

Triglycerides Isolated from *Streptomyces* sp. ZZ035 and Their Nuclear Magnetic Resonance Spectroscopic Characters

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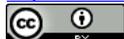
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Abstract

Streptomyces sp. ZZ035 isolated from a folk medicinal soil sample in China showed remarkable antimicrobial activities. During the isolation of secondary metabolites, a white crystal powder (1) was isolated from the broth of this strain. Its nuclear magnetic resonance (NMR) and infrared (IR) spectra indicated that it was a complex composed of triglycerides. Next, six C₁₅₋₁₇ long-chain fatty acids derived from these triglycerides were respectively identified as n-pentadecanoyl, 12-methyltetradecanoyl, 14-methyl pentadecanoyl, palmitoyl, 15-methyl hexadecanoyl and 14-methyl hexadecanoyl using the gas chromatography-mass spectroscopy (GC-MS) technology. Finally, the ¹³C and ¹H assignments of 1 were achieved through the analyses of NMR data. Based on above, their detailed NMR spectroscopic elucidation and meticulous ¹³C, ¹H assignments, especially the split peaks and coupling correlation of protons attached on the glycerol carbons, were performed for distinguishing triglycerides from other glycerides and for the identification of the long-chain fatty acids, and which would be helpful to the qualitative and quantitative analyses of tri-, di- and mono-glycerides.

Keywords

Nuclear Magnetic Resonance, Triglyceride, Glyceride, Fatty Acid, Bacteria

1. Introduction

Around the reservoir for domestic water in Chinese rural areas, a folk medicinal soil in a dark and moist environment is used to prevent infection and accelerating cure by being spread around the wound after dog bite. To reveal the anti-infection reasons of this soil, a sample was collected in Xianjing Countryside in Zhuzhou County, China, and sixty-one actinomycete strains ZZ01 to ZZ061

were selectively isolated from this sample [1]. After chemical analysis were performed for discovering strains producing a series of secondary metabolites, the bioactive evaluation showed that thirteen strains had antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli* and/or *Candida albicans*, and then the classification and identification of targeted seven strains indicated that they belonged to the genus *Streptomyces* [2]. Among them, *Streptomyces* sp. ZZ035 with remarkable antimicrobial activities against *S. aureus*, *E. coli* and *C. albicans* was targeted for discovering antimicrobial metabolites. During the isolation of components from the broth of this strain, a white crystal powder was isolated and identified as triglycerides, and many nuclear magnetic resonance (NMR) data including ^1H , ^{13}C , distortionless enhancement by polarization transfer (Dept), heteronuclear single-quantum correlation (HSQC), ^1H - ^1H correlation spectroscopy (^1H - ^1H COSY) and heteronuclear multiple-bond correlation (HMBC) were obtained for the structural analyses of these triglycerides.

Main ^{13}C , ^1H signals of tri-, di- and mono-glycerides were fully assigned in previous works [3]-[8], while seldom of them presented the detailed elucidation of those signals in ^{13}C , ^1H NMR spectra, especially for those carbons and protons of glyceryl groups, γ -methylene to carbonyl carbons of acyl groups and fatty acid terminals. Another, the chemical shifts (4.10 - 4.35 ppm) of protons attached on the C-1' of 1-monoglycerides, C-1' of 1, 2-diglycerides, C-1' and C-3' of triglycerides (Figure 1(a)) was very close to each other [3] [4] [5], the assignments of those protons easily confused when some of them were showed in the same ^1H NMR spectrum. Moreover, the protons attached on C-1' and C-3' of triglycerides

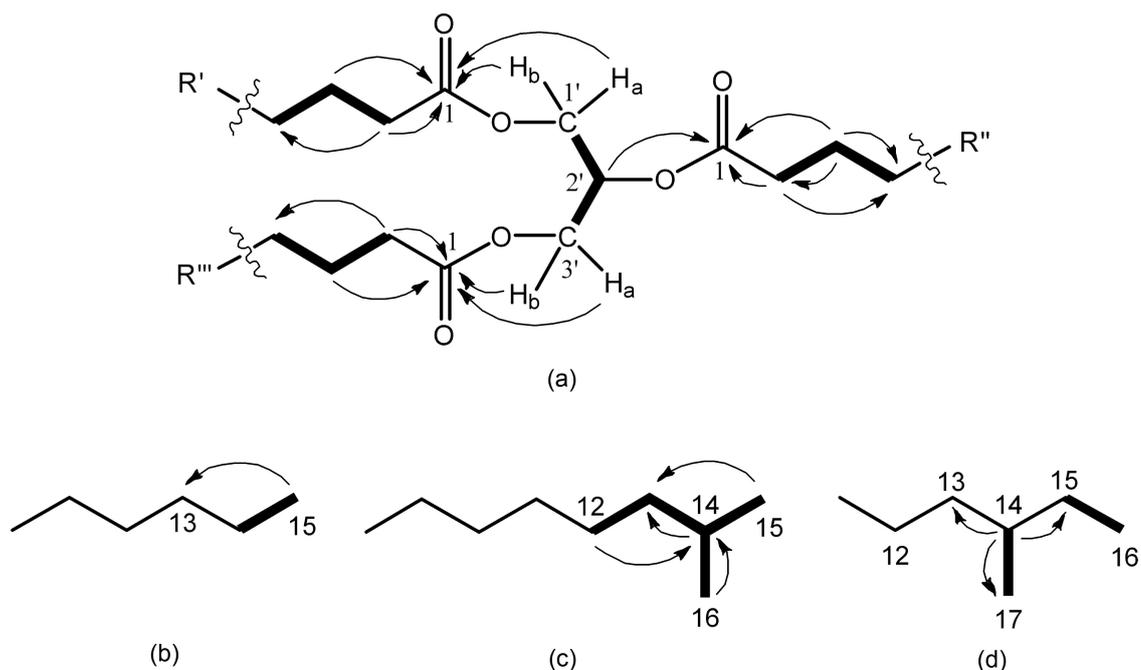


Figure 1. Key hydrogen-hydrogen correlations and heteronuclear multiple-bond correlations of **1**. **R'**, the ester acyl linked the carbon-1' of glycerol; **R''**, the ester acyl linked the carbon-2' of glycerol; **R'''**, the ester acyl linked the carbon-3' of glycerol; **R'**, **R''** or **R'''** was one of six acyl groups identified by gas chromatography-mass spectroscopy technology.

were indistinctly assigned to a doublet of double doublets centered at 4.22 ppm, and the split peaks and meticulous assignments of these protons were not clarified [3] [4] [5] [6] [7]. As many glycerides were widely used in foods, cosmetics and drugs, a variety of analyses in vivo and in vitro usually need to be performed. The detailed NMR spectroscopic elucidation and meticulous ^{13}C , ^1H assignments of these triglycerides would provide a foundation for NMR technology applying for the qualitative and quantitative analyses of tri-, di- and mono-glycerides. Used triglycerides isolated by us as an example, detailed elucidation of NMR data and meticulous ^{13}C , ^1H assignments were performed with one dimension (1D) and two dimensions (2D) NMR technology presented in this paper. Some new NMR data for identifying triglycerides were updated, and provided some references for the identification of fatty acids and glycerides.

2. Materials and Methods

2.1. Strain

Streptomyces sp. ZZ035 was isolated from a soil sample collected in Xianjing Countryside in Zhuzhou County, China (Geographic coordinates: 27°30'N, 113°17'E) [1], and was store at 4°C in College Bioscience and Bioengineering, Jiangxi Agricultural University, China. Polyphasic taxonomy procedure indicated that this strain belonged to the genus *Streptomyces* and was closest to *S. cinnamonensis* [2]. Its 16S deoxyribonucleic acid (16S rDNA) sequence was deposited at NCBI GenBank with accession numbers KJ995739.

2.2. Fermentation and Isolation

The strain of *Streptomyces* sp. ZZ035 was cultured in 5000 mL Erlenmeyer flasks that contained 2000 mL of 2A medium consisting of 1.0% glucose, 3.5% soluble starch, 0.2% yeast, 0.4% casein, 4.6% 3-[N-morpholino] propane sulfonic acid, and 1.8% sodium chloride (W/V) at 28°C for 8 d on a rotary shaker at 190 rpm until 60 L of broth obtained. The broth was centrifuged to obtain mycelium, and which was extracted with methanol (MeOH). The extract was concentrated under decompression, and then freeze-dried to obtain lyophilized powder. The powder successively dissolved in chloroform (CHCl_3) to obtained chloroform-soluble fraction, and which was purified using a silica column eluted with chloroform-petroleum ether (10:4). Finally, the fraction contained identical spots on thin layer chromatography (TLC) plates were combined and concentrated to remove solvent, and then the residue was crystallized with CHCl_3 -MeOH (1:1) at 4°C to give a white crystal powder (1, 547 mg).

2.3. Structural Elucidation and Components Analyses

Thin layer chromatography using for the isolation and analysis was carried out with silica GF_{254} (Qingdao Haiyang Chemical Co., Ltd, China), and iodine vapor were used as chromogenic agents. All NMR experiments were recorded on a Bruker AV-400 NMR spectrometer equipped with a 5-mm PABBO BB-probe head. The chemical shifts were respectively relative to deuteriochloroform

(CDCl₃) at δ_{H} 7.26 ppm and δ_{C} 77.0 ppm. For the gas chromatography-mass spectroscopy (GC-MS) analyses, the methanolysis of **1** was performed by potassium hydroxide in methanol/hexane, and then the fatty acid methyl esters were determined on a Thermo Trace 1300/ISQ GC-MS spectrometer with an electron ionization (EI) ion source (70 eV). The chromatographic peaks were identified by comparing their mass spectra with those in the NIST 11 MS data library. IR spectrum was determined on a Thermo Nicolet 380 FT-IR spectrometer with potassium bromide pellet.

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3. Results

3.1. Structural Elucidation and Components Analyses

1 was obtained as a white crystal powder, and was easily soluble in chloroform. Assigned to many methylene carbons confirmed by its Dept 135° and HSQC spectra, a large peak at 29.26 - 29.95 ppm in its ¹³C NMR and a corresponding peak at 1.20 - 1.36 ppm in its ¹H NMR indicated that **1** was likely a fatty acid ester [3] [9]. This was also deduced by its IR absorbance peaks at 1745 (Ester, C=O), 1171 (Ester, C-O), 2924 (Methylene), 2854 (Methylene) and 721 cm⁻¹ (More than four methylenes) [10].

The ¹³C and Dept 135° NMR spectra of **1** showed two carbonyl carbons at δ_{C} 173.23 and 172.86, one methine carbon at δ_{C} 68.89 and one methylene carbon signal at 62.10 ppm. From the HSQC spectra of **1**, two signals at δ_{H} 4.14 and 4.29 with two double protons observed in its ¹H NMR spectrum were attached on the methylene carbon signal at 62.10 ppm. This deduced that the signal at 62.10 ppm were assigned to two methylene carbons C-1' and C-3' (Figure 1(a)), and which two double protons at δ_{H} 4.14 (H_a-1', H_a-3') and 4.29 (H_b-1', H_b-3') were attached on. Those above together with the IR absorbance peaks at 3446 (br. s), 1745 (s) and 1171 cm⁻¹ deduced that **1** was likely triglycerides, and which was also supported by the ¹H NMR data of triglycerides in previous papers [3] [4] [5] [6] [7]. The important ¹H-¹H COSY correlations between H-1', H-3' and H-2', and H-2, H-4 and H-3 (Figure 1(a)), together with key HMBC correlations between H-2' and C-1 (R''), H_a-1'/3', H_b-1'/3' and C-1 (R'/R'''), and H-2, H-3 and C-1 (R', R'' or R''') (Figure 1(a)), were further confirmed that **1** was triglycerides.

Four methyl carbons at δ_{C} 22.65, 19.21, 14.09 and 11.38 in the ¹³C, Dept 135° and 90° spectra deduced three types of fatty acid terminals shown on Figures 1(b)-(d) [9] [11]. As shown on Figure 1(b), a straight chain fatty acid terminal was deduced by the ¹H-¹H COSY correlation between proton at δ_{H} 1.27 (H-14) and that at δ_{H} 0.88 (H-15), and by the HMBC correlation between H-15 and a

carbon at δ_C 31.92 (C-13). Similarly, the correlations between proton at δ_H 1.53 (H-14) and that at δ_H 0.86 (H-15 or H-16), and between proton at H-14, a proton at δ_H 1.25 (H-12) and δ_H 1.15 (H-13) in the ^1H - ^1H COSY spectrum deduced another fatty acid terminal from C-12 to C-16 shown on **Figure 1(c)**. This was further confirmed by the key HMBC correlations between H-15 or H-16 and two carbons respectively at δ_C 27.97 (C-14), 39.06 (C-13), and between H-12 and C-14 shown on **Figure 1(c)**. According to the ^{13}C NMR data reported [11], two methyl carbons at δ_C 19.21 (C-17) and 11.38 (C-16), two methylene carbons at δ_C 27.11 (C-15) and 36.65 (C-13) and one methine carbon at δ_C 34.40 (C-14) presented the third fatty acid terminal from C-13 to C-17 shown on **Figure 1(d)**. This was confirmed by the ^1H - ^1H COSY correlations between H-14 and H-17, and H-15 and H-16, and by the HMBC correlations between H-14 and C-17, C-15 and C-13.

Many carbon signals at 29.26 - 29.95 ppm and their one-bond related proton signals at 1.20 - 1.36 ppm in the ^{13}C , ^1H and HSQC spectra of **1** were assigned to most methylenes of fatty acid chain [3,9]. To understand the carbon numbers composed fatty acid, GC-MS technology was used for the analyses of the fatty acid methyl esters (FAME) synthesized from **1**, and six FAME were identified as shown in **Table 1**. Thereby, six C_{15-17} long-chain fatty acyl groups composed of **1** were identified as *n*-pentadecanoyl (**1a**), 12-methyltetradecanoyl (**1b**), 14-methyl pentadecanoyl (**1c**), palmitoyl (**1d**) and 15-methyl hexadecanoyl (**1e**) and 14-methylhexadecanoyl (**1f**).

3.2. Nuclear Magnetic Resonance Data of Triglycerides

Comparing previous reports [3]-[9] [12], their detailed and meticulous ^{13}C , ^1H assignments were achieved by the ^{13}C , ^1H , HSQC, ^1H - ^1H COSY and HMBC spectra, and shown in **Table 2** and **Table 3**.

4. Discussion

These triglycerides were isolated from the broth of *Streptomyces* sp. ZZ035 derived from a folk medicinal soil sample. Their detailed NMR spectroscopic

Table 1. Fatty acid methyl esters synthesized from **1** detected by gas chromatography-mass spectroscopy (GC-MS) technology.^a

No.	RT (min)	SI	RSI	Compounds	Formula
1	26.47	848	868	Pentadecanoic acid methyl ester	$\text{C}_{16}\text{H}_{32}\text{O}_2$
2	26.61	900	900	12-Methyl tetradecanoic acid methyl ester	$\text{C}_{16}\text{H}_{32}\text{O}_2$
3	28.19	859	879	14-Methyl pentadecanoic acid methyl ester	$\text{C}_{17}\text{H}_{34}\text{O}_2$
4	28.80	827	868	Palmitic acid methyl ester	$\text{C}_{17}\text{H}_{34}\text{O}_2$
5	29.83	846	896	15-Methyl hexadecanoic acid methyl ester	$\text{C}_{18}\text{H}_{36}\text{O}_2$
6	29.97	829	830	14-Methyl hexadecanoic acid methyl ester	$\text{C}_{18}\text{H}_{36}\text{O}_2$

^aThe GC-MS analyses were determined on a Thermo Trace 1300/ISQ GC-MS spectrometer with an electron ionization ion source (70 eV), and the chromatographic peaks were identified by the NIST 11 MS data library. RT, Retention time; SI, Similarity index; RSI, Reversed search index.

Table 2. ^{13}C and ^1H nuclear magnetic resonance assignments of glyceryl and part long-chain fatty acyl groups (δ , ppm in deuteriochloroform).^a

Position	R^{b}			R^{c}			R^{d}		
	δ_{C}	Dept ^c	δ_{H} (Int, mult., <i>f</i> in Hz)	δ_{C}	Dept	δ_{H} (Int, mult., <i>f</i> in Hz)	δ_{C}	Dept	δ_{H} (Int, mult., <i>f</i> in Hz)
1	173.28	C	-	172.86	C	-	173.28	C	-
2	34.06	CH_2	2.31 (2H, t, 7.6 Hz)	34.22	CH_2	2.31 (2H, t, 7.6 Hz)	34.06	CH_2	2.31 (2H, t, 7.6 Hz)
3	24.87	CH_2	1.61	24.91	CH_2	1.61	24.87	CH_2	1.61
4	29.12	CH_2	1.34	29.08	CH_2	1.34	29.12	CH_2	1.34
5- n^{d}	29.26 - 29.95 (CH_2) _{<i>n-4</i>}		1.20 - 1.36	29.26 - 29.95 (CH_2) _{<i>n-4</i>}		1.20 - 1.36	29.26 - 29.95 (CH_2) _{<i>n-4</i>}		1.20 - 1.36
1'	62.10	CH_2				4.14 (H_{a} , dd, 6.0, 11.9) 4.29 (H_{b} , dd, 4.2, 11.9)			
2'	68.89	CH				5.26 (H, tt, 4.2, 6.0)			
3'	62.10	CH_2				4.14 (H_{a} , dd, 6.0, 11.9) 4.29 (H_{b} , dd, 4.2, 11.9)			

^a400 MHz for ^1H shifts relative to deuteriochloroform (CDCl_3) at δ_{C} 7.26; 100 MHz for ^{13}C shifts relative to CDCl_3 at δ_{C} 77.0. ^b R' , R'' or R''' was one of six acyl groups **1a-1f** identified by gas chromatography-mass spectroscopy technology. ^cDept is the abbreviation of distortionless enhancement by polarization transfer spectrum. ^d*n* was equal to 9 for acyl **1b**, 11 for acyls **1c** and **1e**, 12 for acyls **1a** and **1f**, and 13 for acyl **1d**.

Table 3. ^{13}C and ^1H nuclear magnetic resonance assignments of long-chain fatty acyl terminals (δ , ppm in deuteriochloroform).^a

Position ^b	1a^c			1c			1f		
	δ_{C}	Dept ^d	δ_{H} (Int, mult., <i>f</i> in Hz)	δ_{C}	Dept	δ_{H} (Int, mult., <i>f</i> in Hz)	δ_{C}	Dept	δ_{H} (Int, mult., <i>f</i> in Hz)
12				27.41	CH_2	1.25	27.22	CH_2	1.15
13	31.92	CH_2	1.26	39.06	CH_2	1.15	36.65	CH_2	1.08, 1.26
14	22.68	CH_2	1.27	27.97	CH	1.53	34.40	CH	1.29
15	14.09	CH_3	0.88 (3H, t, 6.8)	22.65	CH_3	0.86 (3H, d, 6.8)	27.11	CH_2	1.25
16				22.65	CH_3	0.86 (3H, d, 6.8)	11.38	CH_3	0.85 (3H, t, 7.0)
17							19.21	CH_3	0.84 (3H, d, 6.7)

^a400 MHz for ^1H shifts relative to deuteriochloroform (CDCl_3) at δ_{C} 7.26; 100 MHz for ^{13}C shifts relative to CDCl_3 at δ_{C} 77.0. ^bPositions were numbered according to **Figures 1(b)-(d)** for acyl terminals **Figure 1(a)**, **Figure 1(c)** and **Figure 1(f)**. ^c**Figure 1(a)**, *n*-pentadecanoyl terminal (that same to palmitoyl one); **Figure 1(c)**, 14-methyl pentadecanoyl terminal (same to 15-methyl hexadecanoyl one), **Figure 1(f)**, 14-methylhexadecanoyl terminal (same to 12-methyltetradecanoyl one). Dept is the abbreviation of distortionless enhancement by polarization transfer spectrum.

elucidation and key ^{13}C , ^1H assignments were performed for distinguishing triglycerides from other glycerides and for the identification of the long-chain fatty acid terminals. More and more glycerides isolated from bacteria and other bioresources, they showed a variety of bioactivities [8] [11] [13] [14] [15]. Some of them showed antimicrobial [8], and some were platelet aggregation inhibitors or antagonists of cannabinoid receptor [11] [13] [14]. Our antimicrobial experiments indicated that **1** contained C_{15-17} fatty acyl groups had no antimicrobial activities against *S. aureus* and *C. albicans*. Same to most glycerides [8] [9] [11] [12], triglycerides also present a large peak at 29.26 - 29.95 ppm in their ^{13}C NMR spectra and a large peak at 1.20 - 1.36 ppm in their ^1H spectra NMR spectra. The acyl terminals of glycerides were deduced from the chemical shifts of methyl carbons in their ^{13}C NMR spectra, such as a methyl carbon at δ_{C} 14.09 for a straight chain acyl terminals, two methyl carbons at δ_{C} 22.65 for an *iso*-fatty acid terminal

(Figure 1(c)), and two methyl carbons at δ_C 11.38 and 19.21 for an *anteiso*-fatty acid terminal (Figure 1(d)).

For distinguishing triglycerides from other glycerides, peaks due to protons attached on the glycerol carbons are of primary importance. The chemical shifts of protons on C-1' or C-3', and C-2' for triglycerides were respectively 4.22 and 5.27 ppm in $CDCl_3$ according to previous reports [3] [4] [5], while two nonequivalent protons attached on C-1' or C-3' of these triglycerides were deduced from the 1D and 2D NMR data of **1**. The chemical shift of one proton was 4.14 ppm, and another was 4.30 ppm. Each proton attached on C-1' or C-3' presented a geminal coupling ($J = 11.9$ Hz) with another and a vicinal coupling ($J = 6.0$ or 4.2 Hz) with H-2', and which gave a double doublets for each proton on C-1' or C-3' due to an AMX coupling system. Their ^{13}C and 1H assignments were up to date in Table 2, and the relative split peaks in the 1H NMR spectrum were amplified in Figure 2.

Another, an A_2MX_2 coupling system led H-2' to split triple-triplets centered at 5.26 ppm, among which three middle ones mostly overlapped to form an abnormal peak (Figure 2). These ^{13}C , 1H assignments and split analyses of triglycerides were first confirmed by their HMBC and HSQC spectra shown on Figure 1(a) and Figure 3. Moreover, the chemical shifts of protons attached on the C-1' of 1-monoglycerides ($\delta_{H-1'}$ 4.14/4.18), C-1' of 1, 2-diglycerides ($\delta_{H-1'}$ 4.28), C-1' and C-3' of triglycerides (Figure 1(a)) was very close to each other [3]-[8], and the assignments of those protons easily confused when some of them were showed in the same 1H NMR spectrum. The clarification of these ^{13}C , 1H assignments would be helpful to distinguish triglycerides from other glycerides

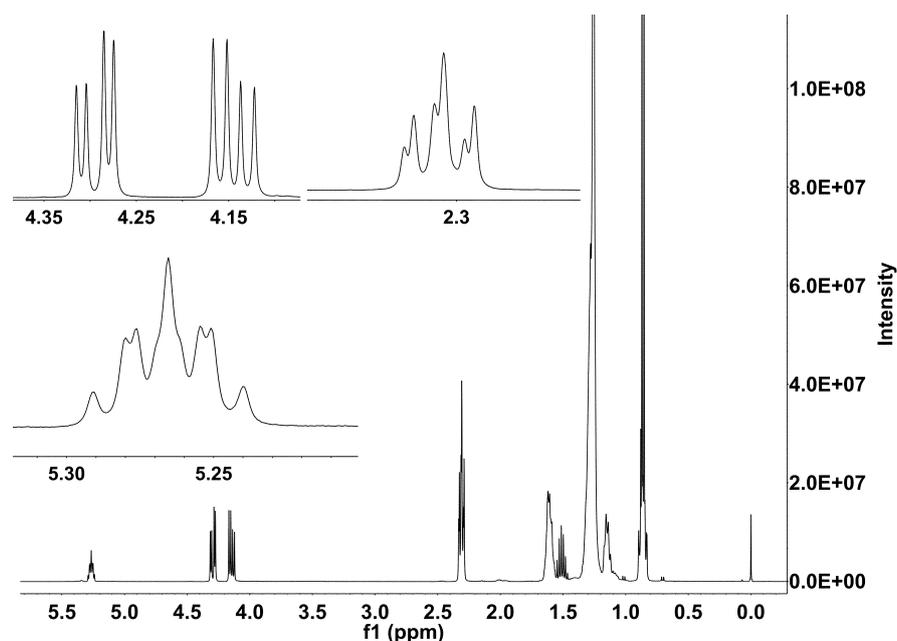


Figure 2. Hydrogen nuclear magnetic resonance spectrum of **1**. a, nine split peaks centered at 5.27 ppm were assigned to hydrogen-2 (H-2'); b, two double doublets centered 4.14 and 4.30 ppm were respectively assigned to $H_a-1'(3')$ and $H_b-1'(3')$; c, protons attached carbon-2 of R' or R''' (higher field) and carbon-2 of R'' (lower field).

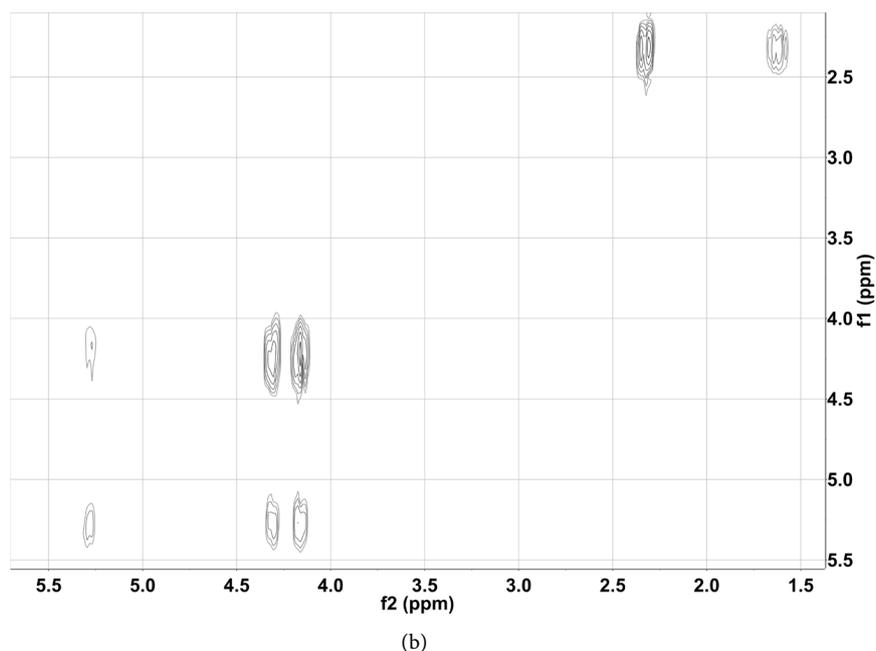
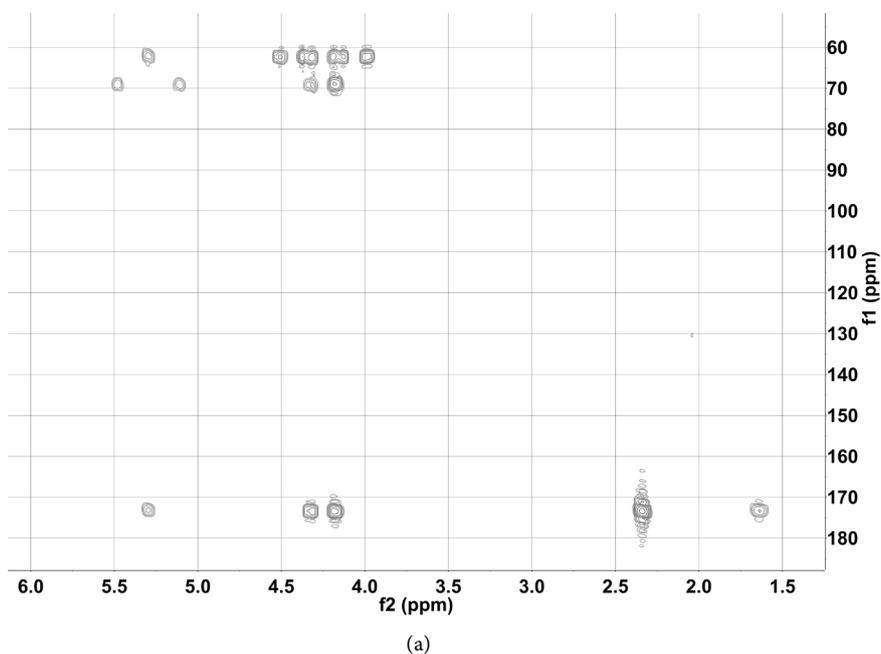


Figure 3. Part hydrogen-hydrogen (^1H - ^1H) correlation spectroscopy (a) and heteronuclear multiple-bond correlation spectrum (b) for the ^{13}C and ^1H assignments of glyceryl of **1**.

with NMR technology.

As many glycerides were widely used in foods, cosmetics and drugs, a variety of analyses involved determination, metabolism and transformation *in vivo* or *in vitro* usually need to be performed [3] [4] [5] [16]. Further, ^{13}C and ^1H NMR was proved to be a very useful technique in monitoring the extent of lipid hydrolysis in digestion processes and to determine the bioaccessibility and bioavailability of lipophilic compounds [3] [4] [5] [16] [17]. Thereby, the detailed and meticulous ^{13}C , ^1H assignments of these triglycerides would provide a foundation for NMR

technology applying for the qualitative and quantitative analyses of tri-, di- and mono-glycerides.

5. Conclusion

A white crystal powder (1) composed of several triglycerides was isolated from the broth of *Streptomyces* sp. ZZ035, and analyzed by GC-MS, IR and NMR technology. The important ^{13}C and ^1H assignments of these triglycerides were achieved through the analyses of NMR data. Moreover, their detailed NMR spectroscopic elucidation and meticulous ^{13}C , ^1H assignments, especially the split peaks and coupling correlation of protons attached on the glycerol carbons, were performed for distinguishing triglycerides from other glycerides and for the identification of the long-chain fatty acids, and which would be helpful to the qualitative and quantitative analyses of tri-, di- and mono-glycerides.

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