Isolation of griseofulvin from the endolichenic fungus *Cubamyces menziesii* (Berk.) Lücking inhabiting *Parmotrema rampoddense* (Nyl.) Hale

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ABSTRACT

Endolichenic fungi represent valuable sources for the discovery of diverse and biologically relevant natural products with significant applications in pharmaceuticals, agriculture, and industry. In this study, *Cubamyces menziesii* (Berk.) Lücking, an endolichenic fungus isolated from the lichen *Parmotrema rampoddense* (Nyl.) Hale, was subjected to extraction and chromatographic purification to obtain griseofulvin (**1**) and acetyl tributyl citrate (**2**). The antimicrobial activity of the isolated compounds was assessed using the microtiter plate antimicrobial assay showing 125 – 250 µg/mL minimum inhibitory concentration and minimum bactericidal concentration in *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923. This is also the first report of griseofulvin (**1**), a commercially available antifungal drug, from the genus *Cubamyces*. The findings highlight the potential of *C. menziesii* as a promising resource for bioactive compounds, with implications for the development of novel antimicrobial agents for medical and industrial applications.

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INTRODUCTION

Endolichenic fungi, often seen as similar to fungal endophytes in plants, thrive within the thalli of lichens, forming a symbiotic relationship. They are as diverse as fungal endophytes with single species reported in a lichen host indicating some level of host specificity (Oh et al. 2020). Certain endolichenic fungi adapt to particular lichen hosts, similar to the host specificity observed in fungal endophytes in plants. The functions of endolichenic fungi within the lichen symbiosis are still limitedly studied. However, they are recognized as valuable sources of bioactive metabolites (dela Cruz and Santiago 2021). They have

KEYWORDS

Cubamyces, Endolichenic fungi, Griseofulvin, *Trametes*

been reported to produce compounds distinct from their lichen hosts (Santiago et al. 2021) which can aid and protect the lichen symbionts from other invading microorganisms (Galinato et al. 2021; Santiago et al. 2021). It is plausible that these endolichenic fungi contribute to the overall health and function of the lichen thalli, playing roles in nutrient exchange, protection, or other aspects of symbiotic relationship.

Species within the lichen *Parmotrema* have been reported to exhibit biological activities (Saha et al. 2021). Due to its leafy thalli, *Parmotrema* is considered ideal for the isolation of endolichenic fungi. Several studies reported *Parmotrema*-associated fungi and their biological activities, e.g., *Lecythophora* sp. isolated from *Parmotrema tinctorum* had ATPase inhibitory activity (Kithsiri Wijeratne et al. 2016). An endolichenic *Aspergillus niger* associated with *Parmotrema ravum* has novel antimicrobial metabolites (Padhi et al. 2020), and possibly a novel fungal species with photoprotective properties has been reported in *Parmotrema austrosinense* (Zhao et al. 2017). Our previous study with three endolichenic taxa, *Fusarium proliferatum*, *Nemania primolutea*, and *Daldinia eschscholtzii*, isolated from *Parmotrema rampoddense*, reported antibacterial activities against ESKAPE bacterial pathogens, and the isolation and identification of bis(2-ethylhexyl)terephthalate, acetyl tributyl citrate, and fusarubin (Tan et al. 2020). Basidiomycetous fungi have already been reported as endolichenic fungi with *Trametes versicolor* as among the dominantly isolated species in *Parmotrema tinctorum* (Yang et al. 2021). Compounds derived from fruiting bodies of *Trametes cubensis* have shown anti-inflammatory activities (Li et al. 2021) while an endophytic strain from leaves of *Hevea* spp. showed cellulolytic activity (De Oliveira Amaral et al. 2022).

We herein report the isolation of griseofulvin (**1**) and acetyl tributyl citrate (**2**) from the endolichenic fungus *Cubamyces menziesii* [=*Trametes menziessi* (Berk.) Ryvarden], sourced from *Parmotrema rampoddense*, and their antimicrobial activity against *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923.

MATERIAL AND METHODS

General Experimental Procedure

^{1}H- and ^{13}C-NMR were measured on an ECZ 600 FT-NMR spectrometer using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Mass spectrometry was recorded on a JEOL AccuTOF LC-plus JMS-T100LP spectrometer. Optical rotation was measured using a JASCO P-2200 polarimeter. Thin layer chromatography was performed in Merck 60 F254 aluminum-backed precoated silica gel plates and visualized in UV_{254} followed by vanillin-sulfuric acid with heating. Column chromatography was performed in Merck silica gel 7734 or Merck silica gel 9385. Analytical grade solvents (> 98% purity) were used in extraction and chromatography.

Molecular Identification of the Endolichenic Fungus

The endolichenic fungus *C. menziesii* was isolated from the foliose lichen *P. rampoddense*, which was previously collected from Sagada, Mountain Province, Philippines. Detailed method for the isolation was earlier described in our previous study (Tan et al. 2020). Identification of species was done through molecular methods. Briefly, live cultures of the endolichenic fungi stain ELF1B were sent to Macrogen, South Korea for sequencing of the ITS genes with the primer pair, ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). Sequences were then assembled and searched using BLAST. Phylogenetic trees were constructed using maximum likelihood and Bayesian analysis. To do this, sequences were aligned using MAFFT (Katoh and Standley 2013) and cleaned up by BMGE (Criscuolo and Gribaldo 2010). Using Smart Model Selection, the best model of evolution was determined to be GTR+G+I. Maximum likelihood was performed using PhyML with 1000 bootstrap replications. All of these procedures were run in NGPhylogeny.fr. For Bayesian analysis, MrBayes 3.2 was used to run six simultaneous Markov chains for 1,000,000 generations, sampled every 100th generation, generating 10,000 trees. Burn-in phase discarded the first 2,000 trees and the remaining trees were used to calculate the posterior probability. MrBayes 3.2 was run using the CIPRES Science Gateway. Generated phylogenetic tree (Figure 1) was viewed by using FigTree.

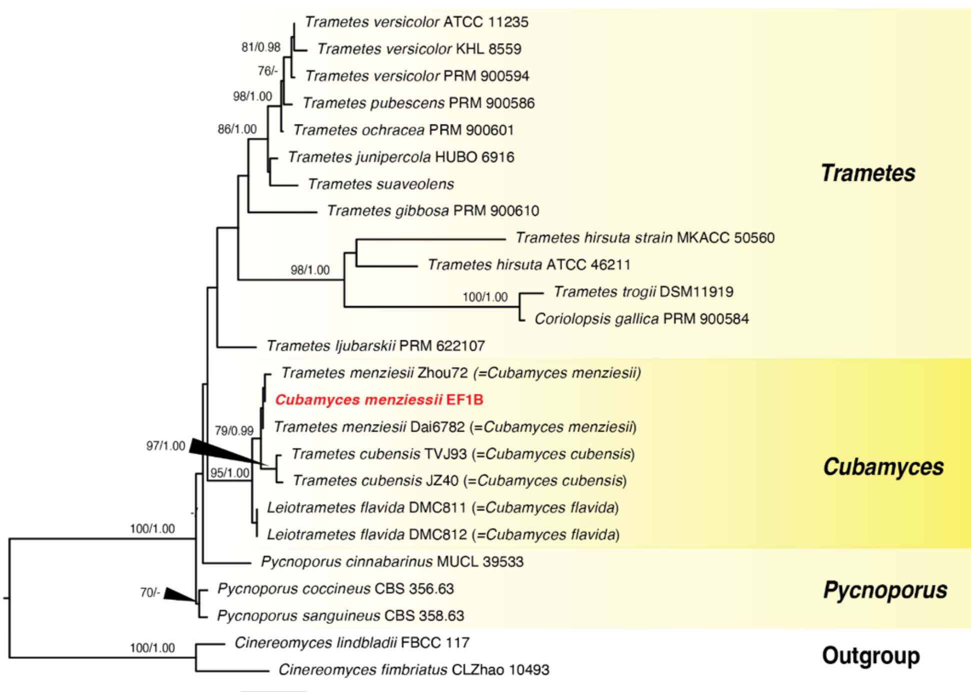


Figure 1: Phylogram of *Cubamyces* and closely-related genera based on ITS sequence data. Values above the branches indicate bootstrap support from Maximum Likelihood (ML), bootstrap support and Bayesian posterior probability (PP). Only values equal to 70 or higher for ML and 0.80 or higher for PP are shown. The isolate in this study is marked in bold red. The current accepted names for *Cubamyces* species are indicated inside the parentheses (Lücking et al. 2020). The tree is rooted with *Cinereomyces lindbladii* (Berk.) Jülich FBCC 117 and *Cinereomyces fimbriatus* C.L. Zhao 10493.

Mass Production of Endolichenic Fungi *Cubamyces menziesii*

Sterilized glass bottles containing malt extract and peptone were inoculated with *C. menziesii* ELF1B agar blocks. These were incubated in a slant position at room temperature and were allowed to grow for 3 weeks. The mycelia were then separated from the broth culture. An equal volume of ethyl acetate was added to the broth and was made to stand for 24 h. The separated mycelia were ground and submerged in ethyl acetate, with occasional shaking for 24 hours, and filtered. The ethyl acetate fractions from broth and mycelia were combined, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure yielding the *C. menziesii* crude extract.

Purification of the *Cubamyces menziesii* Crude Extract

C. menziesii crude extract (402.3 mg) was subjected to silica gel column chromatography using gradient increments of hexane in ethyl acetate (5%, 15%, 25%, 40%, 70%), neat ethyl acetate, and 1:1 ethyl acetate/methanol as eluents. Thin layer chromatography of the collected fractions visualized under UV_{254} and sprayed with vanillin-sulfuric acid, followed by charring afforded eight pooled fractions, Fr. A – H. Fr. B (**2**, colorless oil, 11 mg) was deemed TLC-pure having a single spot in various solvent systems and was identified as acetyl tributyl citrate (Tan et al. 2020). The NMR and MS spectra of compound **2** are given in the Supplementary Material. Fr. G was re-chromatographed in silica gel by isocratic elution using hexane/ethyl acetate (1:1) to yield griseofulvin (**1**, white solid, 14.3 mg).

Compound **1** (Griseofulvin): See Table 1.

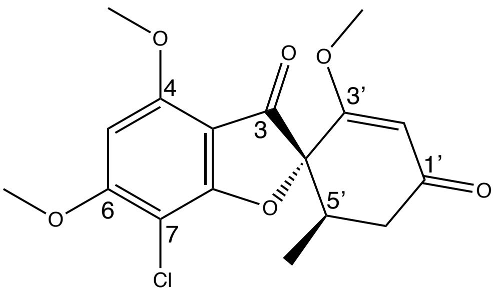
Compound **2** (Acetyl tributyl citrate): ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H, integration, mult. (*J* in Hz): 4.15, 2H, t (*J* = 8) H-1; 4.08, 4H, t (*J* = 8), H-2; 3.29, 2H, d (*J* =16), H-3; 3.19, 2H, d (*J* =16), H-4; 2.05, 3H, s, H-5; 1.61, 6H, m, H-6; 1.37, 6H, m, H-7; 0.92, 9H, t (*J* =8), H-8. ^{13}C (125 MHz, $CDCl_3$): δ 169.6; 169.2; 78.3; 66.1; 64.9; 38.8; 30.7; 30.4; 21.0; 19.2; 13.8.

Microtiter Plate Antimicrobial Assay

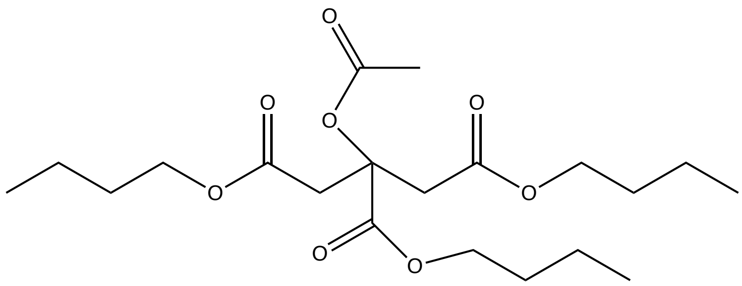
Determination of the minimum inhibitory concentration and minimum bactericidal concentration in triplicate was done using the microtiter plate method as previously published (Tan et al. 2020), utilizing *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923 in 1 x 10^6 CFU/mL. Briefly, 1000 μg/mL stock solution of compounds **1** and **2** was serially diluted to obtain 500, 250, 125, 62.5, 31.3, 15.6, 7.8, and 3.9 μg/mL concentrations in a 96-well plate. Incubation was done at 37°C for 24 hours. The wells without visible bacterial growth after 24 hours were labeled as MIC, and were inoculated onto a Mueller-Hinton agar (MHA) plate to determine the MBC. The plates without visible bacterial growth after 24 hours were the MBC of the sample. Ciprofloxacin (150 μg/mL) was used as the positive control (MIC and MBC ≤ 3.91 μg/mL), while DMSO was used as the negative control (MIC and MBC >500 μg/mL).

RESULTS AND DISCUSSION

In our continuous search for biologically active compounds from microbial sources (Bungihan et al. 2011; Bunguihan et al. 2013; Tan et al. 2015; Tan et al. 2020), we have embarked on the chemical characterization of the endolichenic fungus *C. menziesii*. The endolichenic fungus was determined with high support using molecular techniques as shown in Figure 1 (*vide supra*). Two compounds, griseofulvin (**1**) and acetyl tributyl citrate (**2**) (Figure 2), were isolated and purified from the combined ethyl acetate extract of the broth and mycelia of *C. menziesii*. Their structures were elucidated based on 1D and 2D NMR, mass spectrometry, and in comparison with published data. We have previously identified compound **2** from the endolichenic fungus *Fusarium proliferatum* (Tan et al. 2020). The ^{1}H and ^{13}C NMR spectra of compound 2 are shown in Figures S7 – S9 (Supplementary Material).



Griseofulvin (**1**)



Acetyl tributyl citrate (**2**)

Figure 2: Isolated compounds from the ethyl acetate extract of *Cubamyces menziesii*.

Griseofulvin (**1**) was isolated as white solid with an observed optical rotation of $[\alpha]_D^{25}$ +363^0 (*c* 1.0, $CHCl_3$) and a molecular formula of $C_{17}H_{17}ClO_6$ (HR-ESIMS: $[M+Na]^+$ *m/z* 375.1405, calculated for $[C_{17}H_{17}ClO_6Na]^+$ *m/z* 375.1431, mass difference of Δm = –0.0026 amu or -6.9 ppm). The ^{1}H-NMR (Table 1) showed an aromatic proton (δ_H 6.12, 1H, s, H-5), an olefinic proton (δ_H 5.53, 1H, s, H-2’), three methoxy groups (δ_H 4.02, 3H, s; δ_H 3.97, 3H, s; δ_H 3.60, 3H, s), and a secondary methyl (δ_H 0.94, 3H, d, 6.8 Hz). The ^{13}C-NMR (Table 1) showed characteristic signals attributed to a benzene (δ_C 89.6, 97.4, 105.0, 157.8, 164.7, 169.5) fused with the cyclopentanopyranone ring (δ_C 90.8, 192.5), an α,β-unsaturated lactone (δ_C 105.2, 170.8, 197.0), a secondary methyl (δ_C 14.3), and three methoxy groups (δ_C 56.5, 56.8, 57.1). As shown in Figure 3, heteronuclear multiple bond correlations (HMBC) of H-5’ (δ_H 2.84) with the C-3 carbonyl (δ_C 192.5) and the quaternary carbon at C-2 (δ_C 90.8) have elucidated the cyclopentanopyranone ring. The α,β-unsaturated lactone moiety was deduced based on the HMBC correlation of H_2-6’ (δ_H 3.03 and δ_H 2.43) with δ_C 197.0 and δ_C 105.2; and H-2’ (δ_H 5.53) with δ_C 197.0 and δ_C 170.8. Significant COSY and HMBC correlations in **1** are shown in Figure 3. The 2D NMR spectra of compound **1** are shown in Figures S3-S5 (Supplementary Material). The ^{1}H NMR spectrum and optical rotation of **1** are also in agreement with the published spectrum of griseofulvin (Oliveira et al. 2015; Townley et al. 1979). Hence, the structure of **1** was deduced as griseofulvin.

Table 1: ^1H and ^{13}C NMR data of Griseofulvin (1) in $CDCl_3$

Position	^{13}C NMR (150 MHz)	^1H NMR (600 MHz, δ in ppm, *J* in Hz)
2	90.8	
3	192.5	
3a	105.0	
4	164.7	
5	89.6	6.12, 1H, s
6	157.8	
7	97.4	
7a	169.5	
1'	197.0	
2'	105.2	5.53, 1H, s
3'	170.8	
5'	36.5	2.84, 1H, m
6'	40.1	3.03, 1H, dd (*J* = 13.6, 16.4) 2.43, 1H, dd (*J* = 4.8, 16.4)
5'-CH_3	14.3	0.94, 3H, d (*J* = 6.8)
3'-OCH_3	57.1	3.63, 3H, s
4-OCH_3	56.8[a]	3.97, 3H, s
6-OCH_3	56.5[a]	4.02, 3H, s

[a]- interchangeable

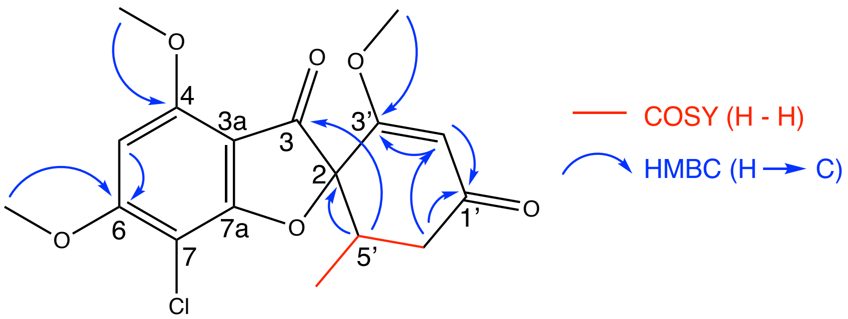


Figure 3: COSY and HMBC correlations in griseofulvin (1).

The two compounds showed antimicrobial activity in *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* using the microtiter plate method. A 250 µg/mL MIC and MBC were observed in *S. aureus* and *K. pneumoniae* for both compounds. In *P. aeruginosa*, compound **1** showed 125 µg/mL MIC and MBC, while compound **2** gave an MIC of 125 µg/mL and MBC of 250 µg/mL.

Interestingly, griseofulvin (**1**) is a well-known antifungal drug being used to treat ringworm and dermatophytosis in humans and animals (Aris et al. 2022; Petersen et al. 2014). It was previously isolated from the genus *Penicillium* and other ascomycetes such as *Xylaria flabelliformis*, *Abieticola koreana*, and *Stachybotrys levispora* (Aris et al. 2022). To the best of our knowledge, this is the first report on the isolation of griseofulvin from the genus *Cubamyces*. In griseofulvin biosynthetic gene cluster, several fungi species have identified its gene cluster distribution including genus *Penicillium* species (*P. coprophilum*, *P. griseofulvum*, *P. capsulatum*, and *P. aethiopicum*), *Aspergillus alliaceus*, *Memnoniella echinata*, and *Xylaria carpophila* (Aris et al. 2022). Hence, this is a pioneering study on the genus *Cubamyces* as a new source of griseofulvin (**1**). The biosynthetic pathway of griseofulvin (**1**), based on the gene cluster analysis using *P. aethiopicum*, has elaborated the *in vitro* synthesis of spirocyclic or grisan moiety, a scaffold responsible for the biological activity of griseofulvin (Cacho et al. 2013).

CONCLUSION

Endolichenic fungi are acknowledged for their crucial contribution in the discovery of diverse biologically active natural products. These fungi have proven to be highly sustainable for drug discovery, given their abundant nature and the ability to be cultivated within shorter periods under controlled conditions. In this study, griseofulvin (**1**), an antifungal drug, and acetyl tributyl citrate (**2**), were isolated from the endolichenic fungus *Cubamyces menziesii*. Both compounds exhibited weak antimicrobial activity against *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*. This study also represents the first report on the isolation of griseofulvin within the genus *Cubamyces*. Hence, this study highlights the importance of endolichenic fungi in drug discovery, emphasizing their vital applications in this field.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Conceptualization, MAT and TEDC; Methodology, JDDC, MAT; Validation, MAT, TEDC, JDDC; Formal analysis, MAT, CCSA, TEDC, HDM, Writing- original draft preparation, MAT, CCSA, TEDC; Writing – revision, MAT, TEDC, HDM.

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SUPPLEMENTARY INFORMATION

Isolation of griseofulvin from the endolichenic fungus *Trametes cubensis* inhabiting *Parmotrema rampoddense*

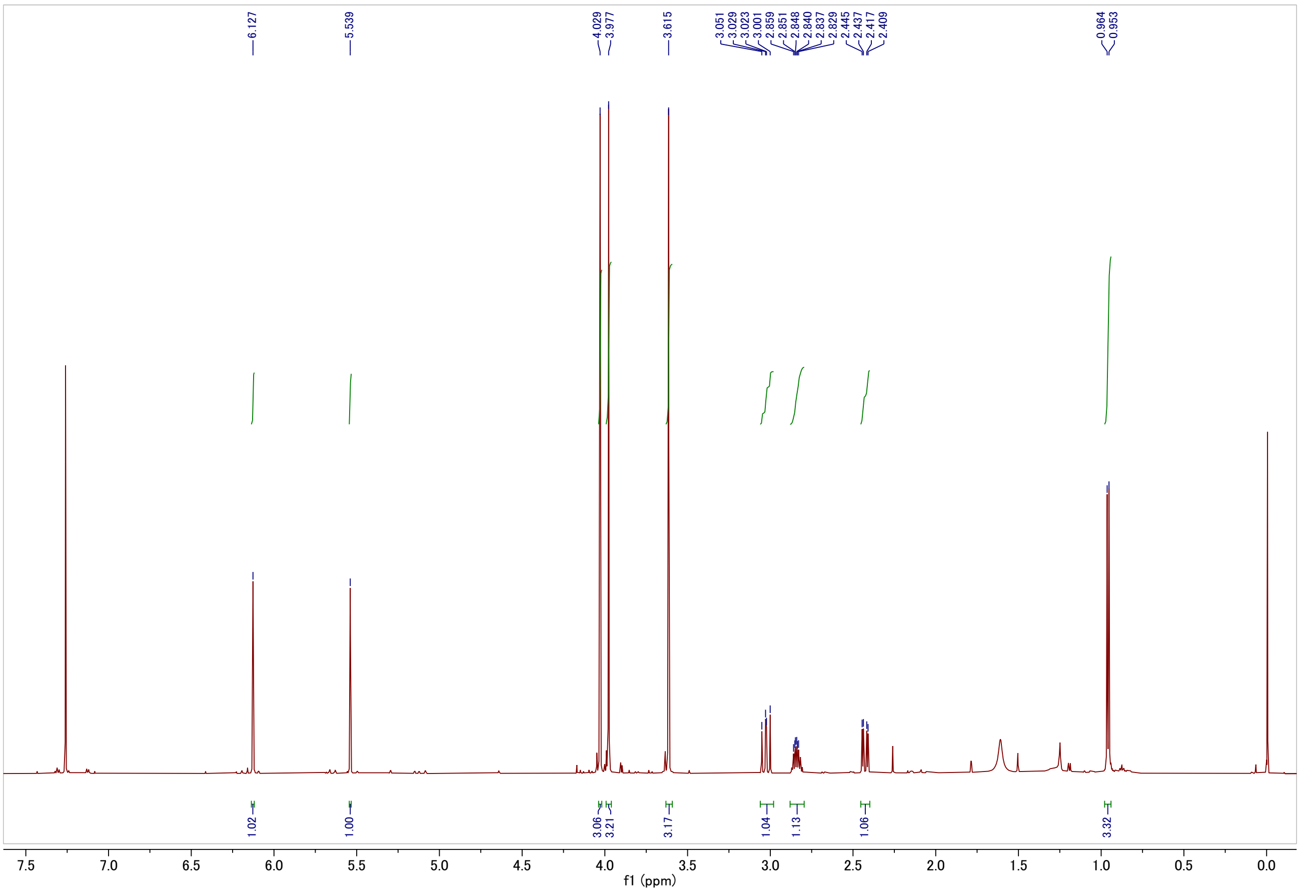

Figure S1: ^{1}H-NMR (600 MHz) of Compound 1 (Griseofulvin) in $CDCl_3$

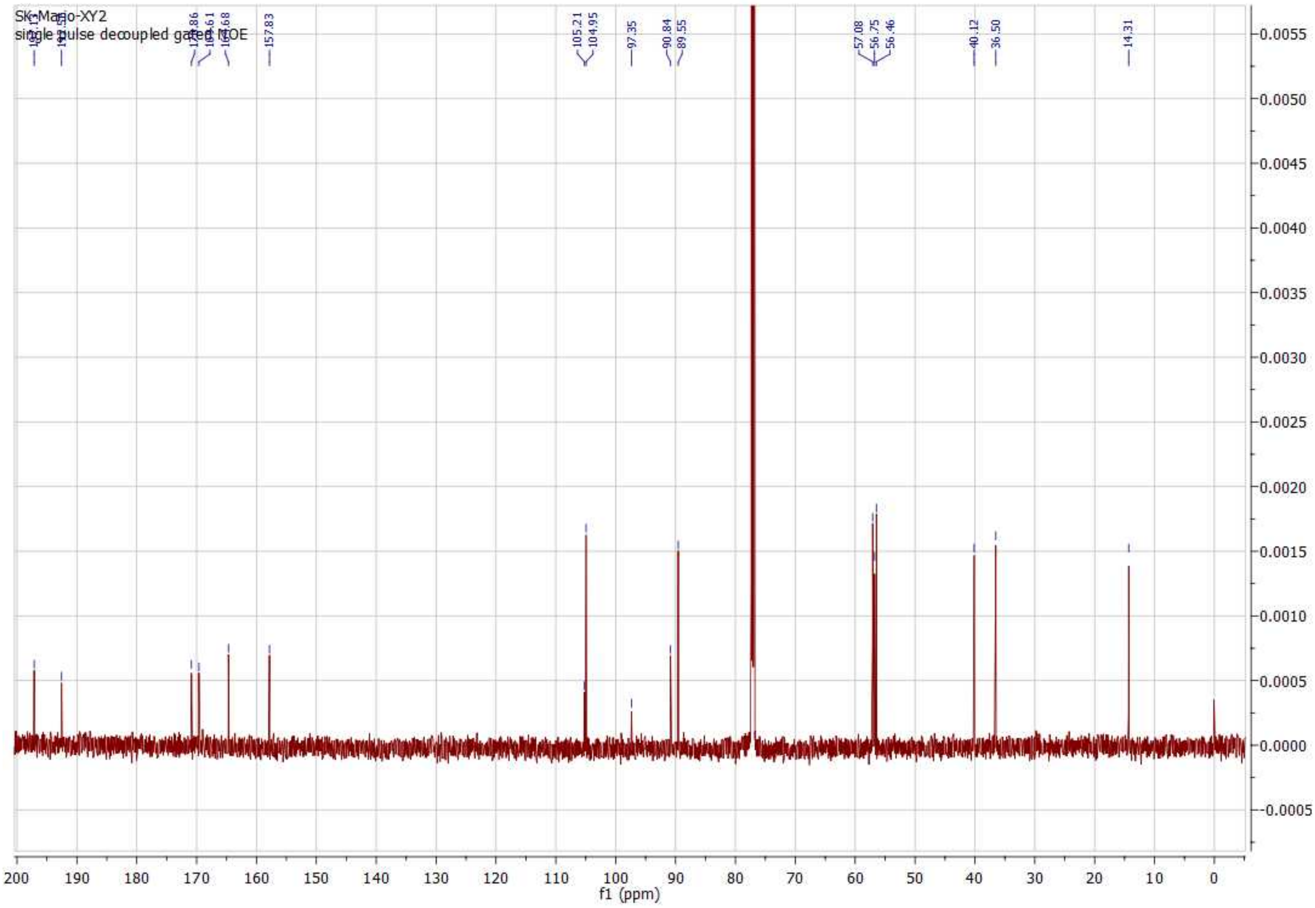

Figure S2: ^{13}C-NMR (150 MHz) of Compound 1 (Griseofulvin) in $CDCl_3$

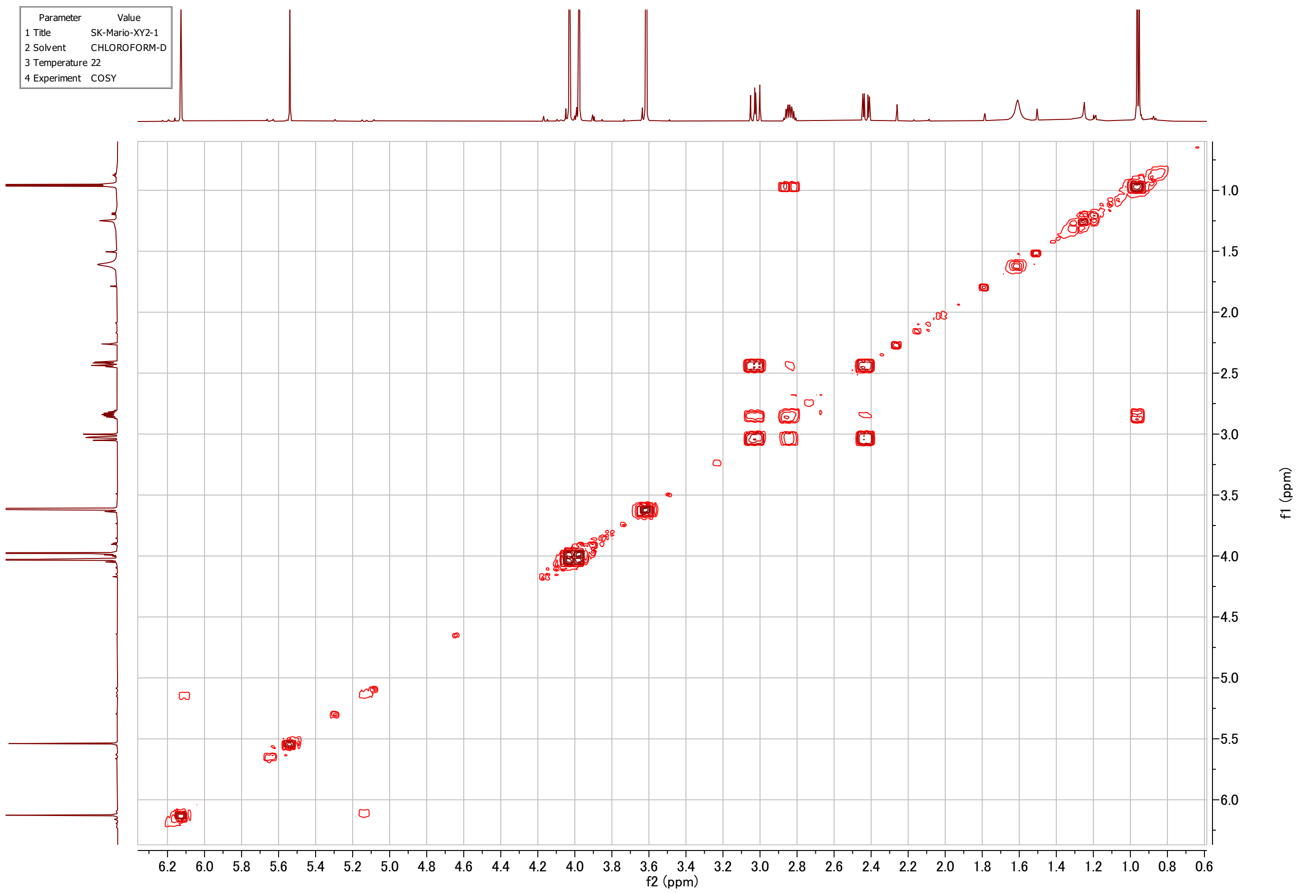

Figure S3: COSY of Compound 1 (Griseofulvin)

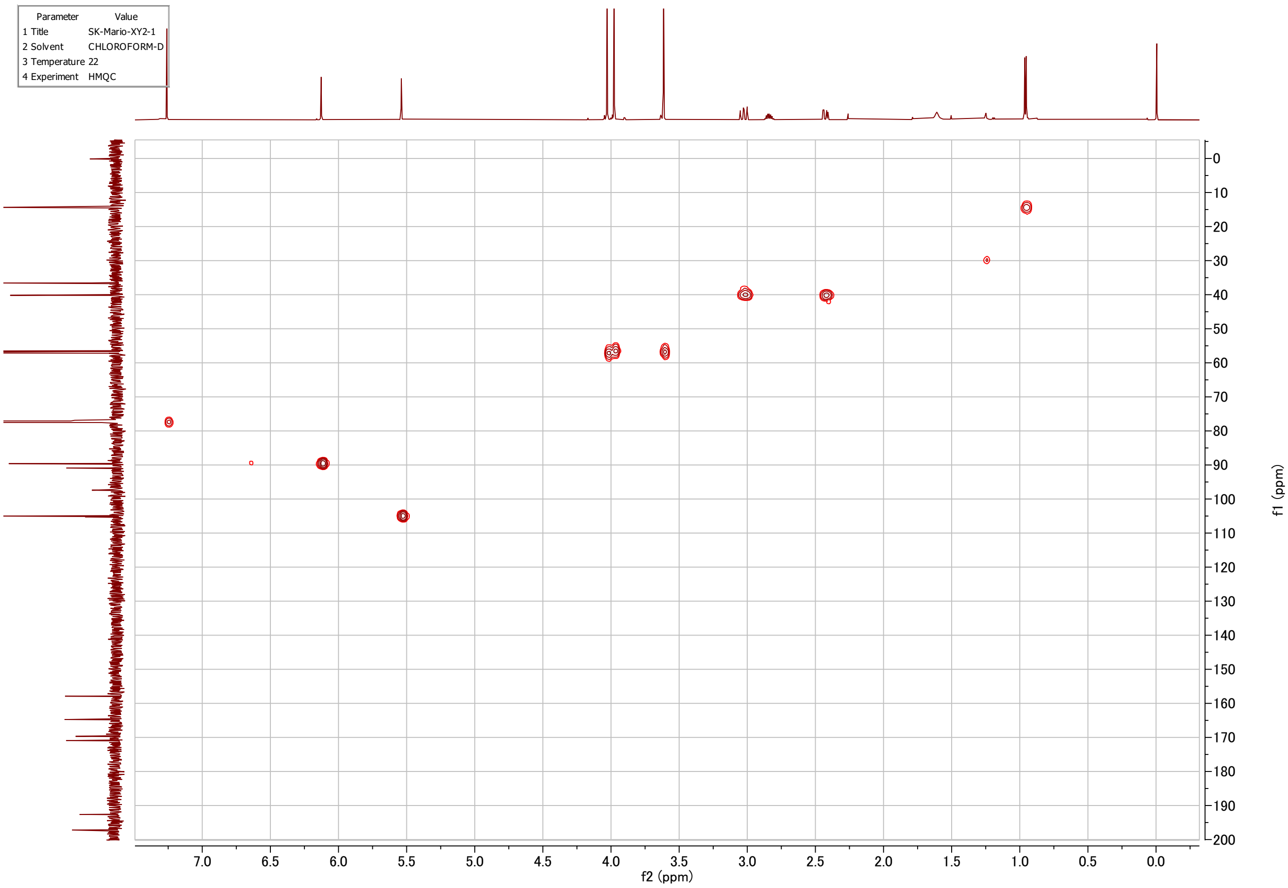

Figure S4: HMQC of Compound 1 (Griseofulvin)

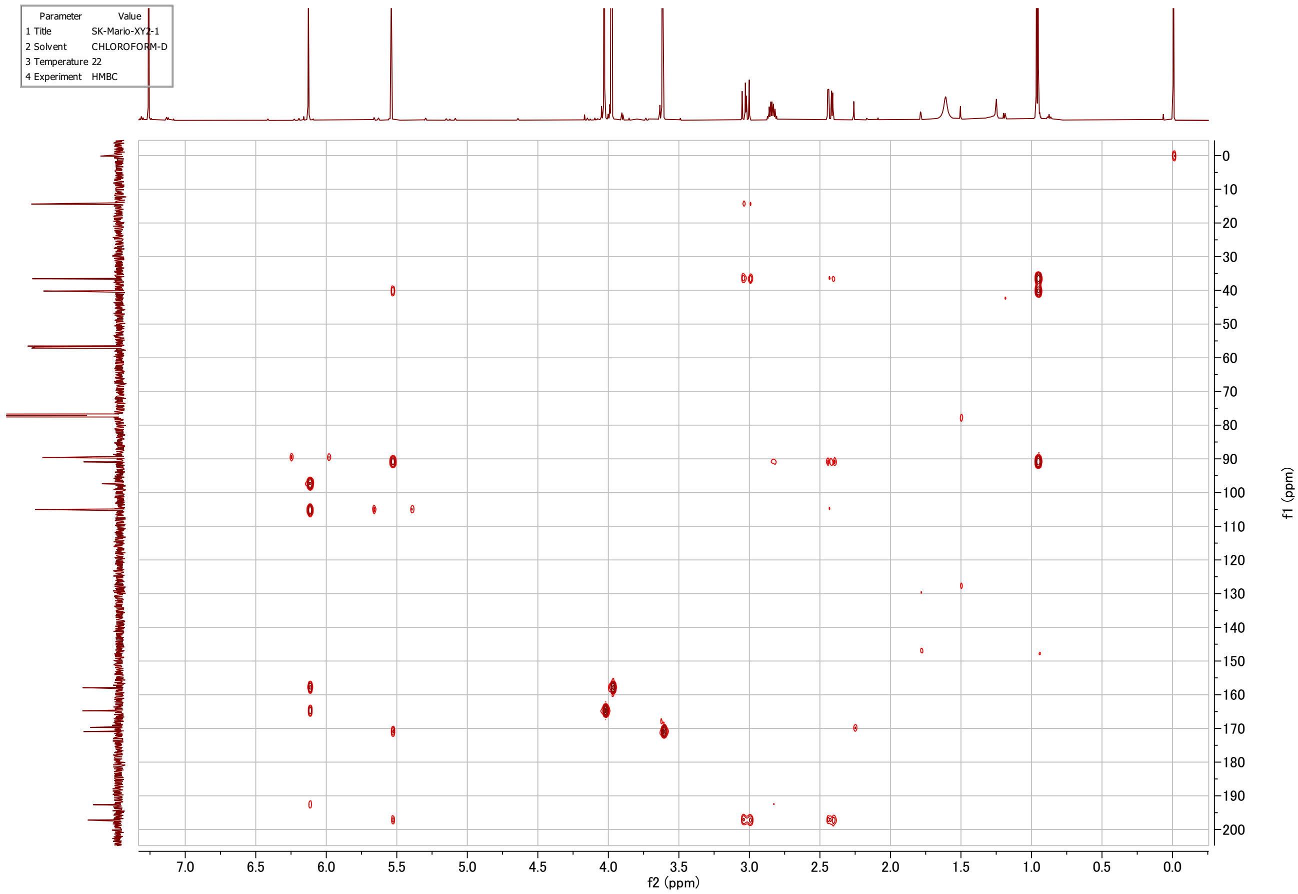

Figure S5: HMBC of Compound 1 (Griseofulvin)

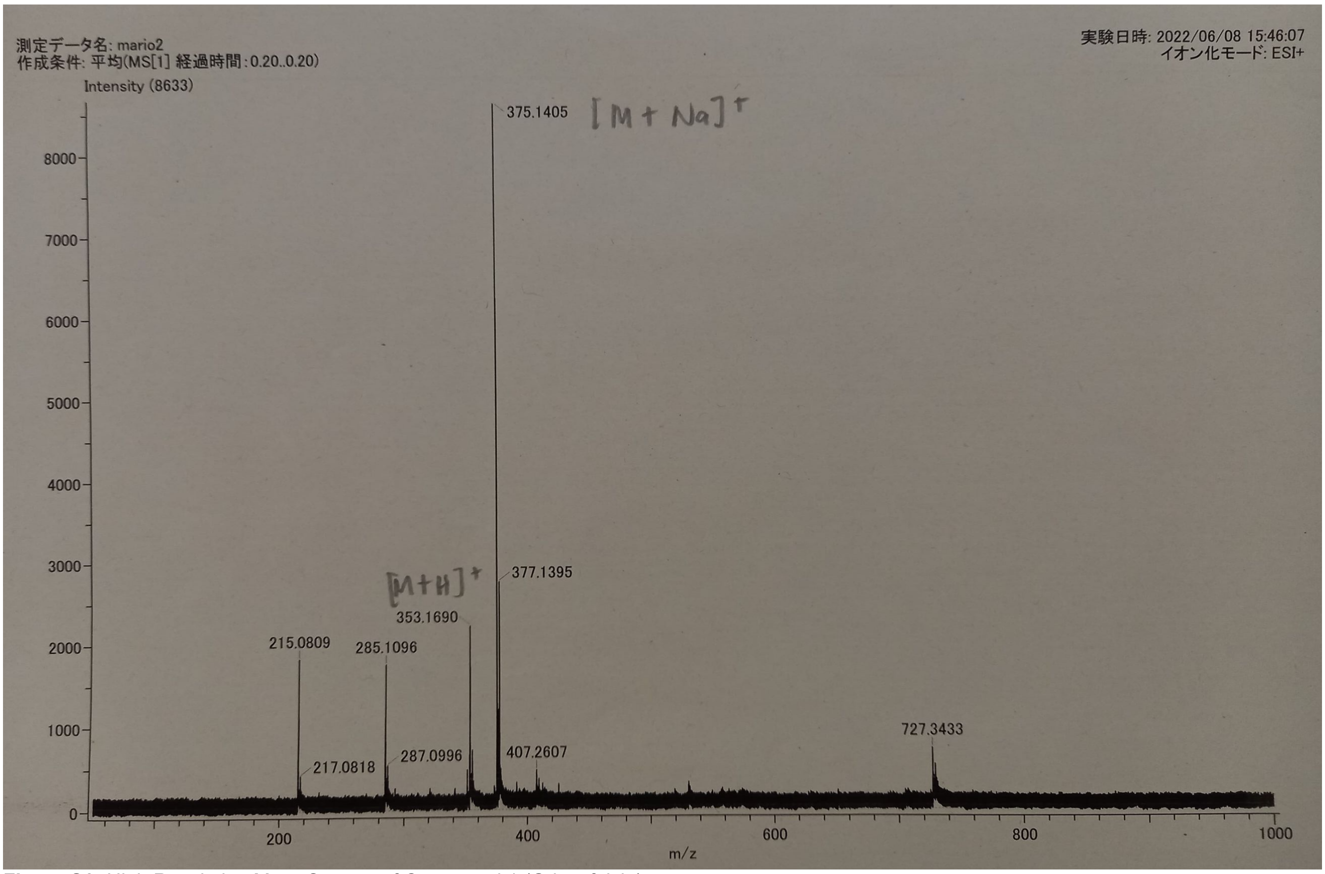

Figure S6: High Resolution Mass Spectra of Compound 1 (Griseofulvin)

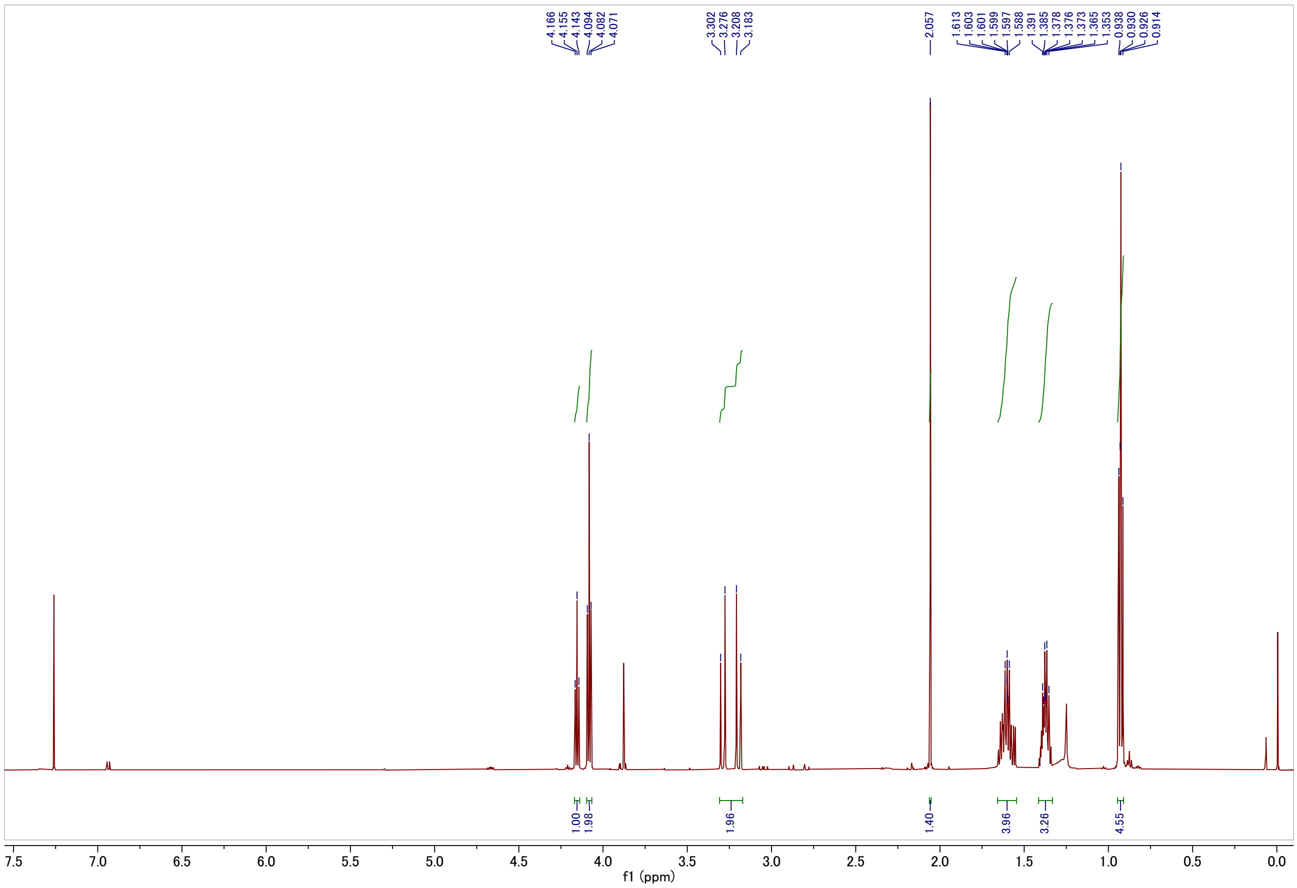

Figure S7: ^{1}H-NMR ($CDCl_3$, 600 MHz) of Compound 2 (Acetyl butyl citrate)

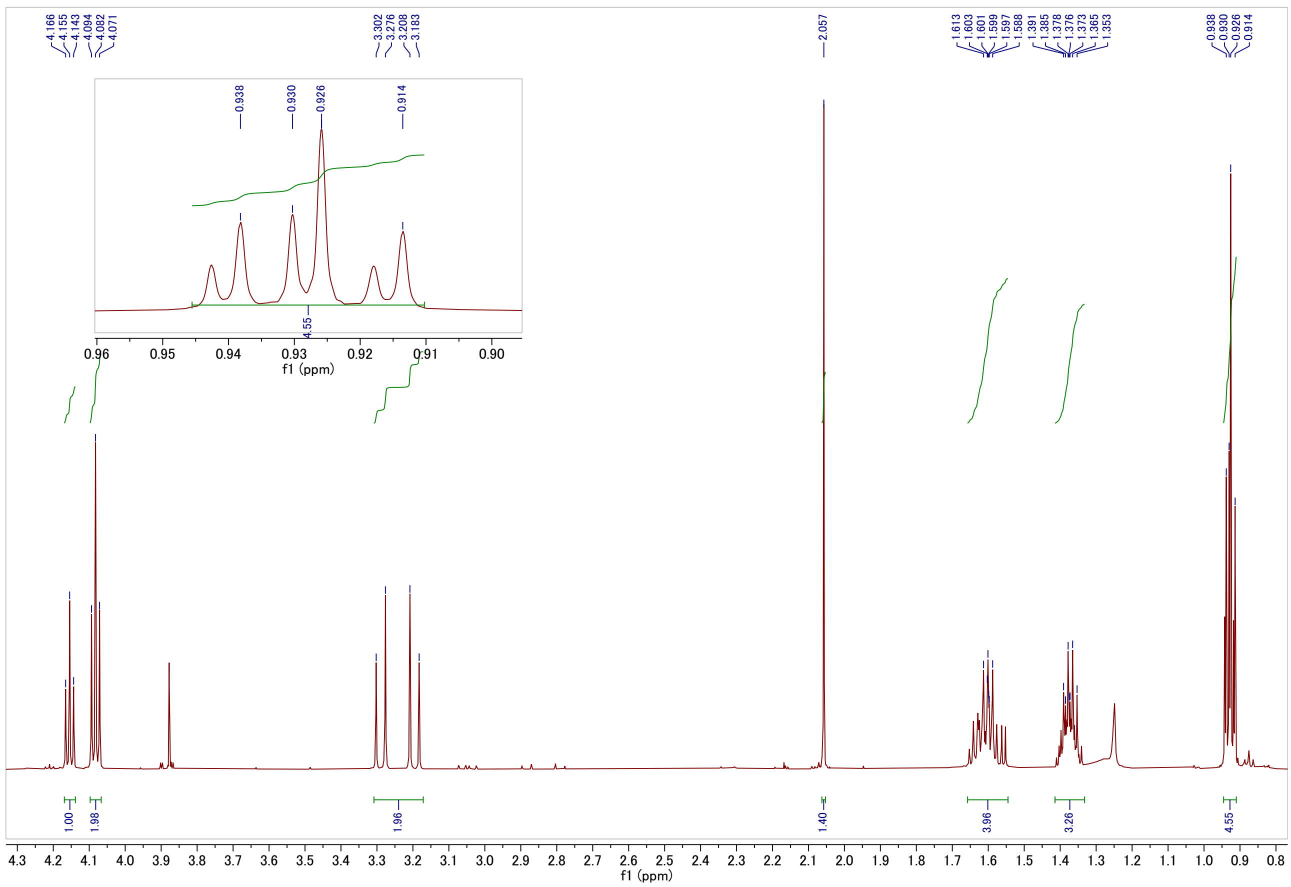

Figure S8: ^{1}H-NMR Expansion of Compound 2 (Acetyl butyl citrate)

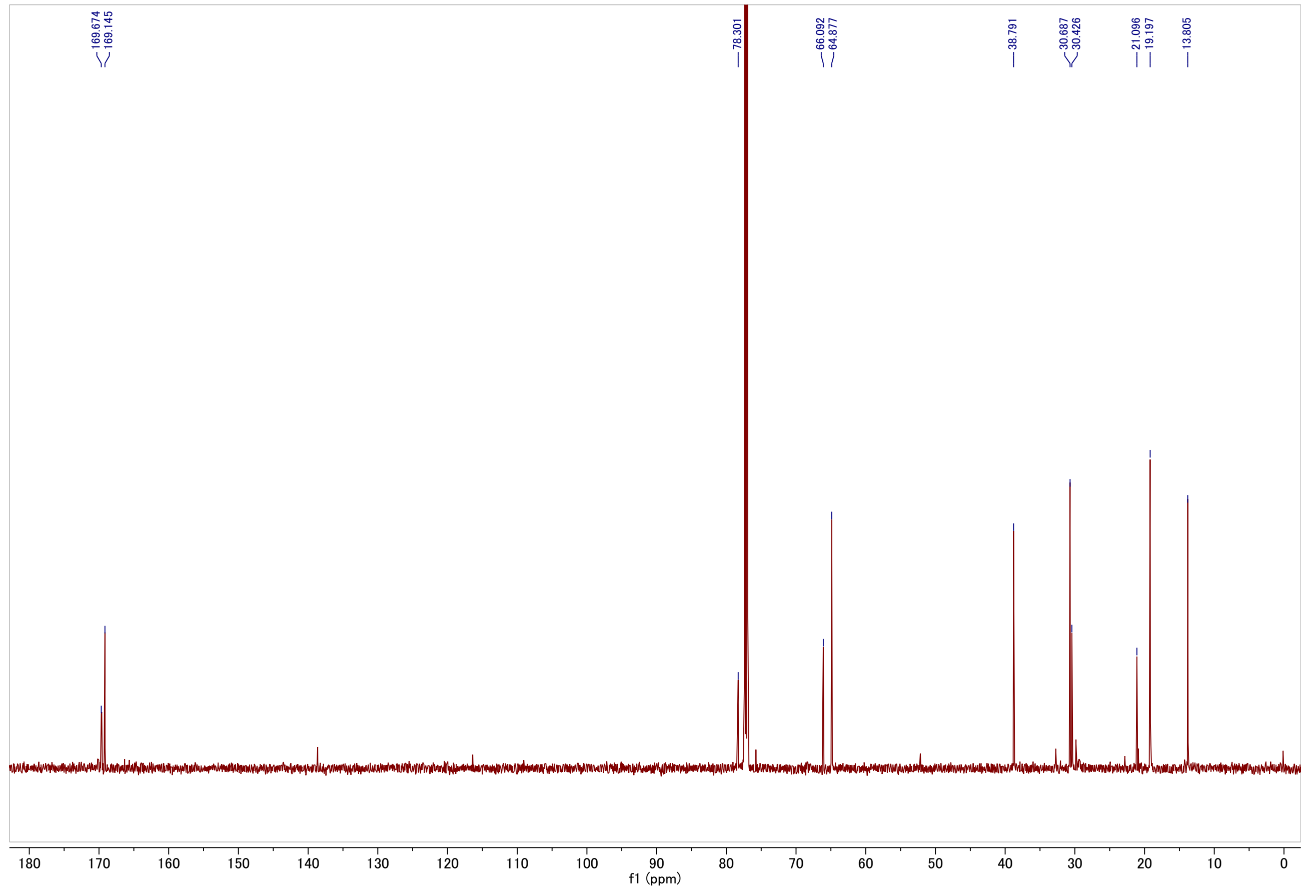

Figure S9: ^{13}C-NMR (150 MHz) of Compound 2 (Acetyl butyl citrate)