AL072, A Novel Anti-*Legionella* Antibiotic Produced by *Streptomyces* sp.

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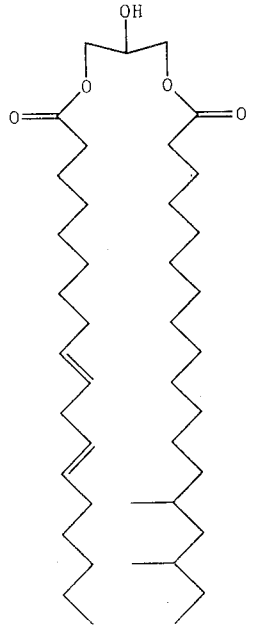
AL072 is a potent anti-*Legionella* antibiotic produced by *Streptomyces* strain AL91. The compound was isolated from the fermentation broth with 1 volume of isopropyl alcohol, followed by an ethyl acetate extraction and subsequent concentration under reduced pressure. Purification was performed on an octadecyl silica gel column followed by preparative HPLC. AL072 purified as mentioned above showed extremely specific activity only towards *Legionella pneumophila*. No antibacterial activity against any other bacteria tested was demonstrable. Its molecular weight was determined by FAB-MS (*m/z* 648) and the compound was identified as a novel 1,3-diacyl glycerol with the molecular formula $C_{41}H_{76}O_5$. One of the two acyl groups is linoleyl and the other is 3,5-dimethyl octadecanoyl.

Legionella pneumophila is a Gram-negative bacterial pathogen and the causative agent of Legionnaires' disease or Pontiac fever.[1] This organism is a facultative intracellular parasite that multiplies in the human macrophage.[2,3] The ubiquity of *Legionella* in lakes and rivers throughout the world is well known, and nearly 100% of lakes and rivers in the southern United States are reported to be contaminated.[4,5] Despite the extensive aerosolization that may take place, for instance, as in natural waterfalls, no known case of Legionellosis related to this type of exposure has ever been reported. This is because the concentration of *Legionella* in these waters is usually low.[4]

Man-made environments are the major sources of outbreaks of Legionellosis. Many hospitals as well as other large buildings are contaminated with *Legionella*.[6,7] This bacterium grows well in the biofilms that are formed in the water plumbing systems and subsequently can be spread by aerosol from a shower.[8] Cooling towers are also frequently contaminated with *Legionella*.[9~11] In some cases, the number of *Legionella pneumophila* found in these cooling towers were extremely high (up to 2×10^7/liter). Cooling towers not only provide an excellent amplifier for *Legionella*, but also are efficient disseminators of these bacteria.

Although Legionnaires' disease is a relatively uncommon type of pneumonia it is potentially serious, often causing disability and having a fatality rate of around

Fig. 1. Structure of AL072.



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10%, even in the cases for healthy individuals.[12] *Legionella pneumophila* is sensitive to erythromycin, rifampicin, aminoglycosides and other antibiotics[13~15] in *in vitro* tests, but *in vivo*, only erythromycin, sometimes combined with rifampicin is effective. Recently, attention has also been drawn to the quinolones and macrolides for the treatment of *Legionella* infections.[16]

In the course of our screening for a specific anti-*Legionella* antibiotic from various soil microorganisms, we found a novel and potent substance, AL072 (Fig. 1), with antibacterial activity only against *Legionella pneumophila*. The producing microorganism, named as *Streptomyces* strain AL91, was isolated from a soil sample collected in Pohang, Kyongsangnam-Do, Korea, and identified to be a new type of *Streptomyces* species.

Materials and Methods

Strains

Antibiotic producing microbial strain AL91 was isolated from a soil sample collected in Pohang, Kyongsangnam-Do, Korea and the test microorganism *Legionella pneumophilla* ATCC33152 was obtained from Korean National Institute of Health (KNIH).

Taxonomy

Methods adopted by the International Streptomyces Project (ISP) were used for the taxonomic studies. The cultural characteristics were determined on the media recommended by the ISP. All of the cultures were incubated at 27°C for 14 days. Cell-wall preparations were analyzed by the method of BECKER *et al.*[17], and whole-cell hydrolysates were prepared and examined using the chemotaxonomic techniques of LECHEVALIER.[18] The lipid composition of cell-wall extracts was determined by the method LECHEVALIER *et al.*[19]

Susceptibility to Antibiotics

Susceptibility of strain AL91 to fourteen kinds of antibiotics was conducted *in vitro* by the agar diffusion method. Filter paper discs impregmented with specific concentrations of antibiotics (BBL Senci Disc, Becton Dickinson) were applied to the surface of Bennett's agar inoculated with 0.5% (v/v) of 48 hour cultured broth of the strain AL91 and the plates were incubated at 28°C for 5 days. After incubation, the zones of inhibition surrounding the discs were measured.

Cultivation

Seed Culture

A loopful of the producing microorganism was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium (0.1% glucose, 2.4% soluble starch, 0.3% peptone, 0.5% malt extract and 0.4% $CaCO_3$, pH 7.0) and incubated for 72 hours at 27°C on a rotary shaker (300 rpm).

Main Culture

The seed culture broth was transferred into a 2,000-ml Erlenmeyer flask containing 400 ml of producing medium (2% sucrose, 1% glucose, 0.5% corn steep liquor, 0.49% yeast extract, 2% soybean meal, 0.4% $CaCO_3$, 0.2% NaCl and 0.005% K_2HPO_4, pH 7.0) to 3% (v/v) and incubated for 96 hours at 27°C with an agitation rate of 300 rpm.

Test Microorganism Culture

Liquid Medium

Medium containing yeast extract 11.5 g, charcoal 1.5 g, ACES 6.0 g, α-ketoglutaric acid 1.0 g/liter and filter sterilized L-cysteine 0.2 g and ferric pyrophosphate 0.125 g (BCYEα, pH 7.1) was used to culture the test microorganism.

Solid Medium

BCYEα with 1.5% of agar was used to culture the test microorganism on solid medium.

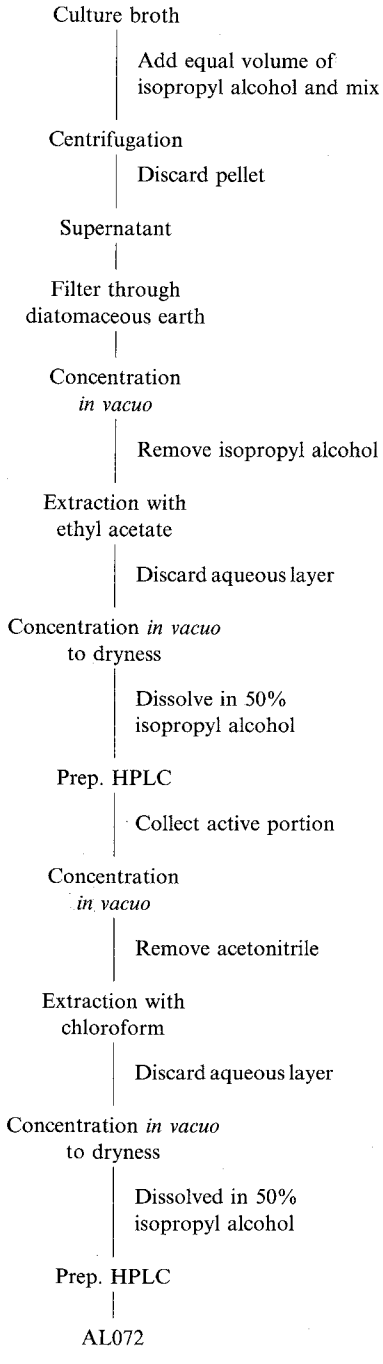
Media for Bioassay

BCYEα solid medium covered with Mueller-Hinton soft agar containing 2% of overnight culture broth of *Legionella pneumophila* was used for the bioassay.

Purification of the Active Compound

AL072 was isolated and purified with the procedure shown in Fig. 2. An equal volume of isopropyl alcohol was added to the 6 liters of culture broth and mixed. The supernatant after centrifugation was filtered through diatomaceous earth. Isopropyl alcohol was removed by concentration *in vacuo*. The concentrated culture broth was extracted 3 times with equal volumes of ethyl acetate and the organic phase was evaporated to dryness under reduced pressure. The solid residue was dissolved in 100 ml of 50% isopropyl alcohol in water and concentrated *in vacuo* to remove isopropyl alcohol. The solution was applied to an octadecyl silica gel column (Waters, μ-Bondapak C_{18}, 200 × 25 mm) for chromatography. A fraction eluted with 70% aqueous ethyl alcohol was concentrated to dryness and redissolved in 30 ml of 50% isopropyl alcohol. The solution was applied on a preparative Waters μ-Bondapak C_{18} (200 × 25 mm) HPLC column and developed with acetonitrile - water (56 : 44). The fraction containing anti-*Legionella* activity was collected and concentrated *in vacuo* to remove the acetonitrile. The residue was dissolved in 50 ml of 50% isopropyl alcohol and concentrated. The aqueous solution was extracted 3 times with equal volume of chloroform and the organic phase was concentrated to dryness under reduced pressure. The residue was redissolved in 20 ml of 50% isopropyl alcohol and was rechromatographed on a preparative Waters μ-Bondapak C_{18}

Fig. 2. Purification process for AL072.



(200 × 25 mm) HPLC column and developed with acetonitrile - water (56 : 44). The fraction showing anti-*Legionella* activity was collected and concentrated to give pure AL072.

Results and Discussion

Taxonomic Studies

A summary of the culture characteristics of strain AL91 on various media is presented in Table 1. White aerial mycelium was formed on yeast extract - malt extract agar, inorganic salts starch agar and Bennett's agar, while yellow to brown aerial mycelium was formed on the other ISP media. Poor growth was noted on peptone - yeast extract iron agar. Substrate mycelium ranged from colorless to brown depending upon the ISP medium employed. Soluble pigments were produced on tryptone - yeast extract and peptone - yeast extract iron agar. Optimum temperature for growth was 20 ~ 37°C.

Physiological characteristics of strain AL91 and its utilization of various carbon sources are shown in Table 2. The culture utilized all carbon sources tested except L-arabinose and inositol. The culture degraded casein, xanthine, but not tyrosine, and was able to hydrolyse starch but not gelatin.

Scanning electron microscopic examination of the organism revealed an exclusively branching substrate mycelium as well as abundant aerial mycelium, which is then transformed into spirals of subspherical, smooth arthrospores. No fragmentation of the substrate mycelium was noted. The aerial mycelium had 15 ~ 20 spores per chain. The spores, as examined by a scanning electron microscopy, were subspherical in shape with smooth surfaces. Sporangia, zoospores and sclerotia were not observed (Fig. 3).

Cell wall and whole-cell sugar analysis showed that strain AL91 contained the LL isomer of diaminopimelic acid (DAP) and only glucose, respectively. The strain

Table 1. Cultural characteristics of strain AL91.

Media	Growth	Aerial mycelium color	Reverse color	Soluble pigment
Trypton - yeast extract agar (ISP No. 1)	Good	Brown	Brown	Brown
Yeast extract - malt extract agar (ISP No. 2)	Good	White	Brown	None
Oatmeal agar (ISP No. 3)	Good	Yellow to pink white	Colorless	None
Inorganic salts starch agar (ISP No. 4)	Good	White	Colorless	None
Glycerol - asparagine agar (ISP No. 5)	Good	Dark yellow	Dark yellow	None
Peptone - yeast extract iron agar (ISP No. 6)	Poor	Dark brown	Brown	Dark brown
Tyrosine agar (ISP No. 7)	Good	White brown	Dark brown	None
Bennett's agar	Good	White	Yellow brown	None

Observation after incubation at 27°C for 14 days.

Table 2. Physiological characteristics and carbon utilization of strain AL91.

Melanin production (ISP No. 6)	+
Solubilization of Tyrosine	−
Casein	+
Xanthine	+
Hydrolysis of Gelatin	−
Starch	+
Optimum growth temperature	20~37°C
Utilization of D-Glucose	+
L-Arabinose	−
Sucrose	+
D-Xylose	+
Inositol	−
D-Mannose	+
D-Fructose	+
Rhamnose	+
Raffinose	+
Cellulose	+

−: Negative, +: positive.

Fig. 3. Scanning electron micrograph of the isolated *Streptomyces* sp AL91. (Philips SEM 515, ×17,000).

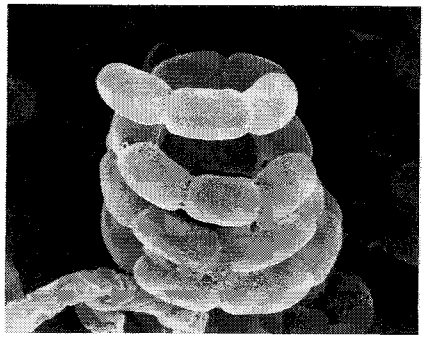


Table 3. Sensitivity of strain AL91 to various antibiotics.

Antibiotics	Concentration (μg/ml)	Sensitivity (Inhibition zone, φmm)
Carbenicillin	100	—
Chloroamphenicol	30	27.0
Neomycin	30	12.0
Nalidixic acid	30	—
Vancomycin	30	22.0
Clindamycin	2	—
Ampicillin	10	12.0
Kanamycin	30	17.0
Tetracyclin	30	17.0
Cephalothin	30	24.0
Erythromycin	15	40.0
Rifampin	5	—
Gentamycin	10	10.0
Streptomycin	10	17.0

−: Not affected.

Table 4. Anti-bacterial activitiy of AL072. (Unit: φmm)

Microorganism	Strain	φ
Legionella pneumophila	ATCC33152	108
Staphilococcus aureus	[a]C4063	11
S. aureus	C4037	24
S. aureus	C4038	12
S. aureus	C4039	13
Streptococcus pyogenes	C4003	—
S. pyogenes	C4033	20
S. aronson	C4032	20
S. agalatiae	C4029	—
S. equi	C4030	21
S. durans	C4035	24
Listeria monocytogenes	C4042	12
Corinebacterium diphtheriae	C4084	9
Bacillus subtilis	C4062	8
B. megatherium	C4066	6
Escherichia coli	C4017	6
E. coli	C4072	16
E. coli	C4073	16
E. coli	C4002	15
E. coli	C4018	19
E. coli	C4075	20
E. coli	C4019	21
E. coli	C4020	10
E. coli	C4052	16
E. coli	C4050	14
Citrobacter freundii	C4049	—
Salmonella typhimurium	C4045	22
S. typhimurium	C4046	14
Shigella flexneri	C4077	—
Klebsiella pneumoniae	C4078	—
K. pneumoniae	C4058	11
K. pneumoniae	C4021	—
K. oxytoca	C4022	—
K. aerogenes	C4001	—
Enterobater cloacae	C4023	—
E. cloacae	C4009	—
E. cloacae	C4024	—
E. cloacae	C4008	—
E. aerogenes	C4053	9
Serratia marcescens	C4054	—
S. marcescens	C4025	—
S. marcescens	C4026	—
S. marcescens	C4059	—
Proteus mirabilis	C4012	—
P. mirabilis	C4013	—
P. mirabilis	C4080	19
P. vulgaris	C4010	12
P. vulgaris	C4011	17
P. rettgeri	C4081	—
P. rettgeri	C4014	—
P. morganii	C4015	—
P. morganii	C4016	7
Pseudomonas aeruginosa	C4056	—
P. aeruginosa	C4068	—
P. aeruginosa	C4069	—
P. aeruginosa	C4070	—
P. aeruginosa	C4055	—
P. aeruginosa	C4057	—
P. aeruginosa	C4028	18
P. cepacia	C4071	—

—: No activity.
[a] Culture of Cheil Foods and Chemicals Inc.

contained large portion of phosphatidylethanolamine and trace of phosphatidylinositol and diphosphatidylglycerol as a diagnostic phospholipid, indicating a type II phospholipid pattern. Additionally, sensitivity to various known antibiotics are shown in Table 3.

Based on these characteristics, we concluded that strain AL91 could be assigned to the genus *Streptomyces*, and named it *Streptomyces* sp. strain AL91.

Biological Activity of AL072

The *in vitro* anti-bacterial activity of AL072 against *Legionella pneumophila* and other clinical isolates is shown in Table 4. The activity was determined using the paper disc method on a medium for bioassay. AL072 showed strong activity against *Legionella pneumophila* but no or weak activities against other clinical isolates.

Physico-chemical Properties of AL072

The physico-chemical properties of AL072 are summarized in Table 5. AL072 was isolated as clear oil, and was found to be soluble in various organic solvents including methyl alcohol, ethyl alcohol, isopropyl alcohol, ethyl acetate and chloroform, but was insoluble

Table 5. Physico-chemical Properties of AL072.

Appearance	Clear oil
Molecular formula	$C_{41}H_{76}O_5$
UV λ_{max}^{MeOH} nm ($\log \varepsilon$)	242 (2.56)
IR (KBr) ν_{max} cm^{-1}	2921, 2852, 1700, 1685, 1457, 1420, 1282, 937, 722, 670
TLC Rf Value	0.58
$[\alpha]_{25}^{D}$ (MeOH)	+22.8
FAB-MS $(M+H)^+$	649

Fig. 4. ^1H NMR spectrum of AL072 ($CHCl_3$-*d*, 400 MHz).

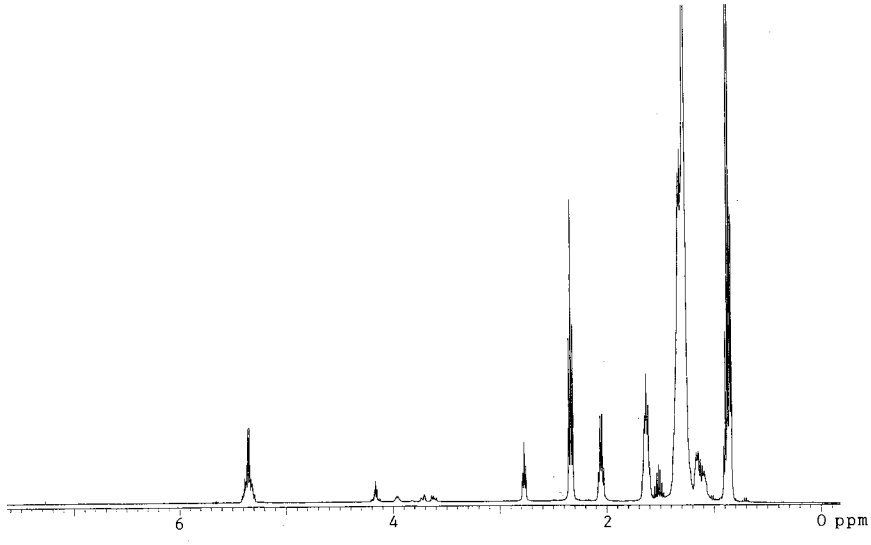


Fig. 5. ^{13}C NMR spectrum of AL072 ($CHCl_3$-*d*, 100 MHz).

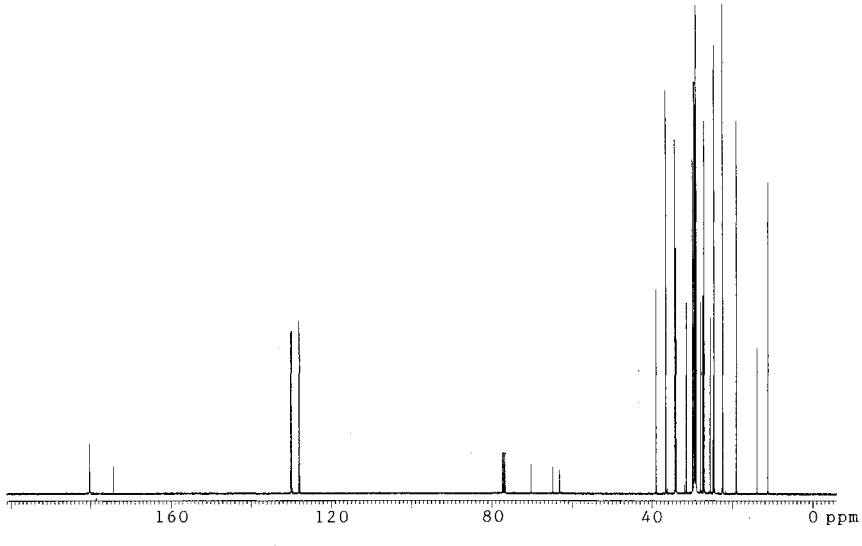


Fig. 6. ^{1}H-^{13}C long range couplings of glycerol part.

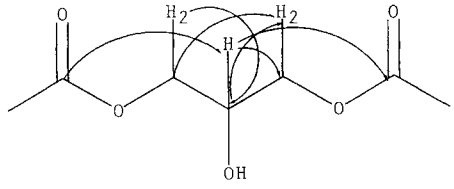


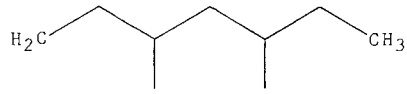
Table 6. ^{1}H NMR and ^{13}C NMR data of AL072.

	^{13}C	Multi-plization	^{1}H	Assign-ment
1)	12.01	q	0.86 (3H, m)	Octa.
2)	14.67	q	0.91 (3H, m)	Lino.
3)	19.85	q	0.87 (3H, m)	Octa.
4)	23.23	t	0.88 (1H, m)	Lino.
			1.32 (1H, m)	
5)	23.28	q		Octa.
6)	25.34	t		Octa.
7)	25.36	t	1.65 (2H, t, 7.0)	Lino.
8)	26.29	t	2.80 (2H, t, 6.3)	Lino.
9)	27.78	t		Octa.
10)	27.84	t		Octa.
11)	27.87	t	1.27 (1H, m)	Lino.
			2.08 (1H, dd, 6.8, 13.6)	
12)	28.10	t		Octa.
13)	28.63	d	1.54 (1H, m, 6.6)	Octa.
14)	29.72	t		Lino.
15)	29.75	t		Lino.
16)	29.82	t		Lino.
17)	29.93	t		Octa.
18)	30.02	t		Lino.
19)	30.17	t		Octa.
20)	30.26	t		Octa.
21)	30.29	t		Lino.
22)	30.33	t		Octa.
23)	30.36	t		Octa.
24)	30.38	t		Octa.
25)	30.62	t		Octa.
26)	30.69	t		Octa.
27)	32.20	t		Lino.
28)	34.79	t	2.37 (2H, t, 7.5)	Lino.
29)	34.81	t		Lino.
30)	35.08	d	1.29 (1H, m)	Octa.
31)	37.33	t	1.1 (1H, m)	Octa.
			1.29 (1H, m)	
32)	39.75	t	1.17 (2H, m)	Octa.
33)	63.90	t	3.62 (1H, dd, 6.1, 11.8)	Gly.
			3.72 (1H, dd, 3.7, 11.8)	
34)	65.59	t	4.16 (2H, m, 6.4, 5.4)	Gly.
35)	70.96	d	3.96 (1H, ddd, 6.1, 3.7, 6.4)	Gly.
36)	128.50	d	5.35 (1H, m)	Lino.
37)	128.70	d		Lino.
38)	130.60	d	5.39 (1H, m)	Lino.
39)	130.70	d		Lino.
40)	174.90	s		Octa.
41)	181.0	s		Lino.

Lino: Linoleic acid, Octa.: 1,3-Dimethyloctadecanoic acid, Gly.: Glycerol.

in water. The Rf value of AL072 on silica gel TLC developed with chloroform-methyl alcohol (19:1) was 0.58. AL072 showed a positive color reaction to ammonium molybdate reagent, but did not react with the ninhydrin agent. The UV spectrum of AL072 dissolved in methyl alcohol showed a maximum absorption peak at 242 nm. AL072 was very stable in 80% alcohol solution between pH 2 and pH 12 and retained almost all of its activity even after 1 hour heating at 95°C. The molecular formula of AL072 was determined to be $C_{41}H_{76}O_5$ by FAB-MS, EI-MS, ^{13}C NMR and ^{1}H NMR. The molecular weight determined was 648.

Fig. 7. Certified partial structure of carboxylic Acid.



Structure Elucidation of AL072

Proton NMR ($CHCl_3$-*d*, 400 MHz) showed that peaks between 0.8~1.0 ppm were attributed to methyl groups and between 1.2~1.3 ppm to methylene groups. The peaks of 5H's between 3.6~4.2 ppm were attributed to the glycerol partial structure. The multiplets between 5.3~5.4 ppm showed the existence of double bonds (Fig. 4).

The study of the ^{13}C NMR spectrum completed the partial structure determination obtained from proton NMR such as the identification of two carbonyl carbons (181.0 ppm and 174.9 ppm), two double bonds (128.5, 128.7, 130.6 and 130.7 ppm) and three hydroxyl groups (63.90, 65.59 and 70.96 ppm) (Fig. 5). The DEPT 45, DEPT 90 and DEPT 135 experiments revealed that AL072 included four methyl groups and three methine groups.

In order to carry out the GC-MSD experiment, AL072 was subjected to methanolysis by methanolic sodium hydroxide and boron trifluoride (BF_3) as the methyl group donor. One of the two main peaks obtained from GC-MSD was identified to be linoleic acid. 17 peaks of ^{13}C NMR obtained from AL072 agreed with those of linoleic acid.

The HETCOR spectrum showed the peaks correlated along 63.90/3.62, 63.90/3.72, 65.59/4.16 and 70.96/3.96 (^{13}C peaks/^{1}H peaks). While HH-COSY gave four cross peaks of 4.16/3.96, 3.96/3.72, 3.96/3.62 and 3.72/3.62, HOHAHA gave two additional peaks of 4.16/3.72 and 4.16/3.62. The analysis of those two spectra revealed the partial structure of glycerol.

HMBC experiments were carried out under two different conditions of the delay of evolution for long range couplings. One was 60 msec and the other, 40 msec.

^{1}H-^{13}C long range couplings of the glycerol component observed by HMBC spectrum are shown in Fig. 6.

The ^{13}C NMR spectrum gave 41 peaks as listed in Table 6. Among those, 18 peaks belong to linoleic acid, 3 peaks are from glycerol and the remaining 20 peaks are assigned to the other carboxylic acids. INADEQUATE and HMBC spectra gave the partial structure of the carboxylic acid (Fig. 7).

Consequently, AL072 is identified as a 1,3-diacyl glycerol. One of the two acyl groups was linoleyl and the other a 3,5-dimethyl octadecanoyl group.

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