A new macrolide isolated from the endophytic fungus *Colletotrichum sp.*

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A new macrolide, colletotriolide (**1**), was isolated from the endophytic fungus *Colletotrichum sp.* isolated from *Pandanus amaryllifolius* by a series of chromatographic techniques. Its structure was determined based on HR-MS, 1D and 2D NMR. In addition, tyrosol C (**2**) isolated from the fungus *Colletotrichum gloeosporioides*, and dothiorelone C (**3**) and cytosporone (**4**), both isolated from the fungus *Chaetomium globosum*, were also identified in this study. All three fungi were isolated from the leaves of *Pandanus amaryllifolius*. Biological evaluation showed that **1** has a low activity against *Escherichia coli*, while **4** is moderately active. Compounds **1**, **3** and **4** were inactive against the A549, HT29 and HCT116 cell lines.

KEYWORDS

Endophyte, Pandanus, macrolide, polyketide, structure elucidation

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INTRODUCTION

Natural products research has tapped the screening of secondary metabolites from fungal endophytes present in various plant species for pharmaceutical development. Endophyic fungi or endophytes have become a source of novel biologically active secondary metabolites (Schulz et al. 2002; Teles et al. 2006). Their diverse biological activities range from plant growth regulatory activity, phytopathogenesis, and herbicidal activity (Garcia-Pajon and Collado 2003) to human health risks reduction like antitumor (Kumar et al. 2004), antiplasmodial and antiviral (Isaka et al. 2007), antioxidant (Harper et al. 2003), antitubercular (Rukachaisirikul et al. 2007) and antibacterial (Lu et al. 2000) activities.

Pandanus amaryllifolius Roxb. (*Pandanaceae*), commonly known as the fragrant screw pine, is widely distributed in tropical regions. It is one of the 700 species of the genus *Pandanus* that is regarded as a medicinal plant and is known to elaborate the presence of alkaloids (Tan et al. 2010). In the interest of finding biologically-active constituents from the endophytes of *P. amaryllifolius*, we recently reported the new compounds diaportheones A and B from *Diaporthe* sp. P133 (Bungihan et al. 2011) and guignardiol from *Guignardia* sp. (Bungihan et al. 2010). In this paper, we report the identification and biological evaluation of a new macrolide **1** from *Colletotrichum sp.*, the known compounds **2** from *Colletotrichum gloeosporioides*, and **3** and **4** from *Chaetomium globosum*.

MATERIALS AND METHODS

Instrumentation

Melting point (uncorrected) was recorded on a Yanaco Melting Point Apparatus Model MP-500P. The optical rotation was measured on a JASCO P1020 Polarimeter. The UV spectra were recorded on a JASCO V-560 spectrophotometer. The IR spectra (ATR) were recorded on a JASCO FT/IR-230 Spectrophotometer. The ^{1}H and ^{13}C NMR spectra were measured using a JEOL JNM A-500 (500 MHz), JEOL JNM A-400 (400 MHz) or JEOL JNM ECP 400 (400 MHz) spectrometer with CD_3OD or $CDCl_3$ as solvent and TMS as reference. The HR-ESI-MS (positive mode) was recorded on a Thermofisher Scientific Company Exactive mass spectrometer. Silica Gel 60 (Merck, 230–400 Mesh) was used for column chromatography. Medium-pressure liquid chromatography was carried out on a silica gel prepacked column CPS-HS-221-05 (Kusano Kagakukikai).

Fungal Material

The fungi were isolated from the leaves of *Pandanus amaryllifolius* collected from the UST Botanical Garden. Freshly cut mature and healthy leaves were washed in running water to remove debris. Surface sterilization was performed following modifications of the procedure made previously by Petrini et al. (1992). The fungi growing out of the plant tissue were sub-cultured using potato dextrose agar. Identification of the fungal materials were accomplished using DNA extraction protocols provided by Promega® and sequencing at Macrogen, *Inc*. (Korea) using the 18 s rRNA genes with the primers LR1 and SRLR and generating the homology online by BLAST at NCBI.

The fungal isolates were cultured in sterile potato dextrose broth (1.5 L) at room temperature under static conditions in the dark for three weeks. Mycelial biomass were harvested and weighed after three weeks growth. Both the culture broth and the fungal mycelia were repeatedly extracted with EtOAc and

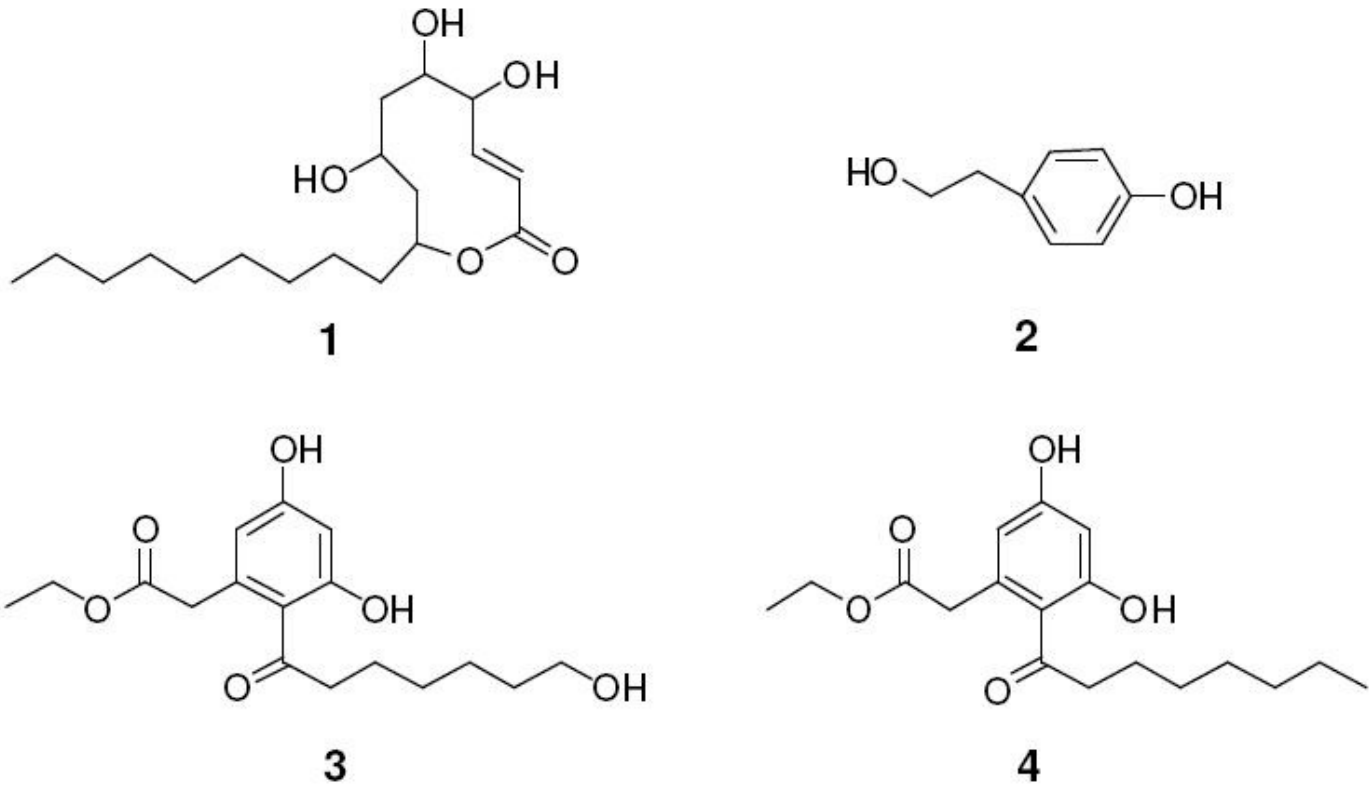

Figure 1. Isolated compounds from the endophytes of *P. amaryllifolius*

combined. The extracts were filtered using Whatman filter paper and concentrated under reduced pressure using rotary evaporator at a temperature ≤ 45 ^{0}C.

Isolation of Secondary Metabolites

Isolation of Colletotriolide (1). The EtOAc extract of *Colletotrichum* sp. (831.0 mg) was chromatographed on a silica gel column chromatography with 10% MeOH in $CHCl_3$ and 10% increments of MeOH in $CHCl_3$ to give 7 fractions (Fr. A – G). Fr. E was further purified on a silica gel column chromatography with $CHCl_3$/MeOH gradient to give Fr. E1 – E3. Fr. E3 was washed with hexane to afford pure compound **1** as white powder (5.1 mg)

Compound 1 (Colletotriolide). White powder; m.p. 146-147 °C; $[\alpha]_D^{24}$ -45.6 (MeOH; *c.* 0.04); UV (MeOH) λ_{max} 211 nm; IR (ATR) ν_{max} 3300, 1702, 1644, 1262 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HR-ESIMS *m/z* 351.2132 $[M + Na]^+$ (calcd for $C_{18}H_{32}O_5Na$, 351.2142).

Isolation of Tyrosol C (2). The EtOAc extract of *Colletotrichum gloeosporioides* (698.7 mg) was subjected to silica gel flash column chromatography using 10% EtOAc in hexane and 10% increments of EtOAc in hexane affording 7 fractions (Fr. A – G). Fr. F was chromatographed on a silica gel column chromatography (50% EtOAc in hexane) to give Fr. F1 – F3. Fr. F2 was purified by MPLC (50% EtOAc in hexane) to afford pure compound **2** (colorless oil, 1.0 mg).

Isolation of Dothiorelone C (3) and Cytosporone B (4). The EtOAc extract of *Chaetomium globosum* (501.9 mg) was subjected to silica gel open column chromatography using gradient 10% acetone in CH_2Cl_2 to give fractions A – F Fraction F was further purified by Sephadex LH20 using neat CH_2Cl_2 and MPLC using neat EtOAc to afford pure compound **3** as brown oil (2.5 mg). Fraction A was purified by MPLC (50% EtOAc in hexane) and silica gel column chromatography (50% EtOAc in hexane) to afford the pure isolate **4** as a yellow oil (1.4 mg).

Biological Evaluation of the Secondary Metabolites

Antibacterial assay was done following the method of Dickson et al. (2007) using the slightly acid-fast bacterium *G. terrae,* the gram-negative bacterium *E. coli* and the gram-positive bacterium *S. aureus*. For the cytotoxicity assay, the isolates were submitted to Yakult, Inc., Japan as previously described by Wu et al. (2009). The cell lines used were the human lung and colorectal cancer cell lines (A549, HT29 and HT116) which were maintained in Dulbecco's modified Eagle's medium (D-MEM) (D6046) with 10% heat–inactivated fetal bovine serum (FBS) and D-MEM/F-12 medium (D8062, Sigma) with 5 mg/mL gentamicin at 37 °C in a humidified atmosphere containing 5% CO_2.

RESULTS AND DISCUSSION

The endophytic fungi *Colletotrichum* sp., *Colletotrichum gloeosporioides*, and *Chaetomium globosum* that were isolated from *P. amaryllifolius* leaves were cultured for three weeks in potato dextrose broth and extracted exhaustively with EtOAc. All three extracts were chosen for further purification as indicated by their good results in a preliminary screening against *Mycobacterium tuberculosis, Gordonia terrae, Staphylococcus aureus* and *Escherichia coli*. The three fungi exhibited a zone of inhibition greater than 40mm for the three bacteria while

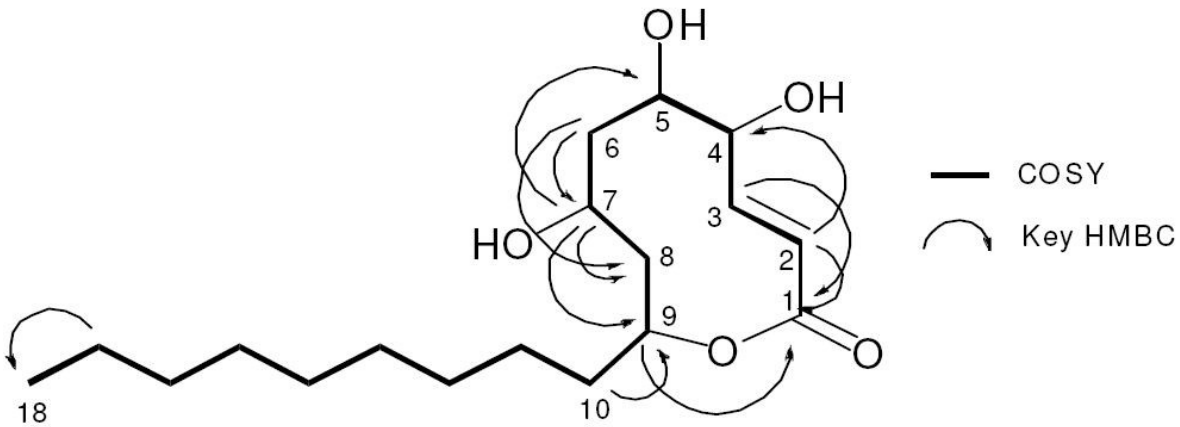

Figure 2. 2D NMR correlations in **1**.

showing an inhibition of greater than 90% at 128 µg/mL for *M. tuberculosis*. Bioassay-guided isolation of each extracts using column chromatography and TLC bioautography afforded a new macrolide, colletotriolide (**1**) from *Colletotrichum sp.*, tyrosol C (**2**) from *Colletotrichum gloeosporioides*, and dothiorelone C (**3**) and cytosporone B (**4**) from *Chaetomium globosum* (Fig. 1).

Compound **1** was obtained as an optically active ($[\alpha]_D^{24}$ – 45.6 (*c* 0.04, MeOH), white powder (*mpt.* 146 – 147 °C). The HR-ESI-MS spectrum exhibited a sodiated molecular ion peak at *m/z* 351.2132 $[M+Na]^+$ (calcd for $C_{18}H_{32}O_5Na$, 351.2142), in agreement with the molecular formula $C_{18}H_{32}O_5$. The IR spectrum showed absorptions for a hydroxyl (3300 cm^{-1}), ester carbonyl (1702 cm^{-1}), and olefinic (1644 cm^{-1}) functional groups. The ^1H NMR spectrum showed the presence of two olefinic protons (δ_H 6.01, dd, J = 1.5, 15.5 Hz, H-2; 6.93, dd, J = 5, 15.5 Hz, H-3), four oxygenated methines (δ_H 4.16, ddd, J = 1.5, 5.5, 7.0 Hz, H-4; 3.67, td, J = 4.0, 7.5 Hz, H-5; 3.82, m, H-7; 4.84, m, H-9), a methyl proton (δ_H 0.81, t, J = 7.0 Hz, H-18) and methylene protons at δ_H 1.54-1.08. The ^{13}C and DEPT NMR spectra showed the presence of 18 carbons attributable to an ester carbonyl (δ_C 167.9, C-1), two sp^2 methines (δ_C 123.4, C-2; 148.6, C-3), four oxygenated sp^3 methines (δ_C 75.6, C-4; 74.2, C-5; 69.0, C-7; 77.4, C-9), a methyl (δ_C 14.3, C-18) and ten sp^3 methylenes. The structure of **1** was further established by ^1H-^1H COSY, HMQC and HMBC spectroscopic analyses. ^1H-^1H COSY

Table 1. ^1H and ^{13}C NMR of Colletotriolide (**1**) in CD_3OD

No.	δ_C (125 MHz)	δ_H (J in Hz, 400 MHz)	HMBC (H → C)
1	167.9		
2	123.4	6.01, 1H, dd (15.5, 1.5)	C-1, C-3, C-4
3	148.6	6.93, 1H, dd (15.5, 5.0)	C-1, C-2, C-4
4	75.6	4.16, 1H, ddd (7.0, 5.5, 1.5)	
5	74.2	3.67, 1H, td (7.5, 4.0)	C-4
6	37.8	1.59, 2H, dd (5.5, 4.0)	C-5, C-7, C-8
7	69.0	3.82, 1H, m	C-5, C-6, C-9
8	36.7	1.54-1.45, 1H, m	
		1.24-1.17, 1H, m	
9	77.4	4.84, 1H, m	C-1
10	36.2	1.44-1.38, 2H, m	C-8, C-9, C-11
11	26.3	1.32, 1H, m	C-12
		1.24-1.17, 1H, m	
12	26.5	1.24-1.17, 1H, m	C-11
		1.08, 1H, m	
13	34.0	1.63, 1H, m	
		1.54-1.45, 1H, m	
14	25.2	1.32, 1H, m	C-13
		1.08, 1H, m	
15	23.6	1.32, 1H, m	
		1.08, 1H, m	
16	29.5	1.24-1.17, 2H, m	C-14, C-16, C-17
17	32.8	1.24-1.17, 2H, m	C-18
18	14.3	0.81, 3H, t (7.0)	

showed contiguous homonuclear connectivity from H-2 to H_3-18. The presence of a C_9 macrocyclic lactone ring was elucidated by key HMBC correlations (Fig. 2). The proton of an oxygenated methine at H-9 (δ_H 4.84) and the olefinic protons at H-2 (δ_H 6.01) and H-3 (δ_H 6.93) showed a long-range coupling with a lactone carbonyl at C-1 (δ_C 167.9). Additionally, the methylene H-6 (δ_H 1.59) and methine H-7 protons (δ_H 3.82) showed a correlation to the C-8 carbon (δ_C 36.7). A *trans* configuration between the olefinic carbons at C-2 and C-3 was also elucidated as evidenced by their large coupling constant ($J_{2,3}$ 15.5 Hz). The presence of the hydroxyl groups at C-4 (δ_C 75.6), C-5 (δ_C 74.2) and C-7 (δ_C 69.0) was determined based on their deshielded chemical shifts indicative of an oxygen-bearing carbons and the additional number of proton and oxygen atoms based on the high resolution mass spectrum. Due to the conformational instability of the macrocyclic lactone ring and the limited amount of sample, the relative stereochemistry of the C-4, C-5, C-7 and C-9 carbons cannot be determined. As evidenced from the above arguments, the structure of colletotriolide was elucidated as in **1**.

The known compounds **2** (tyrosol C, Christophoridou and Dais 2009), **3** (dothiorelone C, (Huang et al. 2009), and **4** (cytosporone B, Brady et al. 2000) were identified by 1D and 2D NMR, MS and in comparison with the literature data.

The isolated compounds (**1**, **3**, **4**) showed no significant cytotoxic activity against A549, HT29 and HCT116 cell lines. Compound **4** showed a moderate antibacterial activity against *E. coli* with an IC_{50} 62.5 µg/mL while **1** exhibited a weak *E. coli* inhibition with an IC_{50} 500 µg/mL. Previous study reported **2** to have antioxidant and anticancer activities (Ahn et al. 2008).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

MEB performed the isolation of fungi and purification of the compounds. MAT wrote the manuscript and measured the NMR and MS. HT, TEEC and MGN conceptualized the study design and helped in the correction of the manuscript.

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Supplementary Material

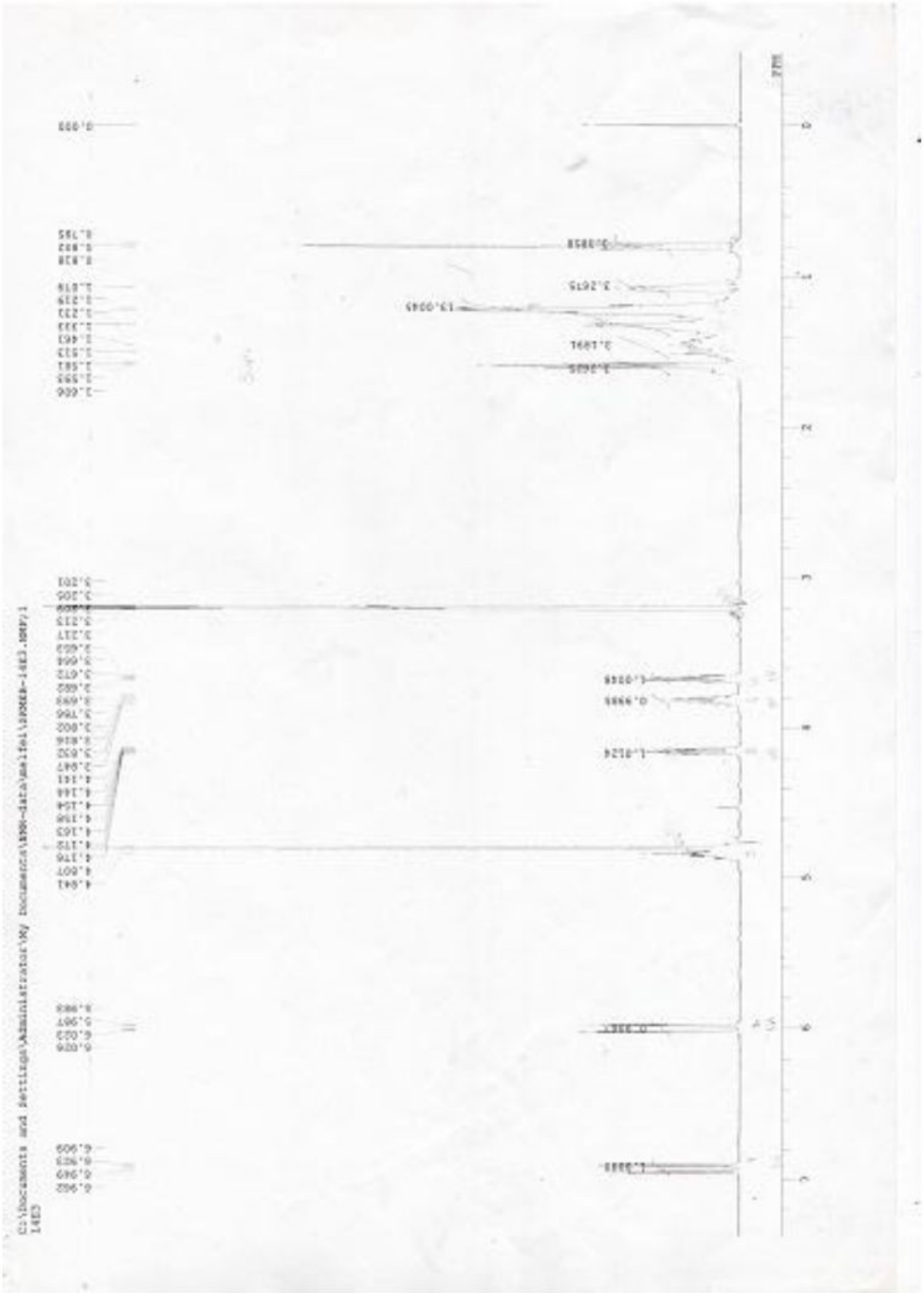

Figure 3. ^{1}H NMR (400 MHz) of Colletotriolide (**1**) in CD3OD

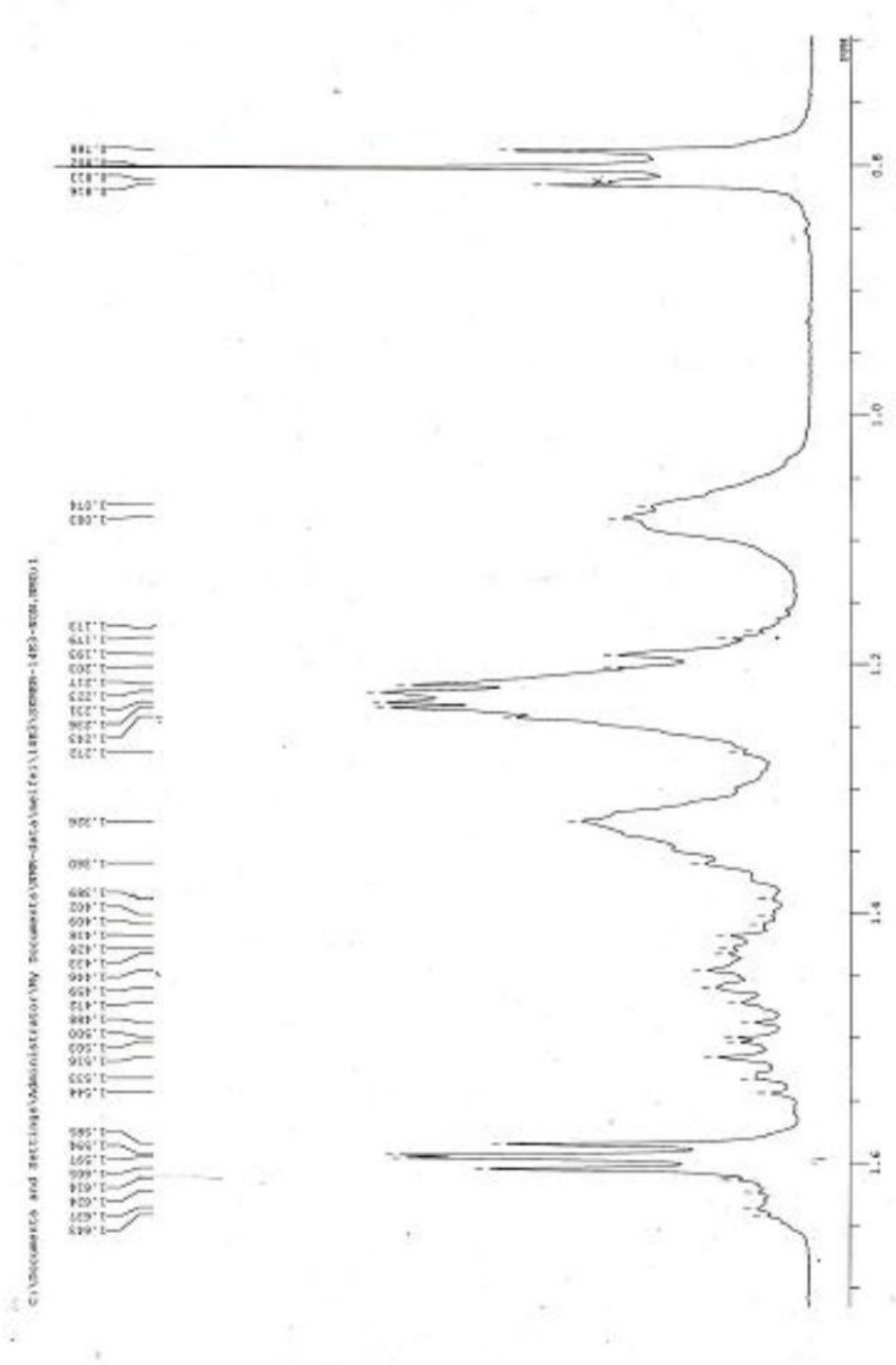

Figure 4. ^{1}H NMR (Expansion) of Colletotriolide (**1**) in CD_3OD

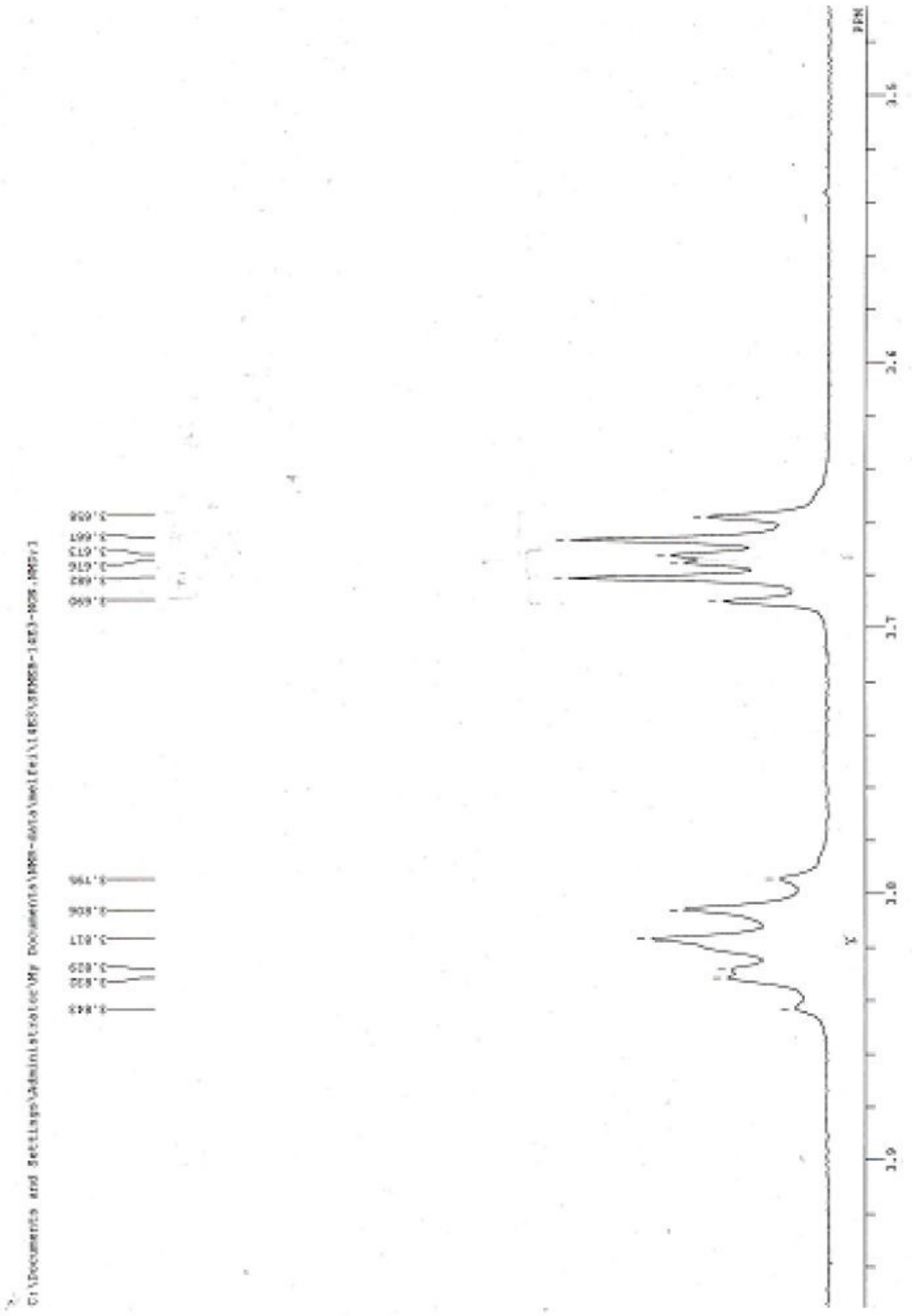

Figure 5. ^{1}H NMR (Expansion) of Colletotriolide (1) in CD_3OD

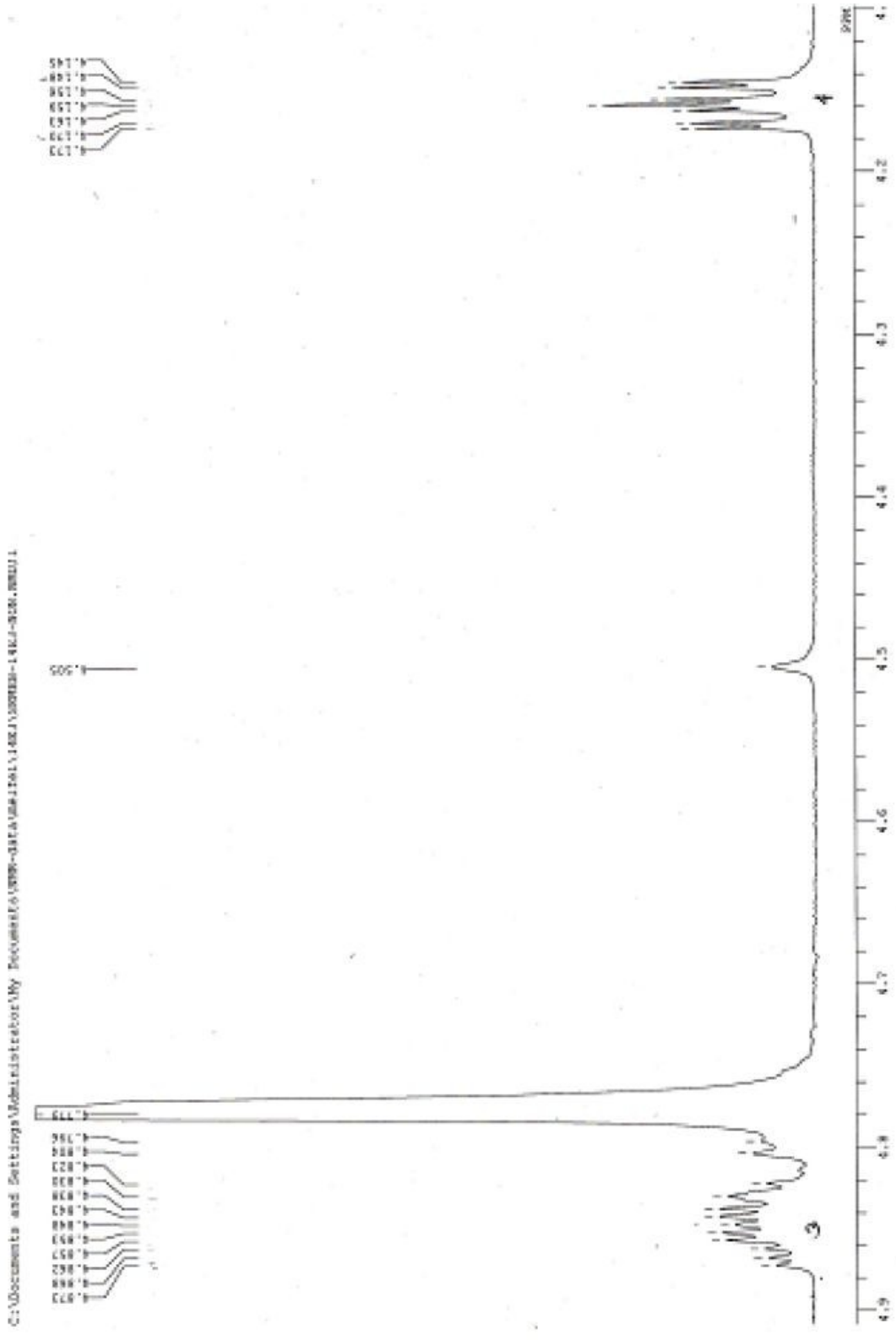

Figure 6. ^{1}H NMR (Expansion) of Colletotriolide (**1**) in CD_3OD

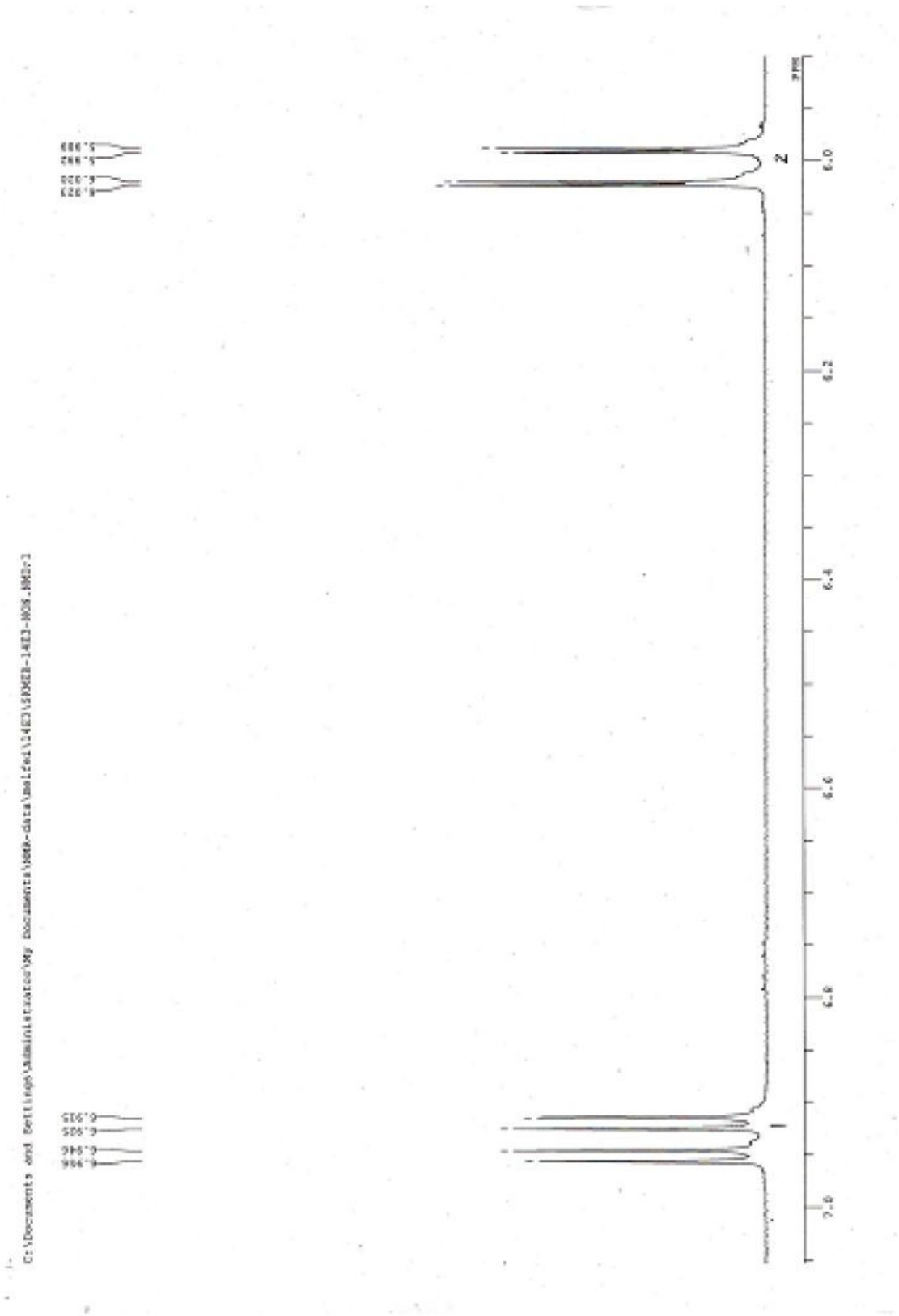

Figure 7. ^{1}H NMR (Expansion) of Colletotriolide (**1**) in CD_3OD

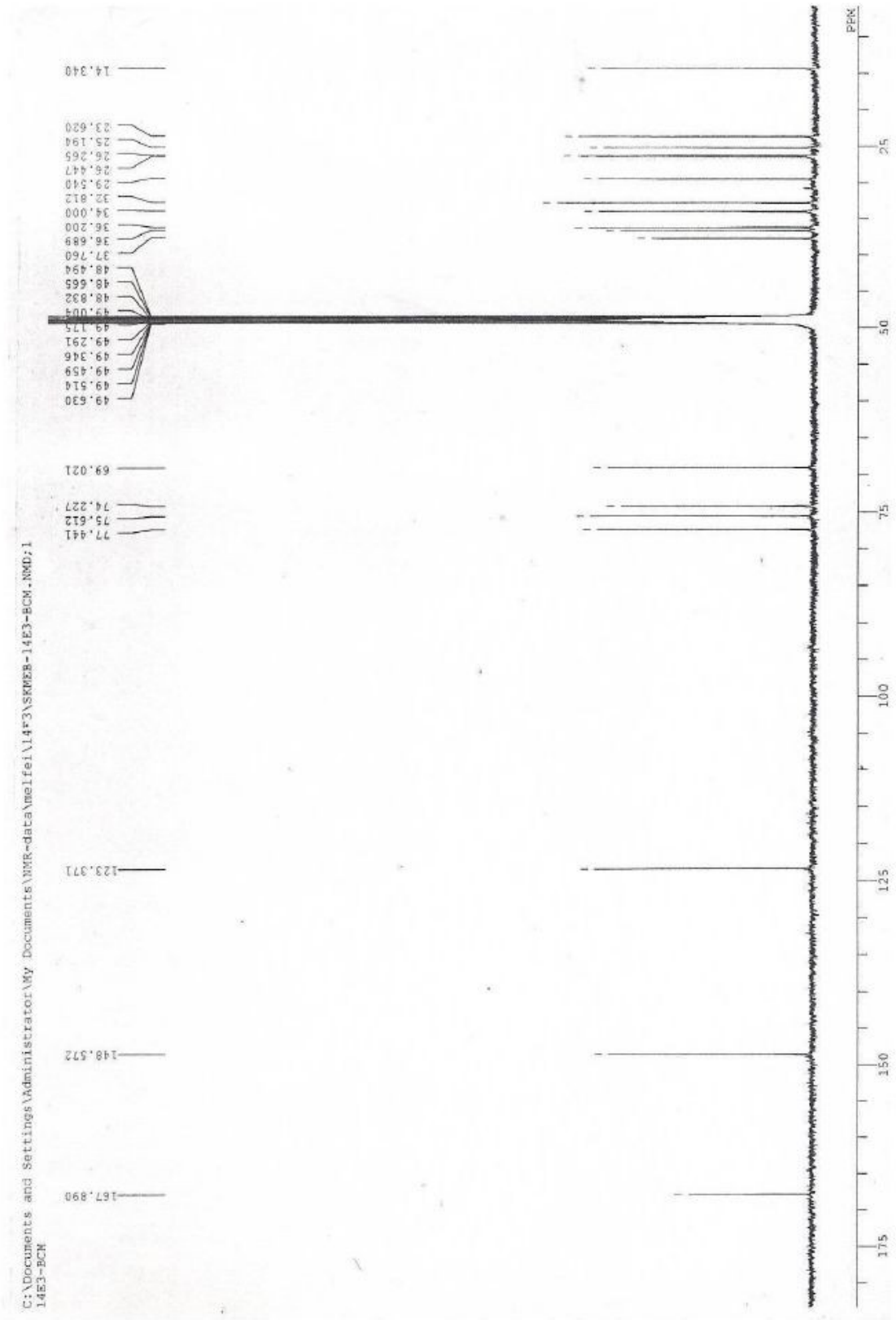

Figure 8. ^{13}C NMR (125 MHz) of Colletotriolide (1) in CD_3OD

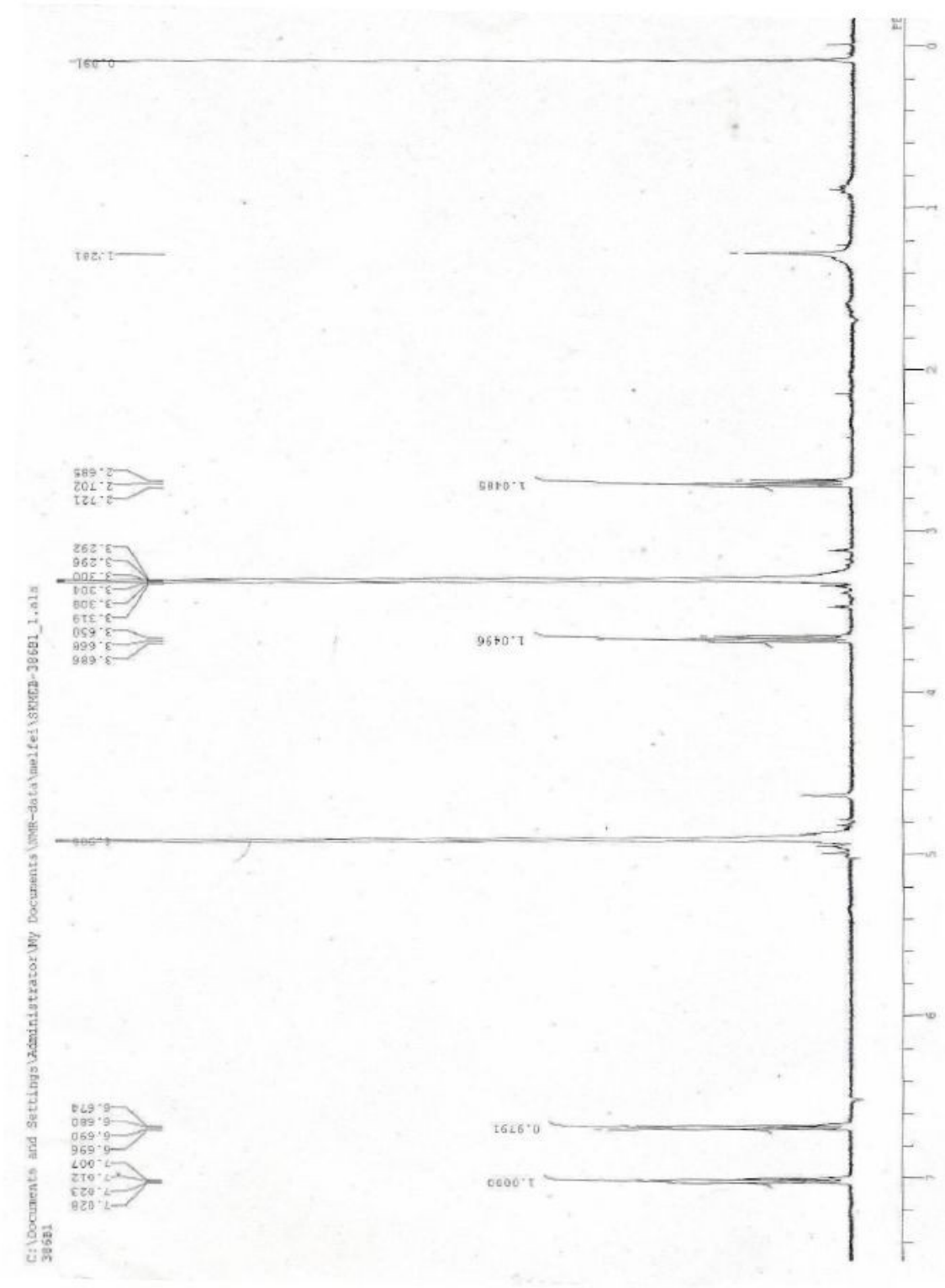

Figure 9. ^{1}H NMR (400 MHz) of Tyrosol C (**2**) in CD_3OD

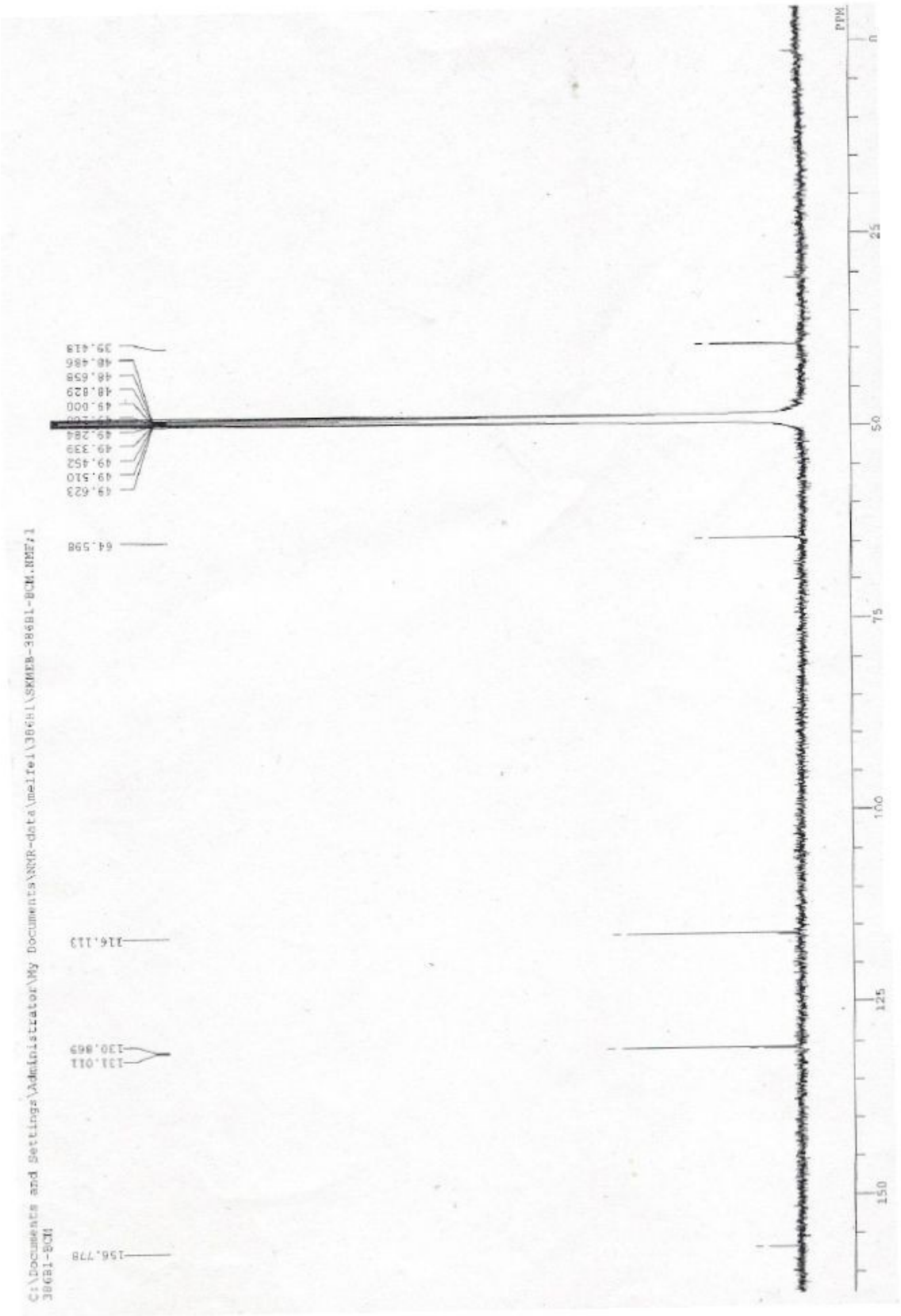

Figure 10. ^{13}C NMR (125 MHz) of Tyrosol C (**2**) in CD_3OD

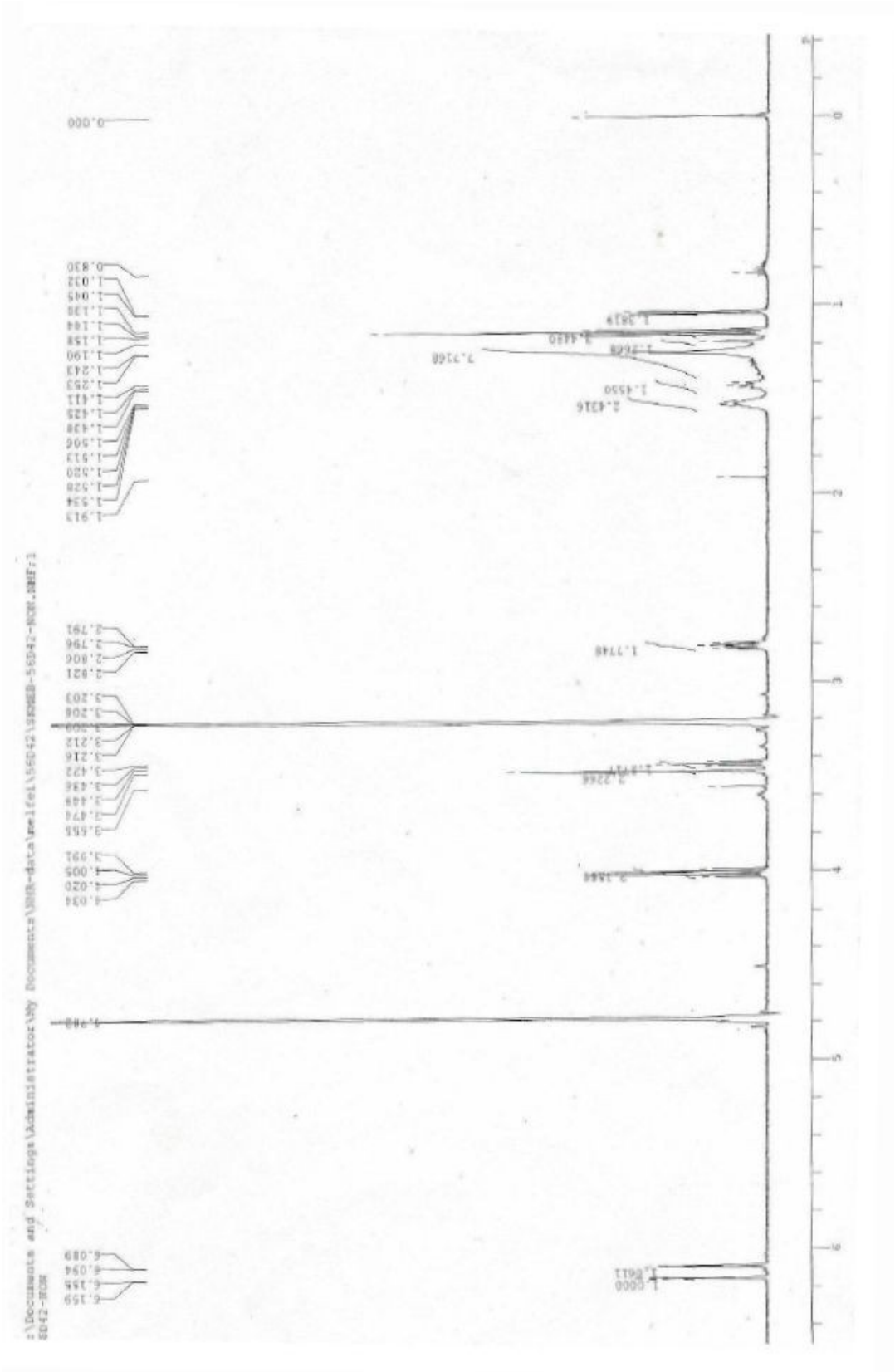

Figure 11. ^{1}H NMR (500 MHz) of Dothiorelone C (**3**) in CD_3OD

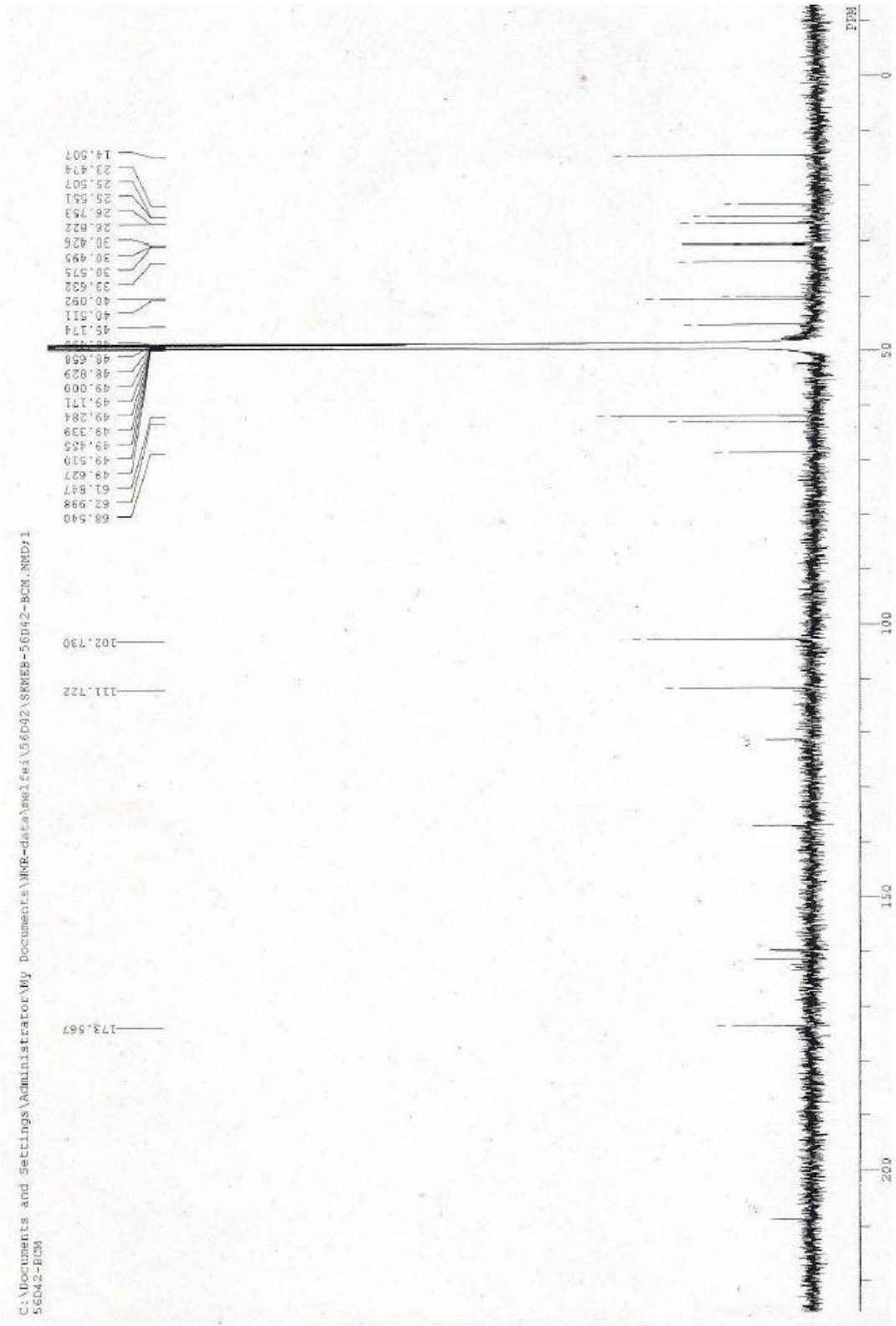

Figure 12. ^{13}C NMR (125 MHz) of Dothiorelone C (**3**) in CD_3OD

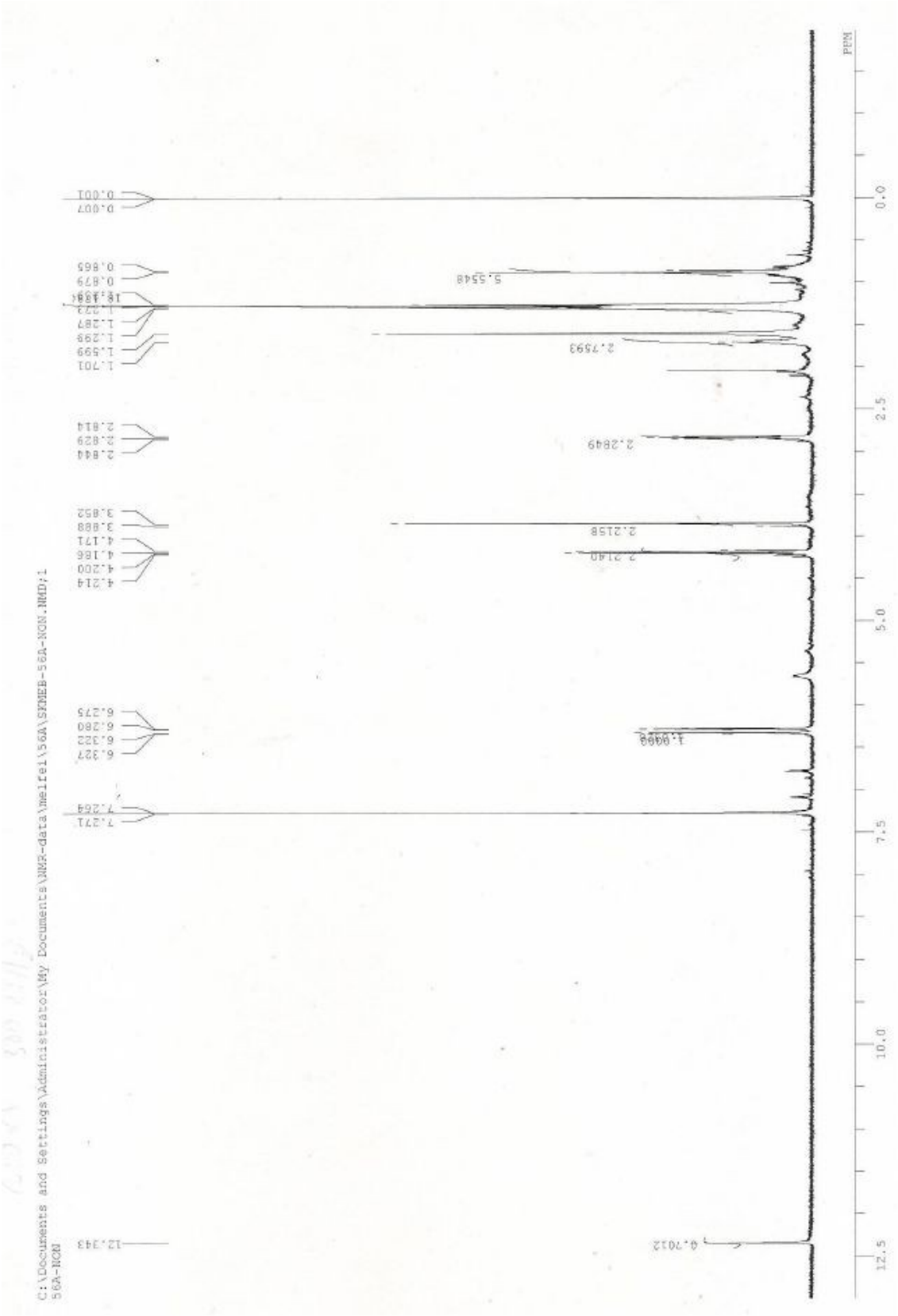

Figure 13. ^{1}H NMR (500 MHz) of Cytosporone B (**4**) in $CDCl_3$

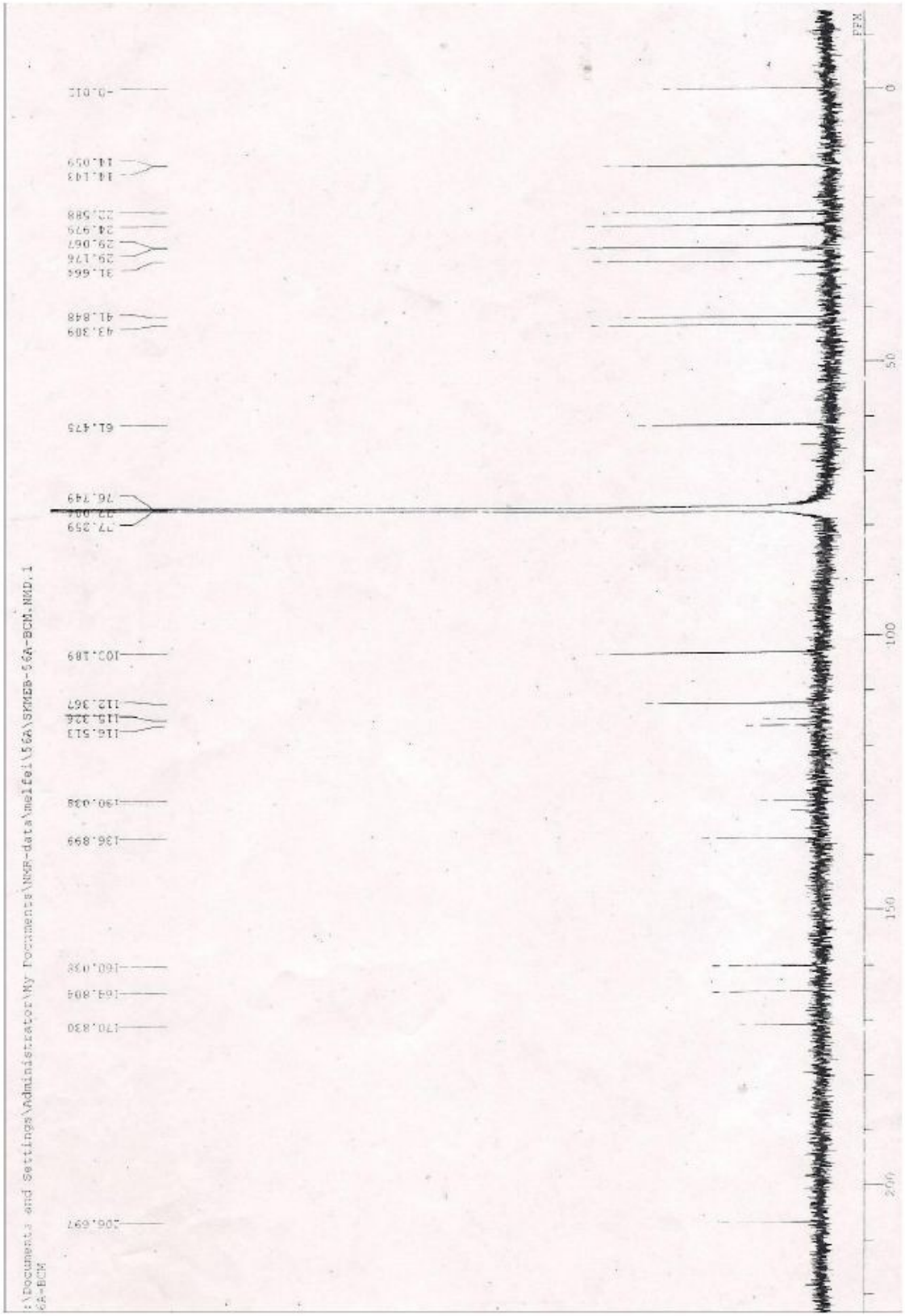

Figure 14. ^{13}C NMR (125 MHz) of Cytosporone B (**4**) in $CDCl_3$