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Graphical Abstract

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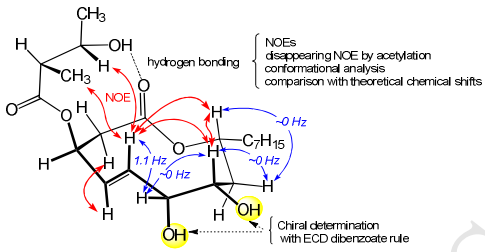

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Achaetolide-II isolated from *Helminthosporium velutinum* TS28

Miki Arayama,[a] Hayato Maeda,[a] Kazuaki Tanaka,[a] Noboru Takada, [a] Tatsuo Nehira,[b] and Masaru Hashimoto*[a]

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ABSTRACT

A C3-*O*-acylated analogue of achaetolide (**2**) was isolated from phytopathogenic fungus *Helminthosporium velutinum* TS28. The relative configuration involving the C3-*O*-acyl side chain was determined by NMR spectroscopic analysis combined with conformational analysis computer-assisted chemical shift calculations. The absolute configuration was established through ECD analysis after conversion into the dibenzoate (**5**).

1. Introduction

Several (*E*)-olefin-containing ten-membered polyketide macrolides have been obtained from fungal sources, and some of these show interesting biological activities, such as antimalarial activity,[1] antifungal activities,[2,3] activation of peroxisome proliferator-activated receptor,[4] and cytotoxicities.[5] Thus, they have become attractive synthetic targets for organic chemists.[6-8] We have recently isolated achaetolide (**1**)[9] from *Ophiobolus* sp. and succeeded in determining its absolute configuration,[10] which had remained unknown. During further exploration of secondary metabolites from ecologically unique microbes,[11-13] we isolated achaetolide-II (**2**), a 3-*O*-acylated analogue of **1**, from the mycelium of *Helminthosporium velutinum* TS28. We have revealed the C14-epimeric isomers of cochlioquinones from the culture broth of this fungus.[14] The structural studies involve configurational determination of asymmetric centers in the side chain based on NOE experiments, theoretical chemical shift computations, and chemical derivatization. The absolute configuration was investigated by means of an electronic circular dichroism (ECD) exciton chirality method after conversion into the 6,7-*O*-dibenzoate (**5**). Degradation of **2** and the following chemical transformations were performed to verify the stereochemical conclusion.

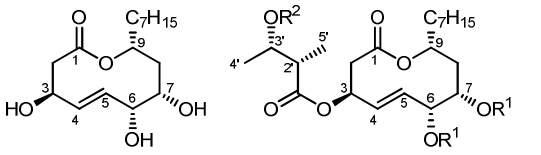

Figure 1. Structures of achaetolide (**1**) and achaetolide-II (**2**)

2. Results and discussion

Achaetolide-II (**2**) was found as a major component of the extract (75 mg/g) from the mycelium of *H. velutinum* TS28. ESI-MS analysis showed a protonated molecular ion at *m/z* = 401.2523, suggesting its molecular formula as $C_{21}H_{36}O_7$ ([M+H]: 401.2539). The ^{1}H NMR spectrum indicated that **2** was comprised of two inseparable components in an approximately 95:5 ratio. These were assigned as tautomeric isomers, because the ratio varied in methanol-d_4 (ca. 80:20 ratio).[15] Structural determination was performed on the main tautomer in $CDCl_3$. The NMR data obtained in $CDCl_3$ solution are summarized in Table 1. The ^{13}C NMR spectrum featured 21 resonances, and measurement of the DEPT spectrum classified these as two carbonyl, eight methine (including five oxymethines), eight methylene, and three methyl carbons, and also revealed 33 protons directly linked to the carbon atoms. The signals of the last three protons were observed at δ 2.19, 2.26, and 3.96 ppm in $CDCl_3$. These were assigned as hydroxy protons because no HMQC correlations were observed and the signals disappeared upon addition of D_2O. The vicinal spin coupling between H-4 and H-5 (J = 15.8 Hz) indicated an (*E*)-configuration for the C4,C5-double bond. Detailed analysis revealed very small vicinal spin couplings (J = 0–1.1 Hz) for H-6/H-7, H-7/Hβ-8, and Hβ-

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Table 1. NMR spectral data of achaetolide-II (**2**) in $CDCl_3$*.

position	δ^{13}C, type	δ^{1}H (*J* in Hz)	HMBC	NOESY
1	170.59, C			
2	41.40, CH_2	α: 2.54, dd (3.5, 12.5) β: 2.76, dd (3.3, 12.5)	1, 3, 1, 3, 4	H-3, H-4 H-3
3	68.15, CH	5.68, m	4	Hα-2, Hβ-2
4	126.00, CH	6.03, ddd (2.2, 3.7, 15.8)	3, 6	Hα-2, Hα-8
5	127.45, CH	5.73, ddd (1.1, 2.2, 15.8)	3, 6	H-6, H-7, H-9, H-3', H_3-5'
6	73.11, CH	4.55, m		H-5, H-7,
7	75.23, CH	3.80 br d (10.2)		H-5, H-6, Hβ-8, H-9
8	36.92, CH_2	α: 2.36, ddd (8.3, 10.2, 15.8) β: 1.53, m	7, 9 7, 9, 10	H-4,
9	73.70	4.80, dt (6.6, 8.3)	1, 8, 10, 11	H-5, H-7, Hβ-8,
10	36.79	1.50, m	7, 8, 9, 11	
11-14	24.98, CH_2, 29.14, CH_2, 29.43, CH_2, 31.74, CH_2,	1.25, m		
15	22.62, CH_2	1.25, m	16	
16	14.07	0.87, t (6.7)	15	
1'	173.71	-		
2'	49.37, CH	2.51, m	1', 4', 5'	H-3'
3'	69.26, CH	3.92,dquint (8.4, 6.2)	4', 5'	H-5, H-2', H_3-4', H_3-5'
4'	20.69, CH_3	1.24, d (6.2)	2', 3'	H-3'
5'	14.67, CH_3	1.20, d (7.2)	1', 2', 3'	H-5, H-3'
6-OH		2.26, d (3.3)	5, 6, 7	
7-OH		2.19, d (6.8)	6, 7, C8	
3'-OH		3.96, d (6.2)	3', 4'	

*The spectrum indicated that **2** is composed of two major conformers in a ca. 95:5 ratio. Only the spectral data for the major conformer .

8/H-9, suggesting almost perpendicular relationship for the dihedral angles between these protons. The ten-membered lactone framework was established by observing a HMBC signal between H-9 and C1. The results suggested that **2** possesses the same framework as achaetolide (**1**). The major set of ^{1}H resonances were in good accordance with those of **1** reported by Bodo *et al*.,[9] except for the H-3 signal. A NOESY spectrum indicated characteristic correlations at Hα-2/H-4, H-5/H-7, H-5/H-9, and H-7/H-9, as shown in Fig. 2, which confirmed the relative configuration as well as the conformation. It was also revealed that the minor component evident in the NMR spectrum was a conformational isomer; although only a few of its signals were analyzable.

The COSY spectrum further revealed a 3-hydroxy-2-methylbutanoate substructure (C1'-C5' side chain) in **2**. A remarkably high frequency for H-3 (5.68 ppm) indicated that C3-alkoxy group is responsible to an ester linkage with the side chain. 3-Hydroxy-2-methylbutanoic acid would be readily eliminated in the mass spectrometer to give a fragment ion at *m*/*z* = 283.1897 ($[M–C_5H_9O_3]^+$) with almost the same intensity as that of $[M+H]^+$ because of the β-acyloxy carbonyl system.

Interestingly, H-3' gave a NOESY correlation with H-5, as shown in Fig. 3. This suggested that the C1'-C5' side chain surrounds the nonalide framework. We assumed a hydrogen bonding between the C3' alcoholic proton and the C1 carbonyl oxygen on the basis of the following observations: (a) the C3'-OH signal appeared at higher frequency (3.96 ppm) than those of the other two alcoholic protons (2.19 and 2.26 ppm) in $CDCl_3$,[16] (b) when 3'-OH was masked in a form of acetate (→ **3**), the NOE between H-5 and H-3' disappeared, and (c) the above transformation significantly changed the conformational ratio (to 75:25) in $CDCl_3$. Acetate **3** was prepared from **2** by successive treatments with (i) *p*-TsOH, acetone (→ **4**), (ii) Ac_2O, pyridine,

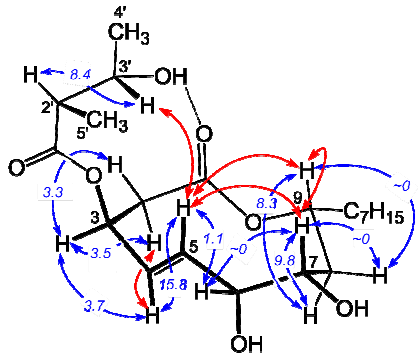


Figure 2. $^{3}J_{HH}$ spin couplings (blue arrows) and crucial NOEs (red arrows) of **2**

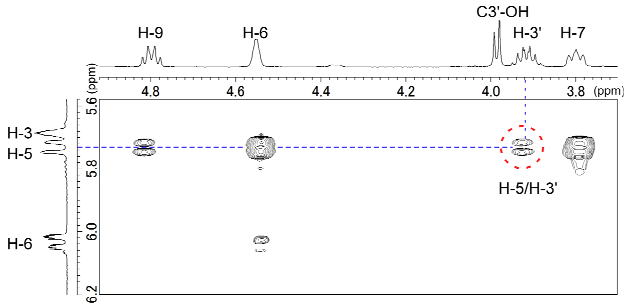


Figure 3. Middle frequency range of NOESY spectrum of **2**

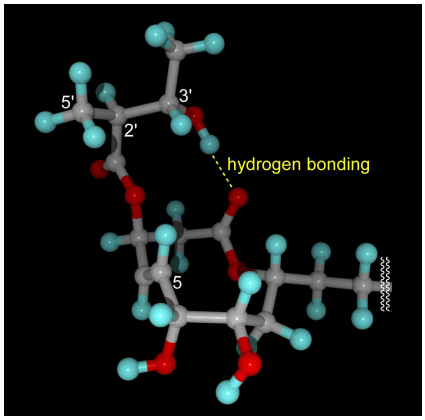


Figure 4. The most stable conformation of the (3*S**,2'*S**,3'*S**)-isomer of **2** based on EDF2/6-31G*. The C9 side chain was manually elongated after the calculations.

and (iii) *p*-TsOH, MeOH.[17]

Molecular modeling calculations also suggested hydrogen bonding, irrespective of the configurations at C2' and C3'. The (3*S**,2'*S**,3'*S**)- and (3*S**,2'*R**,3'*S**)-isomers can explain the NOE between H-5 and H-3'. Calculations were performed with EDF2/6-31G*[18] after a conformational search with MMFF.[19] We replaced the C10-C16 heptyl side chain with a methyl group in the calculations in order to reduce the number of conformers to be considered. Entropic factors (Δ*S*) were taken into consideration with the vibrational analyses in view of the conformational flexibility of the C1'-C5' group.[20] The most stable conformation of (3*S**,2'*S**,3'*S**)-isomer is shown in Fig. 4.

The δ value of C-5' (14.67 ppm) empirically suggested *threo*-configuration for the 3-hydroxy-2-methylbutanoate moiety,[21] *i.e.* (2'*S**,3'*S**)-form in **2**. It was further investigated with chemical shift calculations.[11-13] The sets of stable conformers were subjected to chemical shift calculations with EDF2/6-31G* to obtain the theoretical chemical shifts after a correction based on the Boltzmann distribution. Minor conformers of the nonalide moiety were excluded, because their ^{1}H NMR signals appeared separately. Deviations between theoretical and experimental chemical shifts ($\Delta\delta = \delta_{experimental} - \delta_{calculated}$, ppm) for the relevant carbons were plotted to give Fig. 5. The chemical shifts for C1 in the models were estimated to appear at considerably higher frequency than that observed experimentally. Replacement of the C9-heptyl side chain by a non-bulky methyl group presumably leads to magnetic deshielding of C1 which is a so-called steric compression effect.[22] The (3*S**,2'*S**,3'*S**)-isomer clearly gave the highest scores among the isomers. The ^{1}H chemical shift

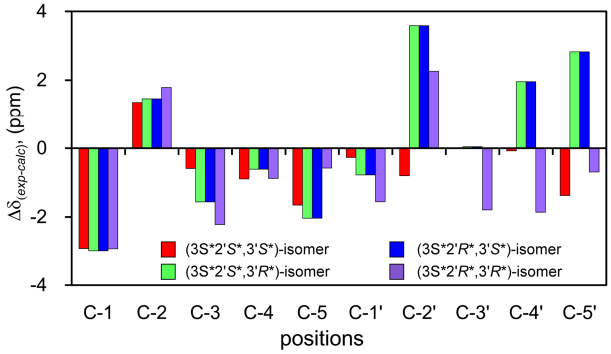


Figure 5. Chemical shift differences (Δδ, ppm) from the experimental data for the respective isomers based on EDF2/6-31G*.

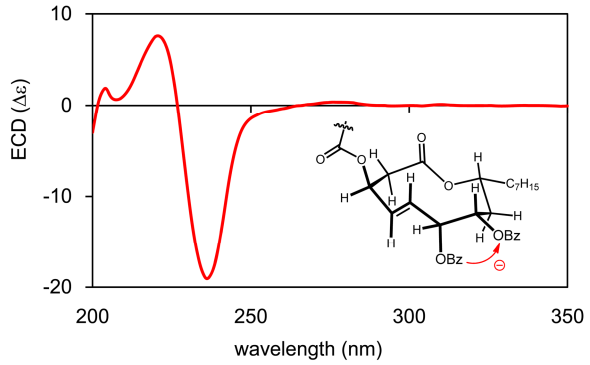


Figure 6. ECD spectrum of dibenzoate **4**.

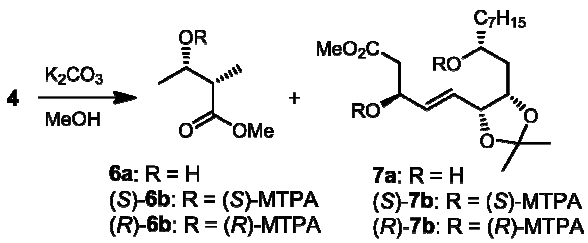
deviations showed a similar tendency, albeit less markedly than for the ^{13}C shifts. These observations were in accordance with the above results. Thus, we determined the total relative configuration of **2** as the (3*S**,6*R**,7*S**,9*R**,2'*S**,3'*S**)-form.

Next, we turned our attention to the absolute configuration. Conversion into **1** under basic conditions was not satisfactory, because these conditions also hydrolyzed the nonalide ring. We applied Nakanishi's dibenzoate rule[23] after conversion into dibenzoate **5**. A UV-insensitive acetyl group was furnished at the C3'-alcohol to avoid undesired ECD interaction.[24] Compound **5** was readily prepared by benzoylation of **3**. The ^{1}H NMR spectrum of **5** showed it to be a 5:1 mixture of conformational isomers. The vicinal spin couplings $^3J_{H\text{-}5/H\text{-}6}$, $^3J_{H\text{-}6/H\text{-}7}$, $^3J_{H\text{-}7/H\beta\text{-}8}$, and $^3J_{H\beta\text{-}8/H\text{-}9}$ for the major conformer of **4** were 0–2 Hz, in good accordance with those observed in **2**. These results suggested that the conformation of **2** is retained in the major conformer of **5**. Molecular modeling calculations supported this interpretation (see Supplementary data). Thus, we can simply apply the benzoate for **5**. As shown in Fig. 6, **5** gave a typical split ECD with a negative Cotton effect at 236 nm (Δε –19) and a positive one at 220 nm (Δε +7.6), revealing a negative chirality for the C6-C7 diol moiety in **2**, that is, a (6*R*,7*S*)-configuration. This was consistent with the absolute configuration of **1**.[10] Accordingly, we conclude that the absolute configuration of **2** is the (3*S*,6*R*,7*S*,9*R*,2'*S*,3'*S*)-form.

Methanolysis of **4** under basic conditions afforded methyl *threo*-3-hydroxy-2-methylbutanoate (**6a**)[25,26] and the previously prepared diol **7a**.[10] These were further converted into (*S*)- and (*R*)-MTPA esters (*S*)-**6b**, (*R*)-**6b**, (*S*)-**7b** and (*R*)-**7b**. Application of the extended-Mosher method[27] disclosed (3*S*,9*R*,2'*S*,3'*S*)-configuration. These experiments verified the conclusion described above.

3. Biological properties

In studies carried out to date, achaetolide-II (**2**) showed no



Scheme 1.

antifungal activity against *Cochliobolus miyabeanus* (IC_{50} >100 μg/mL). A cytotoxicity assay revealed moderate growth inhibition of human colon adenocarcinoma (COLO 201) cells (IC_{50} 370 μg/mL). This molecule also inhibited root growth of *Lactuca sativa* at 500 μg/mL.

4. Experimental

4.1. General

Ultraviolet (UV) spectra were obtained using a Hitachi U-2010 spectrophotometer. Electronic circular dichroism (ECD) spectra were measured on a JASCO J-725 spectrometer. NMR spectra were recorded on a JEOL JNM-ECX500 spectrometer. Tetramethylsilane was used as internal standard (0 ppm) for both ^{1}H and ^{13}C NMR spectra in $CDCl_3$. When deuteriomethanol was used as the solvent, the residual proton signal (CD_2HOD) was used as an internal standard (3.31 ppm) in the ^{1}H NMR spectrum. In ^{13}C NMR spectra recorded in deuteriomethanol, the solvent signal was employed as an internal standard ($^{13}CD_3OD$: 49.15 ppm). Mass spectra were measured in electrospray ionization (ESI) mode on a Hitachi NanoFrontier LD mass spectrometer. In the assay, samples were observed at ×20 magnification using an Olympus CKX31 inverted microscope.

4.2. Fungus.

The fungus was isolated from the surface of dead branches of *Sambucus sieboldiana* around Shinjuku Gyoen National Garden, Tokyo, Japan in 2002 and was identified as *Helminthosporium velutinum* based on its morphological features.[28] The fungus has been deposited at the NIAS (National Institute of Agrobiological Sciences) Genebank, Japan (ID: MAFF 243854).[29]

4.3. Calculations.

Conformational searches and chemical shift calculations were performed using Spartan 14 (Wavefunction, Irvine, CA) installed on a PC (Operating System: Windows 7 Professional, CPU: Intel(Xeon) E5-1660 v2 processor 3.70 GHz, RAM 64 GB). Theoretical ECD spectra were calculated with Gaussian 09 (Revision A.02 by Gaussian Inc., Wallingford, CT) with a PC (Operating System: CentOS a Linux, CPU: 2 Intel Xeon 3 5550 processors 2.67 GHz, RAM 24 GB).

4.4. Isolation.

Helminthosporium velutinum TS28 was cultured in potato-sucrose medium (200 mL in 500 mL baffled Erlenmeyer flask × 40) on a rotary shaker (110 rpm) at 27 °C for 14 days. After filtration *in suction*, the recovered mycelium (820 g, wet weight) was suspended in MeOH (2.2 L) at room temperature for 24 h. After filtration *in* suction, the filtrate was concentrated under reduced pressure until the volume became 300 mL. Water (200 mL) was then added, and the resulting suspension was extracted with EtOAc (2 × 1.0 L). The combined EtOAc extracts were concentrated to give a crude residue (698 mg), which was subjected to column chromatography on silica gel. The fraction eluted with EtOAc/hexane (80:20) was recovered and concentrated to give partially purified **2** (102 mg). A portion of the sample (50 mg) was further purified by medium-pressure column chromatography (Yamazen Universal Column ODS-SM 50 μm 120A, 50–80% MeOH/H_2O for 100 min, 8.0 mL/min flow) to give achaetolide II (**2**, 32 mg). The ^{1}H and ^{13}C NMR spectral data are shown in Table 1. ESI-MS data are shown in the text. Other physical data were follows: $[\alpha]_D^{25}$ –41 (*c* 1.0, $CHCl_3$); UV data are shown in the text. IR (film): ν=3480, 2960, 2930, 2860, 1720, 1270, 1160 cm^{-1}.

4.5. 6,7-O-isopropylidene achaetolide-II (4).

A solution of **2** (49.0 mg, 122 μmol) in acetone (1.5 mL) was left to stand in the presence of *p*-TsOH (1.5 mg) at room temperature for 3 h. Et_3N (one drop) was then added, and the mixture was concentrated in vacuo. Column chromatography of the residue on silica gel (hexane/EtOAc, 70:30) gave **4** (45.4 mg, 84%). The ^{1}H NMR spectrum in $CDCl_3$ indicated that the sample consisted of three conformers in a 3:23:74 ratio. Only representative signals for the major isomer are described: δ 0.87 (3H, t, *J* = 6.7 Hz), 1.18 (3H, d, *J* = 7.1 Hz), 1.22 (3H, d, *J* = 6.4 Hz), 1.25 (10H, m), 1.33, 1.44 (each 3H, s), 1.51 (1H, m), 1.60 (1H, d, *J* = 16.0 Hz), 1.61 (1H, m), 2.21 (1H, dt, *J* =16.0, 10.4 Hz), 2.42 (1H, dd, *J* = 7.8, 13.7 Hz), 2.46 (1H, quint *J* = 7.1 Hz), 3.12 (1H, dd, *J* = 7.8, 13.7 Hz), 3.90 (1H, m), 4.13 (1H, m), 4.61 (1H, dd, *J* = 5.9, 9.1 Hz), 4.90 (1H, m), 5.57 (1H, q, *J* = 7.8 Hz), 5.69 (1H, dd, *J* = 7.8, 16.0 Hz), 5.89 (1H, dd, *J* = 9.1, 16.0 Hz).

4.6. *3'-O-Acetyl-achaetolide-II (**3**).*

A solution of the acetonide **4** thus obtained (45.4 mg, 103 μmol) in a mixture of pyridine (1.0 mL) and Ac_2O (0.1 mL) was left to stand at room temperature for 12 h. After concentration in vacuo, column chromatography of the residue on silica gel (hexane/EtOAc, 90:10) gave the acetate (48.6 mg, 97 %). The ^{1}H NMR spectrum in $CDCl_3$ indicated that the sample was a mixture of two conformational isomers in a 9:91 ratio. Only the signals for the major isomer are described: δ 0.88 (3H, t, *J* = 6.7 Hz), 1.15 (3H, d, *J* = 7.2 Hz), 1.22 (3H, d, *J* = 6.4 Hz), 1.25 (10H, m), 1.33, 1.43 (each 3H, s), 1.51 (1H, m), 1.60 (1H, d, *J* = 16.0 Hz), 1.62 (1H, m), 2.01 (3H, s), 2.21 (1H, dt, *J* = 16.0, 10.4 Hz), 2.33 (1H, dd, *J* = 8.5, 13.6 Hz), 2.69 (1H, quint, *J* = 7.2 Hz), 3.13 (1H, dd, *J* = 8.5, 13.6 Hz), 4.12 (1H, dd, *J* = 5.9, 10.4 Hz), 4.61 (1H, dd, *J* = 5.9, 9.1 Hz), 4.91 (1H, m), 5.09 (1H, dq, *J* = 7.2, 6.4 Hz), 5.58 (1H, q, *J* = 8.5 Hz), 5.68 (1H, dd, *J* = 8.5, 15.9 Hz), 5.89 (1H, dd, *J* = 9.1, 15.9 Hz). A solution of the acetate thus obtained (28.6 mg, 59 μmol) in MeOH (1.0 mL) was left to stand in the presence of *p*-TsOH (1.0 mg) at room temperature for 10 h. After neutralization by adding Et_3N, the mixture was concentrated in vacuo. Column chromatography of the residue on silica gel (hexane/EtOAc, 85:15 → 45:55) gave **3** (13.8 mg, 49%) and recovered alcohol (12.7 mg, 49%), respectively. $[\alpha]_D^{25}$ –28 (*c* 0.5, $CHCl_3$); IR (film): ν=3450, 2950, 2930, 2860, 1740, 1240, 1160 cm^{-1}. The ^{1}H NMR spectrum in $CDCl_3$ indicated that the sample was a mixture of two conformational isomers in a 3:1 ratio. Only representative signals are described: (a 0.75, b 0.25) δ 0.87 (3H, m) 1.15 (3H × b, d, *J* = 7.0 Hz), 1.22 (3H × a, d, *J* = 7.1 Hz), 1.25 (3H × a, d, *J* = 6.3 Hz), 1.25 (10H, m), 1.49 (1H × a, d, *J* = 15.9 Hz), 1.52 (2H, m), 2.00 (3H × a, s), 2.01 (3H × b, s), 2.12 (1H × a, d, *J* = 6.8 Hz), 2.17 (1H × a, d, *J* = 3.4 Hz), 2.22 (1H × b, d, *J* = 6.4 Hz), 2.35 (1H × a, m), 2.36 (1H × b, m), 2.42 (1H × b, d, *J* = 1.8 Hz), 2.55 (1H × a, dd, *J* = 3.9, 12.7 Hz), 2.67 (1H × a, dd, *J* = 3.0, 12.7 Hz), 2.70 (1H × b, m), 2.76 (1H × a, quint., *J* = 7.1 Hz), 3.13 (1H × b, dd, *J* = 7.6, 14.1 Hz), 3.60 (1H × b, m), 3.78 (1H × a, br t, *J* = 8.4 Hz), 4.36 (1H × b, br d, *J* = 7.0 Hz), 4.53 (1H × a, m), 4.75 (1H × a, dt, *J* = 8.2, 6.3 Hz), 5.10 (1H × b, m), 5.12 (1H × a, dq, *J* = 7.2, 6.3 Hz), 5.48 (1H × b, m), 5.49 (1H × b, m), 5.61 (1H × a, m), 5.70 (1H × a, ddd, *J* = 1.0, 2.3, 15.8 Hz), 5.79 (1H × b, dd, *J* = 8.0, 15.5 Hz), 6.04 (1H × a, ddd, *J* = 2.1, 3.7, 15.8 Hz). Only the ^{13}C NMR signals in $CDCl_3$ for the major conformer are described. δ 12.88, 14.08, 16.95, 21.24, 22.63, 25.00, 29.17, 29.45, 31.76, 36.79, 36.93, 41.34, 44.74, 68.66, 71.42, 73.03, 73.22, 75.30, 126.63, 127.17, 168.97, 170.15, 172.66. ESI-MS: *m/z* 443.2643 ($C_{23}H_{38}O_8$, $[M+H]^+$: 443.2639), 465.2459 ($C_{23}H_{38}O_8$, $[M+Na]^+$: 465.2459), 907.5018 ($C_{46}H_{76}O_{16}$, $[2M+Na]^+$: 907.5018).

*4.7. 3'-O-Acetyl-6,7-O-benzoyl achaetolide-II (**5**).*

A solution of **3** (12.7 mg, 29 μmol) and benzoyl chloride (20 μL, 0.17 mmol) in CH_2Cl_2 (0.5 mL) was stirred at room temperature for 12 h. MeOH (50 μL) was then added and the mixture was further stirred at room temperature for 1 h. It was poured into water (15 mL) and extracted with diethyl ether (15 mL × 2). The combined ethereal extracts were washed with saturated aqueous NaCl solution, dried over $MgSO_4$, and then concentrated in vacuo. Column chromatography of the residue on silica gel (hexane/AcOEt, 80:20) gave **5** (15.0 mg, 79%). $[\alpha]_D^{25}$ –32 (*c* 1.0, $CHCl_3$); IR (film): ν=2930, 2860, 1730, 1260, 710 cm^{-1}. The ^1H NMR spectrum in $CDCl_3$ indicated that the sample was a mixture of two conformational isomers in a 5:1 ratio. Only representative signals are described: (a 0.83, b 0.17) × 0.87 (3H, t, *J* = 6.7 Hz), 1.17 (3H × b, d, *J* = 7.1 Hz), 1.26 (3H × a, d, *J* = 7.1 Hz), 1.28 (3H × a, d, *J* = 6.3 Hz), 1.28 (11H, m), 1.60 (1H × a, m), 1.77 (1H × a, d, *J* = 15.6 Hz), 2.02 (3H, s), 2.50 (1H × b, dd, *J* = 8.1, 13.4 Hz), 2.61 (1H × a, dd, *J* = 3.9, 12.6 Hz), 2.69 (1H×a, m), 2.70 (1H × b, m), 2.72 (1H × a, dd, *J* = 3.2, 12.6 Hz), 2.77 (1H × a, dq, *J* = 8.3, 7.1 Hz), 3.12 (1H × b, dd, *J* = 8.1, 13.4 Hz), 4.97 (1H × b, m), 5.08 (1H × a, q, *J*=8.5 Hz), 5.11 (1H × a, dq, *J* = 8.3, 6.3 Hz), 5.25 (1H × a, br d, *J* = 10.1 Hz), 5.56 (1H × b, q, *J* = 8.1 Hz), 5.62 (1H × a, m), 5.82 (1H × b, dd, *J* = 8.1, 16.7 Hz), 5.91 (1H × a, ddd, *J* = 1.0, 2.8, 16.0 Hz), 5.93 (1H × b, dd, *J* = 7.5, 16.7 Hz), 5.98 (1H × a, ddd, *J* = 2.0, 3.5, 16.0 Hz), 6.05 (1H × b, dd, *J* = 3.3, 7.5 Hz), 6.09 (1H × a, m), 7.38 (2H × a, br t, *J* = 8.1 Hz), 7.48 (2H × b, br t, *J* = 8.1 Hz), 7.52 (2H × b, m), 7.53 (2H × a, br t, *J* = 8.1 Hz), 7.60 (1H × b, br t, *J* = 8.1 Hz), 7.65 (1H × a, br t, *J* = 8.1 Hz), 7.91 (2H × b, br d, *J* = 8.1 Hz), 7.93 (2H × a, br dd, *J* = 1.4, 8.3 Hz), 8.07 (2H × b, br d, *J* = 8.1 Hz), 8.16 (2H × a, br dd, *J* = 1.4, 8.1 Hz). Only the ^{13}C NMR signals in $CDCl_3$ for the major conformer are described: δ 13.27, 14.07, 17.23, 21.24, 22.62, 25.12, 29.14, 29.41, 31.77, 35.61, 36.65, 41.35, 44.90, 68.30, 71.51, 72.65, 73.21, 76.09, 123.23, 128.31, 128.41, 128.69, 129.74, 129.77, 133.23, 133.49, 165.02, 165.47, 168.60, 170.24, 172.65. ESI-MS: *m/z* 651.3154 ($C_{37}H_{46}O_{10}$, $[M+H]^+$: 651.3164), 673.2970 ($C_{37}H_{46}O_{10}$, $[M+Na]^+$: 673.2983), 1323.6039 ($C_{74}H_{92}O_{20}$, $[2M+Na]^+$: 1323.6074).

*4.8. Basic methanolysis of **4** giving methyl threo-3-hydroxy-2-methylbutanoate (**6a**) and diol **7a**.*

A suspension of acetonide **4** (83.0 mg, 189 μmol) and K_2CO_3 (40 mg, 289 mmol) in methanol (4.0 mL) was stirred at room temperature for 3 h. The mixture was poured into aqueous 1.0 M HCl solution (50 mL) and extracted with AcOEt (50 mL ×3). The organic layer was combined, washed with brine (100 mL), washed with saturated aqueous NaCl solution, dried over $MgSO_4$, and then concentrated in vacuo. Silica gel column chromatography of the residue eluted successively with 80:20 and 60:40 hexane/AcOEt to give **6a** (ca. 10 mg) and **7a** (32.6 mg). Taking the volatile property into account, **6a** was not fully concentrated. ^1H NMR spectral data **6a**: ($CDCl_3$) δ 1.19 (3H, d, *J* = 7.2 Hz), 1.23 (3H, d, *J* = 6.3 Hz), 2.46 (1H, quint, *J* = 7.2 Hz), 2.65 (1H, br), 3.72 (3H, s), 3.89 (1H, m). The ^1H NMR spectral data of this sample were accorded well with *threo*-methyl 3-hydroxy-2-methylbutanoate but not with the corresponding *erythro*-isomer.[25] ^1H NMR spectral data **7a**: ($CDCl_3$) δ 0.88 (3H, t, *J* = 7.1 Hz), 1.28 (10H, m), 1.37 (3H, s), 1.41 (1H, ddd, *J* = 3.2, 8.8, 14.0 Hz), 1.46 (2H, m), 1.48 (3H, s), 1.63 (1H, ddd, *J* = 2.8, 10.2, 14.0 Hz), 2.57 (2H, m), 3.02 (1H, brd, *J* = 4.2 Hz), 3.72 (3H, s), 3.79 (1H, m), 4.45 (1H, ddd, *J* = 3.4, 6.4, 10.2 Hz), 4.58 (2H, m), 5.72 (1H, dd, *J* = 6.9, 15.5 Hz), 5.80 (1H, dd, *J* = 5.2, 15.5 Hz). The spectral data of this sample were accorded well with that we previously reported.[10]

4.9.** (S)- and (R)-MTPA esters (S)-**6b** and (R)-**6b

A solution of **6a** (ca. 2.0 mg) in pyridine (0.2 mL) was stirred with (*R*)-MTPACl (ca. 10 mg) at room temperature for 2 h. After MeOH (10 mL) was added, the mixture was further stirred at room temperature for 20 min. Diethyl ether (1.0 mL) was added and the resulting suspension was filtered through a cotton pad and the filtrated was concentrated in vacuo. Preparative TLC (hexane/AcOEt, 90:10) gave the MTPA ester (*S*)-**6b** (ca. 2.0 mg). In the similar manner as described for the preparation of (*S*)-**6b**, (*R*)-MTPA ester (*R*)-**6b** (ca. 1.5 mg) was prepared by employing (*S*)-MTPACl in place of (*R*)-MTPACl. The yields of these samples were not accurate because of small quantity. The δΔ values, as shown below, suggested (3*S*)-configuration.

	(*S*)-**6b** (*J* , Hz)	(*R*)-**6b** (*J* , Hz)	δΔ (ppm)
CH_3O-	3.64 (s)	3.54 (s)	+0.10
H-2	2.76 (dq, 7.8, 7.2)	2.74 (dq, 7.9, 7.1)	+0.02
H-3	5.36 (dq, 6.3. 7.8)	5.37 (dq, 6.4, 7.9)	-0.01
H_3-4	1.29 (d, 6.3)	1.37 (d, 6.4)	-0.08
H_3-5	1.19 (d, 7.2)	1.14 (d, 7.1)	+0.05
CH_3O-(MTPA)	3.50	3.54	-

* common signals: 7.4 (3H, m), 7.51 (2H, m).

4.10.** (S)- and (R)-MTPA esters (S)-**7b** and (R)-**7b

In the similar manner as described for the preparation of **6b**, Treatment of **7a** (ca. 3.5 mg) with (*R*)-MTPACl afforded (*S*)-MTPA ester (*S*)-**7b** (1.9 mg). In the similar manner, (*R*)-**7b** (ca. 3.8 mg) was also prepared by employing (*S*)-MTPACl in place of (*R*)-MTPACl. The δΔ values, as shown below, are consistent with those we have reported previously. The Δδ could not obtained for H_2-11 and H_2-12 (*achaetolide numbering*) in the present study because of small quantity of the samples. Signal assignment for these protons requires a series of 2D NMR spectra in high resolution.

	(*S*)-**7b** (*J* , Hz)	(*R*)-**7b** (*J* , Hz)	δΔ (ppm)
CH_3O-(*ester*)	3.59 (s)	3.66 (s)	-0.07
H-2	2.61 (dd, 4.7, 16.0)	2.64 (dd, 4.5, 16.5)	-0.03
	2.77 (dd, 8.9, 16.0)	2.80 (dd, 9.2, 16.5)	-0.03
H-3	5.88 (ddd, 4.7, 6.4, 8.9)	5.86 (ddd, 4.5, 6.5, 9.2)	+0.02
H-4	5.74 (dd, 6.4, 16.0)	5.68 (dd, 6.5, 15.5)	+0.06
H-5	5.80 (dd, 4.9, 16.0)	5.74 (dd, 5.7, 15.5)	+0.06
H-6	4.30 (dd, 4.9, 6.4)	4.47 (t, 5.7)	-0.17
H-7	3.90 (m)	4.15 (m)	-0.25
H_2-8	1.52 (m)	1.61 (m)	-0.09
H-9	5.21 (m)	5.23 (m)	-0.02
CH_3-	1.28 and 1.45	1.32 and 1.43	-
CH_3O-(MTPA)	3.49, 3.57	3.51, 3.54	-

*common signals: 0.86 (3H, t, *J* = 7.1 Hz), 1.2 (10H, m), 1.6 (2H, m), 7.4 (3H, m), 7.51 (2H, m).

4.11. Inhibition assay against C. miyabeanus.

A series of suspensions of spores of *Cochlibolus miyabeanus* and 0.1% sucrose containing 0.01, 0.1, 1, 10, and 100 μg/mL of samples in Petri dishes was prepared and incubated at 25 °C for 24 h. These were observed with a microscope.

4.12. Cytotoxicity assay against human colon adenocarcinoma (COLO 201) cells.

The effect of compounds on human colon adenocarcinoma (COLO 201) cell proliferation was measured by WST-1 assay.[30] COLO 201 cells were cultured in RPMI medium (5×10^3 cells/mL) containing a series of compounds in 96-well tissue culture plates with concentrations of 1.0, 5.0, 10, 50, 100,

500 μg/mL. After 24 h, 10 μL of WST-1 reagent was added to each well and the absorbance was measured at 450 nm using a titer-plate reader.

4.13. Growth inhibition assay against Lactuca sativa.

Samples of 0, 25, 50, 100, 250, 500, and 1000 μg were dissolved in MeOH (1.0 mL). Each solution was loaded on a 55 mmϕ filter paper and the solvent was removed at room temperature. Each filter paper was placed on a Petri dish (70 mmϕ), and distilled water (1.0 mL) and ten sprouts of *L. sativa* were added. After incubation for 3 days at 25 °C, the roots and stems were measured with a ruler.

5. Acknowledgments

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6. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http:

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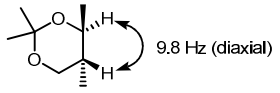

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Supplemental data

Achaetolide-II isolated from *Helminthosporium velutinum* TS28

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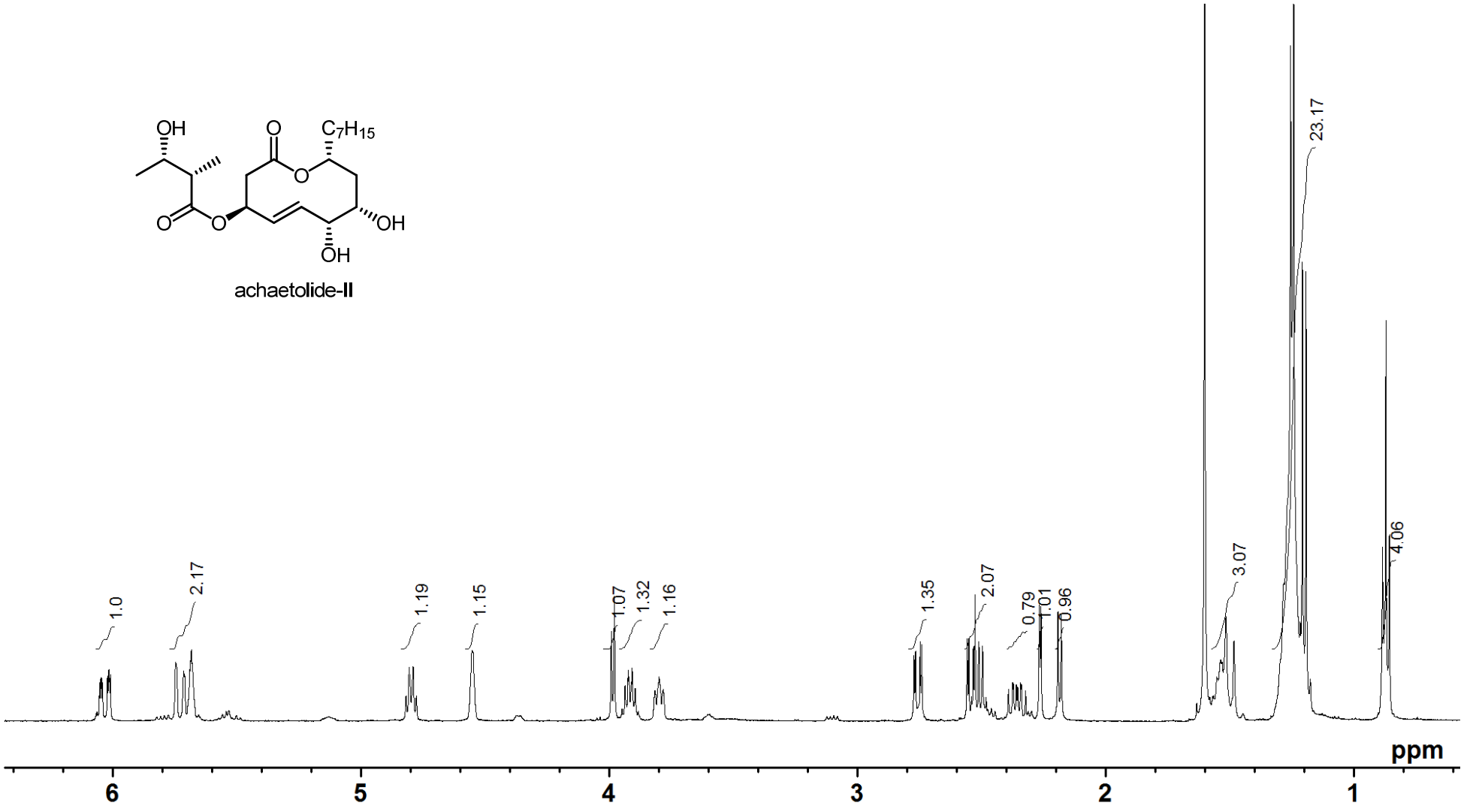
^{1}H NMR (500 MHz) spectrum of achaetolide-II (**2**) in $CDCl_3$



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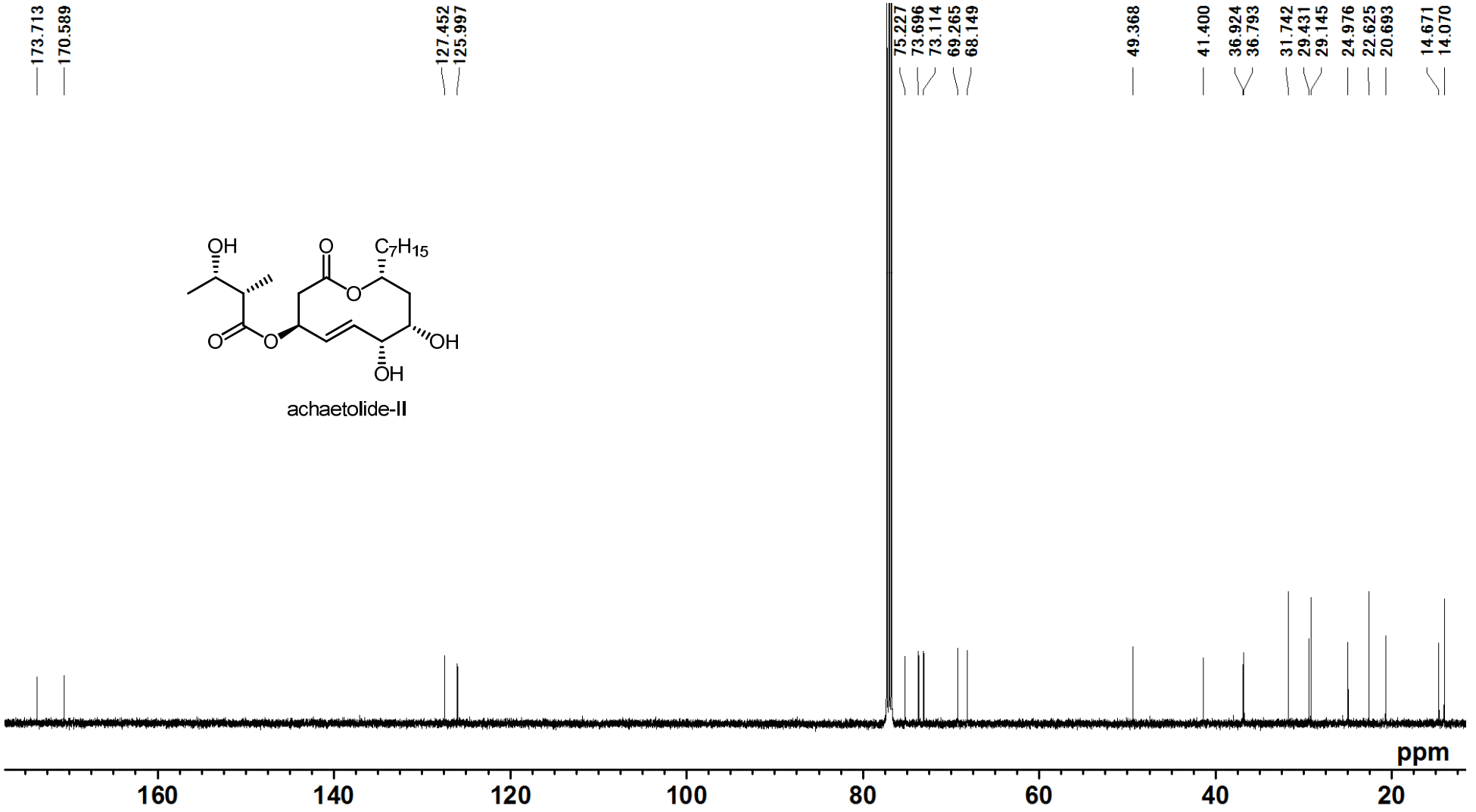

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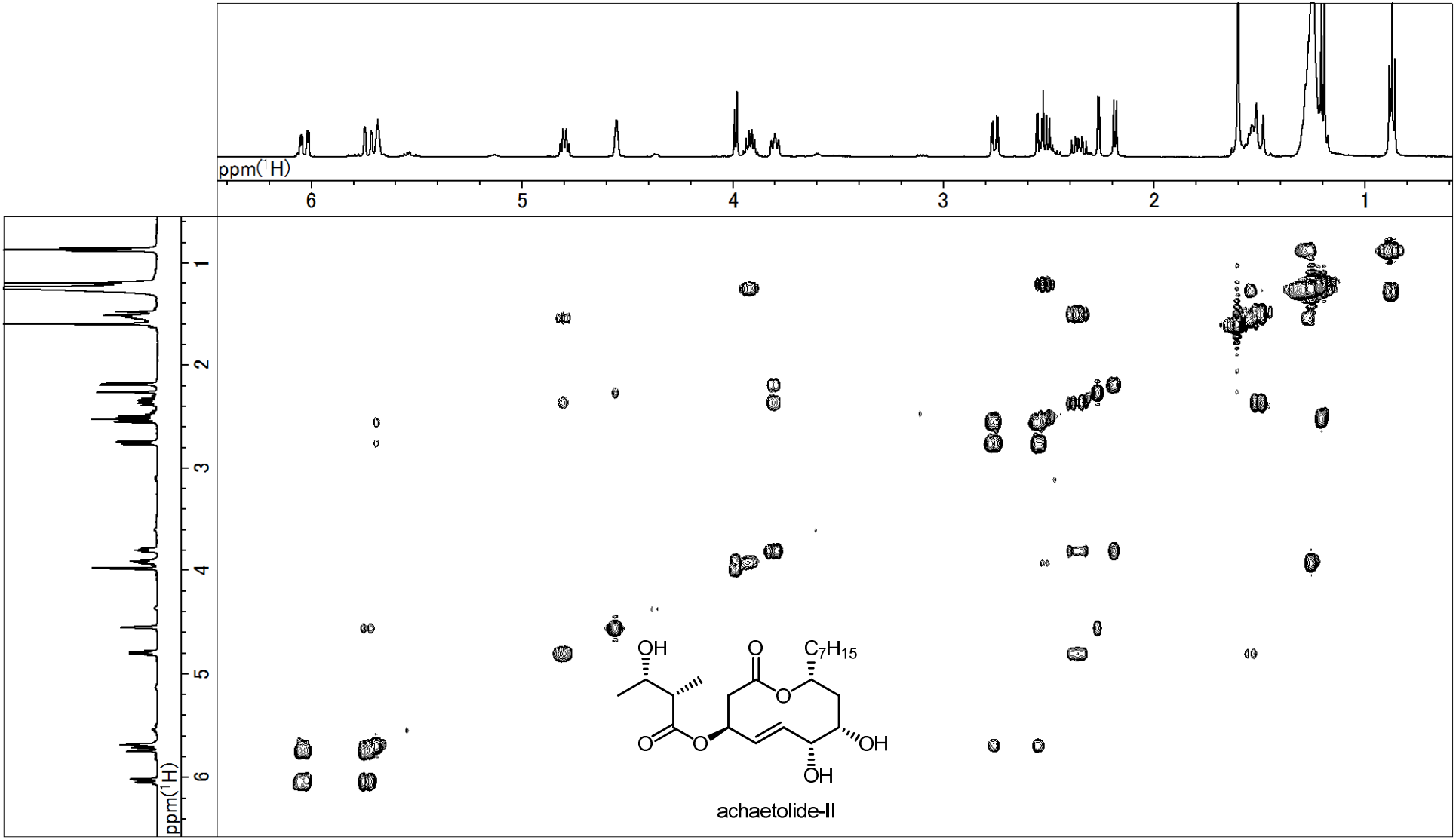

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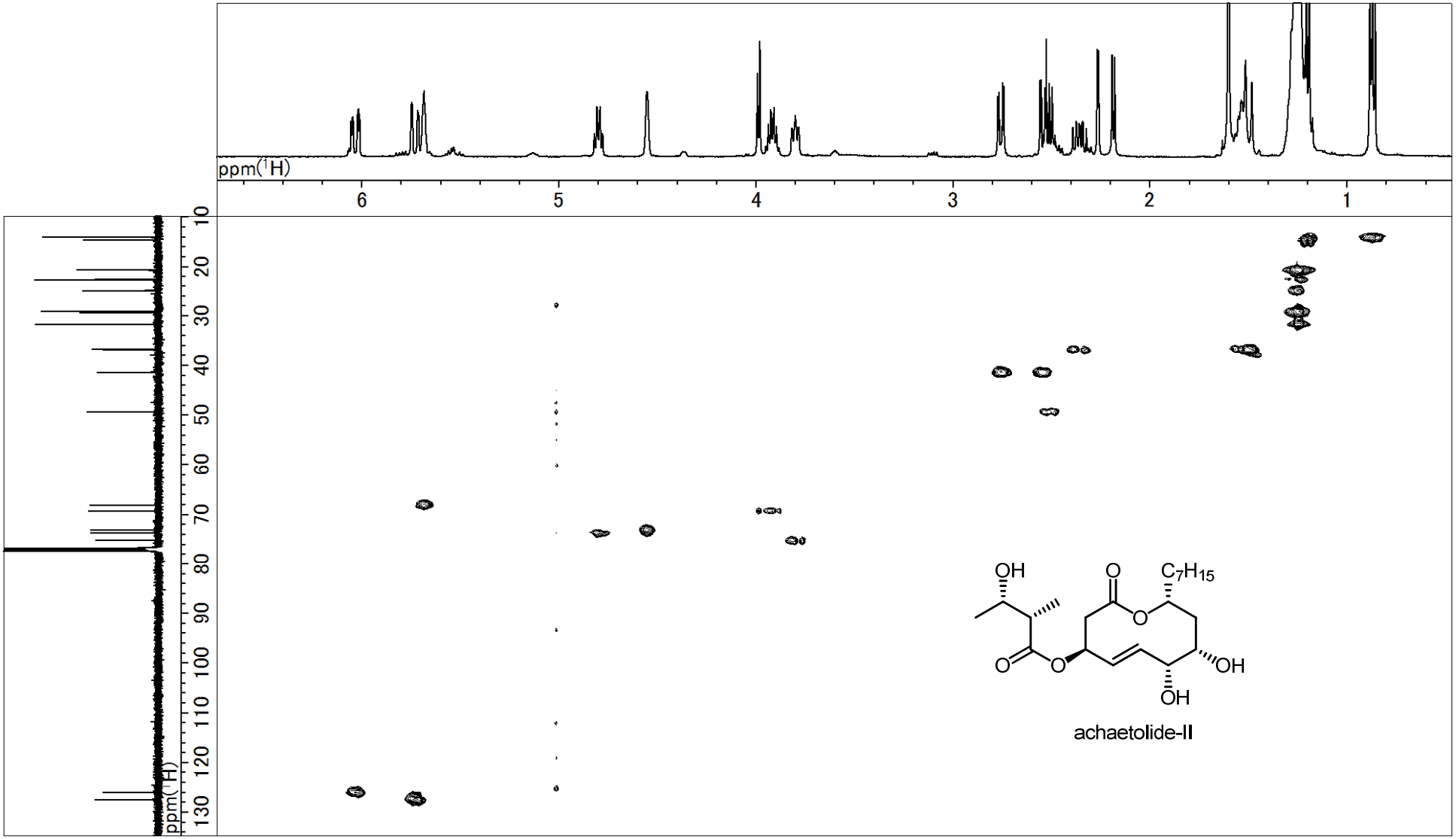

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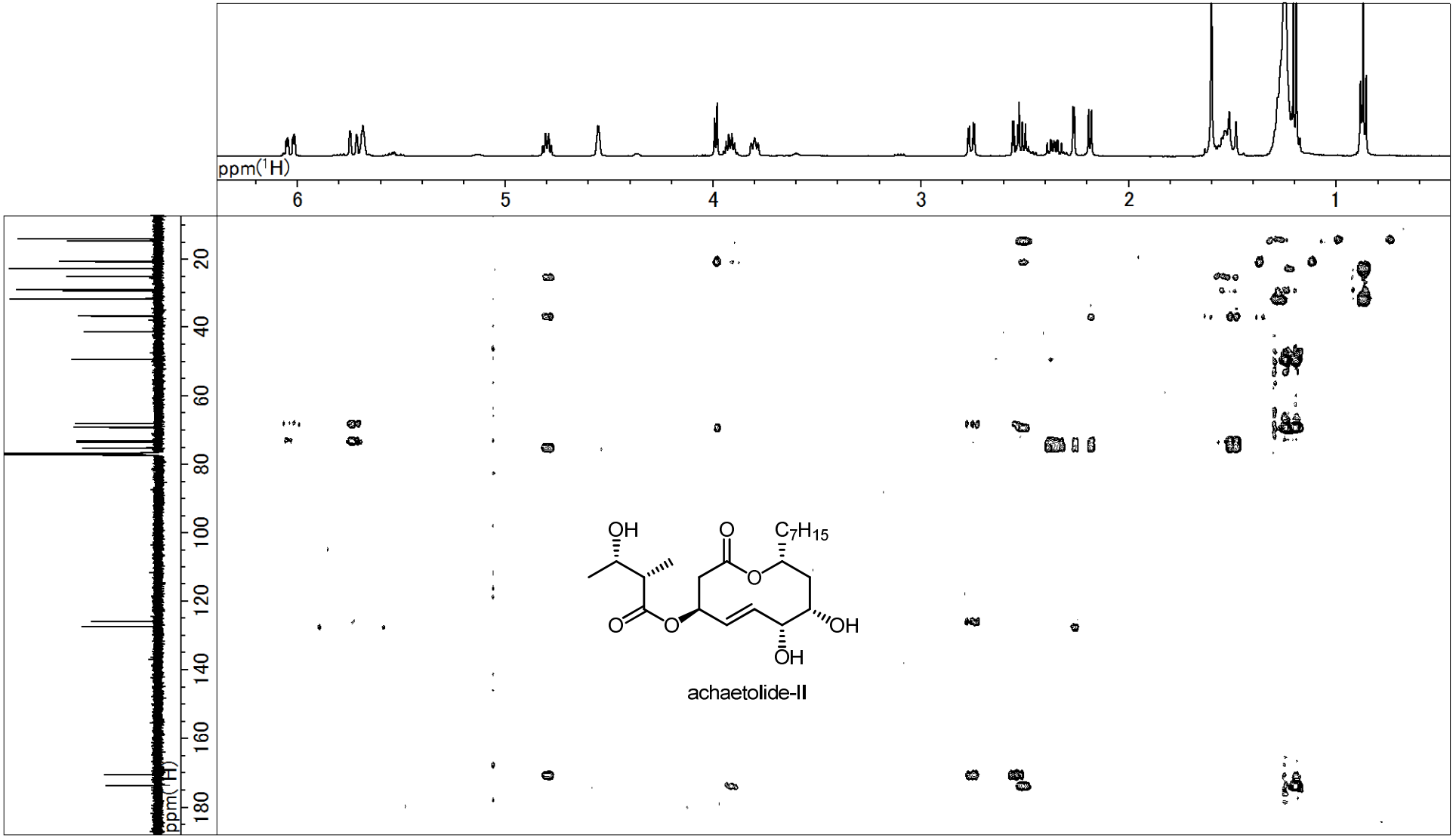

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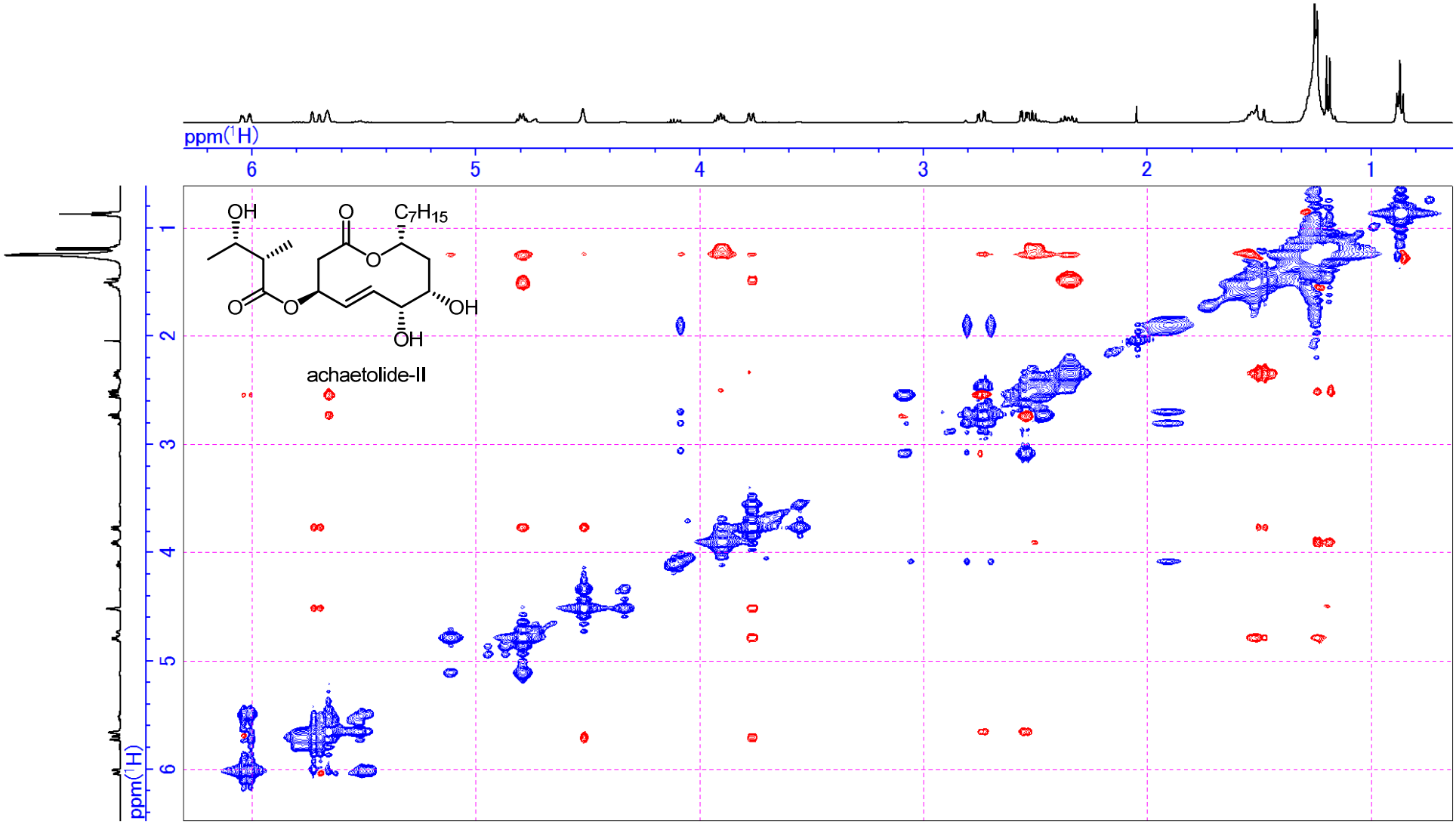

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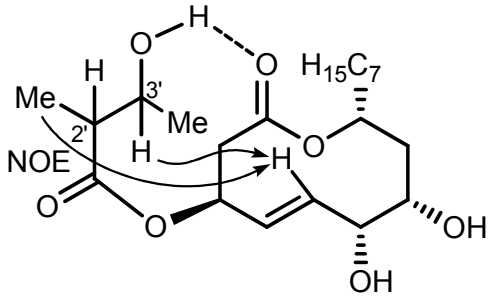
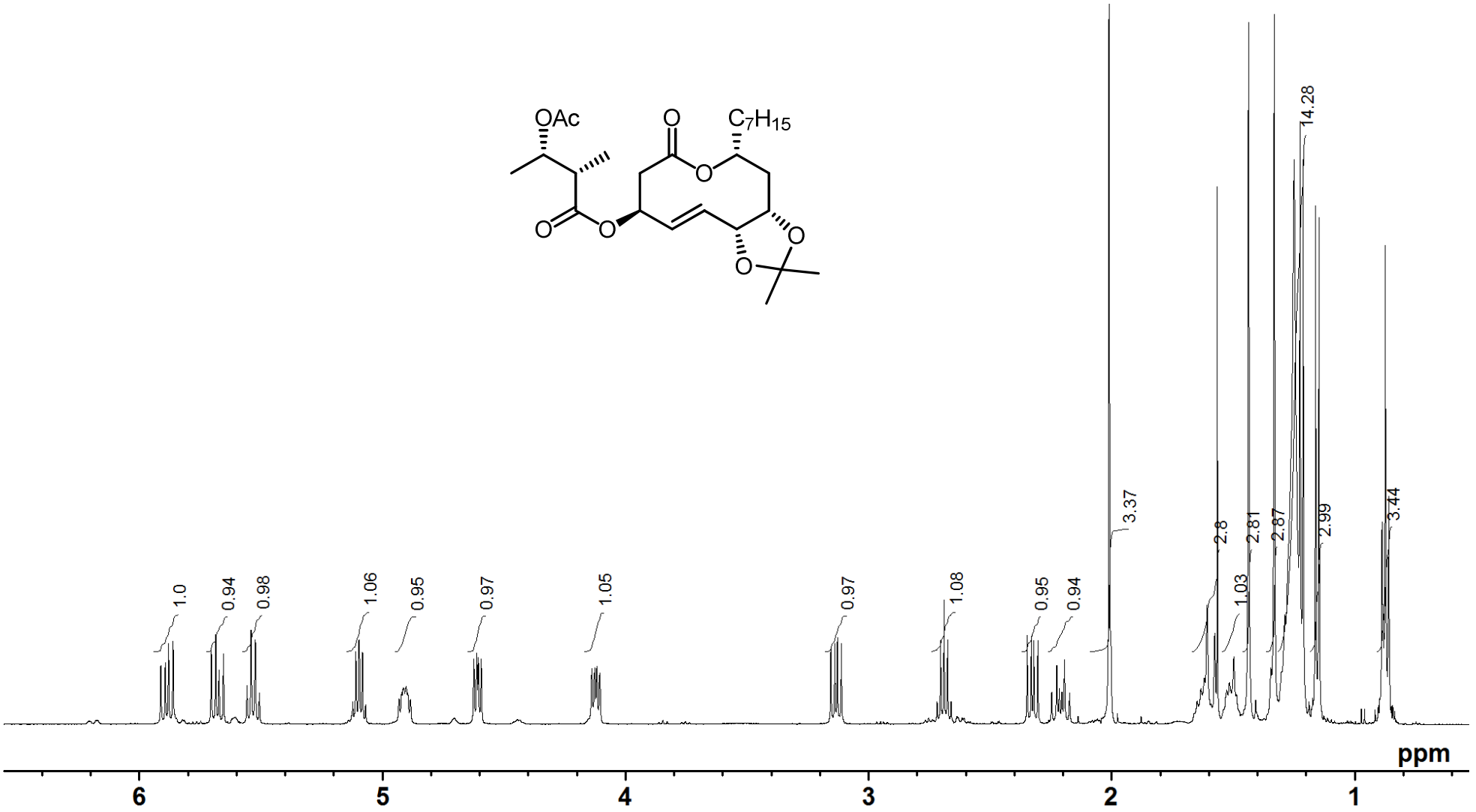
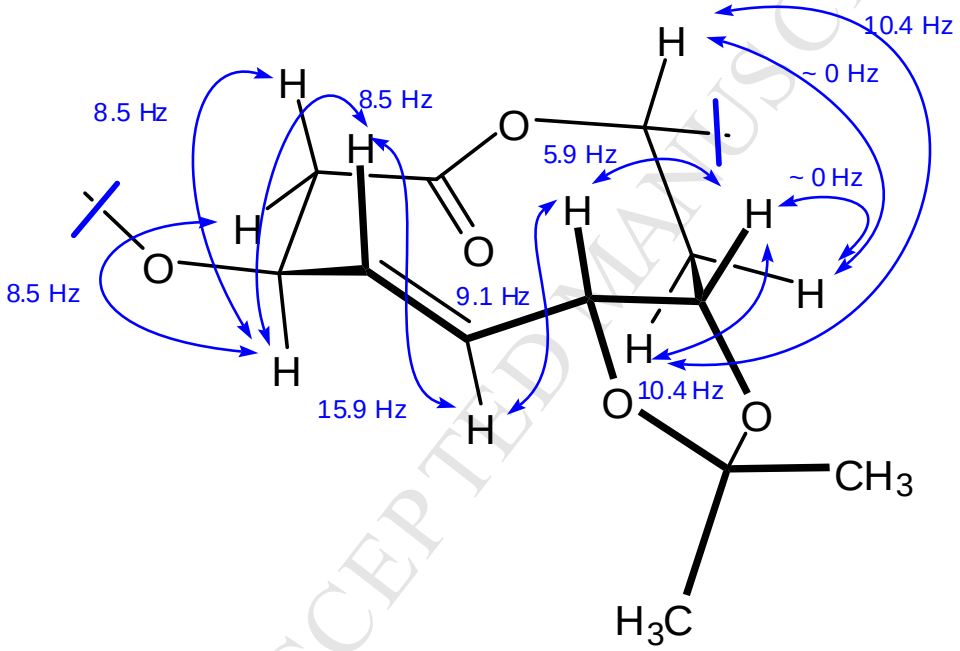


^{1}H NMR spectrum (500 MHz) of 3'-*O*-acetyl-6,7-*O*-isopropylidene-achaetolide-II in $CDCl_3$

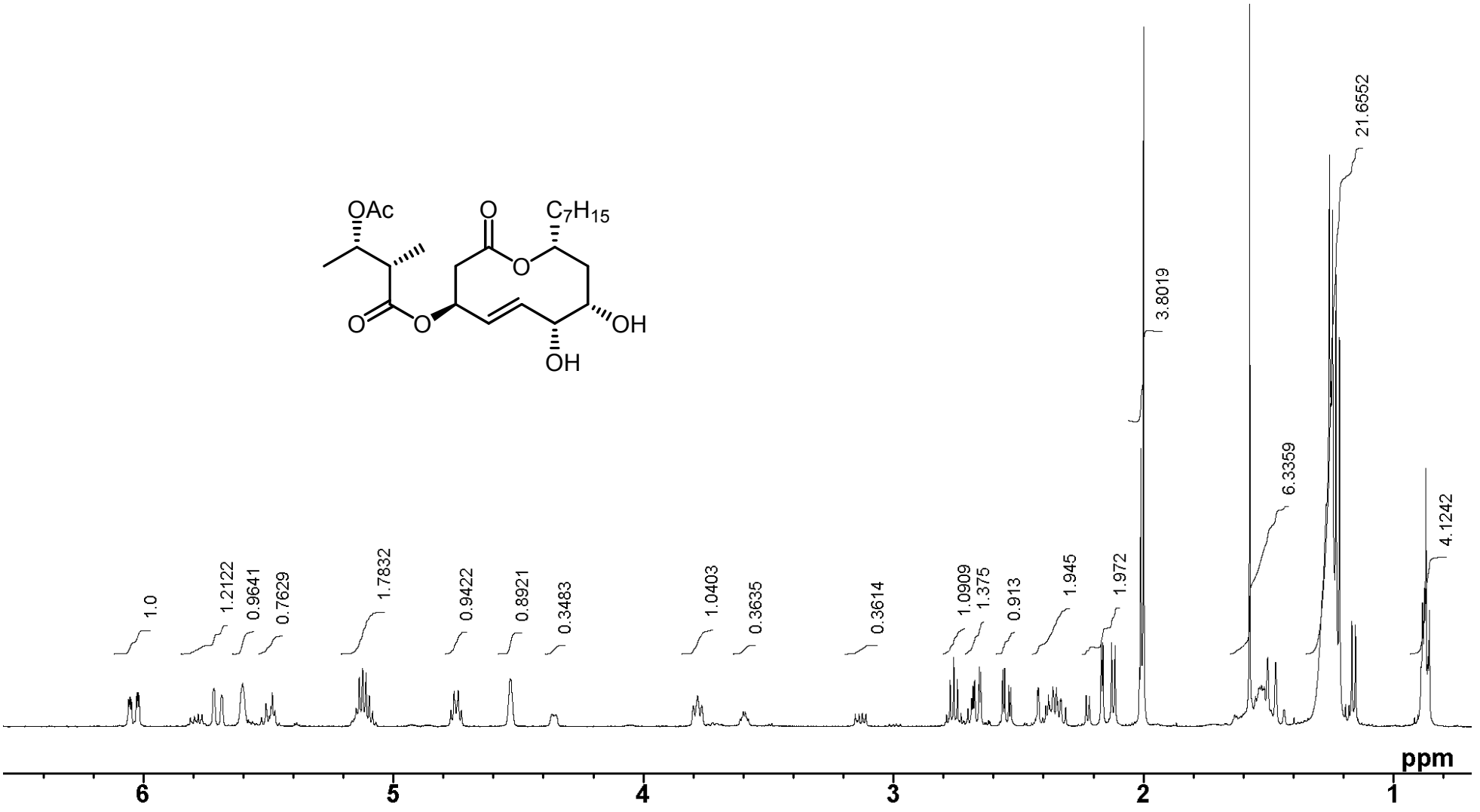


Main conformer of 3'-*O*-acetyl-6,7-*O*-isopropylidene-achaetolide-II and its ^{1}H spin couplings of in $CDCl_3$

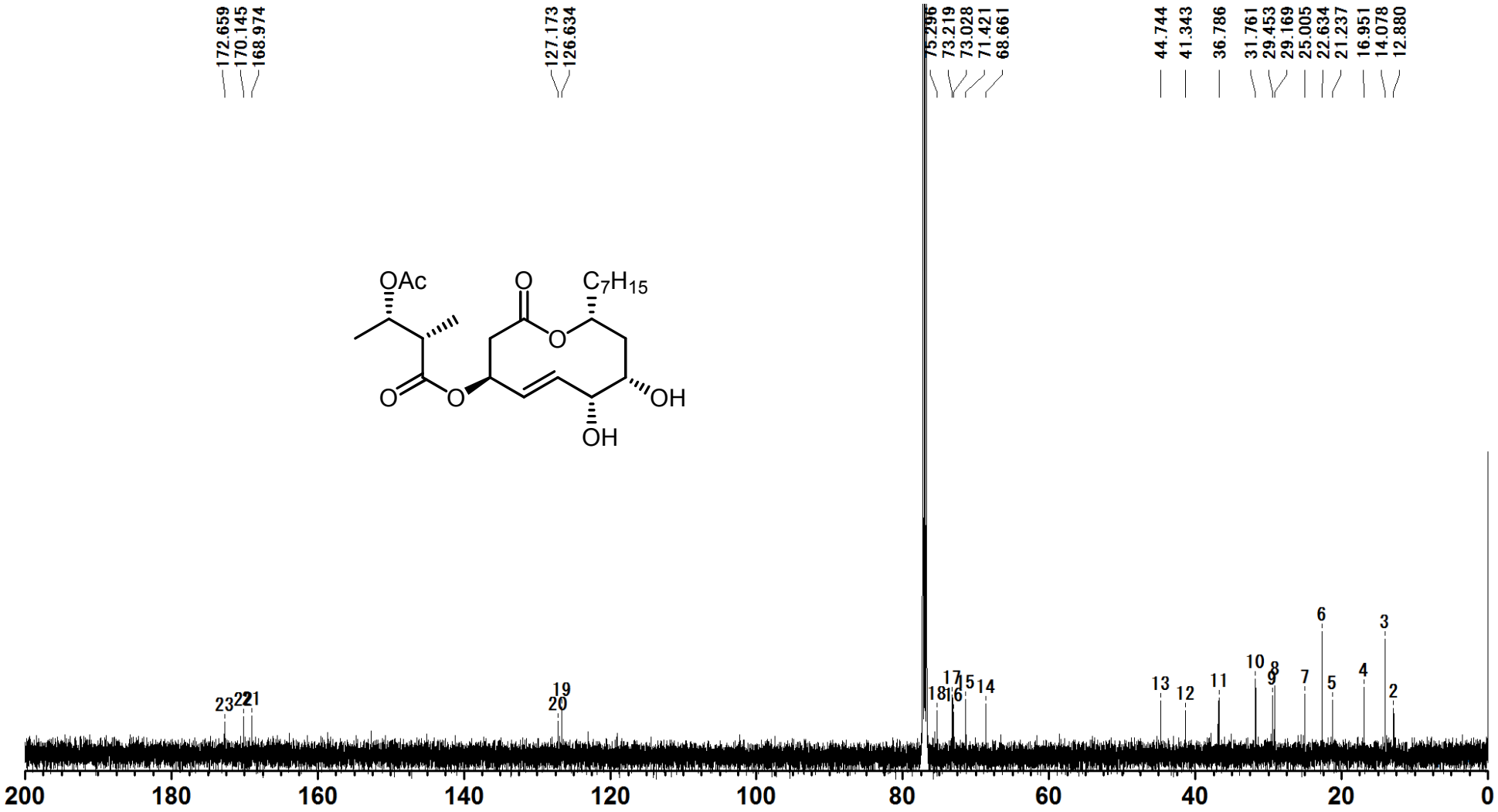


Conformation of 3'-*O*-acetyl-6,7-*O*-isopropylidene achaetolide II

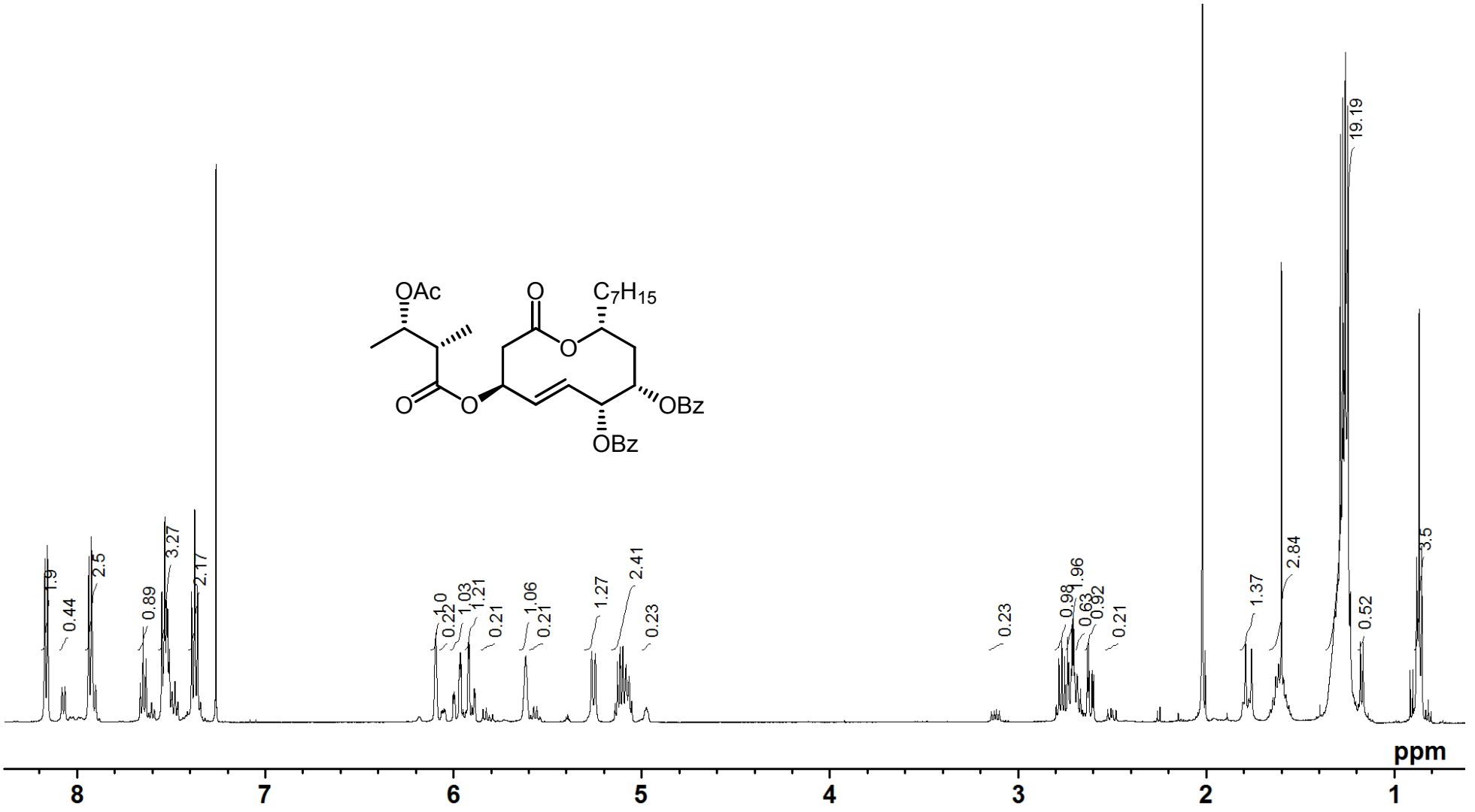
^{1}H NMR spectrum (500 MHz) of 3'-*O*-acetyl-achaetolide-II (**3**) in $CDCl_3$



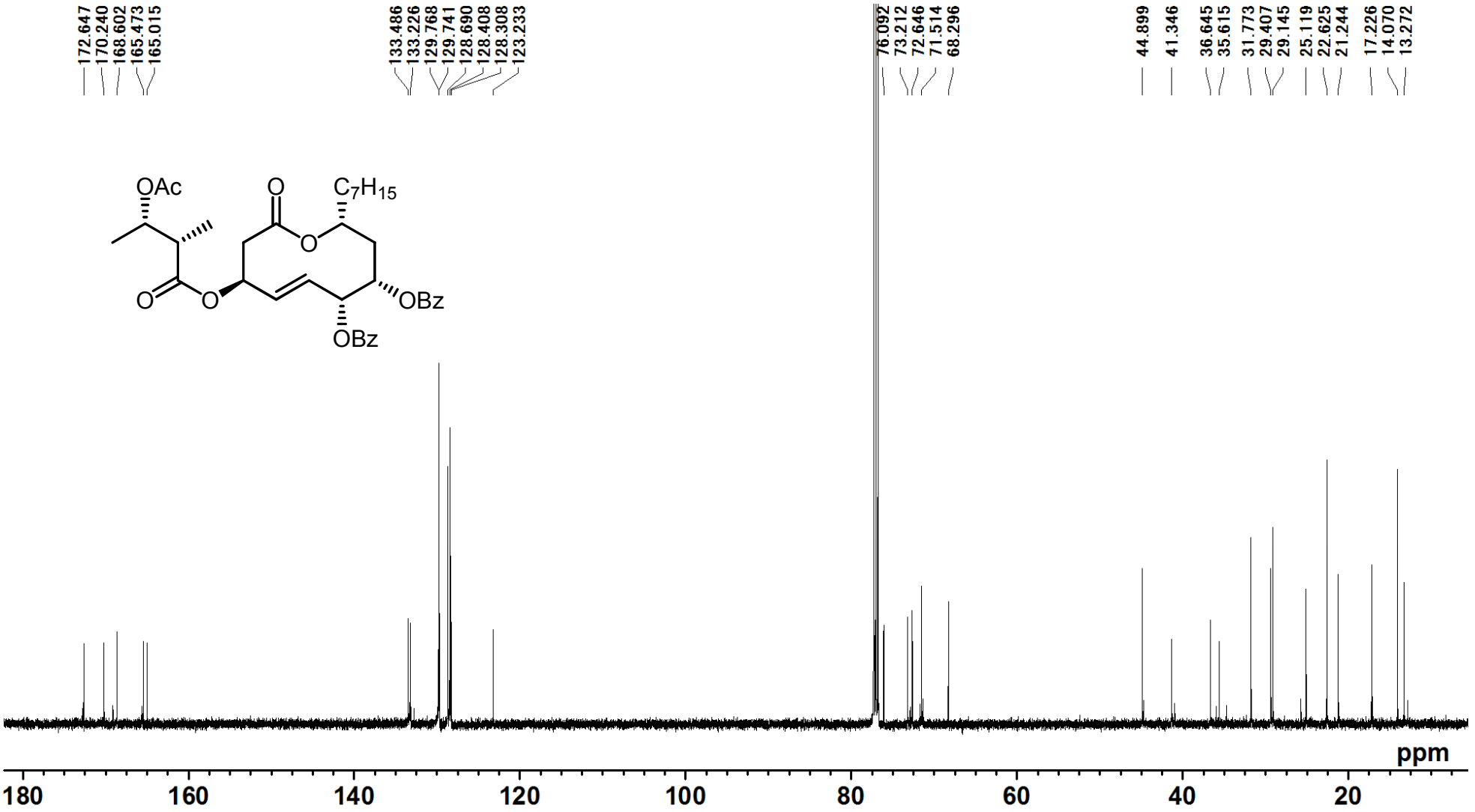
^{13}C NMR spectrum (125 MHz) of 3'-*O*-acetyl-achaetolide-II (**3**) in $CDCl_3$



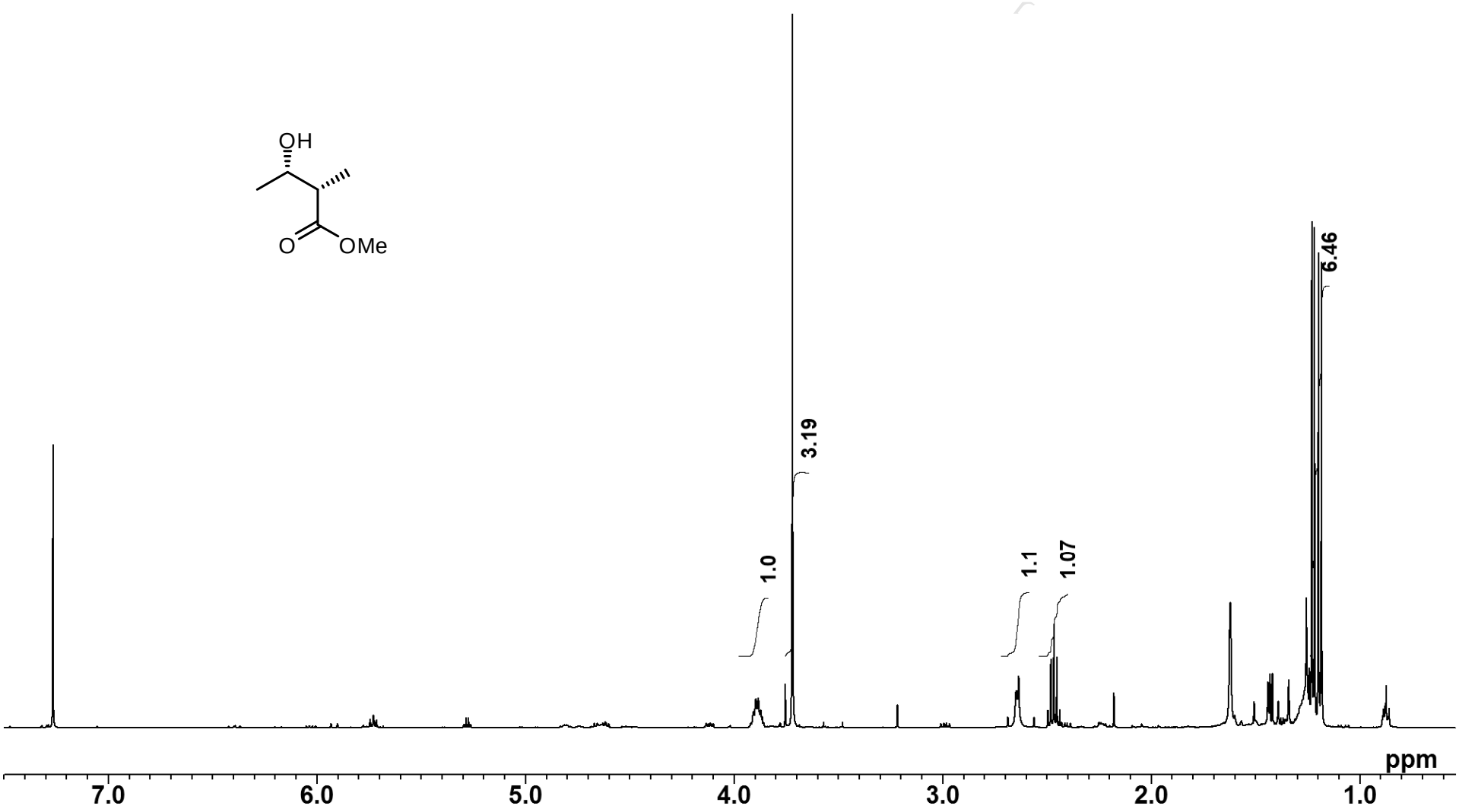
^{1}H NMR spectrum (500 MHz) of 3'-*O*-acetyl-6,7-*O*-dibenzoyl-achaetolide-II (**5**) in $CDCl_3$



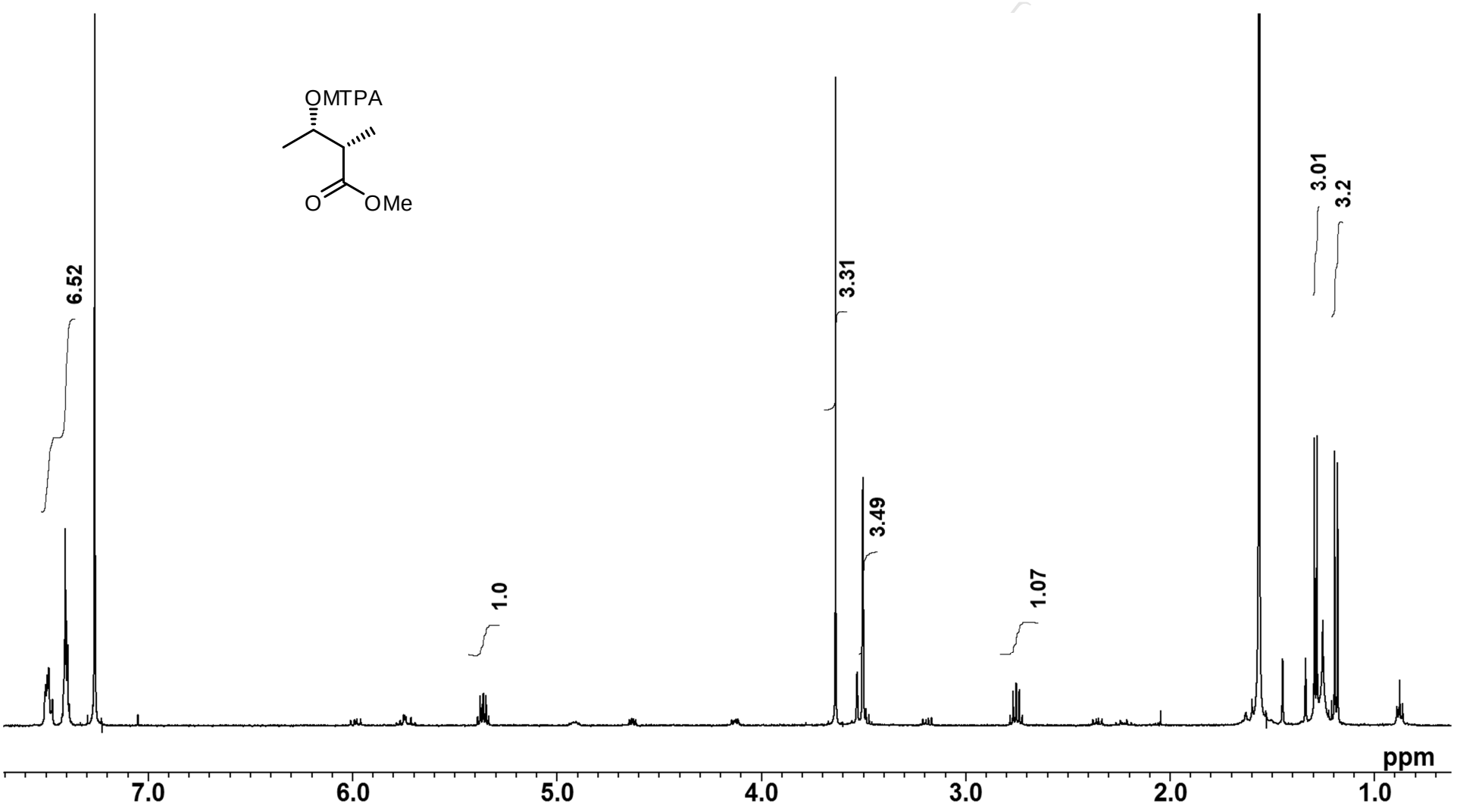
^{13}C NMR spectrum (125 MHz) of 3'-*O*-acetyl-6,7-*O*-dibenzoyl-achaetolide-II (**5**) in $CDCl_3$



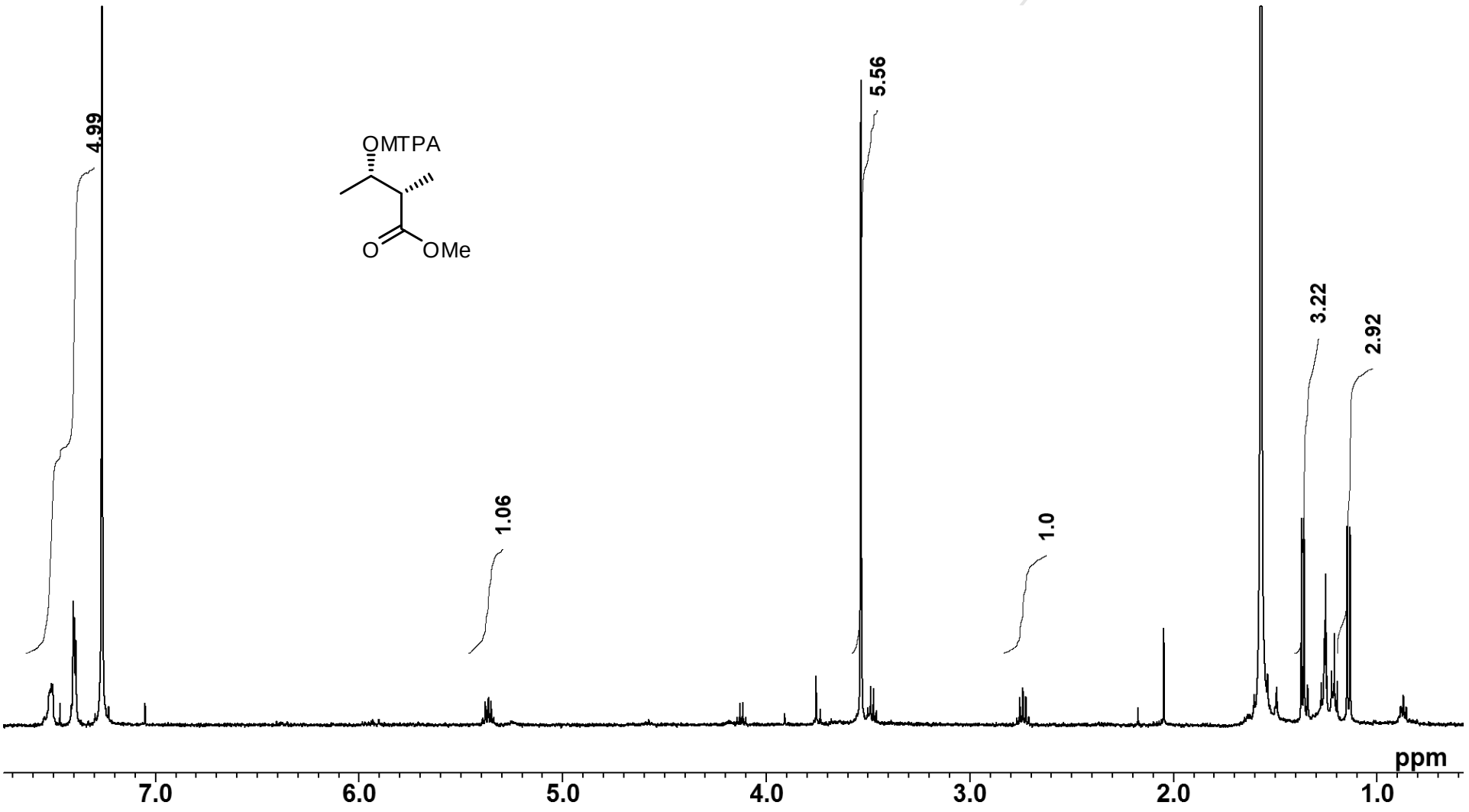
^{1}H NMR spectrum (500 MHz) of **6a** in $CDCl_3$



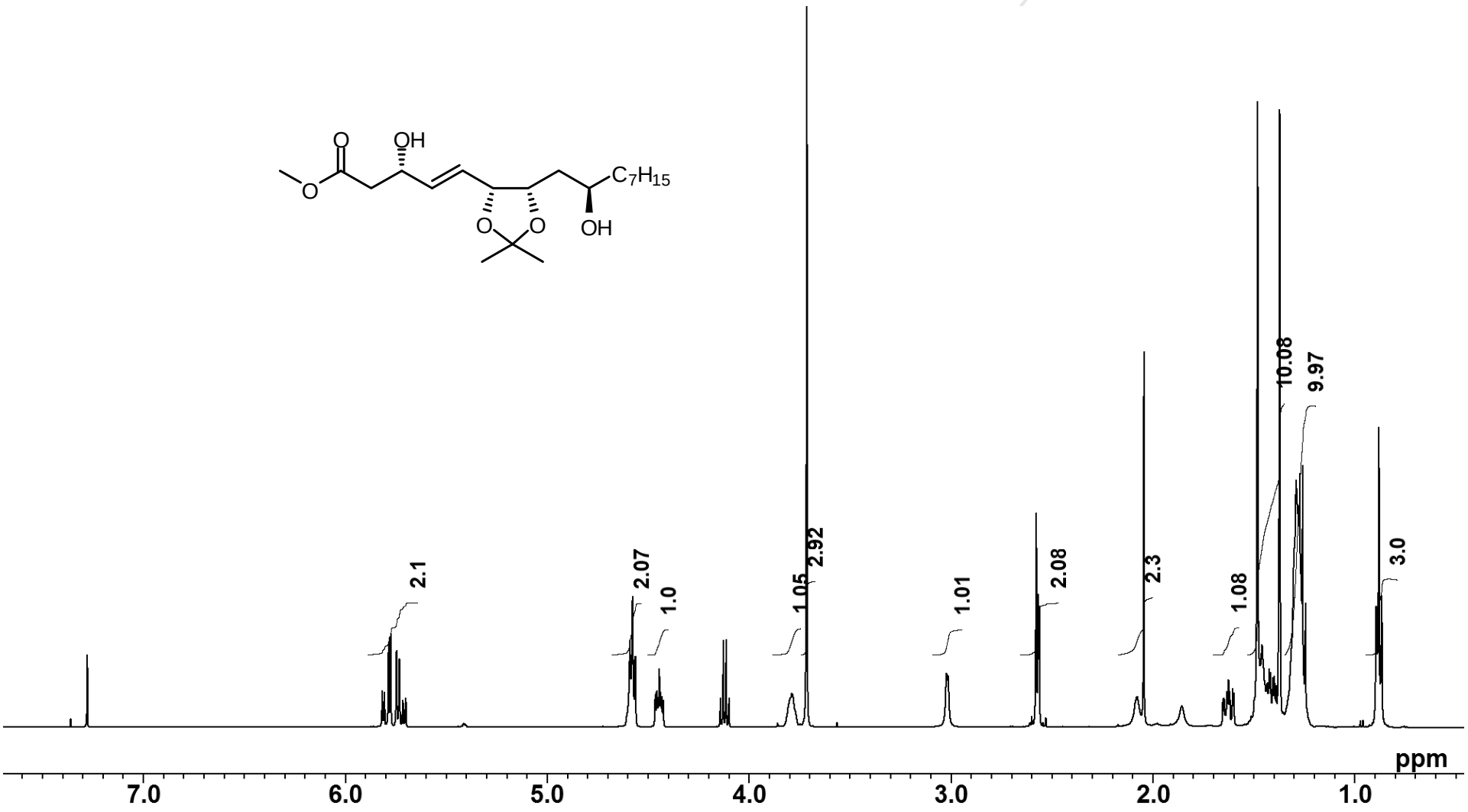
^{1}H NMR spectrum (500 MHz) of (*S*)-MTPA ester (*S*)- **6b** in $CDCl_3$



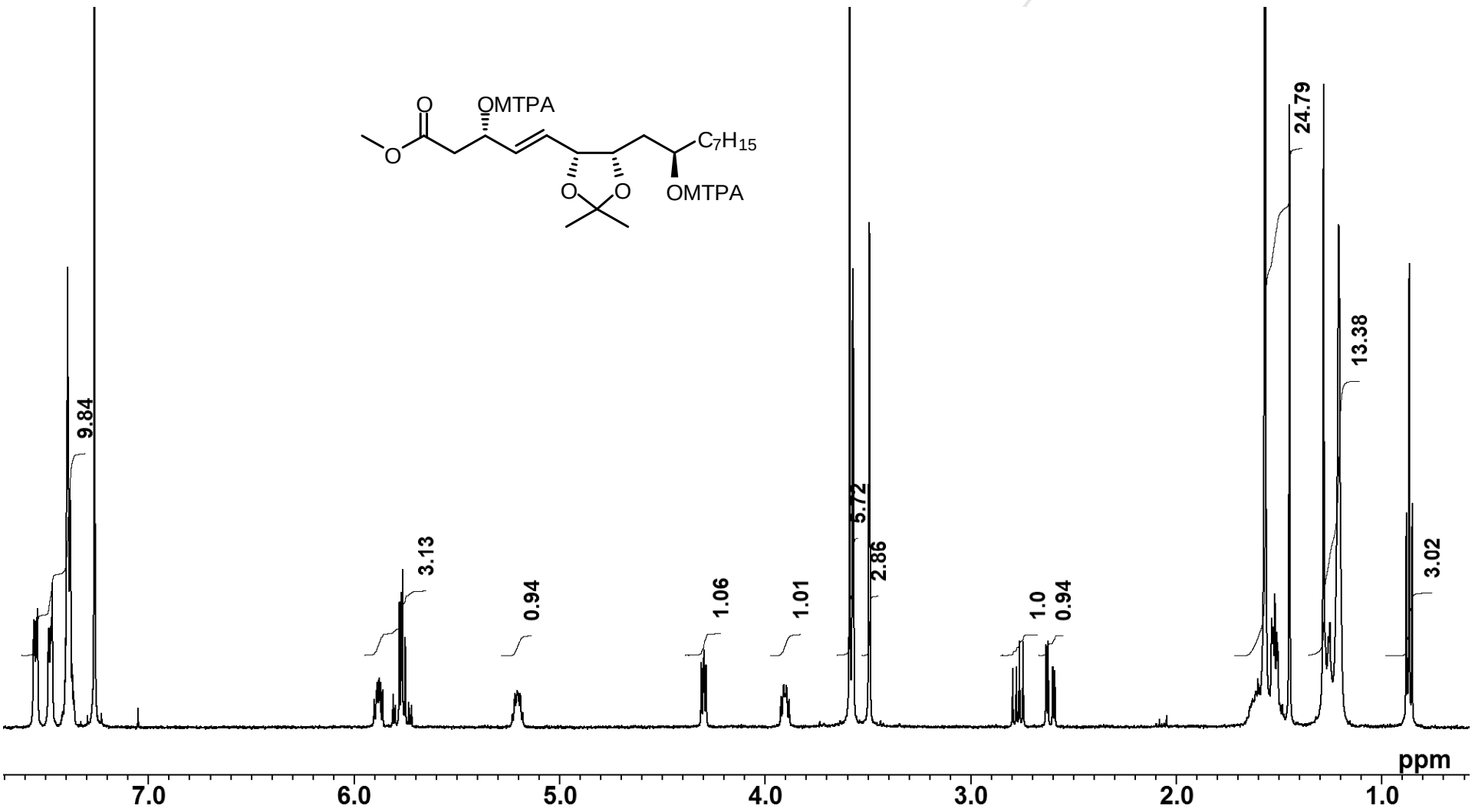
^{1}H NMR spectrum (500 MHz) of (*R*)-MTPA ester (*R*)-**6b** in $CDCl_3$



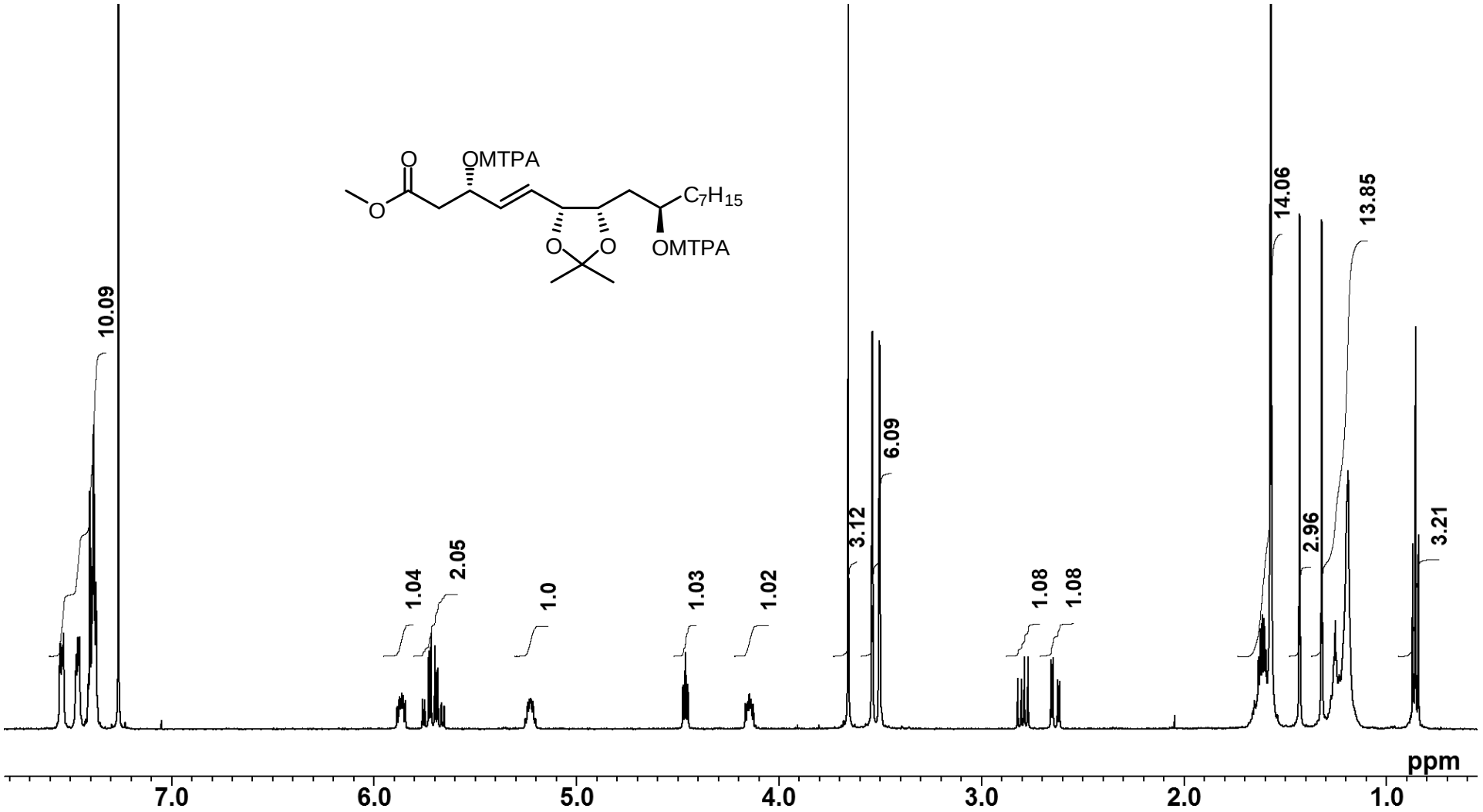
^{1}H NMR spectrum (500 MHz) of **7a** in $CDCl_3$



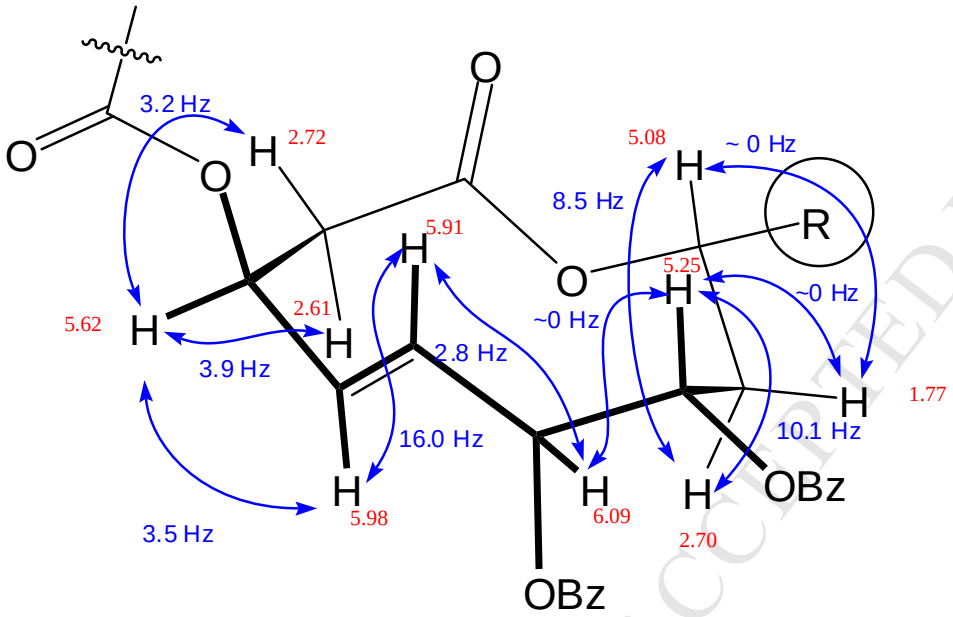
^{1}H NMR spectrum (500 MHz) of (*S*)-MTPA ester (*S*)-**7b** in $CDCl_3$



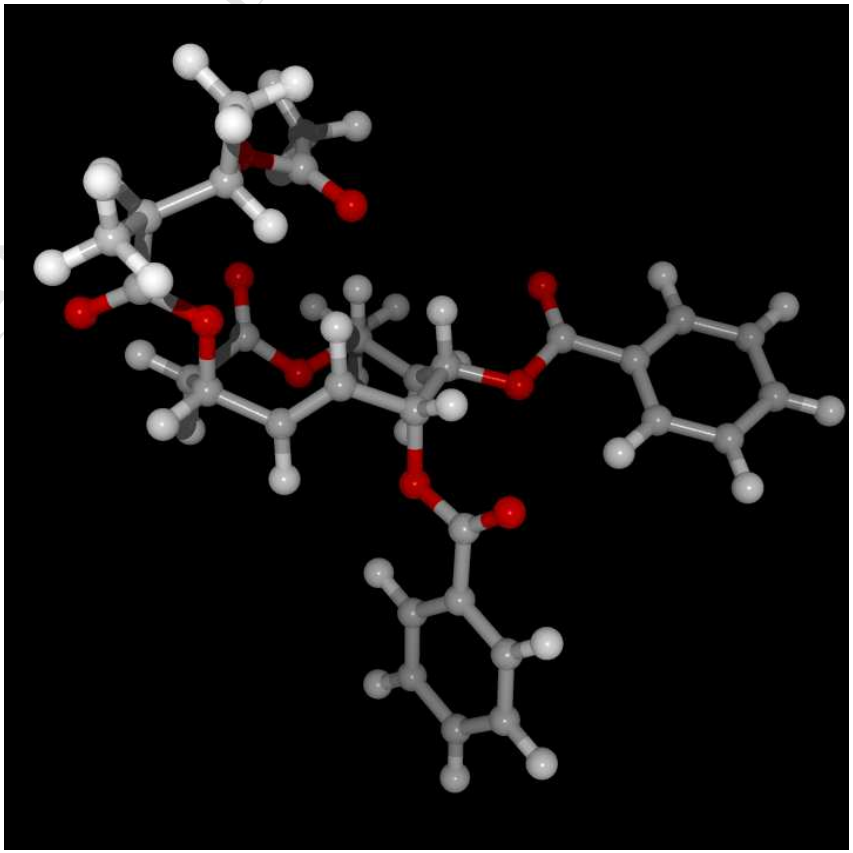
^{1}H NMR spectrum (500 MHz) of (*R*)-MTPA ester (*R*)-**7b** in $CDCl_3$



Stable conformation of 3'-*O*-acetyl-6,7-*O*-dibenzoyl-achaetolide-II (**5**) based on ^{1}H spin coupling (left) and molecular modeling calculation for the model based on EDF2/6-31G* (right)



The most stable conformation based on EDF2/6-31G*



Results of chemical shift calculations with EDF2/6-31G*

	position				δ C-1	δ C-2	δ C-3	δ C-4	δ C-5	δ C-1'	δ C-2'	δ C-3'	δ C-4'	δ C-5'
	ID	ΔH (kJ/mol)	ΔG° (kJ/mol)	Boltz. distr. (%)	**170.59**	**41.40**	**68.15**	**125.99**	**127.45**	**173.71**	**49.37**	**69.26**	**20.69**	**14.67**
(2'S,3'S)-isomer	M0002	0.00	0.00	0.862	173.73	40.15	68.42	126.99	128.65	173.72	50.71	69.07	20.62	15.99
	M0001	5.70	6.67	0.058	173.32	39.40	71.08	125.72	130.41	173.60	47.85	70.93	21.99	16.19
	M0017*	8.46	8.14	0.032	170.28	39.44	70.24	125.74	129.47	179.17	43.67	70.03	22.34	16.73
	M0018	11.17	12.68	0.005	174.07	40.95	68.55	127.39	127.94	171.77	46.20	74.08	22.03	15.56
	M0005	14.21	12.44	0.006	170.34	40.38	69.78	125.89	129.36	177.52	47.26	69.18	19.03	15.36
	M0004	9.98	9.14	0.021	173.92	38.46	69.79	128.55	136.69	174.51	50.55	68.81	20.38	15.99
	M0016	12.73	11.97	0.007	170.46	40.64	73.72	123.92	138.73	178.80	42.97	70.47	22.17	17.72
	M001[1]	14.81	14.31	0.003	172.98	38.02	71.26	128.03	137.07	174.29	50.45	68.97	20.51	16.00
	M0048	14.42	12.50	0.005	169.39	40.39	73.37	125.28	138.77	179.07	43.04	70.42	22.16	17.68
	M005[1]	14.59	16.88	0.001	174.29	39.50	70.45	131.95	132.25	174.44	47.54	70.76	22.17	16.18
	Boltz Avgs				**173.53**	**40.05**	**68.74**	**126.88**	**129.10**	**173.98**	**50.17**	**69.24**	**20.77**	**16.04**
(2'R,3'R)-isomer	M0037	0.00	0.00	0.161	173.77	40.45	68.03	128.32	124.98	173.93	47.94	72.72	22.94	15.28
	M0009	0.19	1.94	0.073	173.85	39.96	70.26	127.06	128.73	173.85	45.64	72.28	23.77	17.49
	M0021	0.21	1.76	0.079	173.73	39.94	70.28	127.01	128.79	173.84	45.65	72.27	23.75	17.48
	M0001	0.59	(0.66)	0.210	173.66	39.23	71.09	125.77	130.56	176.11	47.82	69.46	21.74	14.00
	M0003	1.13	0.29	0.143	173.83	38.96	71.58	127.12	126.89	176.02	47.82	69.57	21.69	14.15
	M0033	1.30	1.52	0.087	173.97	40.56	67.99	127.85	126.14	173.83	47.94	72.76	22.96	15.27
	M0019	2.03	3.07	0.046	174.01	39.88	70.56	128.07	125.61	173.78	45.62	72.26	23.79	17.53
	M0026	2.86	9.40	0.004	173.12	39.12	71.40	124.86	135.69	176.55	47.92	69.98	21.71	14.23
	M0006	3.15	3.12	0.045	173.36	39.25	71.35	123.46	133.01	176.38	48.06	70.00	21.74	14.32
	M0002	3.17	2.10	0.069	173.97	38.94	71.87	126.83	127.94	176.18	47.71	69.81	21.67	14.21
	M0015	3.32	5.01	0.021	174.20	39.77	70.86	127.70	126.61	173.87	45.63	72.26	23.82	17.49
	M0041*	5.15	10.61	0.002	169.24	39.05	71.11	124.31	135.66	179.77	43.67	70.82	22.88	17.54
	M0035	5.55	4.67	0.024	169.78	38.92	71.97	125.76	129.52	179.23	43.59	71.05	22.96	17.63
	M0004	5.56	4.75	0.024	169.78	38.90	71.94	125.74	129.53	179.23	43.59	71.06	22.96	17.64
	M0046	5.99	6.27	0.013	170.01	38.75	72.17	127.00	126.34	179.22	43.57	70.98	23.00	17.60
	Boltz Avgs				**173.54**	**39.61**	**70.37**	**126.87**	**128.03**	**175.26**	**47.11**	**71.05**	**22.55**	**15.36**
(2'R,3'S)-isomer	M0002	(2.06)	0.00	0.488	173.97	40.16	69.05	127.28	128.33	174.59	45.50	66.81	20.36	9.40
	M0016	(0.02)	2.28	0.194	173.28	39.58	70.76	125.69	130.73	174.23	46.37	71.98	17.19	14.64
	M0001	0.00	2.10	0.209	173.38	39.59	70.73	125.68	130.70	174.20	46.42	71.97	17.20	14.68
	M0014	3.60	4.62	0.075	173.25	40.96	68.02	126.95	128.22	174.10	45.06	71.32	15.97	13.55
	M0004*	8.99	8.87	0.013	169.56	39.18	71.19	125.86	129.13	180.13	42.87	66.46	18.71	10.84
	M005[1]	9.17	9.79	0.009	173.93	38.38	70.28	127.66	137.29	175.04	45.22	66.53	20.19	9.26
	M002[1]	9.17	9.11	0.012	173.85	38.46	70.27	127.84	137.23	175.03	45.32	66.53	20.17	9.25
	Boltz Avgs				**173.60**	**39.94**	**69.71**	**126.60**	**129.48**	**174.49**	**45.79**	**69.22**	**18.73**	**11.84**
(2'S,3'R)-isomer	M0001	0	0.00	0.331	173.31	39.43	70.61	125.86	130.00	175.45	47.46	67.22	21.04	9.37
	M0002	2.64	1.50	0.181	173.85	39.21	71.11	126.80	127.82	175.46	47.54	67.26	20.96	9.38
	M0005	2.7	2.00	0.148	172.89	39.54	70.56	123.63	133.09	175.73	47.45	68.07	21.11	9.12
	M0026	3.13	6.98	0.020	172.95	39.42	70.55	124.85	135.72	175.78	47.64	67.60	20.83	9.42
	M0044*	3.3	8.24	0.012	169.71	39.31	69.88	124.16	135.90	180.65	43.14	66.60	18.92	9.88
	M0010	3.73	2.76	0.109	170.36	39.31	70.51	126.91	126.25	180.21	43.20	66.59	19.08	9.89
	M0019	3.76	4.73	0.049	169.92	39.63	69.63	123.19	133.12	180.35	43.24	66.58	19.02	9.94
	M0007	4.6	3.68	0.075	170.73	39.32	70.53	126.40	127.63	180.30	43.12	66.53	19.04	9.88
	M0013	6.4	6.80	0.021	173.59	39.88	70.51	126.88	129.00	174.39	47.53	69.16	17.85	14.98
	M0039	7.02	13.73	0.001	173.52	39.26	72.40	126.19	132.32	175.90	47.69	68.41	20.99	8.95
	M0025	7.72	9.16	0.008	173.83	39.71	70.89	127.95	125.82	174.22	47.56	69.28	17.89	15.04
	M0050	8.45	13.63	0.001	169.44	39.39	70.48	124.46	135.71	177.11	44.10	71.37	17.76	13.76
	M0043	8.74	9.08	0.008	169.98	39.15	71.06	125.81	129.45	176.94	44.16	71.80	17.96	13.77
	M0008	8.76	9.11	0.008	169.98	39.17	71.14	125.77	129.47	176.97	44.17	71.82	18.03	13.73
	M0024	9.41	9.45	0.007	169.74	39.07	70.85	123.41	133.14	177.03	44.20	71.77	18.23	13.61
	M0011	10.35	10.32	0.005	170.49	39.01	71.49	126.58	127.45	176.89	44.10	71.79	18.11	13.70
	M0028	11.13	13.55	0.001	174.51	40.07	70.44	125.32	137.61	176.37	47.81	68.58	21.09	8.96
	M0036	11.3	11.97	0.003	168.92	40.42	68.98	125.60	132.26	179.48	43.32	66.50	18.89	10.89
	M0029	11.67	11.76	0.003	169.42	39.97	70.22	128.13	127.66	176.02	44.42	70.45	17.19	14.02
	M0022	13.14	15.52	0.001	173.12	38.96	71.02	129.50	132.10	175.35	47.41	67.17	21.02	9.57
	M0017	14.49	12.94	0.002	169.55	38.46	71.40	126.80	129.40	173.46	45.12	70.19	18.45	14.65
	M0035	14.78	11.74	0.003	171.73	40.58	72.22	127.26	128.09	170.92	44.08	70.84	16.84	13.87
	M0032	14.88	14.23	0.001	169.32	38.63	70.71	124.60	133.19	173.70	45.35	70.26	18.36	14.69
	M0018	15.19	13.44	0.001	170.06	38.32	71.49	127.76	127.45	173.62	45.27	70.30	18.40	14.73
	Boltz Avgs				**172.48**	**39.40**	**70.63**	**125.72**	**129.77**	**176.69**	**46.29**	**67.43**	**20.32**	**9.81**

*: the extended conformation with the lowest energy