Note

Isogloiosiphone B, a novel acetal, and hydrophobic compounds as β-glucuronidase inhibitors derived from the red alga *Neodilsea yendoana*

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Abstract

A novel acetal named isogloiosiphone B was isolated from the red alga *Neodilsea yendoana*, along with three known hydrophobic compounds as β-glucuronidase inhibitors. The acetal was determined as a naturally occurring compound from the extraction experiments with several kinds of solvent. The acetal showed the highest inhibition against β-glucuronidase among the compounds examined.

Key words: acetal, β-glucuronidase; inhibitor; *Neodilsea yendoana*; alga.

Beta-glucuronidase (EC 3.2.1.31) is not only found in anaerobic intestinal bacteria,[1] but also in mammalian organs and body fluids.[2-5] The bacterial enzyme catalyzes hydrolysis of β-glucuronosyl-*O*-bond[6] to generate β-glucuronic acid and aglycons. The aglycons can be re-absorbed into body to delay eliminating toxic compounds from body as glucuronides.[7] Excess expression of β-glucuronidase in human is related with diverse pathological symptoms.[8-13] Hence, β-glucuronidase inhibitors are highly expected to sustain healthy condition. *Neodilsea yendoana* Tokida (Dumontiaceae, Gigartinales) is a red macroalga abundantly distributed along the coast of North Japan. Several studies have revealed that *N. yendoana* comprises amino acids,[14] rhodoic acid,[15] polyunsaturated fatty acid,[16] and yendolipin.[17] The present paper aims to isolate novel potential β-glucuronidase inhibitors from *N. yendoana*.

Methanol extract of air-dried *N. yendoana* (1.73 kg), collected along the coast of Hakodate, Japan in 2016, was separated to ethyl acetate-soluble fraction (1.30 g) by organic solvent partitioning with guidance of results by slightly modified *Escherichia coli* β-glucuronidase inhibition assay using *p*-nitrophenyl β-D-glucuronide (Supplemental method 1).[18] The fraction was chromatographed twice on silica gel and Sephadex LH-20 to obtain three inhibitory fractions. Final purification was performed by using HPLC (ULTRON VX-ODS, 5 μm; 250 × 4.6 mm) eluted with MeCN-H_2O=15:85 and MeCN-H_2O=9:1 to obtain novel compound **1** (1.43 mg), and phytal (**2**, 2.02 mg), which was first isolated from this alga, cholesterol (**3**, 3.08 mg) and 22-dehydrocholesterol (**4**, 3.31 mg), respectively (Supplemental method 2). Compounds **2**-**4** were identified by comparison of literature data.[19,20]

Novel compound **1** was obtained as colorless oil. Physicochemical data for **1** were as follows: UV λ_{max} (log ε) (MeOH) 206 (2.60); IR ν_{max} ($CHCl_3$): 1769, 1081 cm^{-1}; $[\alpha]_D^{27}$= 0° (*c*1.32, $CHCl_3$); FI-MS *m/z* 146.07 (M^+); FI-HR-MS *m/z* 146.05861 (M^+, calcd 146.05791 for $C_6H_{10}O_4$); ^{1}H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6): see Table 1. IR spectrum of **1** showed C=O and C-O stretching peaks. ^{1}H NMR spectrum of **1** indicated

the presence of one hydroxy, three methylene and one methoxy proton signals, where the hydroxy proton was deduced from no cross-peak in HSQC spectrum. ^{13}C NMR spectrum of **1** revealed one carbonyl, one quaternary, three methylene and one methoxy carbon signals which were assisted with HSQC experiment. DQF COSY experiment of **1** revealed two couples of correlations (H-4/H-5 and H-6/hydroxy proton). The latter correlation was assigned as a hydroxymethyl group. All the proton and carbon signals of compound **1** were assigned according to HMBC cross-peaks of C-2 with Hs-5 and 6, and methoxy protons; ketonic carbon (C-3) with Hs-4, 5 and 6; C-4 with Hs-5; C-5 with H-4; and C-6 with the hydroxy proton. Consequently, compound **1** was elucidated as an isomer of known compound gloiosiphone B (**5**)[21], a structural analogue of laurencione,[22] isolated from the red alga *Gloiosiphonia verticillaris* (Gloiosiophoniaceae, Gigartinales). The ^{13}C NMR signal at C-2 of **1** was shifted to down-field compared to the signal of gloiosiphone B while the signal at C-6 of **1** was shifted to up-field. This difference was contributed to substituting position of methoxy group. Thus, compound **1** was identified as 2-hydroxymethyl-2-methoxyoxolan-3-one, a novel acetal named isogloiosiphone B (Figure 1). Compound **1** possesses an asymmetric center in its structure. However, **1** might be a racemic mixture because specific rotation of **1** was optically inactive. With the consideration that compound **1** might be an artefact from its simple acetal structure, the alga was extracted with methanol, ethanol and acetone. Compound **1** was isolated from all the extracts using different solvents (Figures S7-S9). Thus compound **1** was determined as a naturally occurring compound, not an artefact. Plausible precursor of isogloiosiphone B and gloiosiphone B would be 1,5-dihydroxypentane-2,3-dione. After cyclization of the precursor to corresponding hemiacetal, both compounds may be derived by methylation at different positions.

The isolated compounds **1**-**4** were evaluated for inhibitory effects against *Escherichia coli* β-glucuronidase (Table 2, Supplemental method 3). Isogloiosiphone B (**1**) showed the highest inhibitory activity in a competitive manner (Figure S19) among the isolated compounds and the positive control D-glucaro-1,4-lactone.[23] Compound **1** might

easily access to active site of the enzyme. Additionally, this is the first report that phytal (**2**), cholesterol (**3**) and 22-dehydrocholesterol (**4**) exhibited β-glucuronidase inhibitory activity even though their inhibitory activities were weak.

In summary, we isolated compounds **1**-**4** as β-glucuronidase inhibitors from the red alga *N. yendoana*. Compound **1** was determined as a novel naturally acetal and showed strong β-glucuronidase inhibitory activity. In addition, this is the first report on isolation of phytal from the alga and elucidation of β-glucuronidase inhibitory activity by the hydrophobic compounds.

Acknowledgment

We thank to Dr. Eri Fukushi and Mr. Yusuke Takata, GC-MS & NMR Laboratory, Faculty of Agriculture, Hokkaido University, for measurements of MS and NMR spectra.

Author contribution

D. Z. performed all experiments and prepared draft. H. K. made experimental plans and organized all of the studies. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by authors.

Supplemental material

The supplemental material for this paper is available at https://doi.org/XXX.

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Figure captions

Figure 1. Compounds **1**-**4** isolated from *N. yendoana* as β-glucuronidase inhibitors and gloiosiphone B (**5**).

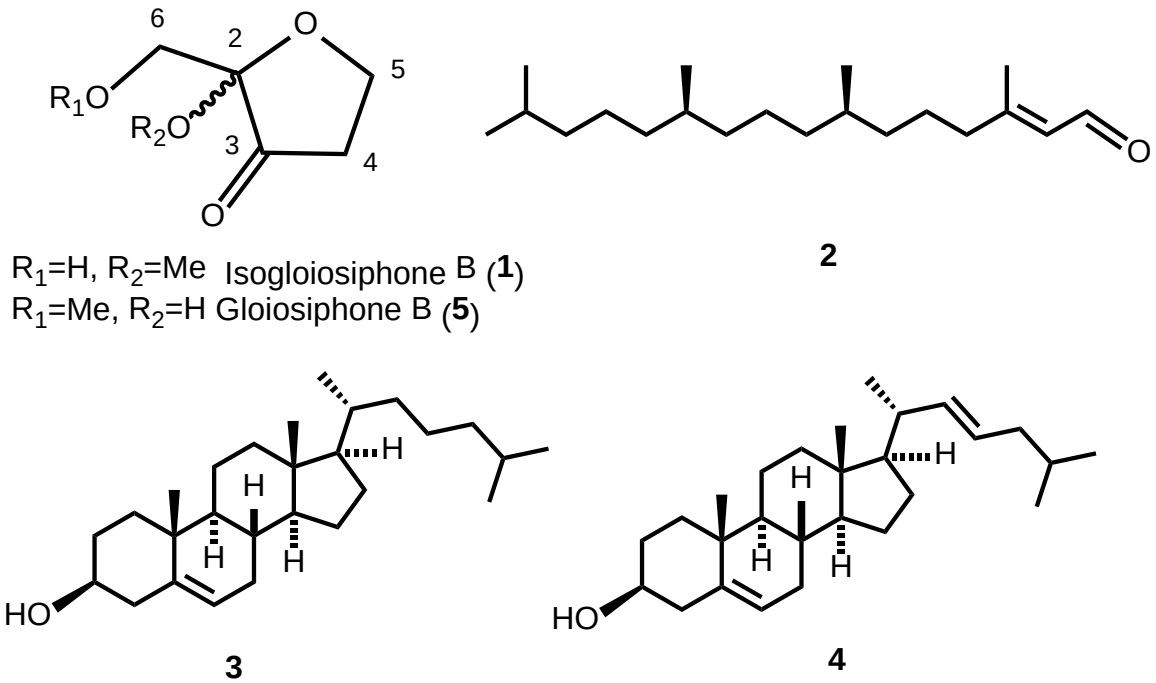

Figure 1. Compounds **1**-**4** isolated from *N. yendoana* as β-glucuronidase inhibitors and gloiosiphone B (**5**).

Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of compound **1** and literature data[21] of gloiosiphone B (**5**).

	Compound **1**[a]		Gloiosiphone B (**5**)[b]	
Position	δ_C	δ_H (mult, *J* in Hz)	δ_C	δ_H (mult, *J* in Hz)
2	101.9		96.0	
3	211.7		208.8	
4	36.3	2.55 (2H, m)	34.2	2.88 (2H, m)
5	64.2	4.33 (1H, m)	62.5	4.32 (1H, m)
		4.27(1H, m)		4.33 (1H, m)
6	63.1	3.62 (2H, d, 5.7)	72.8	3.62 (1H, d)
				3.90 (1H, d)
-OCH_3	50.6	3.12 (3H, s)	59.9	3.45 (3H, s)
-OH		3.87 (1H, t, 5.7)		3.40 (1H, s)

[a] measured in acetone-d_6.

[b] measured in chloroform-*d*.

Table 2. β-Glucuronidase inhibitory activities of compound **1**-**4**.

Compound	IC_{50} (μM)	*Ki* (μM)	Inhibition mode
1	57.0 ± 0.56	8.3 ± 1.3	Competitive
2	103.7 ± 1.02	210.7 ± 13.7	Competitive
3	> 250	421.2 **±** 7.1	Competitive
4	89.5 ± 0.44	32.7 **±** 5.5	Non-competitive
D-Glucaro-1,4-lactone	77.9 **±** 1.40	2.5 **±** 2.1	Competitive

Supplemental Online Materials for

Isogloiosiphone B, a novel acetal, and hydrophobic compounds as β-glucuronidase inhibitors derived from the red alga *Neodilsea yendoana*

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Supplemental method 1.

β-Glucuronidase inhibition assay for separation of the inhibitors

β-Glucuronidase activity was determined by absorptiometric method using *p*-nitrophenyl β-D-glucuronide as a substrate. Reaction mixture contained 10 mM phosphate buffer (pH 7.0, 440 μL), 10 mM *p*-nitrophenyl β-D-glucuronide (Sigma Chemical Co., St. Louis, MO, USA) in the buffer (20 μL) and test substance in methanol (final concentration of 10 μg/mL, 20 μL). The mixture was pre-incubated at 37 ºC for 5 min. Reaction was initiated by adding 80 units/mL *Escherichia coli* β-glucuronidase (type IX-A, Sigma Chemical Co., St. Louis, MO, USA, 20 μL) and kept at 37 ºC for 7 min. The reaction was terminated by adding 1 M sodium carbonate (300 μL). Produced *p*-nitrophenolate anion was measured at 400 nm. All assays were conducted in triplicate. Inhibitory activity was calculated from absorbance as following equation because all assays were performed under the same experimental condition.

$$\text{Inhibitory activity (\%)} = \{(C-C_0)-(T-T_0)\} / (C-C_0) \times 100$$

Where C is the absorbance of negative control (no test substance), C_0 is the absorbance of negative control's blank (no test substance nor enzyme), T is the absorbance of test experiment and T_0 is the absorbance of test experiment's blank (no enzyme).

Supplemental method 2.

Separation and isolation of β-Glucuronidase inhibitors from Neodilsea yendoana

The EtOAc soluble fraction (1.30 g) was chromatographed over silica gel eluted with a combination of $CHCl_3$ and MeOH to afford inhibitory fractions E2 ($CHCl_3$), E3 and E4 ($CHCl_3$-MeOH=29:1, v/v). The fraction E4 (253 mg) was re-chromatographed over silica gel eluted with *n*-hexane-EtOAc (1:2, v/v) to afford inhibitory subfraction E4S3 (40.37 mg). The subfraction was separated on Sephadex LH-20 eluted with MeOH to afford inhibitory fractions. The fractions were combined and finally purified by using Shimadzu LC-10ATvp HPLC equipped with Shimadzu SPD-M10Avp diode array detector and ULTRON VX-ODS column (Shinwa Chemical industries, Ltd, Kyoto, Japan; 5 μm; 250 × 4.6 mm) eluted with MeCN-H_2O (15:85, v/v) to afford the novel compound **1** (1.43 mg). Fraction E2 (34.7 mg) was loaded on a silica gel Sep-Pak cartridge eluted with *n*-hexane-EtOAc (19:1, v/v) and then purified by ULTRON VX-ODS HPLC eluted with MeCN-H_2O (9:1, v/v) to afford compound **2** (2.02 mg). Fraction E3 was separated by preparative TLC developed with *n*-hexane-acetone (6:1, v/v) and then finally purified by ULTRON VX-ODS HPLC eluted with MeOH-H_2O (9:1, v/v) to provide compounds **3** (3.08 mg) and **4** (3.31 mg).

Supplemental method 3.

β-Glucuronidase inhibition assay for kinetic study of the isolated inhibitors

β-Glucuronidase activity was determined by absorptiometric method using *p*-nitrophenyl β-D-glucuronide as a substrate. Reaction mixture contained 10 mM phosphate buffer (pH 7.0, 440 μL), 2 to 8 mM substance in methanol (final concentration of 0 to 500 μM, 20 μL). The mixture was pre-incubated at 37 ºC for 5 min. Reaction was initiated by adding 80 units/mL *Escherichia coli* β-glucuronidase (type IX-A, Sigma Chemical Co., St. Louis, MO, USA, 20 μL) and kept at 37 ºC for 7 min. The reaction was terminated by adding 1 M sodium carbonate (300 μL). Produced *p*-nitrophenolate anion was measured at 400 nm. D-Glucaro-1,4-lactone (Sigma Chemical Co., St. Louis, MO, USA) was used as a positive control and all assays were conducted in triplicate. Inhibitory activity was calculated from absorbance as following equation because all assays were performed under the same experimental condition.

$$\text{Inhibitory activity (\%)} = \{(C-C_0)-(T-T_0)\} / (C-C_0) \times 100$$

Where C is the absorbance of negative control (no test substance), C_0 is the absorbance of negative control's blank (no test substance nor enzyme), T is the absorbance of test experiment and T_0 is the absorbance of test experiment's blank (no enzyme).

Inhibition modes and K_i values of the inhibitors were determined from Lineweaver-Burk and Dixon plots, respectively. We employed reciprocals of absorbance values instead of reciprocals of velocity because we performed all the experiments under the same temperature and time condition.

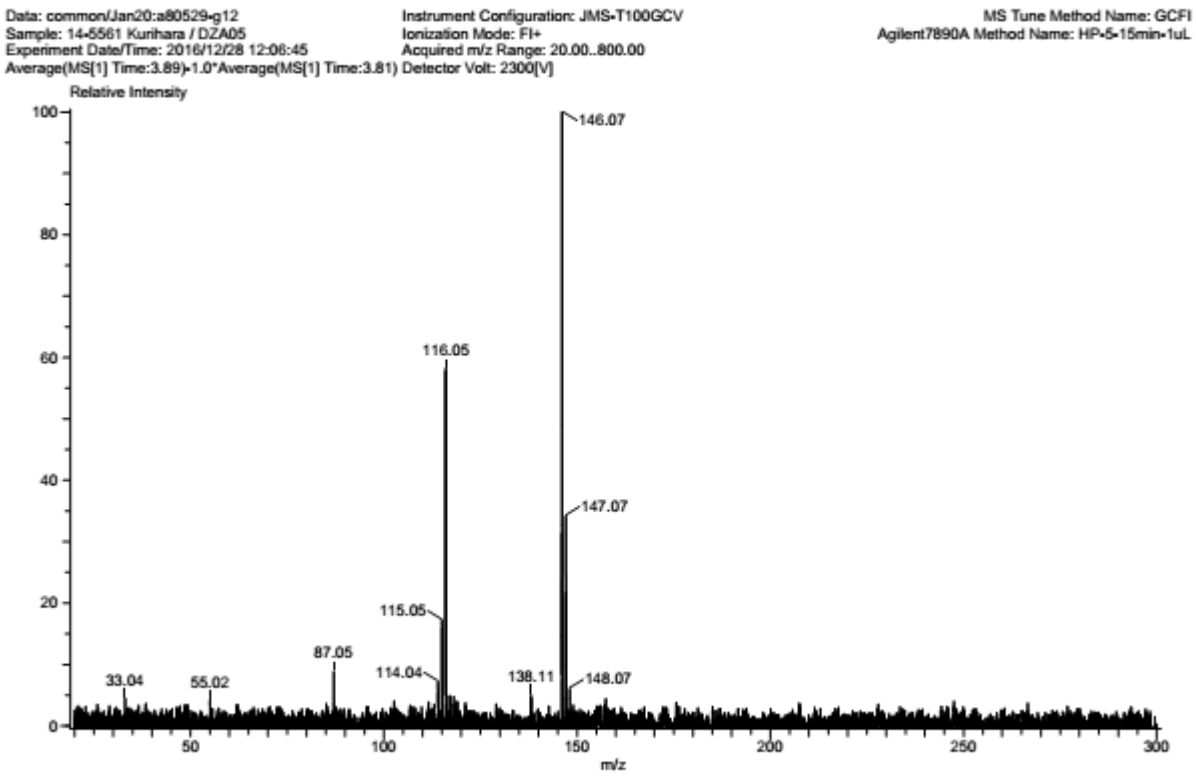


Figure S1. FI-MS spectrum of compound **1**.

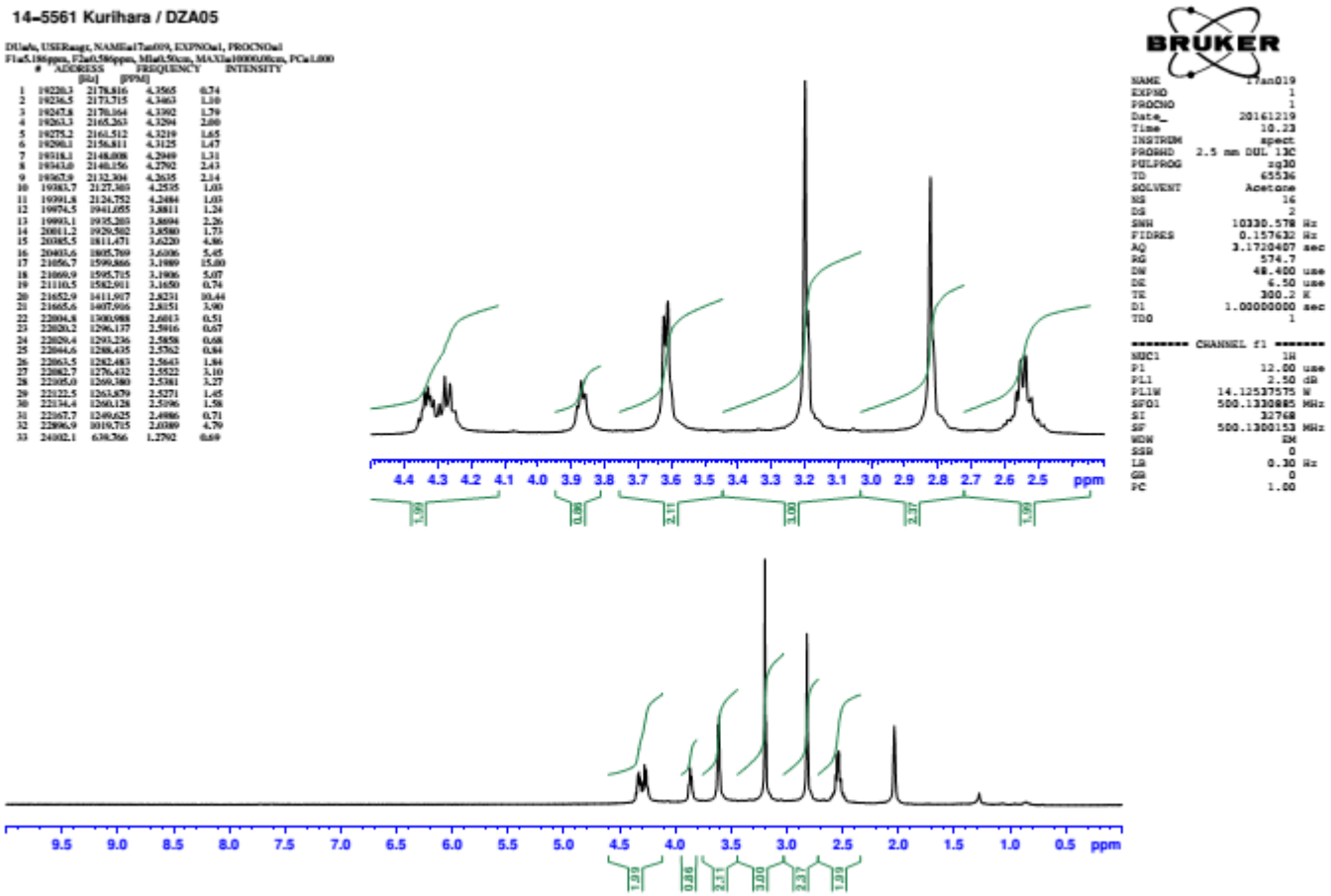


Figure S2. ^{1}H NMR spectrum of compound **1**.

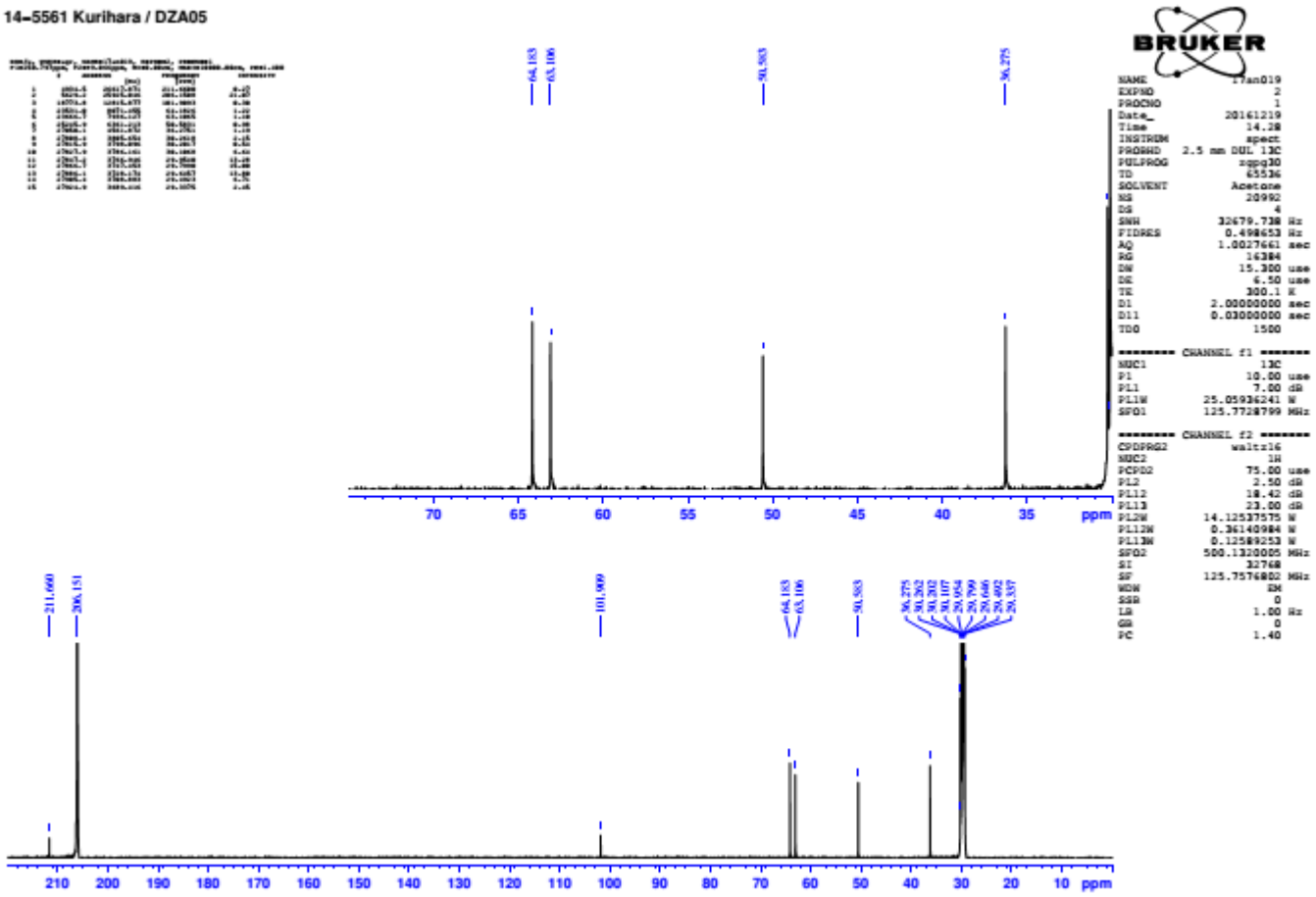


Figure S3. ^{13}C NMR spectrum of compound **1**.

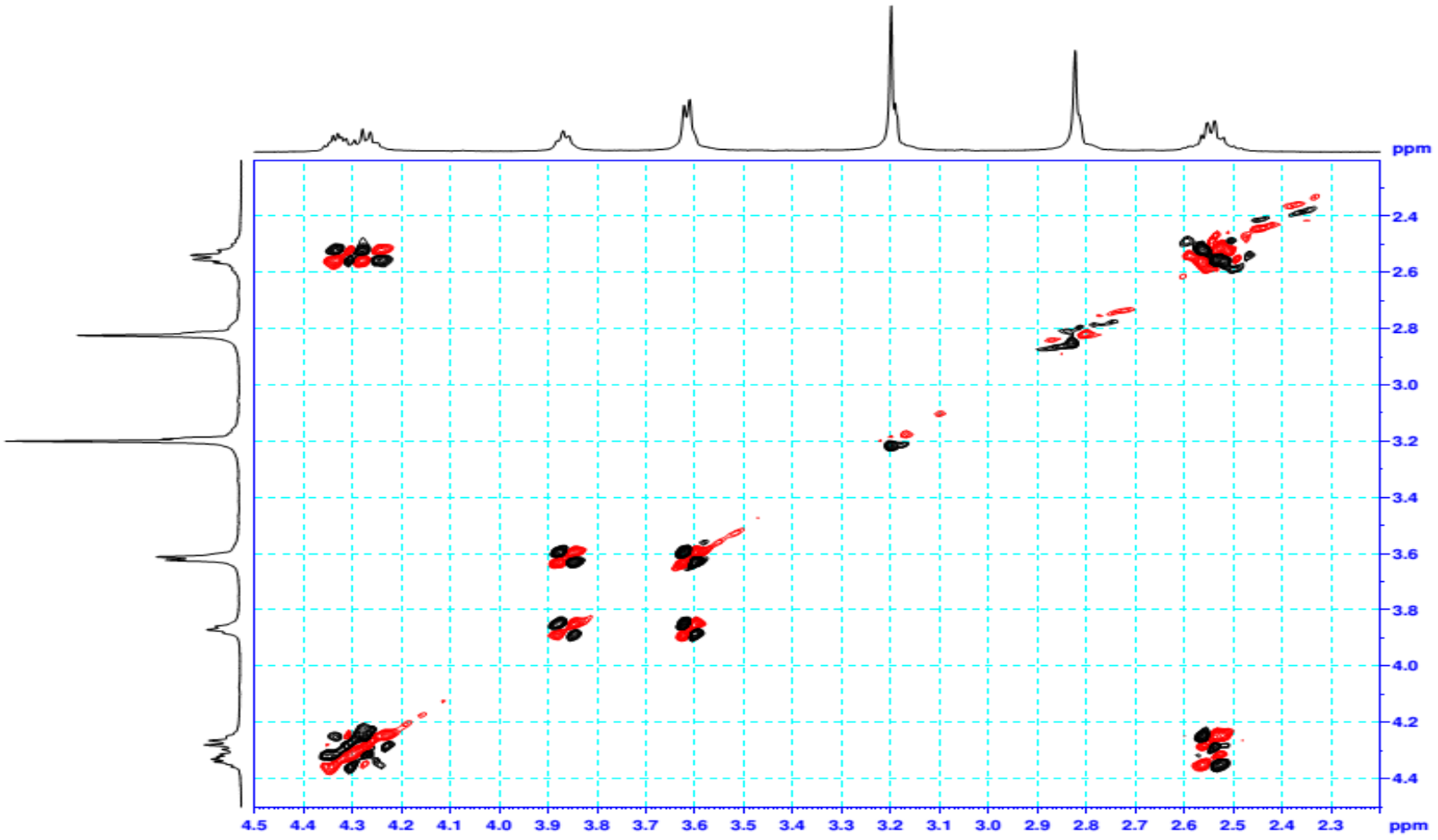


Figure S4. DQF COSY spectrum of compound **1**.

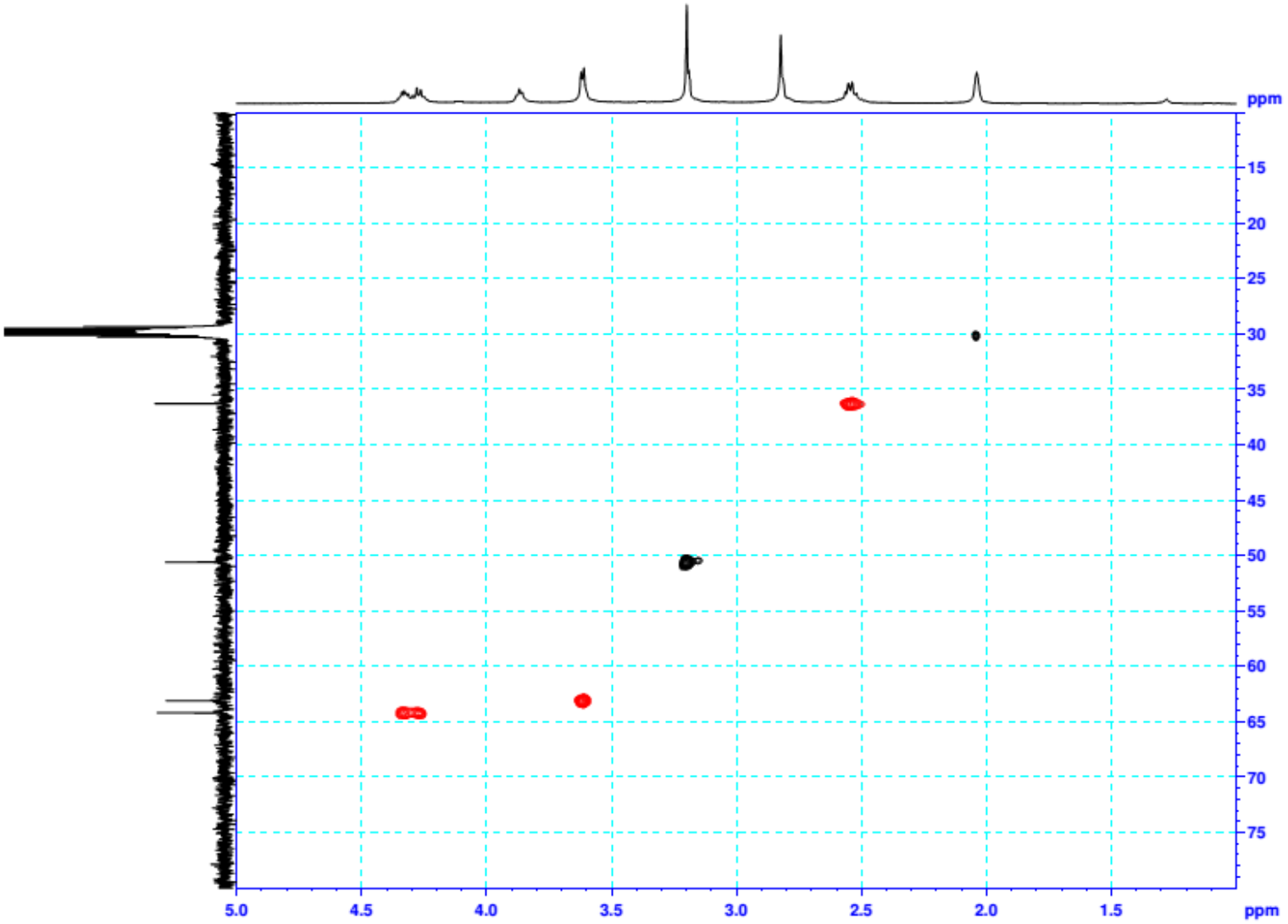


Figure S5. Editing HSQC spectrum of compound **1**.

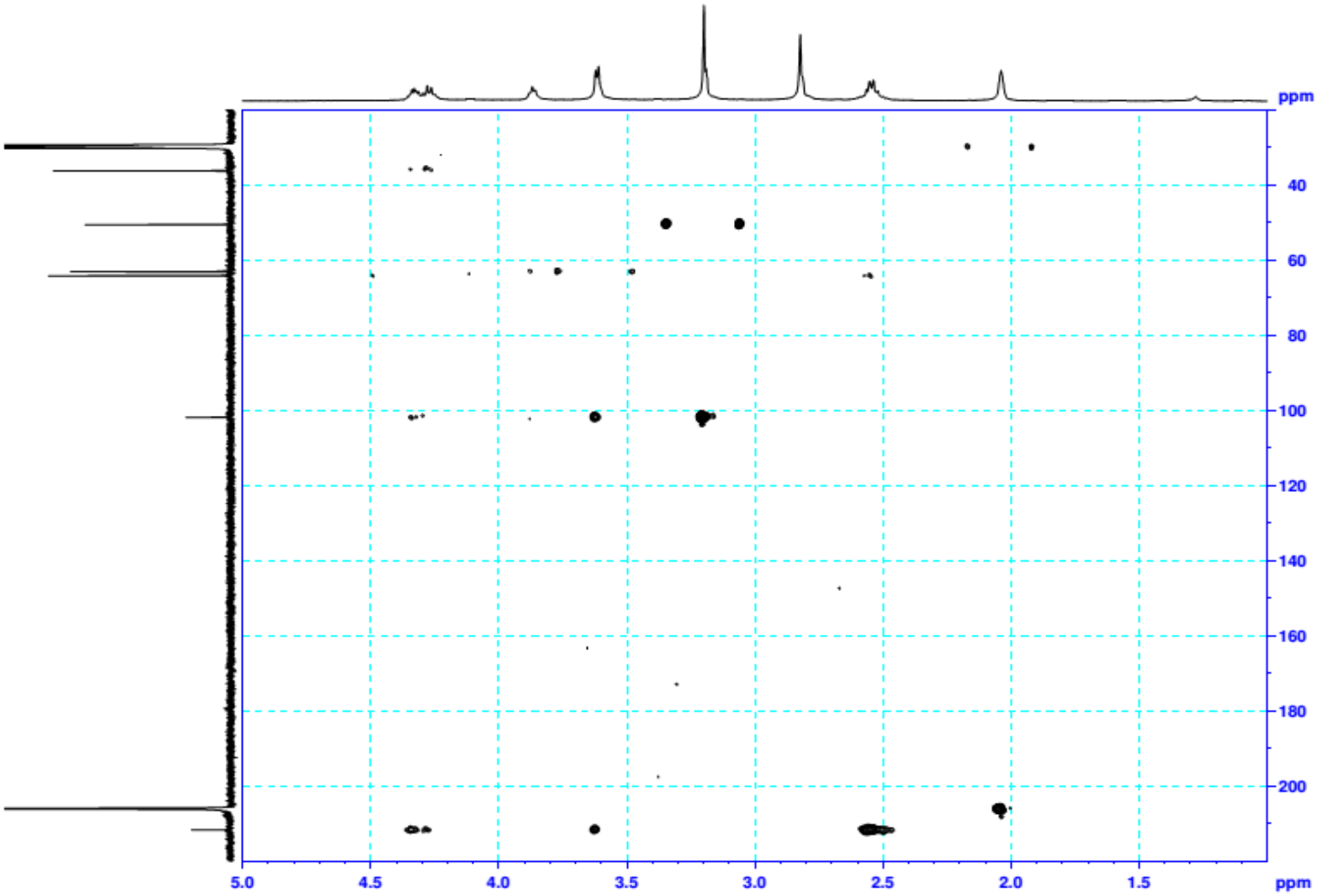


Figure S6. HMBC spectrum of compound **1**.

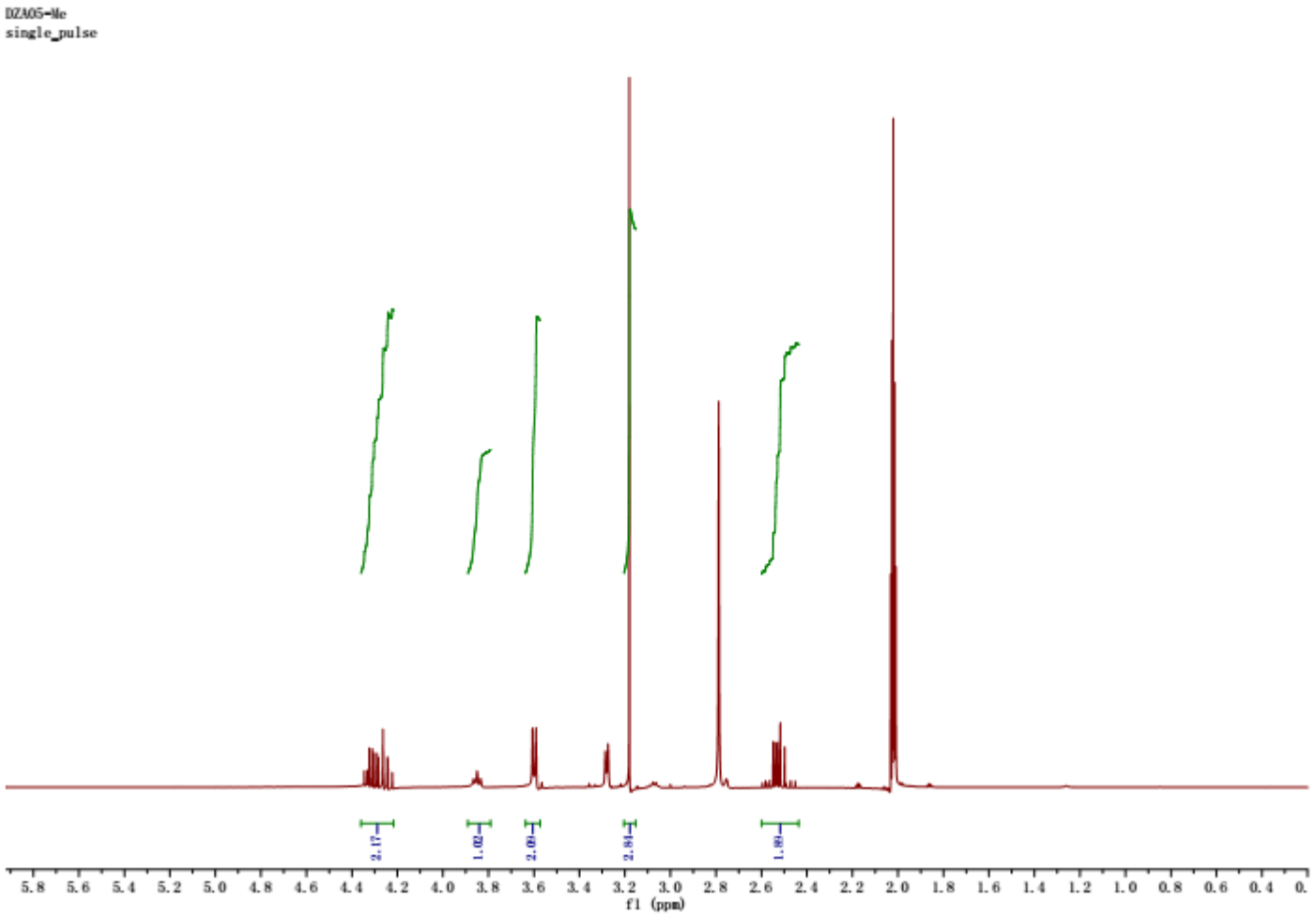


Figure S7. ^{1}H NMR spectrum of compound **1** extracted with methanol.

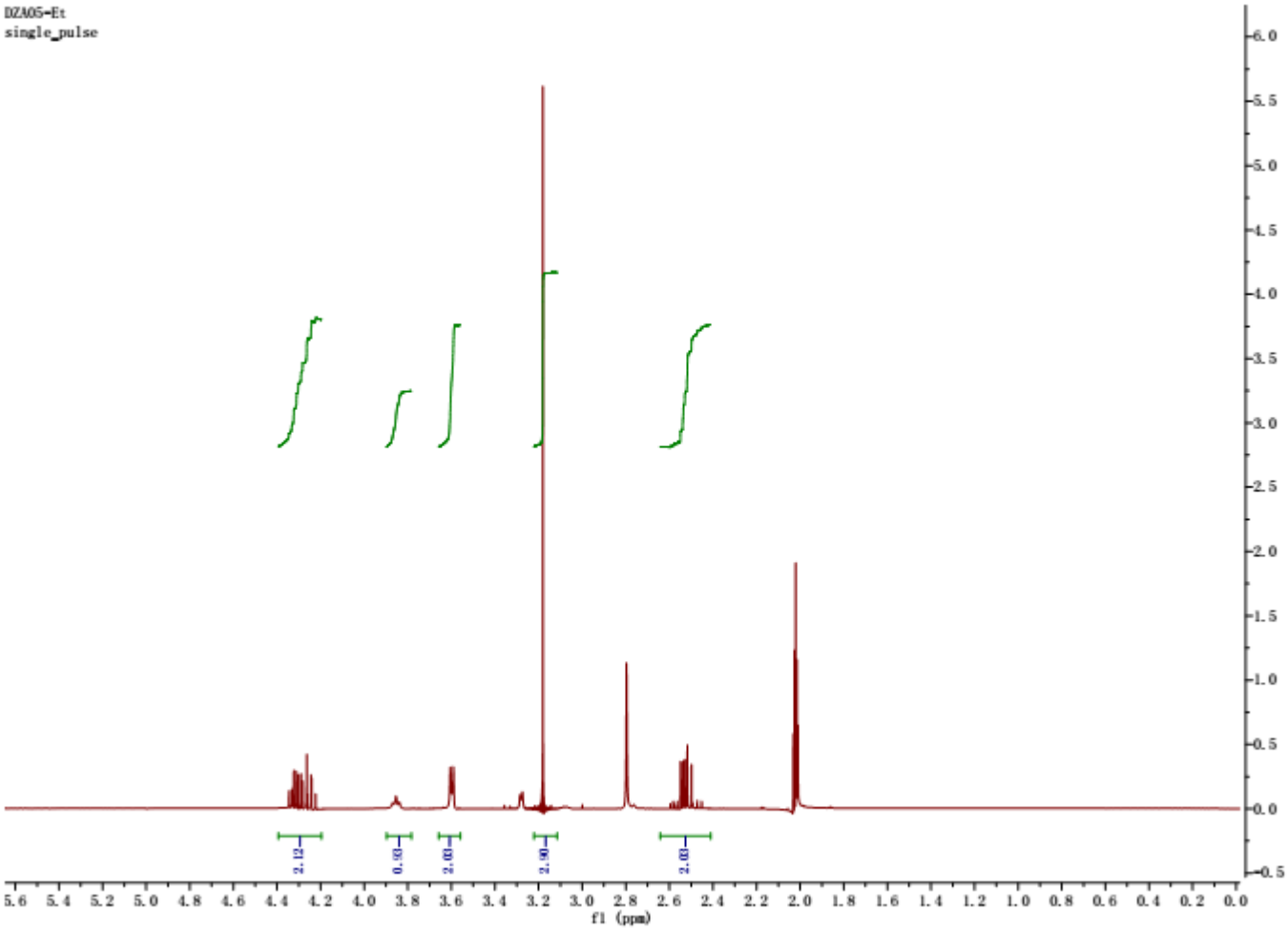


Figure S8. ^{1}H NMR spectrum of compound **1** extracted with ethanol.

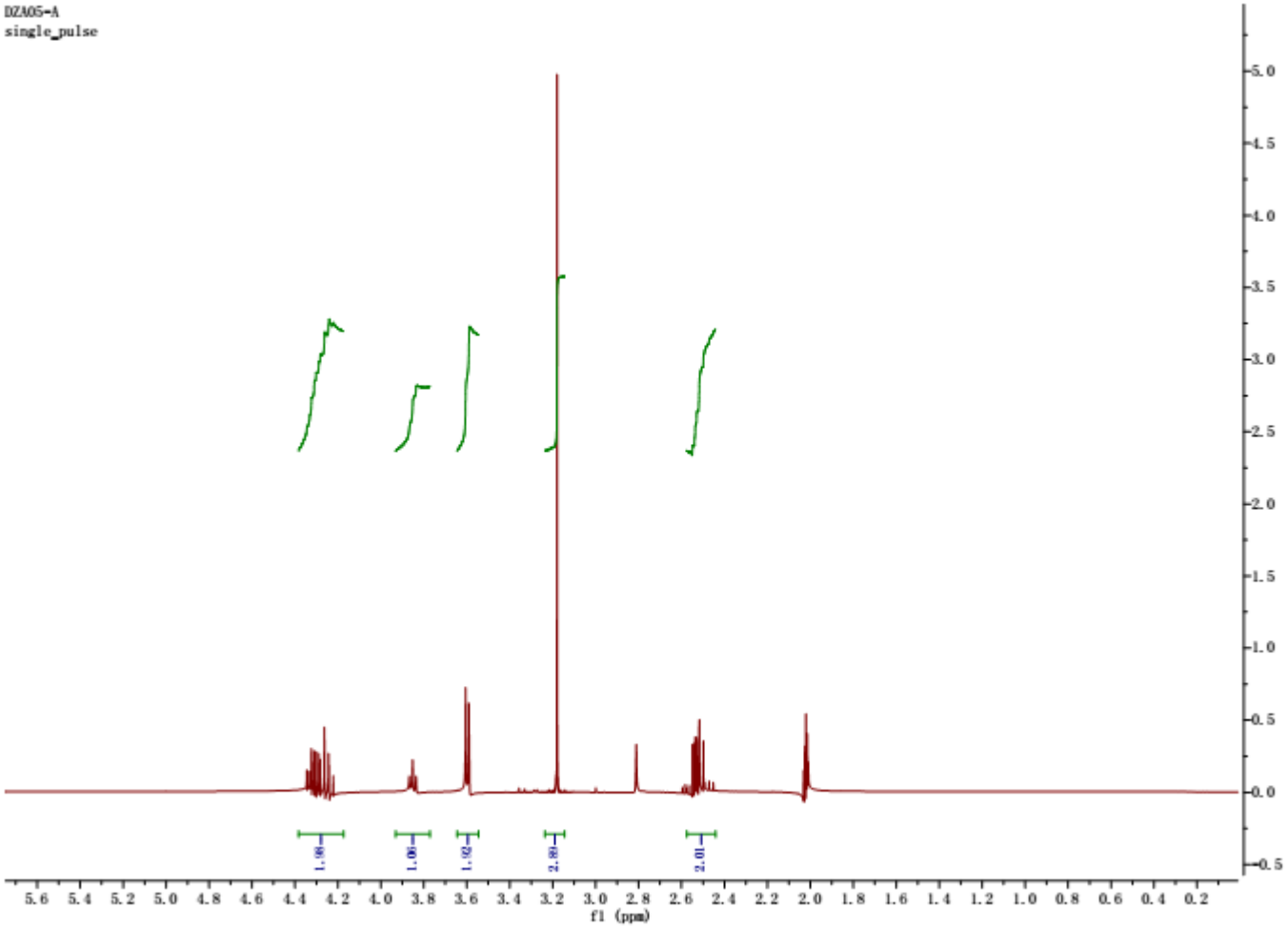


Figure S9. ^{1}H NMR spectrum of compound **1** extracted with acetone.

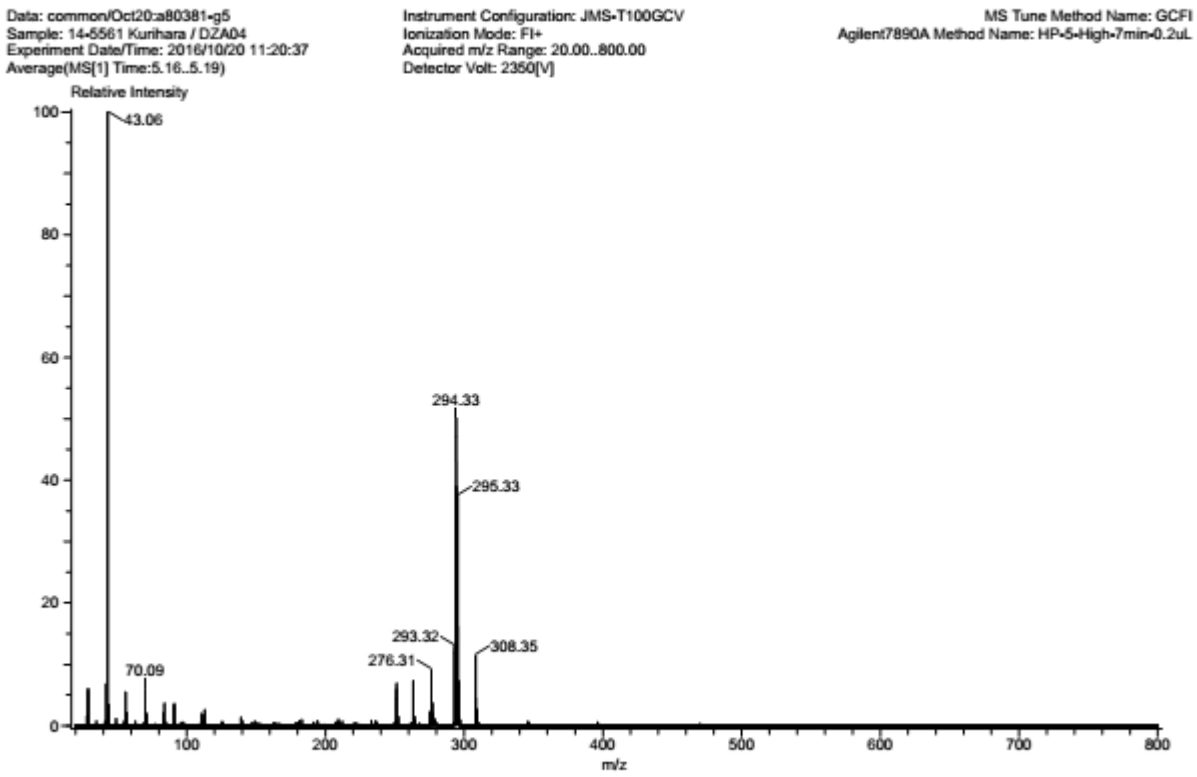


Figure S10. FI-MS spectrum of compound **2**.

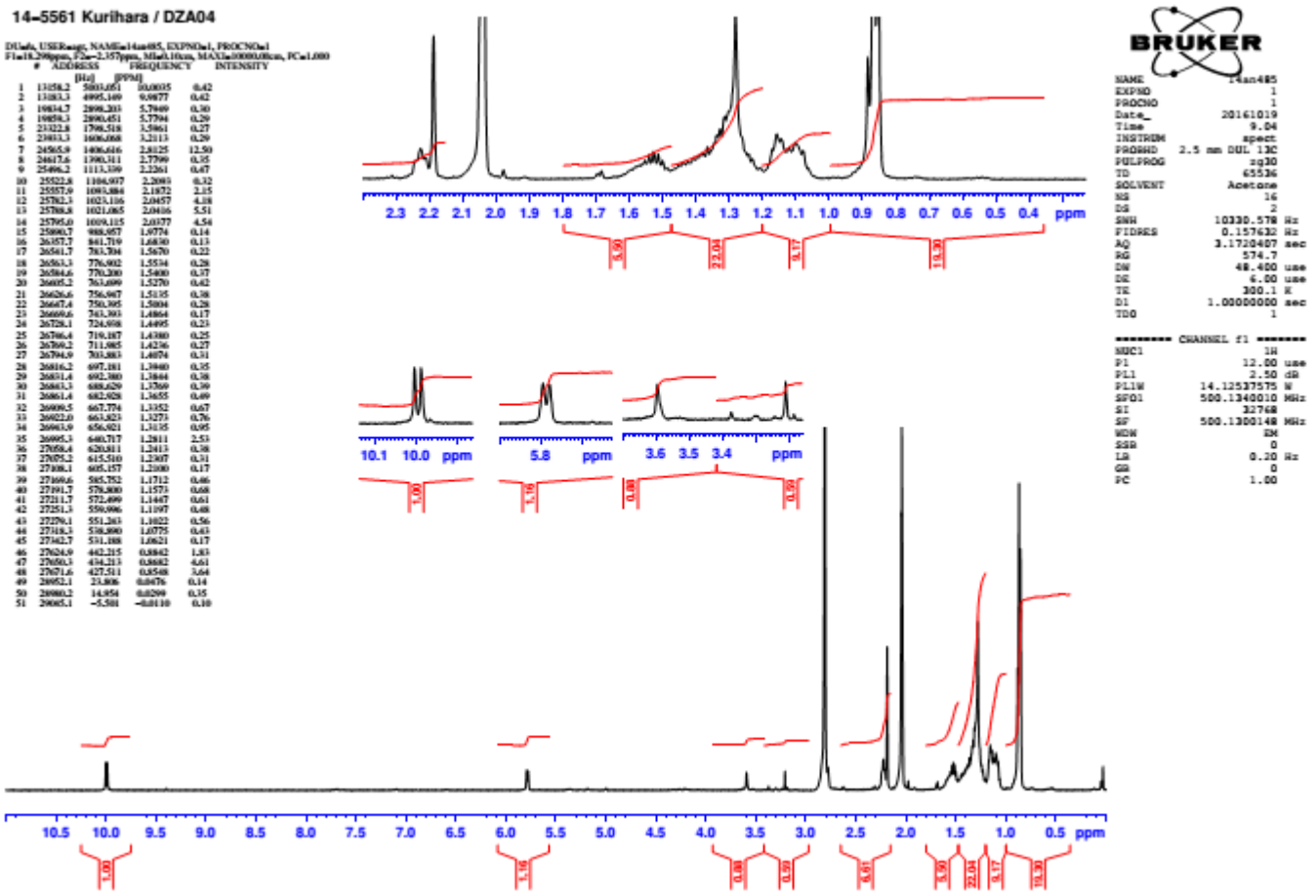


Figure S11. ^{1}H NMR spectrum of compound **2**.

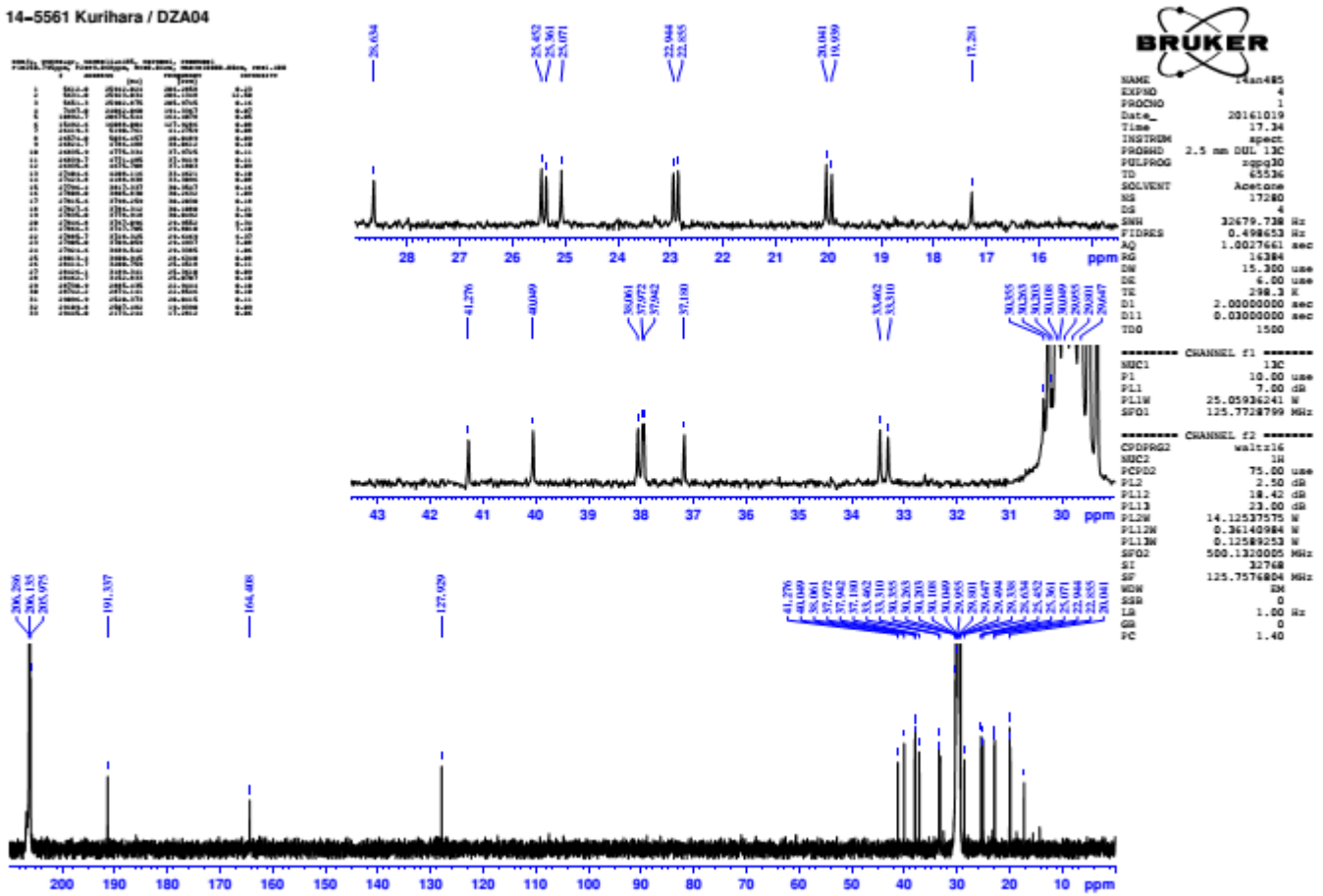


Figure S12. ^{13}C NMR spectrum of compound **2**.

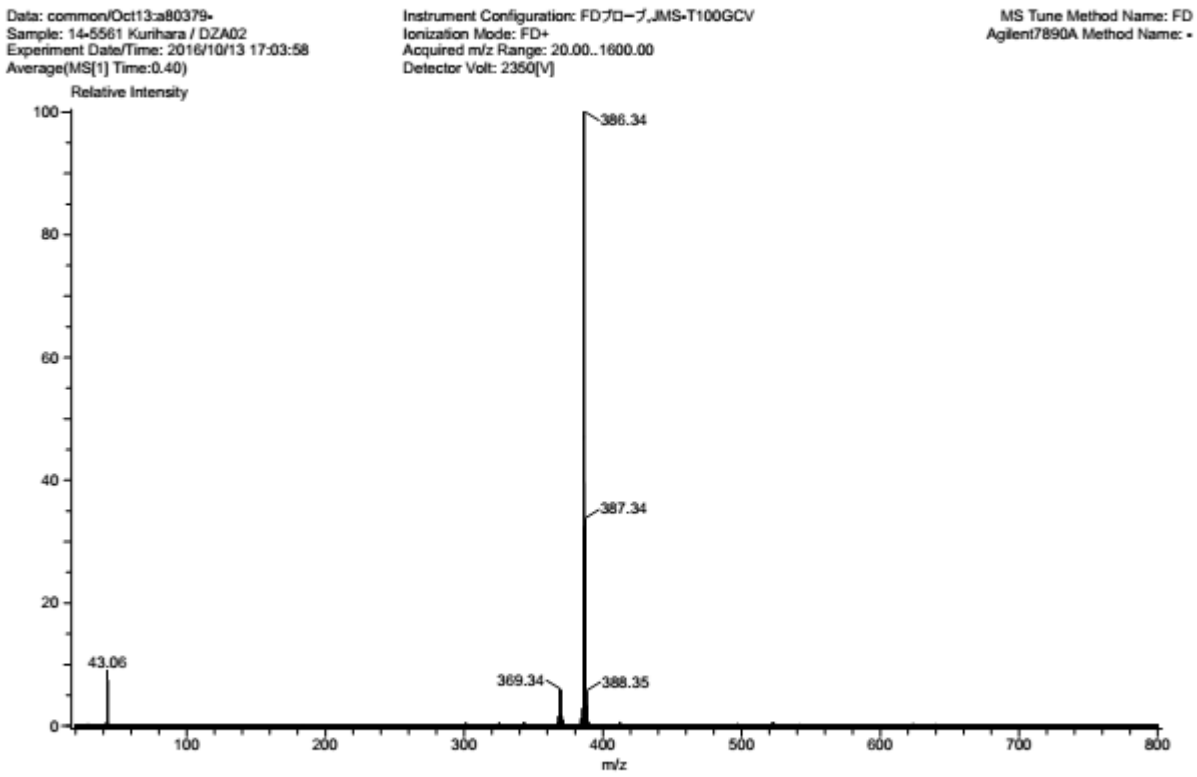


Figure S13. FD-MS spectrum of compound **3**.

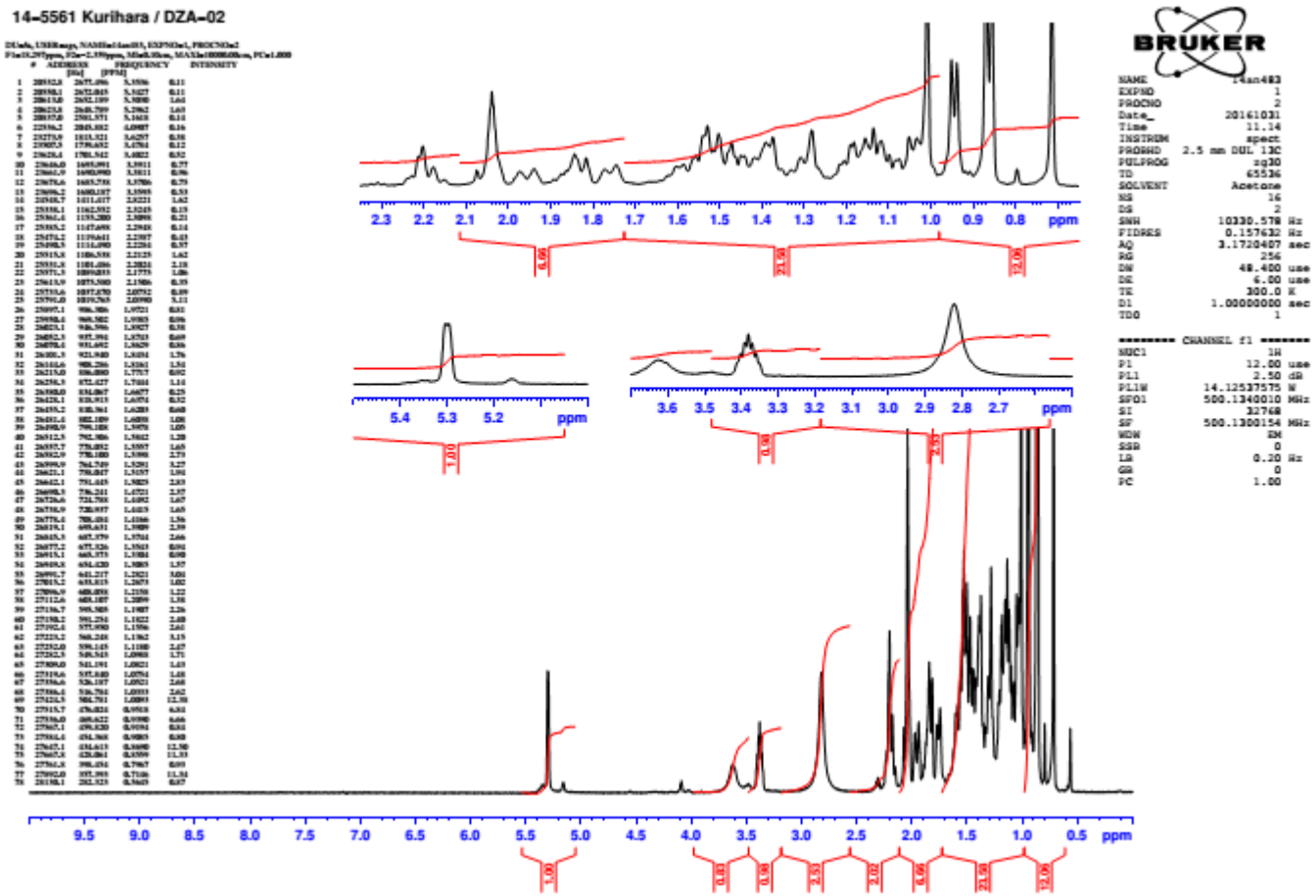


Figure S14. ^{1}H NMR spectrum of compound **3**.

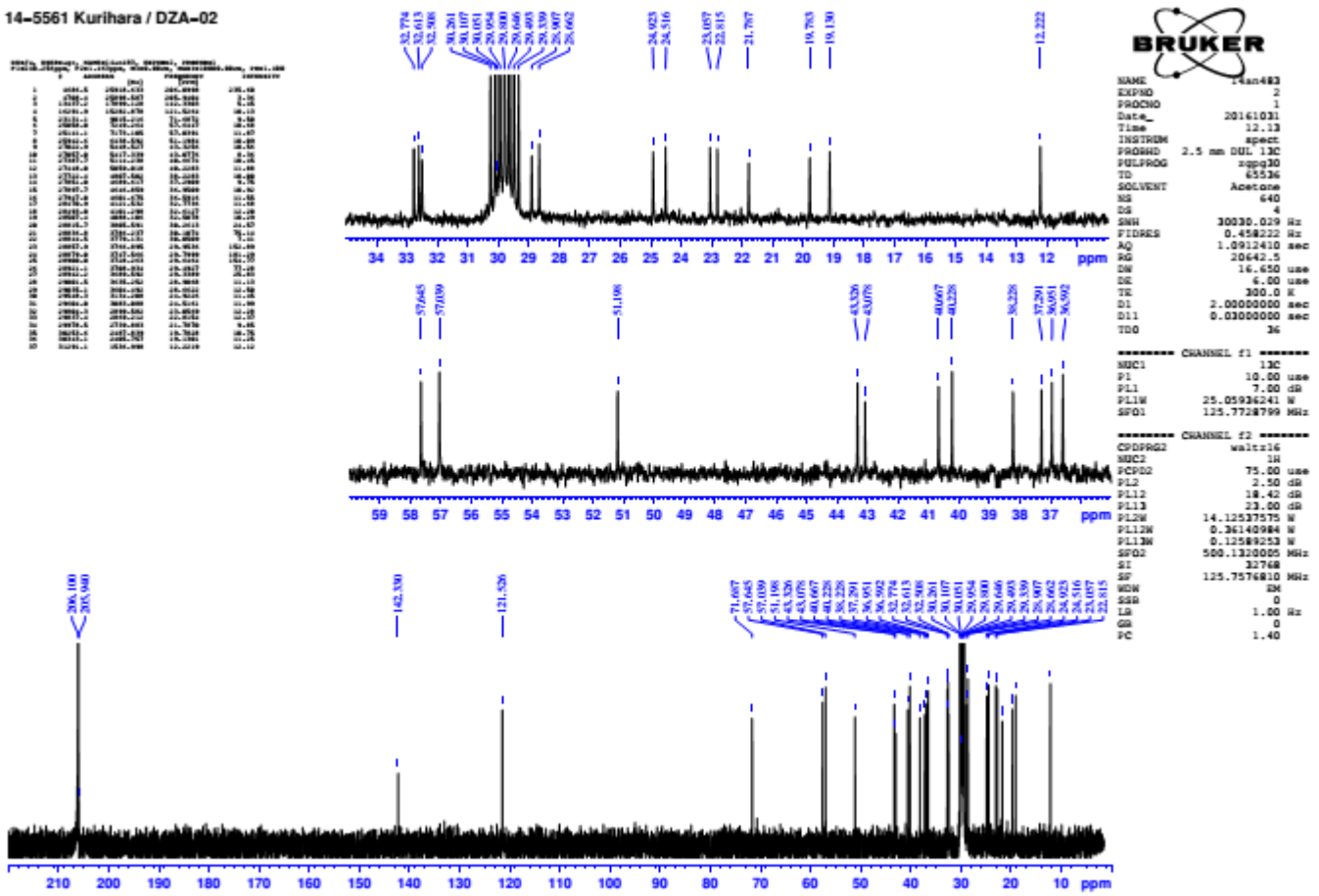


Figure S15. ^{13}C NMR spectrum of compound **3**.

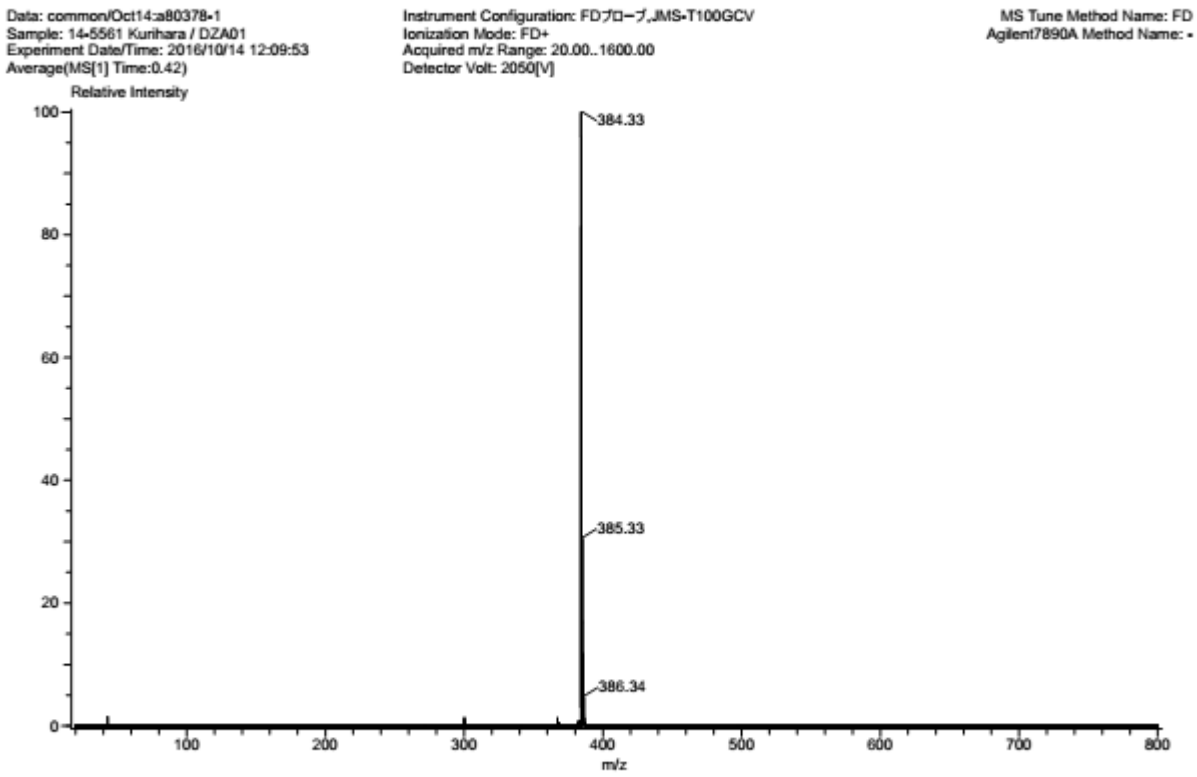


Figure S16. FD-MS spectrum of compound **4**.

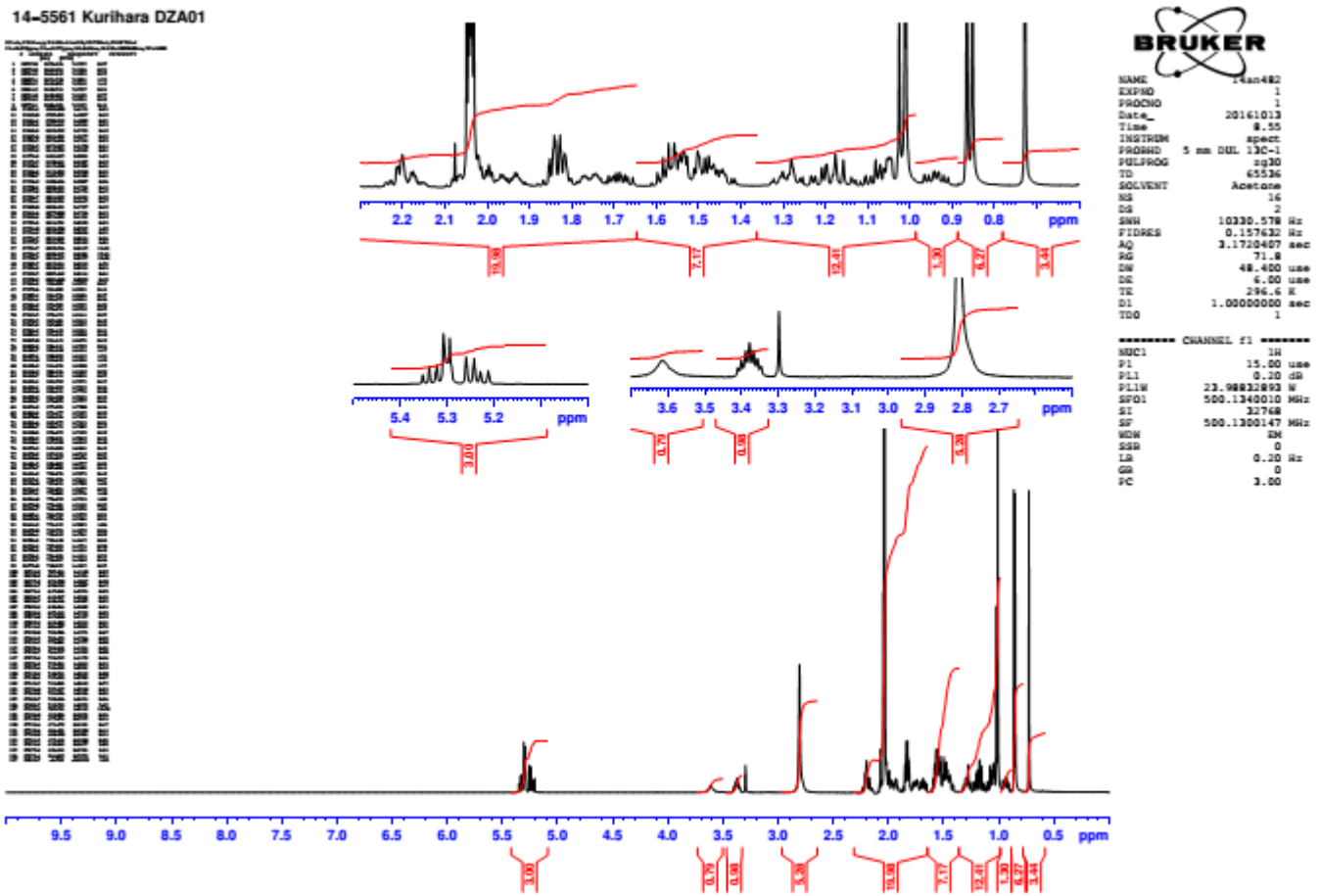


Figure S17. ^{1}H NMR spectrum of compound **4**.

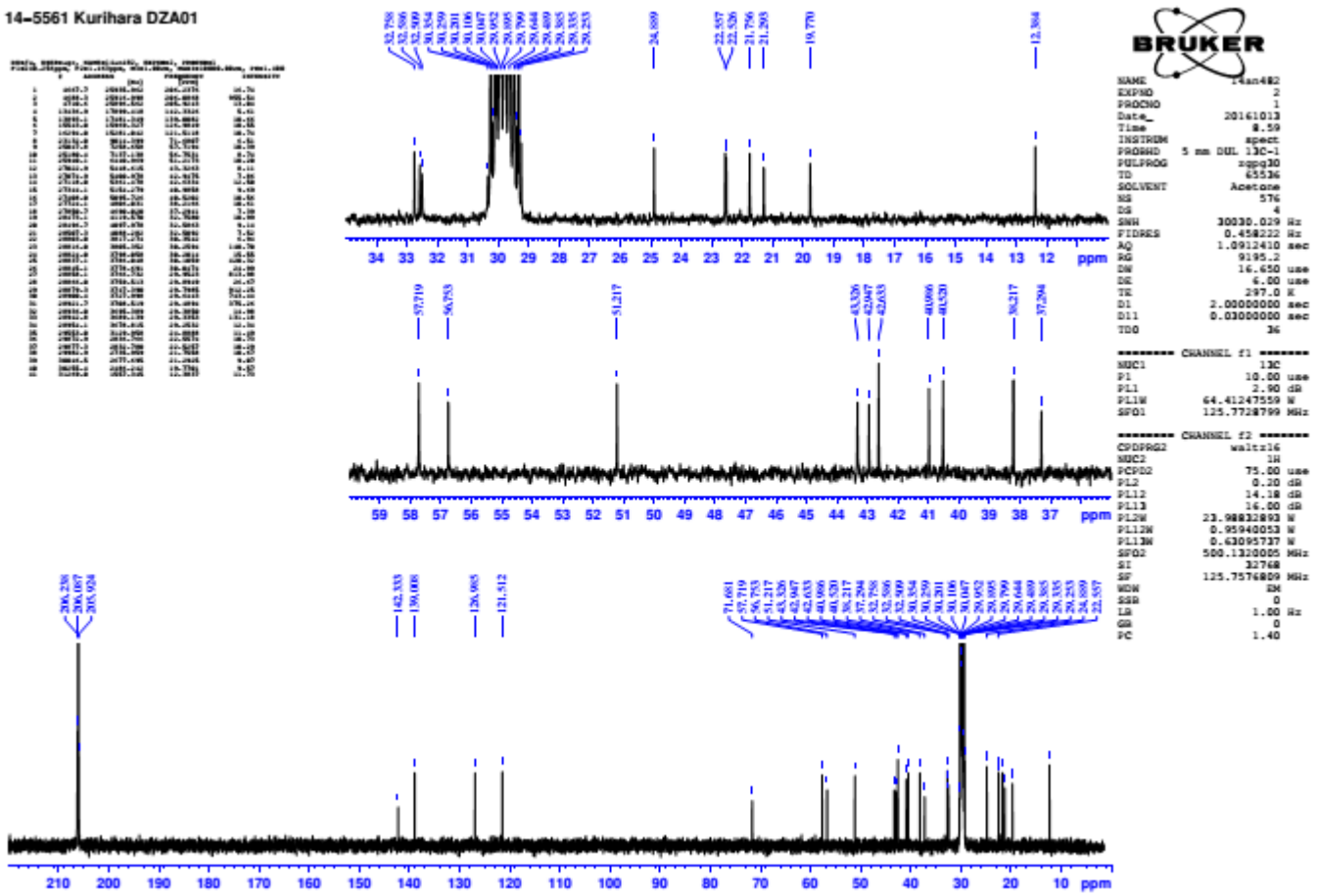
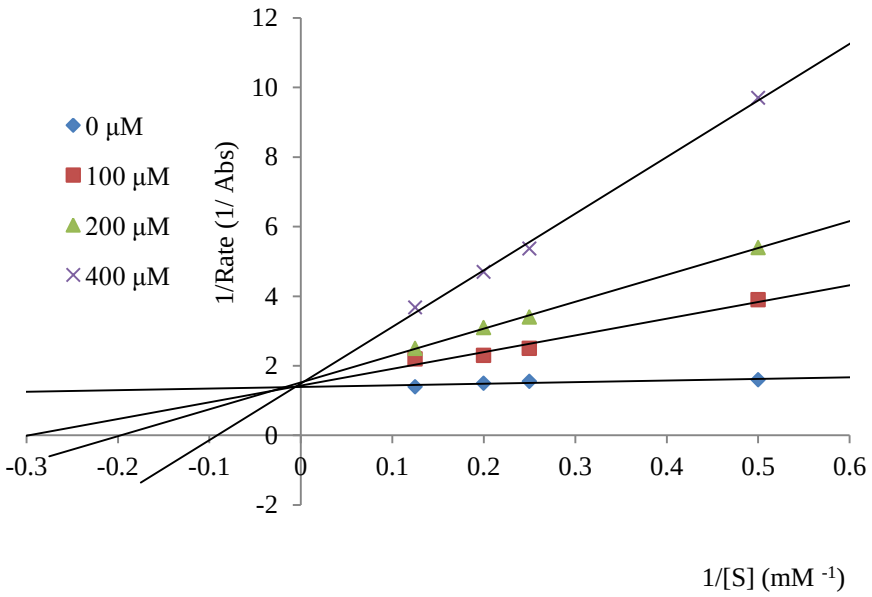
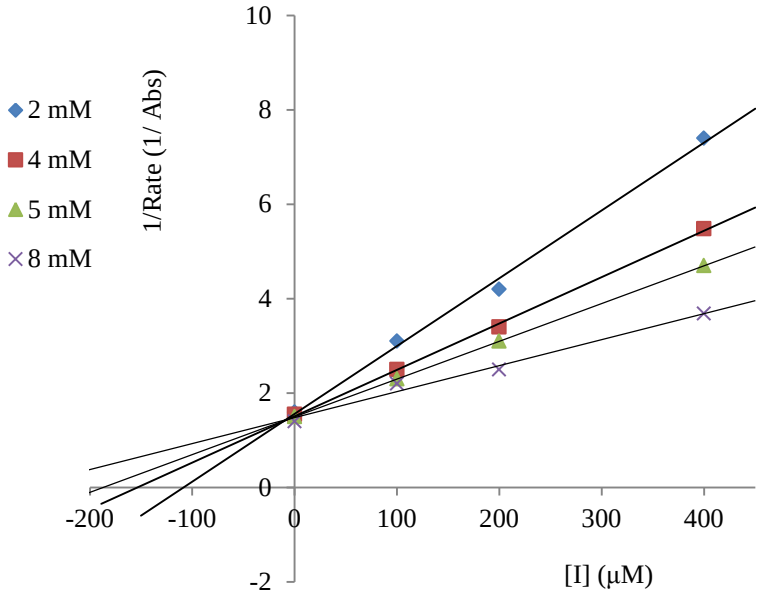


Figure S18. ^{13}C NMR spectrum of compound **4**.

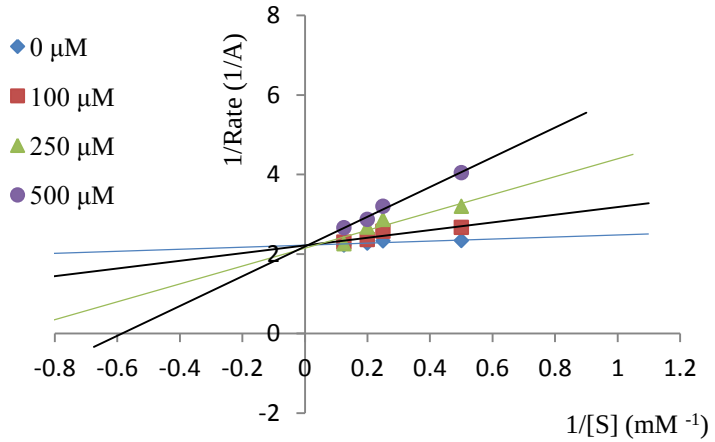


(A)

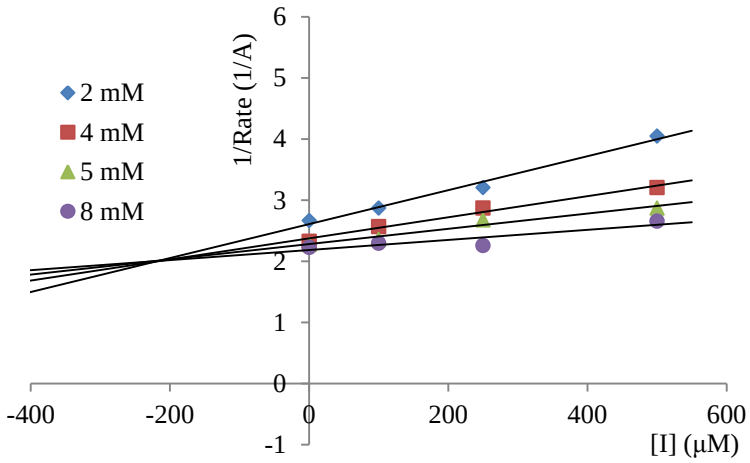


(B)

Figure S19. Lineweaver-Burk plot (A) and Dixon plot (B) of compound **1**.

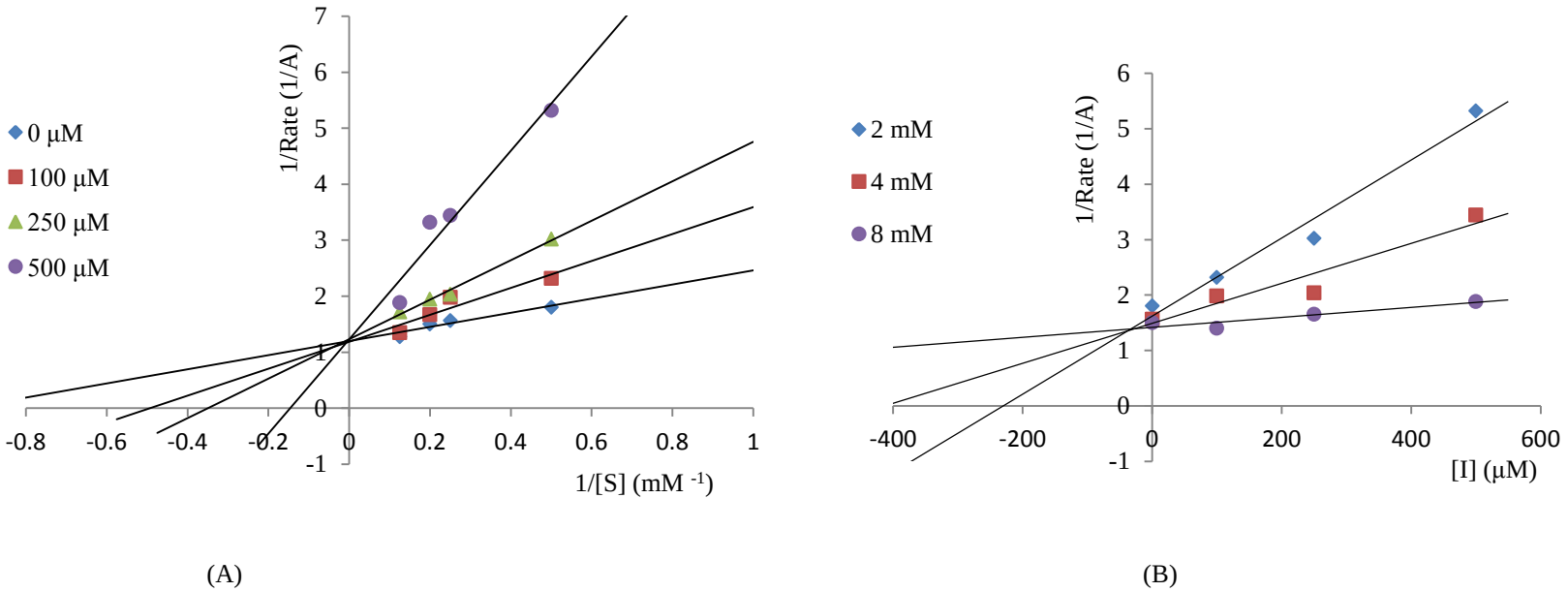


(A)



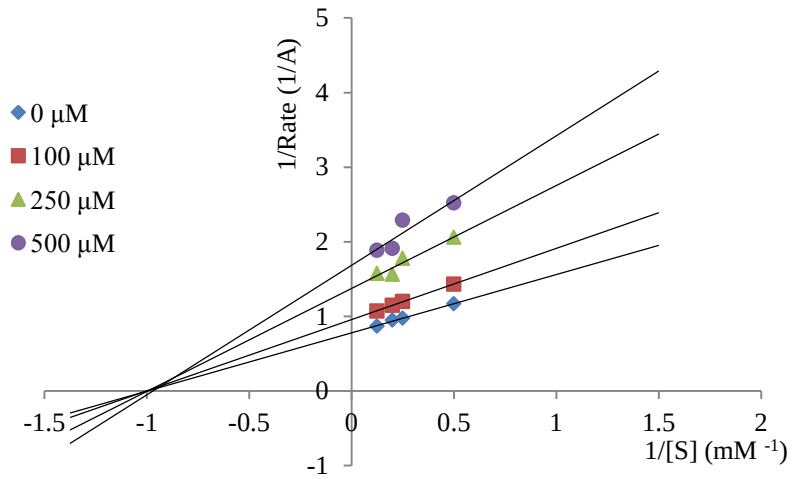
(B)

Figure S20. Lineweaver-Burk plot (A) and Dixon plot (B) of compound **2**.

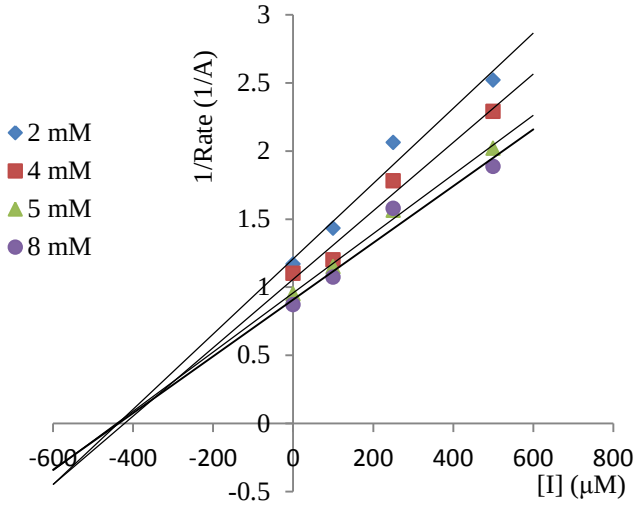


(A) (B)

Figure S21. Lineweaver-Burk plot (A) and Dixon plot (B) of compound **3**.



(A)



(B)

Figure S22. Lineweaver-Burk plot (A) and Dixon plot (B) of compound **4**.

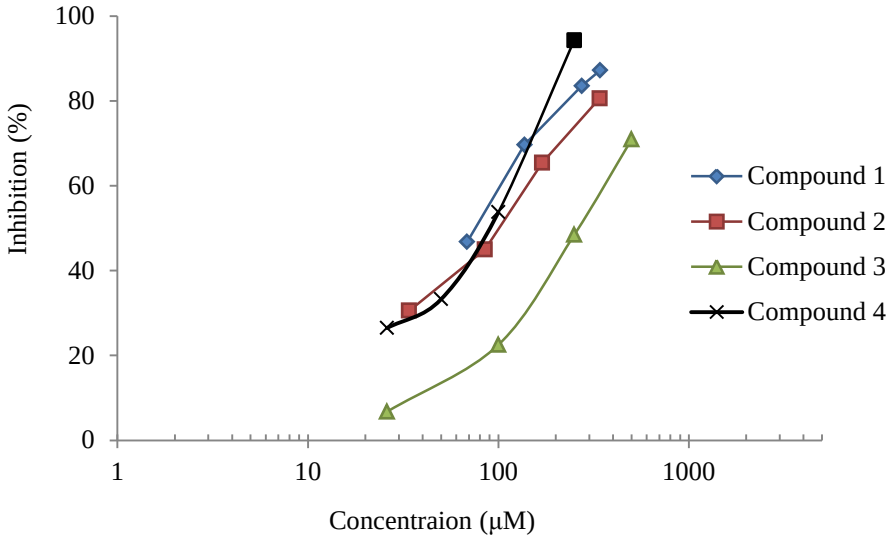


Figure S23. Inhibition of compounds **1-4** against β-glucuronidase.