



## NMR study on secondary metabolites isolated from an identified tunicate

Jung-Rae Rho

Department of Marine Information Science, Kunsan National University, Kunsan 573-701, Korea

Received September 14, 2004

**Abstract** : Four secondary metabolites from an unidentified tunicate were isolated by treatment with trichloroethyl chloroformate(TECF) or acetic anhydride in pyridine. Their structures were determined by an extensive NMR analysis and the configuration of diacetyl derivatives(**3a**, **4a**) was assigned by comparing with NMR data of a similar compound. Three new naturally occurring compounds (**1**, **3**, **4**) showed potent brine shrimp lethality and antifungal effect against *Candida albicans*.

Key word: 1D and 2D NMR, tunicate, amino alcohol, antifungal activity

### INTRODUCTION

Tunicates have been investigated as a source of bioactive marine metabolites for a few decade years.<sup>1</sup> Secondary metabolites from tunicates vary in structure from a common alkaloid to cyclic peptides which are dominantly derived from amino acids.<sup>2-3</sup> In addition, the aliphatic amino alcohol which is biogenetically derivable from alanine was recently isolated from the tunicate *Pseudodistoma* sp.<sup>4-6</sup> In a biological assay, this compound was known to have cytotoxicity against p388 murine leukemia and activity against bacteria *Escherchia* and *Bacillus subtilis* and inhibited the growth of the fungi *Candida albicans*, *Cladosporium resinae*.<sup>7</sup> In our screening of cytotoxic compounds from marine invertebrates, we found significant activity on brine shrimp lethality in the methanol extract of an unidentified tunicate collected at 15-25m depth off the coast of Bac-Do near Keomun Island in 1995.

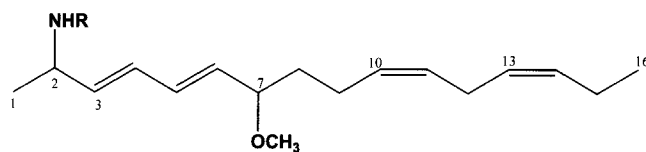
\* To whom correspondence should be addressed. E-mail: jrrho@kunsan.ac.kr

The methanolic extract of this specimen was partitioned between BuOH and H<sub>2</sub>O, and then chromatographed on a reversed phase column to give amino alcohol fractions which also exhibited strong antifungal activity against *Candida albicans*. In an activity-guided separation, the amphiphilic amino alcohols (**1-4**) were obtained as their trichloroethoxy carbonyl or acetyl derivatives (**1a-4a**), not as naturally occurring compounds. The compound **1a** was identified as a new amine structure with the C-16 tetraene unit, and **3** and **4** as new enantiomers with the known planar structure. In this report, we describe the isolation and structural elucidation of compound (**1a**)-(4a), their derivatives of (**1**)-(4), respectively.

## EXPERIMENTAL

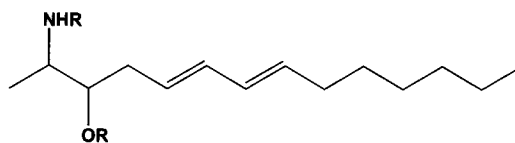
### Collection, Extraction and Isolation

An identified tunicate (sample no. 95B-11) was collected by hand using SCUBA at a depth of 20-30m at Bacdo near Keomun island, Korea in 1995. The freeze-dried tunicate (2.5Kg) was repeatedly extracted with MeOH and CH<sub>2</sub>Cl<sub>2</sub> at room temperature. The crude extract was first partitioned between H<sub>2</sub>O and BuOH and then the latter layer re-partitioned between hexane and 15% aqueous MeOH solvent. The 15% MeOH phase was in turn subjected to reversed phase vacuum flash chromatography eluting with stepwise gradients of MeOH in H<sub>2</sub>O(50%, 60%, 70%, 80%, 90%, 100%). Both 60% and 70% MeOH fractions showed the cytotoxic effect on brine shrimp and the activity against the fungi *Candida albicans*. Firstly, the 60% MeOH fraction was further separated by reversed phase HPLC(YMC ODS-A column, 250mm × 10mm, Shodex RI detector) using a solvent system (H<sub>2</sub>O/MeOH=45/55) to yield a mixture containing compound **1** and **2** whose purification attempts were unsuccessful. Following the treatment of this mixture with trichloroethyl chloroformate(TECF), compound **1a** and **2a** were purified by reversed-phase HPLC with an eluant (H<sub>2</sub>O/MeOH=10/90). In the same way, the 70% MeOH fraction was also separated by reversed phase HPLC using a solvent system (H<sub>2</sub>O/MeOH=40/60) to obtain pure compounds showing the significant brine shrimp lethality. However, purified compounds could readily be not obtained due to their amphiphilic property like above. The mixture was



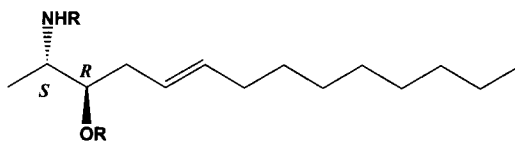
(1) R = H

(1a) R = COOCH<sub>2</sub>CCl<sub>3</sub>



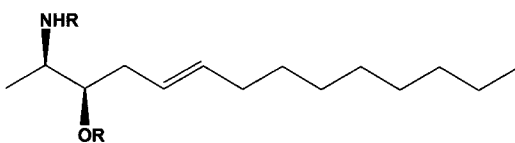
(2) R = H

(2a) R = COOCH<sub>2</sub>CCl<sub>3</sub>



(3) R = H

(3a) R = Ac



(4) R = H

(4a) R = Ac

reacted with acetyl anhydride in pyridine to facilitate separation, followed by the purification of the acetylated compounds by reversed phase HPLC using the solvent (H<sub>2</sub>O/MeOH=5/95) to afford **3a** and **4a**.

### NMR experiment

The 1D and 2D NMR spectra were obtained on a Varian UNITY500 spectrometer working at 500MHz for proton and 125MHz for carbon. The <sup>1</sup>H and <sup>13</sup>C NMR chemical shifts refer to CDCl<sub>3</sub> at 7.26 and 77.0 ppm, respectively. For all experiments, the temperature was stabilized at 297K. The parameters used for 2D NMR spectra were as follows; The gradient COSY spectra were collected with a spectral width 2567 Hz in a 512(t1) × 1024 (t2) matrix applying the pulse gradient of 1ms duration with a strength 10G/m and processed with a sinebell function. The gradient HSQC spectra were measured in a 128(t1) × 1024(t2) matrix with  $J_{CH}$ =140 Hz and processed in a 256(t1) × 1024(t2) matrix by a linear prediction method for a higher resolution. The gradient HMBC experiment was optimized for the long-range coupling constant of 7Hz. The HSQC and HMBC experiments were utilized by the pulse gradients of 1ms duration and 10G/m strength to reduce the artifacts in the spectra.

### Derivation with Trichloroethyl chloroformate(TECF)

A portion of the mixture fraction (ca 30mg) containing compound **1** and **2** was reacted with 1, 4-dioxane (1ml), pyridine (0.2ml) and 2,2,2-TECF (0.1ml) for overnight. The blow of N<sub>2</sub> removed the solvent of the reaction mixture.

### Acetylation of compound **3** and **4**

A portion of the mixture fraction (ca 5.0mg) containing compound **3** and **4** was treated with pyridine (300μl) and distilled acetic anhydride (300μl) at room temperature for 8 h. The solvent of the reaction mixture was removed *in vacuo* and the residue partitioned between H<sub>2</sub>O and EtOAc. The organic layer was evaporated to yield an oil (3.5mg), followed by reversed phase HPLC for further purification.

## RESULTS AND DISCUSSION

Four natural compounds **1-4** were converted to derivatives (**1a**, **2a**) with TECF and diacetyl amino alcohols (**3a**, **4a**) during purification. Compound **1a** was isolated as an amorphous oil which deduced to be  $C_{20}H_{30}Cl_3NO_3$  by HREIMS measurement and carbon spectrum. The planar structure of **1a** was determined by a combination of 1D and 2D NMR experiments and the assignment was listed in Table 1. The  $^{13}C$  NMR spectrum and DEPT experiment indicated the presence of three methyl, five methylene, ten methine and two quaternary carbon signals. And also the carbon chemical shifts in the olefinic region ( $\delta$  127.4-134.7) revealed that **1a** contained four double bonds which were confirmed by one bond correlations with proton signals between  $\delta$  5.30 and  $\delta$  5.39 in the  $^1H$  NMR spectrum.

The COSY experiment showed that the overlapped proton signals centered about  $\delta$  5.35 were coupled to the signals at  $\delta$  2.12, 2.78 and 2.07 indicative of allylic methylene protons. The signal at  $\delta$  2.07 was additionally coupled to a methyl triplet on a terminus and the allylic proton signal at  $\delta$  2.12 coupled to the methylene proton signals (H-8) at  $\delta$  1.67 and 1.51, implying the presence of two double bonds interrupted by the methylene group at  $\delta$  2.78. The upfield carbon chemical shifts ( $\delta$  23.2, 25.7 and 20.7) corresponding to the allylic methylenes assigned the geometries of the two double bonds as *Z*, *Z*.<sup>8</sup> On the other hand, the remaining double bonds were characterized as a conjugated diene from the sequential correlations from H-3 to H-6 in the COSY spectrum and the absorption band in the UV spectrum ( $\lambda_{max} = 225\text{nm}$  in MeOH), and their geometries were established as *E*, *E* by observed coupling constants,  $J_{3,4}=14.2$ , and  $J_{5,6}=14.7$ . The vinyl proton on one end of the diene was correlated with a methine proton signal at  $\delta$  4.38, which, in turn, was correlated with a methyl doublet at  $\delta$  1.31 and an amide proton at  $\delta$  4.95. The vinyl proton on the other end of the diene was connected with two methylene protons on C-8 via a methine proton signal at  $\delta$  3.56 assigned to the proton of a methine bearing oxygen. Attachment of methoxy group at the position of C-7 was confirmed by a correlation between a methyl singlet resonating at  $\delta$  3.25 and the carbon signal at  $\delta$  81.4 in the HMBC.

In addition, the connectivities of one carbonyl, one methylene and quaternary carbon which appeared by treatment of TECF (trichloroethoxy chloroformate) could be determined by HMBC cross peaks, thereby revealing the gross structure of **1a**. Based on this deduction,

Table 1. NMR spectral data for compound 1 in CDCl<sub>3</sub>

position	<sup>1</sup> H	<sup>13</sup> C	COSY	HMBC(H)
1	1.31 ( 3H, d, 6.9)	21.0, q	H-2,	H-2, -3
2	4.38 ( 1H, m)	48.6, d	H-1, -3, NH	H-1, -3
3	5.68 (1H, dd, 14.2, 5.4)	134.3, d	H-2, -4	H-5
4	6.21 ( 1H, dd, 14.2, 10.3)	129.6, d	H-3, -5	
5	6.17 ( 1H, dd, 14.7, 10.3)	131.7, d	H-4, -6	H-7
6	5.54( 1H, dd, 14.7, 8.3)	134.7, d	H-5, -7	H-4, -7
7	3.56(1H, m)	81.4, d	H-6, 8	H-5, -8, OCH <sub>3</sub>
8	1.67(1H, ddd, 7.8, 7.0, 6.8)	35.6, t	H-7, -9	H-6, -7,
	1.51(1H, ddd, 7.8, 6.8, 6.4)			-9, -10
9	2.12(2H, ddd, 7.0, 6.8, 6.4)	23.2, t	H-8, -10	
10	5.36(1H, m)	128.9, d	H-9	H-12
11	5.36(1H, m)	129.4, d	H-12	H-9
12	2.78(2H, dd, 5.9, 5.9)	25.7, t	H-11, -13	H-10, -13, -14
13	5.30(1H, m)	127.4, d	H-12, -14	
14	5.39(1H, m)	132.1, d	H-13, -15	
15	2.07(2H, q, 7.3)	20.7, d	H-14, -16	H-13, -14, -16
16	0.96(3H, t, 7.3)	14.5, q	H-15	H-14, 15
OCH <sub>3</sub>	3.25(3H, s)	56.5, q		H-7
NH	4.95(1H, br d, 5.9)		H-2	
C=O		153.8, s		CH <sub>2</sub>
CH <sub>2</sub>	4.74(2H, m)	74.7, t		
C		96.2, s		

Table 2. <sup>1</sup>H NMR data for compound **2a**, **3a** and **4a** in CDCl<sub>3</sub>.

position	<b>2a</b>	<b>3a</b>	<b>4a</b>
1	0.88 (3H, d, 6.8)	1.11(3H, d, 6.8)	1.11(3H, d, 6.8)
2	4.01(1H, m)	4.18(1H, m)	4.23(1H, m)
3	4.92(1H, m)	4.85(1H, ddd, 6.9, 5.9, 3.4)	4.84(1H, ddd, 6.9, 6.3, 3.9)
4	2.46(1H, m)/2.37(1H, m)	2.25(2H, m)	2.23(2H, dt, 6.2, 6.2)
5	5.47(1H, dt, 14.7, 7.3)	5.31(1H, br dt, 15.1, 6.8)	5.31(1H, br dt, 15.1, 6.8)
6	6.10(1H, d, 14.7, 10.8)	5.50(1H, 15.1, 6.8)	5.50(1H, br dt, 15.1, 7.1)
7	5.97(1H, dd, 10.8, 15.1)	1.97(2H, dt, 6.8, 7.3)	1.97(2H, dt, 7.1, 7.2)
8	5.62(1H, dt, 15.1, 7.3)	1.25-1.30(2H, m)	1.25-1.30(2H, m)
9	32.6(2H, dt, 7.3, 6.8)	1.25-1.30(2H, m)	1.25-1.30(2H, m)
10-13	1.25-1.35(8H, m)	1.25-1.30(8H, m)	1.25-1.30(8H, m)
14	0.88(3H, t, 6.8)	0.87(3H, 3H, 6.8)	0.88(3H, t, 6.8)
NH	5.07(1H, d, 9.2)	5.78(1H, d, 8.3)	5.53(1H, 7.8)
C=O			
CH <sub>2</sub>	4.71, 4.77(4H, m)		
C			
OAc		2.08(3H, s)	2.08(3H, s)
NHAc		1.95(3H, s)	2.00(3H, s)

the natural compound **1** was proposed as 7-methoxy-(3*E*, 5*E*, 10*Z*, 13*Z*)-hexadeca-3, 5, 10, 12-tetraenylamine. To our best knowledge, it has been reported that the planar structure of **1** was unknown. However, further study related with chemical reaction will need for the absolute stereochemistry at the position of C-2, 7 in this molecule.

Compound **2a** was isolated as a colorless oil, following the reaction with TECE. The <sup>1</sup>H NMR spectrum displayed a broad signal at δ 1.25–1.35 and well-separated olefinic signals between δ 5.40 and 6.10, indicative of a methylene envelope and a diene respectively. *E*, *E*-configurations for the diene were assigned from coupling constants,  $J_{5,6}=14.7$  and  $J_{7,8}=15.1$  Hz. The COSY correlations from a methyl doublet at δ 0.88 to the

Table 3.  $^{13}\text{C}$  NMR data for compound **2a**, **3a**, and **4a** at 125MHz in  $\text{CDCl}_3$ .

position	2a	3a	4a
1	14.6, q	14.8, q	18.4, q
2	49.0, d	47.1, d	46.7, d
3	80.4, d	76.2, d	76.0, d
4	34.3, t	34, 8, t	35.0, t
5	124.0, d	124.1, d	123.8, d
6	134.5, d	134.5, d	134.9, d
7	129.5, d	32.6, t	32.6, t
8	134.9, d	22.7-31.9, t	22.7-31.9, t
9	32.6, t	22.7-31.9, t	22.7-31.9, t
10-13	22.6-31.7, t	22.7-31.9, t	22.7-31.9, t
14	14.1, q	14.1, q	14.1, q
C=O	153.9, 153.7, s		
CH <sub>2</sub>	74.5, 76.7, t		
C	95.4, 94.5, s		
OAc		23.5, q	23.5, q
		171.2, s	170.6, s
NHOAc		21.1, q	21.0, q
		169.2, s	169.4, s

intense proton signals at  $\delta$  1.25-1.35 and HMBC correlations of two methine protons on C-2, -3 with carbonyl carbons delineated the structure of **2a**. Thus, the corresponding natural compound **2** was 2-aminotetradeca-5, 7-dien-3-ol, a known structure, which was in good agreement with NMR data reported in the literature.<sup>9-10</sup>

Compound **3a** was given as a molecular formula of  $\text{C}_{18}\text{H}_{33}\text{NO}_3$  by HREIMS and  $^{13}\text{C}$  NMR experiments. The  $^1\text{H}$  NMR spectrum of **3a** exhibited overlapped signals at  $\delta$  1.25-1.30 which represented an aliphatic carbon chain. And two acetate carbonyl and two olefinic



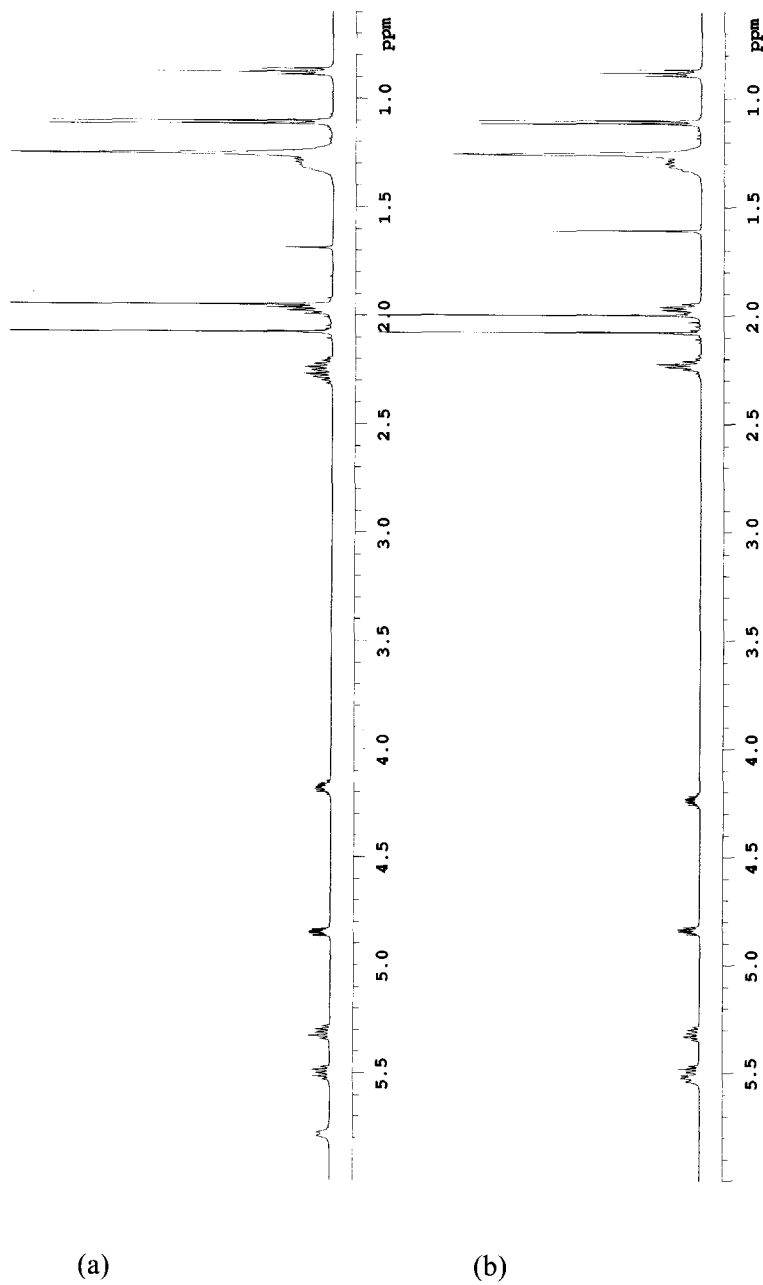


Fig. 1. Comparison of <sup>1</sup>H NMR spectrum for compound **3a** (a) and **4a** (b).

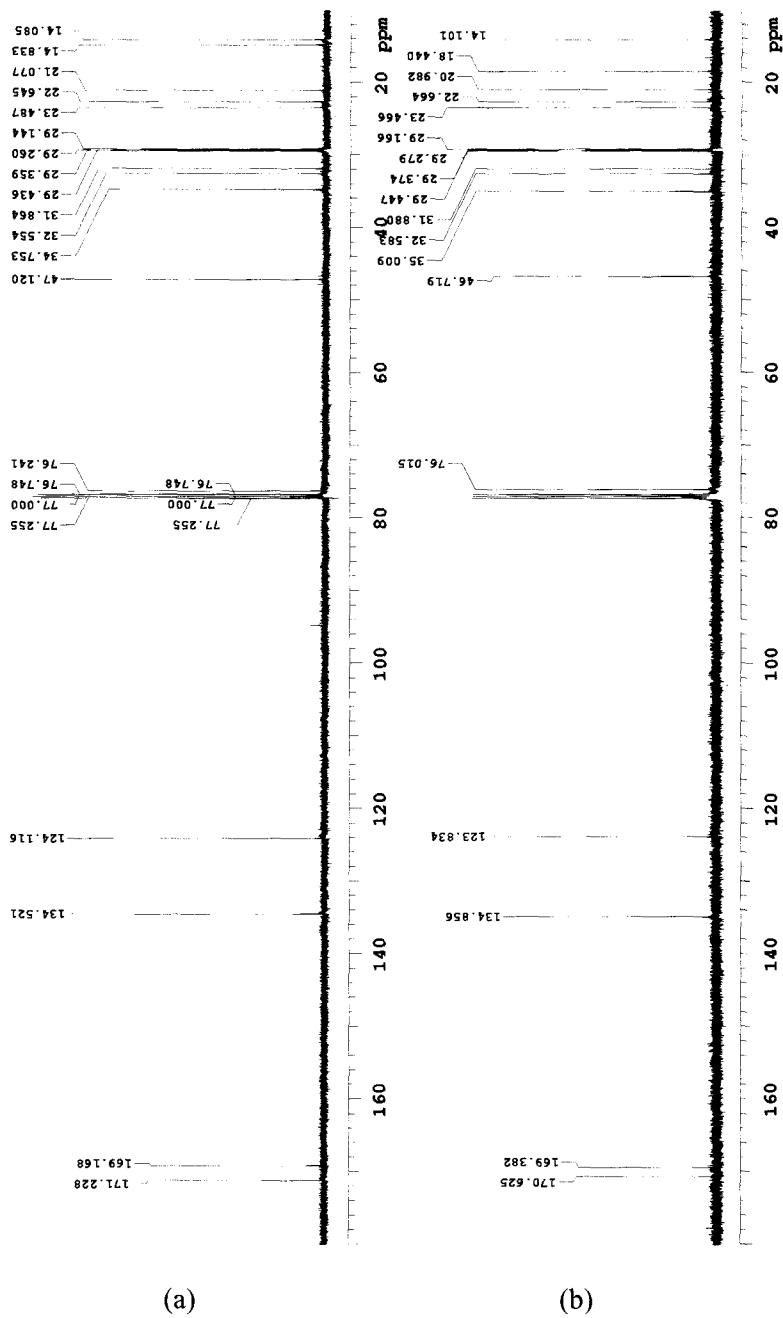


Fig. 2. Comparison of  $^{13}\text{C}$  NMR spectrum for compound 3a (a) and 4a (b).

resonances in the  $^{13}\text{C}$  NMR spectrum accounted for a simple, acyclic diacetylated tetradecaene structure. The key connectivities of **3a** were established by detailed analysis of the correlation peaks in the COSY and HMBC experiments. Table 3 and 4 displayed the assignments for the proton and carbon of **3a** respectively. The geometry of the double bond at the position of C-5 was determined as *E* by a vicinal coupling constant,  $J_{5,6}=15.1$  Hz and the downfield carbon chemical shifts ( $\delta$  34.8 and 32.6) for two allylic methylene moieties. The literature survey showed that the planar structure of **3a** was already published, but NMR data for this molecule was not provided completely. In addition, the absolute stereochemistry for **3a** was different from that of already known structure. Comparison with NMR data for a similar structure reported in the literature allowed compound **3a** to have a (2*S*, 3*R*)-configuration.<sup>9-11</sup> In particular, the  $^1\text{H}$  and  $^{13}\text{C}$  NMR data in the position of C-2, C-3 and NH were in good agreements. Therefore, **3a** was defined as diacetyl 2(*S*)-amino-5-tetradecen-3(*R*)-ol, a new enantiomer to the known structure of diacetyl 2(*S*)-amino-5-tetradecen-3(*S*)-ol.

Compound **4a** had highly similarity by comparison with the NMR spectral data of compound **3a**. The only difference between compound **3a** and **4a** was the  $^{13}\text{C}$  chemical shift at C-1, -2 and  $^1\text{H}$  data at C-2 and amino group as shown in Fig. 1 and 2, suggesting that two compounds were C-2 epimers. From this information, compound **4a** were deduced to be diacetyl 2(*R*) amino-5- tetradecen-3(*R*)-ol.

#### Acknowledgement

This work was supported by a Grant from the Kunsan National University

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