHz ROESY NMR spectrum of compound **1** in CD_3OD.

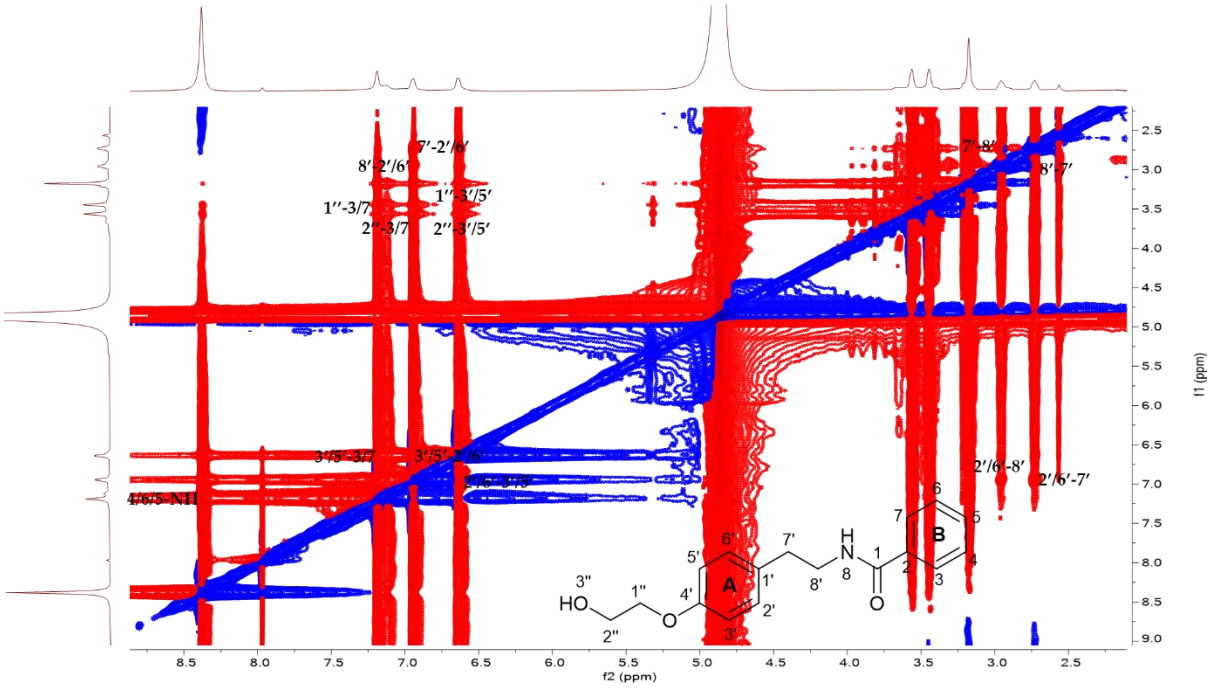

Figure S19: 500 MHz ROESY NMR spectrum of compound **1** in CD_3OD.

Preparation of the compound for bioassay

A 10 mM stock solution of compound **1** was prepared by weighing the dried sample using a mass balance (AND GH-120, A and D Company Limited, Tokyo, JAPAN) and dissolving in an appropriate volume of dimethyl sulfoxide (DMSO) to obtain the concentration. The solution was vortexed (MSI Minishaker, IKA Company, Osaka, JAPAN), filter-sterilized through 0.45 µm Millipore filters into vials and stored at −20 °C until used. Subsequently, the desired concentration was prepared from the stock for biological activity assays.

Cell viability assay for bloodstream forms *Trypanosoma brucei*

The IC_{50} of compound **1** defined as the concentration that inhibits the growth of 50% of *Trypanosome* parasites, was determined as previously described by Amisigo et al. 2019 [9] by incubating trypanosomes at a density of 4×10^3 cells in triplicate without or with the compound at varying concentrations (100–0.196 µg/mL) and kept at 37 °C for 72 hours. AlamarBlue test was used to determine parasite viability. At an excitation wavelength of 530 nm and an emission wavelength of 590 nm, fluorescence was measured using a Varioskan Lux Elisa microplate reader (Thermo Fischer Scientific, USA). The fluorescence counts were plotted against drug concentrations, and the IC_{50} was calculated using GraphPad Prism version 9 dose-response curve analysis. The data represents the mean of three independent experiments (Figure 4).

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