



New dammarane-type glucosides as potential activators of AMP-activated protein kinase (AMPK) from *Gynostemma pentaphyllum*

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ARTICLE INFO

Article history:

Received 8 August 2011

Revised 7 September 2011

Accepted 7 September 2011

Available online 10 September 2011

Keywords:

Gynostemma pentaphyllum (Gp)

Damulins A and B

AMP-activated protein kinase (AMPK)

ABSTRACT

AMP-activated protein kinase (AMPK) is a key sensor and regulator of glucose, lipid, and energy metabolism throughout the body. Activation of AMPK improves metabolic abnormalities associated with metabolic diseases including obesity and type-2 diabetes. The oriental traditional medicinal herbal plant, *Gynostemma pentaphyllum*, has shown a wide range of beneficial effects on glucose and lipid metabolism. In this study, we found that *G. pentaphyllum* contains two novel dammarane-type saponins designated as damulin A (**1**), 2 α ,3 β ,12 β -trihydroxydammar-20(22)-E,24-diene-3-O- β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranoside, and damulin B (**2**), 2 α ,3 β ,12 β -trihydroxydammar-20,24-diene-3-O- β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranoside, that strongly activate AMPK in cultured L6 myotube cells. Damulins A and B also increased β -oxidation and glucose uptake with increasing GluT4 translocation to the plasma membrane in L6 myotube cells. Taken together our results indicate that activation of AMPK by damulins A and B may contribute to beneficial effect of *G. pentaphyllum* on glucose and lipid metabolism.

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1. Introduction

AMP-activated protein kinase (AMPK) is an evolutionally conserved, intracellular energy sensor that increases ATP production and decreases energy consumption by responding to cellular stress that increases the intracellular AMP:ATP ratio.¹ Numerous studies have revealed that AMPK activation improves obesity, type-2 diabetes, and dyslipidemia by stimulating β -oxidation and glucose uptake in skeletal muscle tissues and inhibiting fat and cholesterol synthesis in the liver.^{2–4}

AMPK is a serine/threonine kinase and is a heterotrimeric enzyme consisting of catalytic α and regulatory β and γ subunits.^{4,5} AMPK activation occurs mainly via phosphorylation of Thr¹⁷² in the AMPK α subunit by the upstream kinase (LKB1).^{6,7} AMP was originally reported to promote phosphorylation of Thr¹⁷² in AMPK α by LKB1. When ATP consumption increases the AMP concentration during intensive exercise or prolonged starvation, AMP is coupled to the γ subunit of AMPK and makes AMPK a better substrate for LKB1.^{6,8} Conversely, inactivation of AMPK occurs when AMPK phosphatases, protein phosphatase 2C α (PP2C α), dephosphorylate Thr¹⁷².^{9,10} Moreover, AMP and ADP binding to the regulatory γ subunit were shown to protect AMPK from dephosphorylation

catalyzed by AMPK phosphatases and to promote Thr¹⁷² phosphorylation by LKB1.^{5,11,12}

Once activated, AMPK phosphorylates downstream substrates to switch off ATP-consuming pathways, including fatty acid and cholesterol synthesis, and to switch on ATP-generating pathways, including fatty acid oxidation and glycolysis.^{4,13} Furthermore, activation of AMPK decreases blood glucose levels by stimulating translocation of glucose transporter 4 (GluT4) to the cell membrane as well as upregulating GluT4 expression independent of insulin signaling.^{14,15} These beneficial effects of AMPK activation during glucose and lipid metabolism highlight AMPK as an emerging drug target for metabolic syndromes. Therefore, AMPK activators are considered promising candidates for the discovery of anti-obesity and anti-diabetes agents as well as drugs for treatment of other metabolic diseases.

Gynostemma pentaphyllum (Gp) has been widely used in Asian countries including Korea, China, Japan, and Vietnam as a traditional herbal medicine or tea. Total extract or total saponins from Gp have shown a wide range of beneficial effects such as cholesterol-lowering, hypoglycemic, and antitumor effects,^{17–19} which likely overlap with the diverse downstream effects of AMPK activation.^{3,16,20} We therefore investigated whether these beneficial effects of Gp-extract are due to AMPK activation. A phytochemical study on this Gp-extract was found that the activation of Gp-extract on AMPK is due to two new dammarane-type glycosides named damulins A and B.

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2. Results and discussion

Repeated column chromatography (HP-20, silica gel, RP-18, and semi-preparative HPLC) of the 50% EtOH-soluble extract of the leaves of *G. pentaphyllum* resulted in the isolation of two new dammarane-type glycosides named damulins A and B, respectively.

Compound **1** was obtained as white amorphous powder. Its HRFABMS showed a quasi-molecular ion peak at m/z 805.4717 $[M+Na]^+$, establishing a molecular formula of $C_{42}H_{70}O_{13}$. The IR spectrum suggested the presence of OH functional groups at 3400 cm^{-1} , olefinic C=C double bonds at 1658 to 1631 cm^{-1} , and C–O stretching vibrations at 1077 – 1045 cm^{-1} . The C–H bending vibration by *gem*-dimethyl groups was determined by the band at 1381 cm^{-1} . The ^{13}C NMR spectrum gave 42 signals, of which 12 were assigned to the sugar units and 30 to a triterpene moiety

with typical resonances of two olefinic carbons (δ_C 124.7 and 124.4) and two quaternary carbons (δ_C 140.5 and 132.2). The ^1H NMR spectrum of compound **1** showed eight singlet peaks that were assigned to the aglycon tertiary-methyl groups at δ_H 0.92–1.67, three of which were diagnostic for methyls linked to an sp^2 carbon (δ_H 1.61, 1.63, and 1.67). Two signals for trisubstituted olefinic protons were also displayed at δ_H 5.08 (1H, br, *t*-like, $J = 7.5\text{ Hz}$) and δ_H 5.26 (1H, br, *t*-like, $J = 7.0\text{ Hz}$). Two oxymethines at δ_H 3.63 and 3.75 (each 1H, m), one axial oxygenated methine proton at δ_H 3.02 (1H, d, $J = 9.3\text{ Hz}$), and two anomeric protons at δ_H 4.45 (1H, d, $J = 7.5\text{ Hz}$) and 4.75 (1H, d, $J = 7.5\text{ Hz}$) assigned to the two sugar moieties were also determined from its ^1H NMR spectrum.

The comparison of the ^1H and ^{13}C NMR spectra of compound **1** with those of ginsenoside Rh₃²² and ginsenoside F₄^{23,24} suggested

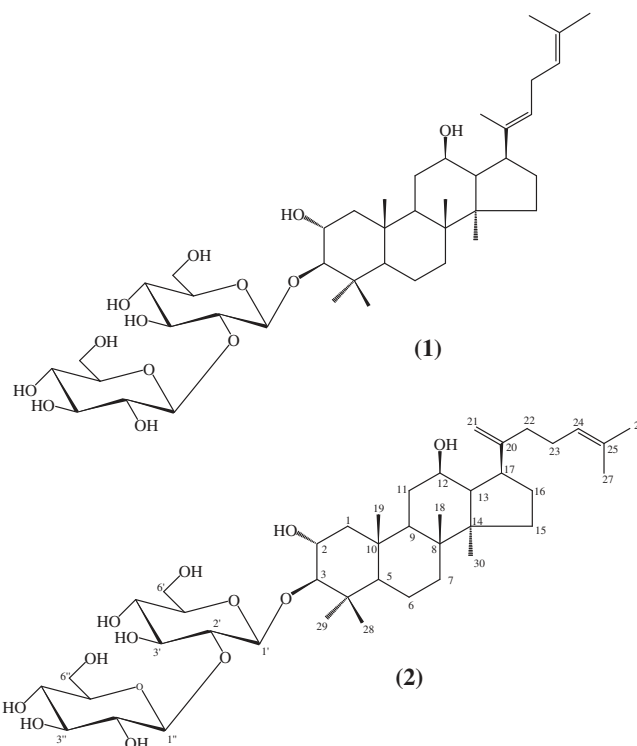
Table 1
 ^1H (500 MHz) and ^{13}C (125 MHz) NMR Data for damulins A (**1**) and B (**2**) in CD_3OD

Position	Damulin A (1)		Damulin B (2)	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
<i>Aglycone</i>				
1	48.1	0.92, m	48.1	0.94, m
2	68.3	2.10, dd, 5.5, 12.5	68.3	2.11, m
3	96.8	3.75, m	96.8	3.75, m
4	42.0	3.02, d, 9.5	42.0	3.01, d, 9.5
5	57.4	0.89, m	57.4	0.88, m
6	19.4	1.54, m 1.59, m	19.4	1.53, m 1.59, m
7	36.1	1.35, m 1.58, m	36.1	1.35, m
8	41.3		41.3	1.58, m
9	51.9	1.52, m	52.0	1.52, m
10	39.1		39.1	
11	32.7	1.28, m 1.82, m	33.1	1.28, m
12	74.1	3.63, m	73.9	1.81, m
13	51.5	1.80, m	53.0	3.59, m
14	52.1		52.3	1.85, m
15	33.5	1.11, m 1.69, m	33.3	1.11, m
16	29.6	1.44, m 1.89, m	31.8	1.70, m
17	51.4	2.59, m	49.6	1.42, m
18	16.3	1.05, s	16.3	2.00, m
19	18.1	0.99, s	18.1	2.58, m
20	140.5		156.3	1.06, s
21	13.1	1.63, s	108.7	0.99, s
22	124.7	5.26, t, 7.0	35.1	4.69, br, s 4.88, br, s
23	28.1	1.68, m	27.8	2.02, m
24	124.4	2.67, m	125.9	2.15, m
25	132.2	5.08, t, 7.5	132.2	2.22, m
26	26.0	1.67, s	26.0	2.14, m
27	17.9	1.61, s	17.9	5.17, m
28	17.3	0.93, s	17.3	1.69, s
29	28.8	1.14, s	28.8	1.63, s
30	18.1	0.92, s	18.0	0.93, s
<i>Sugar moieties</i>				
<i>Glu'</i>				
1'	105.0	4.45, d, 7.5	105.0	4.45, d, 8.0
2'	80.7	3.69, m	80.7	3.69, m
3'	78.8	3.59, m	78.8	3.60, m
4'	71.4	3.37, m	71.3	3.36, m
5'	78.2	3.20, m	78.2	3.22, m
6'	62.5	3.66, m 3.87, br, d, 12.5	62.5	3.62, m 3.84, br, d, 12.0
<i>Glu''</i>				
1''	104.5	4.75, d, 7.5	104.5	4.75, d, 8.0
2''	76.3	3.24, m	76.3	3.24, m
3''	78.4	3.25, m	78.4	3.26, m
4''	72.2	3.19, m	72.2	3.21, m
5''	78.1	3.36, m	78.0	3.36, m
6''	63.4	3.62, m 3.84, br, d, 10.5	63.3	3.66, m 3.87, dd, 2.5, 12.0

that its aglycon moiety is similar with except for the absence of a hydroxy functional group at C-2. The comparison between compound **1** and the known compounds, gypenoside LXXVII²¹ and gypenosides XLVI,²⁶ also showed that all three compounds have an identical skeleton with 2 α ,3 β ,12 β -trihydroxy substitutions. The 2 α ,3 β -dihydroxy substitutions of compound **1** were also confirmed from the carbon chemical shifts of both positions at δ_C 68.3 (C-2) and δ_C 96.8 (C-3), and from the J value of the proton, which was ascribable to H-3 at δ_H 3.02 (1H, d, J = 9.3 Hz). When the ^{13}C NMR data for compound **1** (Table 1) were compared with ginsenoside Rg₈,²⁷ ginsenoside F₄,²³ and ginsenoside Rh₅,²⁸ the stereochemistry of the double bond at C-20(22) of compound **1** was determined to be the *E*-configuration because the chemical shift at C-21 of compound **1** was observed at δ_C 13.1 ppm.^{25,28,29}

From its hydrolysis and ^1H and ^{13}C NMR spectra data, compound **1** was suggested to contain two units of *D*-glucose. The chemical shifts, the multiplicity of the signals, the values of the coupling constants in the ^1H , and ^{13}C NMR data (See Table 1) indicated the presence of two β -configurations at the anomeric positions for the glucosyl units in compound **1**. One β -configuration for the first glucosyl unit with [δ_H 4.45 (1H, d, J = 7.5 Hz, H-1'); δ_C 105.0 (C-1')], and the other for the second glucosyl unit [δ_H 4.75 (1H, d, J = 7.5 Hz, H-1''); δ_C 104.5 (C-1'')] were displayed. All ^1H and ^{13}C NMR signals of the two sugar moieties were also assigned using two-dimensional NMR spectra including ^1H - ^1H COSY, HMQC, and ^1H - ^{13}C HMBC correlations (see also Supplementary data and Fig. 1). The linkage sites and the sequences of the two saccharides and the aglycone were confirmed from an HMBC experiment. HMBC correlations were observed from H-1' (δ_H 4.45) of the first glucose to C-3 (δ_C 96.8) of aglycon and from H-3 (δ_H

3.02) of aglycon to C-1' (δ_C 105.0). Other correlations from H-1'' (δ_H 4.75) of the second glucose to C-2' (δ_C 80.7) and from H-2' (δ_H 3.69) to C-1'' (δ_C 104.5) suggested the connectivity of the sugar moiety (Fig. 1). Thus, the structure of compound **1** was established to be 2 α ,3 β ,12 β -trihydroxydammar-20(22)-*E*,24-diene-3-*O*-[β -*D*-glucopyranosyl-(1 $\rightarrow$ 2)- β -*D*-glucopyranoside], and named damulin A.



Compound **2** was also purified as amorphous powder. The molecular formula of compound **2** was determined as $\text{C}_{42}\text{H}_{70}\text{O}_{13}$ from ^{13}C NMR and the quasi-molecular ion peak at m/z 783.4905 in a positive-mode HRESIMS ($[\text{M}+\text{H}]^+$; calcd. 783.4895). Comparison of the ^1H and ^{13}C NMR spectra of compounds **1** with **2** indicated that they had a similar aglycon moiety. However, compound **2** was a disappearance of a tertiary CH_3 group at C-20 and the double bond of the olefinic methine group at C-22 in the aglycon of compound **1**. The methyl proton signal at δ_H 1.63 (3H, s) in compound **1** was replaced by two broad singlet peaks at δ_H 4.69 and 4.88 (each 1H, br, s) in compound **2**, and one typical signal of the olefinic proton at δ_H 5.26 (1H, br, *t*-like, J = 7.0 Hz) in the ^1H NMR spectrum of compound **1** was disappeared. These results were also supported by the ^{13}C NMR spectrum of compound **2**. The olefinic carbon signal at δ_C 124.7 (C-22) and the methyl carbon signal at δ_C 13.1 (C-21) in compound **1** were replaced by a methylene peak at δ_C 35.1 and a terminal methylene peak at δ_C 108.7 in compound **2**, respectively.³⁰ In addition, the quaternary carbon at δ_C 140.5 (C-20) in the ^{13}C NMR spectrum of compound **1** was also shifted to downfield (δ_C 156.3) in compound **2**. ^1H and ^{13}C NMR data (Table 1), as well as the two-dimensional NMR experiments including ^1H - ^1H COSY, HMQC, and ^1H - ^{13}C HMBC spectroscopic data (see also Supplementary data and Fig. 1), supported that compound **2** (Table 1) showed two glucosyl units with β -configuration as in compound **1**. The linkage sites and the sequences of the two saccharides and the aglycon were also determined using HMBC experiments (Fig. 1). HMBC correlations from the anomeric proton

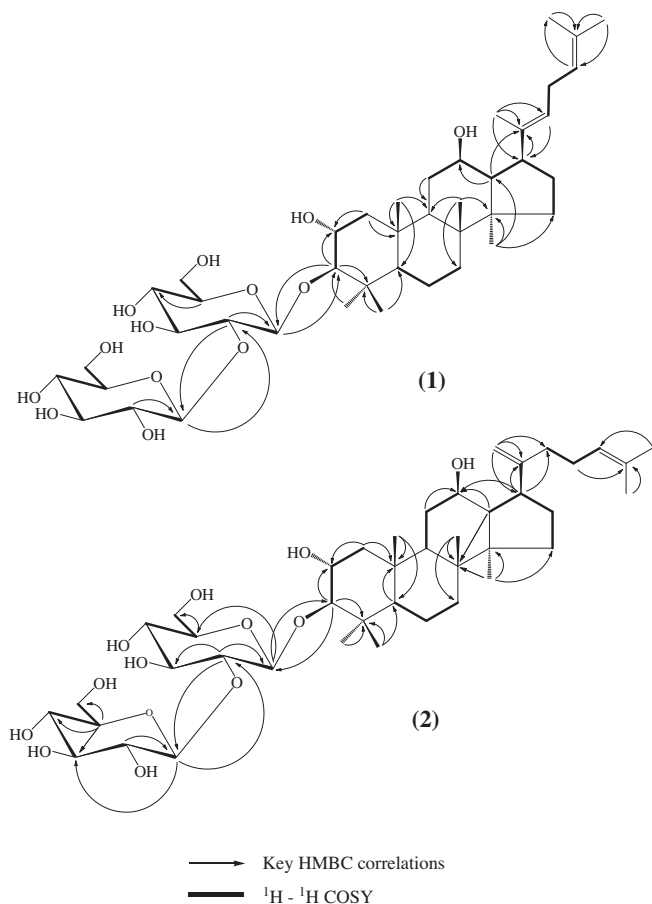


Figure 1. ^1H - ^1H COSY and HMBC correlations of damulins A (1) and B (2).

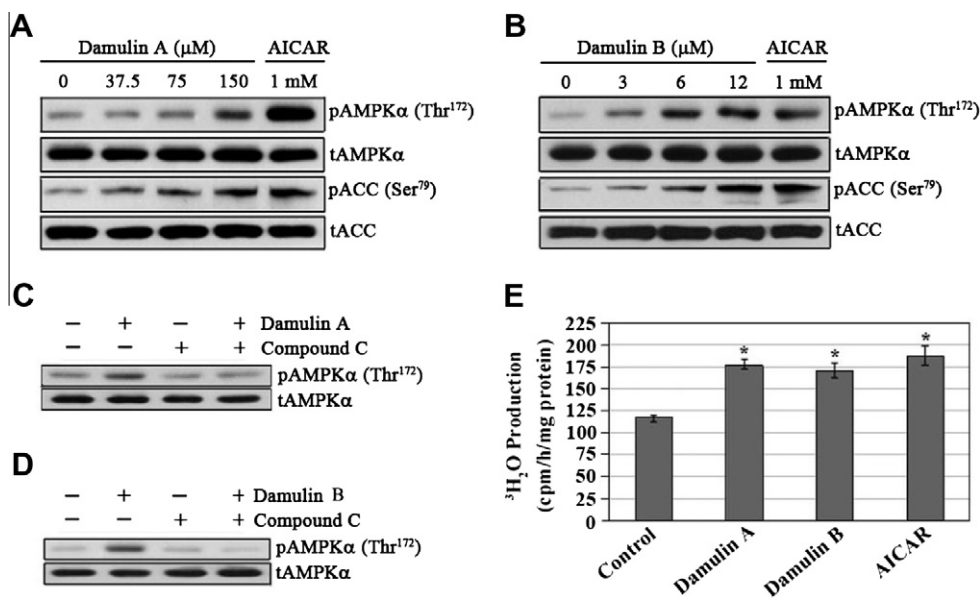


Figure 2. Effect of damulins A (1) and B (2) on AMPK activation and fatty acid oxidation in L6 myotube cells. L6 myotube cells were exposed to different doses of damulins A and B for 2 h or AICAR (1 mM) for 1 h, and phosphorylation of AMPK was analyzed with Western blotting. Damulins A (A) and B (B) increased phosphorylation of AMPK and ACC in a dose-dependent manner. Increased phosphorylation of AMPK induced by 150 μM damulin A (C) and 12 μM damulin B (D) was abrogated by pretreatment with compound C (10 μM) for 10 min. (E) Increased β-oxidation in cells by damulins A (150 μM) and B (12 μM). DMSO was used as a vehicle (control). AICAR (1 mM) was pretreated for 1 h. **P* < 0.05 compared with control.

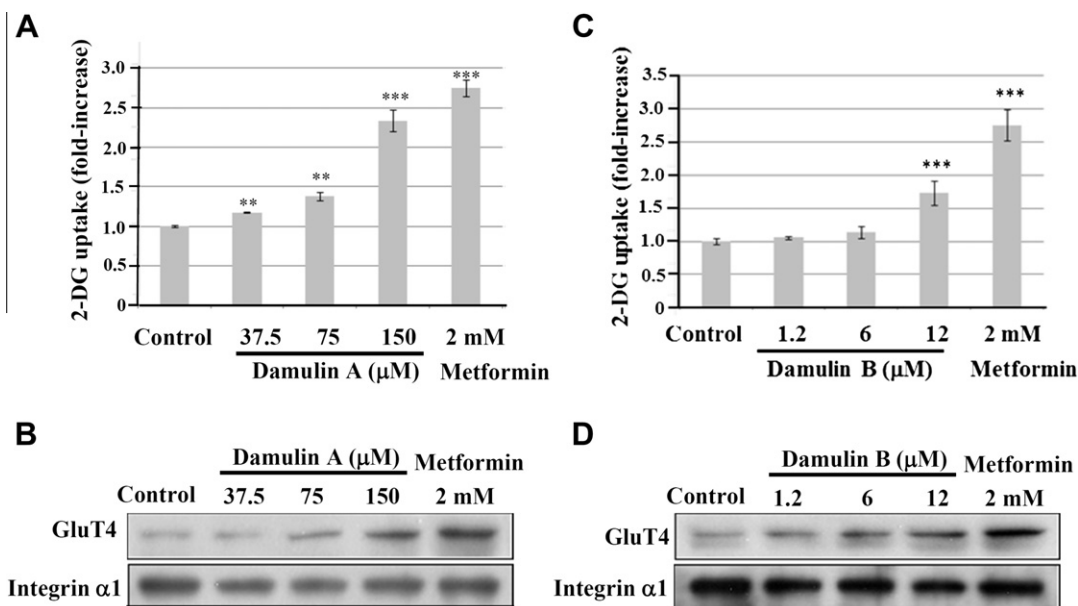


Figure 3. Dose-dependent increment of 2-DG uptake and GluT4 translocation to the cytoplasmic membrane in L6 myotube cells by damulins A and B. 2-DG uptake was increased by damulins A (A) and B (C). Stimulation of GluT4 translocation to the cytoplasmic membrane by damulins A (B) and B (D). Damulins A and B were treated to cells for 2 h and metformin was treated for 1 h. As a control DMSO was treated to cells. Cytoplasmic membrane fraction of cell lysates (30 μg) was subjected to Western blot analyses. Data are the means ± S.D. of three experiments. ***P* < 0.01; ****P* < 0.001 versus control.

at δ_H 4.45 (1H, d, J = 8.0 Hz, H-1') to δ_C 96.8 (C-3) and the other at δ_H 4.75 (1H, d, J = 8.0 Hz, H-1'') to δ_C 80.7 (C-2') are a strong evidence for this conclusion. Based on the above results, the structure of compound 2, damulin B, was elucidated as 2 α ,3 β ,12 β -trihydroxydammar-20,24-diene-3-*O*-[β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranoside].

In L6 myotube cells, the 50% ethanol extract of *G. pentaphyllum* increased Thr¹⁷² phosphorylation of the AMPK α subunit (data not shown). To investigate whether purified compounds are activators

of AMPK, L6 myotube cells were treated with damulins A and B in different concentrations. As expected, treatment with damulins A and B led to a dose-dependent increase in phosphorylation of AMPK and acetyl-CoA carboxylase (Fig. 2A and B). To verify the specificity of damulins A and B for AMPK phosphorylation, we tested the effect of the AMPK inhibitor, compound C, on the phosphorylation of AMPK. As seen in Figure 2C and D, increased AMPK phosphorylation in the damulins A- and B-treated L6 myotube cells was almost completely abrogated by pretreating cells with 10 μM

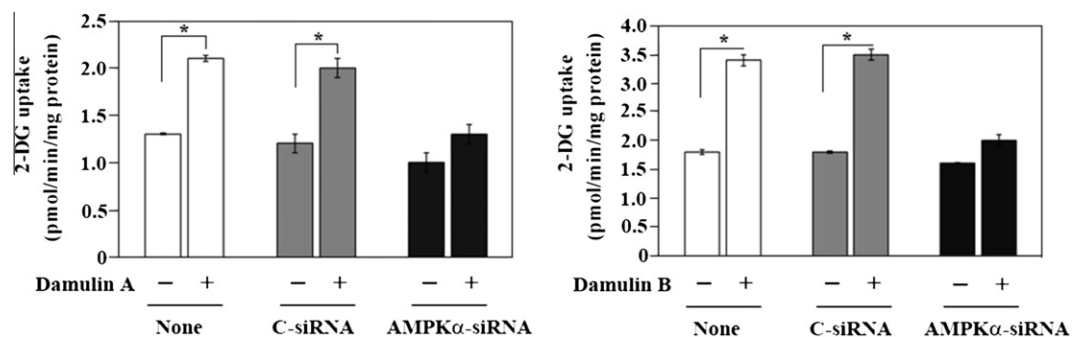


Figure 4. Effect of AMPK α 2 knockdown on 2-DG uptake in L6 myotube cells. The increments of 2-DG uptake by damulins A and B were reduced by knockdown of AMPK α 2 (AMPK α -siRNA). Non-specific siRNA (C-siRNA) was transfected as a negative control. Data are the means $\pm$ S.D. of three independent experiments. * P < 0.05 compared with control.

compound C. Accordingly, β -oxidation was also increased in L6 myotube cells by treatment of damulins A and B (Fig. 2E). Similarly, glucose uptake as determined by 2-deoxy-[3 H]-glucose (2-DG) uptake was also stimulated by damulins A and B in a dose-dependent manner with increasing GluT4 translocation to the plasma membrane in L6 myotube cells (Fig. 3), and this increase of 2-DG uptake was abrogated by knockdown of AMPK α 1/2 (Fig. 4). These results together indicate that damulins A and B stimulate glucose uptake via AMPK activation. *G. pentaphyllum* has been used in Asian counties as a folk herbal medicine with various therapeutic properties such as lowering blood cholesterol and glucose levels.^{17,18} Furthermore, saponins from *G. pentaphyllum* also exert an apoptotic activity against various cultured cancer cells,^{19,31,32} although its mechanism of action is poorly understood. However, these diverse biological effects of saponins from *G. pentaphyllum* overlap with well-documented downstream effects of AMPK activation,^{3,16,20} prompting this research. In this study, we found that the 50% ethanol extract of *G. pentaphyllum* exerted AMPK activation and stimulated its downstream targets in vitro. We also characterized two novel dammarane-type saponins, damulins A and B, from *G. pentaphyllum* extract as active ingredients that increase phosphorylation of AMPK. As expected, both damulins A and B increased glucose uptake in L6 myotube cells via AMPK activation, explaining how the *G. pentaphyllum* extract lowered blood glucose levels in *db/db* diabetic mice, an effect that was seen previous report.¹⁸

Taken together, our results reveal a molecular mechanism underlying the beneficial effects of *G. pentaphyllum* on type-2 diabetes and obesity. Further investigation and optimization of these derivatives may enable the preparation of new AMPK activators with potential applications in the treatment of type-2 diabetes, obesity, and metabolic disorders.

3. Experimental section

3.1. General experimental procedures

IR spectra (KBr) were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo electron Corp.), and the UV spectra in MeOH using a Shimadzu spectrometer. NMR spectra were obtained on Varian Unity Inova 500 MHz spectrometer at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). HRFABMS and HRESIMS data were recorded on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Ion exchange resin (Diaion HP-20) was purchased from Mitsubishi Chemical Co. Silica gel (Merck, 63–200 μ m particle size), RP-18 (Merck, 150 μ m particle size) were used for column chromatography. TLC was carried out using Merck silica gel 60 F₂₅₄ and RP-18 F₂₅₄ plates. HPLC was car-

ried out using a Gilson system with an UV detector and Optima Pak C₁₈ column (10 $\times$ 250 mm, 10 μ m particle size, RS Tech, Korea).

3.2. Chemicals and reagents

HPLC solvents were obtained from Burdick & Jackson, USA. AICAR (5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside), BAPTA/AM (1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. Metformin, insulin, and protease inhibitor cocktail set 1 were purchased from Calbiochem. The active forms of recombinant AMPK α 1, β 1, γ 1 protein and antibodies against phospho-AMPK α 1/2 (Thr¹⁷²), total AMPK, phospho-ACC (Ser⁷⁹), and total ACC antibodies were purchased from Cell Signaling Technology (New England Biolabs). GLUT4 and β -actin antibodies were from Santa Cruz Biotechnology, Inc. Reagents for polyacrylamide gel electrophoresis were from Bio-Rad. 2-Deoxy-[3 H]-glucose was from Perkin-Elmer Life Sciences. All other reagents were of the highest analytical grade.

3.3. Plant material

The cultivated whole plants of *G. pentaphyllum* were purchased from Wonkwang Food Co. in Geochang County, Gyeongsangnam-do, Republic of Korea, and authenticated by Professor Jae-Hong Pak, Department of Biology, Kyungpook National University. A voucher specimen (TLH 1001) was deposited in the herbarium (KNU) of Kyungpook National University, Republic of Korea.

3.4. Extraction and isolation of AMPK activators

After dried *G. pentaphyllum* leaves (5 kg) were extracted with 50% EtOH (8 L) twice for 6 h, the first and second supernatants were combined, filtered with gauze, and centrifuged (1500 g) for 30 min to remove insoluble matter. To isolate compounds that activate AMPK, the 50% EtOH extract of *G. pentaphyllum* was subjected to HP-20 ion exchange resin column chromatography (20 $\times$ 65 cm). Each fraction was collected by sequential elution with H₂O (10 L), 50% MeOH in H₂O (10 L), and acetone (10 L). Then, the acetone fraction was dried, suspended in H₂O (2 L), and successively partitioned with *n*-hexane (3 $\times$ 2 L), EtOAc (3 $\times$ 2 L), and *n*-BuOH (3 $\times$ 2 L). The partial BuOH fraction (50 g) was subjected to silica gel column (15 $\times$ 65 cm; 63–200 μ m particle size) chromatography with a gradient solvent system of CH₂Cl₂/MeOH/H₂O (from 5/1/0.1 to 0/5/0.1) to yield five subfractions (B.1–B.5). The combined B.3 and B.4 fraction (B.34) was then subjected to reverse phase ODS-A column chromatography (6.5 $\times$ 65 cm; 150 μ m particle size) with a gradient solvent system of MeOH/H₂O (from 2/1 to 2/0) to yield six subfractions (B.34–1 to B.34–6). Further purifi-

cation of sub-fraction B.34–5 (502 mg) by Gilson HPLC (Optima Pak C₁₈ column, 10 × 250 mm, 10 μm particle size; UV detectors at 205 and 254 nm, flow rate 2 mL/min) with an isocratic solvent system (75% MeOH in 0.1% formic acid in H₂O for 45 min, then increased to 100% MeOH over 15 min) resulted in the isolation of compounds **1** (damulin A, 18 mg, *t_R* = 42 min) and **2** (damulin B, 14 mg, *t_R* = 31.5 min). The chemical structures of the pure compounds were elucidated with 1D (including ¹H and ¹³C) and 2D (including gHSQC, COSY, HMBC, and NOESY) NMR spectra as well as mass data analyses.

3.5. Damulin A (1)

White amorphous powder; IR *v*_{max} (KBr): 3400 (OH), 2943 (C–H), 1658, 1641, 1631 (C=C), 1381(C–H), 1077, 1045 (C–O) cm^{−1}; HRFABMS *m/z* 805.4717 [M+Na]⁺, calcd. for C₄₂H₇₀O₁₃Na; 805.4714; ¹H (500 MHz) and ¹³C (125 MHz) NMR data in MeOH-*d*₄ (see Table 1).

3.6. Damulin B (2)

Amorphous powder; IR *v*_{max} (KBr): 3386 (OH), 2943 (C–H stretching), 1641, 1631 (C=C), 1453, 1379 (C–H bending), 1166, 1078, 1041(C–O), 887, 669 cm^{−1}; HRESIMS *m/z* 783.4905 [M+H]⁺, calcd. for C₄₂H₇₀O₁₃; 783.4895; ¹H (500 MHz) and ¹³C (125 MHz) NMR Data in MeOH-*d*₄ (see Table 1).

3.7. Cell culture

L6 cells were obtained from the American Type Culture Collection. L6 cells were differentiated into myotube cells and adipocyte cells, respectively, as previously described.^{33,34}

3.8. Measurements of fatty acid oxidation and glucose uptake

Fatty acid oxidation was carried out, and ³H₂O content was measured in a scintillation counter as previously described.³³ Glucose uptake was measured as the method described.³⁵ Cultured L6 myotube cells were suspended in glucose-free transport buffer containing 20 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO₄, 1 mM CaCl₂, and 1% BSA. Cells (~2 × 10⁵) were transferred to plastic tubes and incubated at 37 °C in the presence or absence of damulins A and B and AICAR. Subsequently, 2-deoxy-[³H]p-glucose was added to the cells for 10 min, and the incubations were terminated by washing three times with ice-cold 0.9% NaCl (w/v). Non-specific transport was measured in the same conditions, except that cytochalasin B (10 μM) was added to the medium before the addition of cells. Non-specific values were subtracted from all conditions. Cells were collected in 0.05 N NaOH by centrifugation for 30 s (6000 g), and cell-associated radioactivity was determined by scintillation counting. The results were expressed as pmol 2-DG/min/mg protein or fold increase compared to control.

3.9. Knock-down of AMPK in cultured cells

For targeting AMPKα2 isoforms in L6 cells, ON-TARGETplus SMARTpool small interfering RNA (siRNA) for rat AMPKα2 and negative control siRNA were purchased from Dharmacon. For gene knockdown experiments, differentiated L6 cells were transfected with siRNAs according to the manufacturer's instructions, cultured in α-Minimum Essential Media (MEM) without antibiotics and fetal bovine serum for 48 h, and then chemicals were added to the cells.

3.10. Western blot analysis

Cultured L6 myotube cells were lysed with cell lysis buffer A (50 mM Tris–HCl, 1% Nonidet P-40, 5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF and 1× protease inhibitor cocktail, pH 8.0). Isolated mouse soleus muscle tissues were homogenized with a glass homogenizer in HEPES buffer (20 mM HEPES, 250 mM sucrose, 1 mM EDTA, pH 7.4) containing protease inhibitor cocktail. Total membrane fractions from L6 myotube cells were prepared as we previously described. Western blot analysis was carried out as previously described.³³

3.11. Statistical analysis

All in vitro results are representative of at least three independent experiments, and data are the means ± S.D.

Acknowledgment

This work was supported by a Grant PF06212-00 from the Plant Diversity Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology and of the Korean government.

Supplementary data

Supplementary data (1D and 2D (including COSY, HSQC, and HMBC) NMR spectra of the new compounds damulins A (**1**) and B (**2**)) associated with this article can be found, in the online version, at [doi:10.1016/j.bmc.2011.09.013](https://doi.org/10.1016/j.bmc.2011.09.013).

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Fig. S1.17. HMBC NMR spectrum of damulin A (**1**) in CD_3OD

SUPPORTING INFORMATION AVAILABLE

New dammarane-type glucosides as potential activators of AMP-activated protein kinase (AMPK) from *Gynostemma pentaphyllum*

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Fig. S1.18. HMBC NMR spectrum of damulin A (**1**) in CD₃OD

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Fig. S2.25. HMBC NMR spectrum of damulin B (**2**) in CD₃OD

Fig. S2.26. HMBC NMR spectrum of damulin B (**2**) in CD₃OD

00-5
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 KJ1400-1024500
 Archive directory:
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 Sample directory:
 File: gCOSY
 Pulse Sequence: gCOSY
 Solvent: CDCl₃
 Temp: 25.0 C / 298.1 K
 Relax: delay 2.000 sec
 Acq. time 2.110 sec
 Width: 5505.6 Hz
 F2 width: 5505.6 Hz
 # repetitions: 8
 SSB: noSSB
 DESPQ: F1, ANS-5264104 Hz
 DATA PROCESSING:
 Sqr: 2.00 Hz / 0.025 sec
 F1 data PROCESSING:
 Sqr: 2.00 Hz / 0.025 sec
 F1 size 2048 x 1024
 Total time 2 min

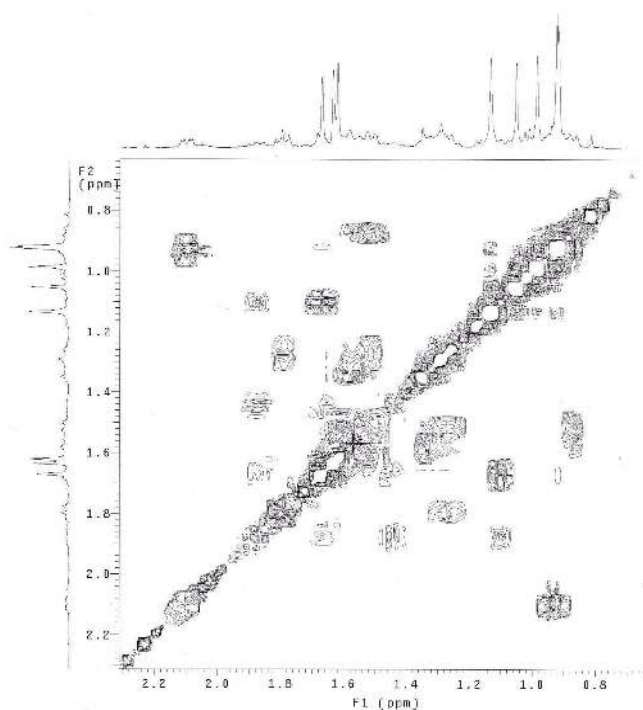


Fig. S1.7. COSY NMR spectrum of damulin A (1)

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 Temp: 25.0 C / 298.1 K
 User: J14-02
 Relax: delay 2.000 sec
 Acq. time 2.110 sec
 Width: 5505.6 Hz
 F2 width: 5505.6 Hz
 # repetitions: 8
 SSB: noSSB
 DESPQ: F1, ANS-5264104 Hz
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 Sqr: 2.00 Hz / 0.025 sec
 F1 data PROCESSING:
 Sqr: 2.00 Hz / 0.025 sec
 F1 size 2048 x 1024
 Total time 2 min

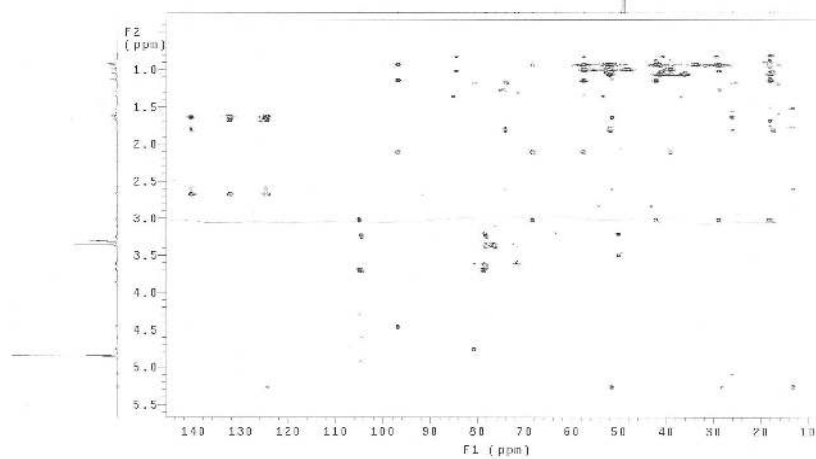


Fig. S1.8. HMBC NMR spectrum of damulin A (1)

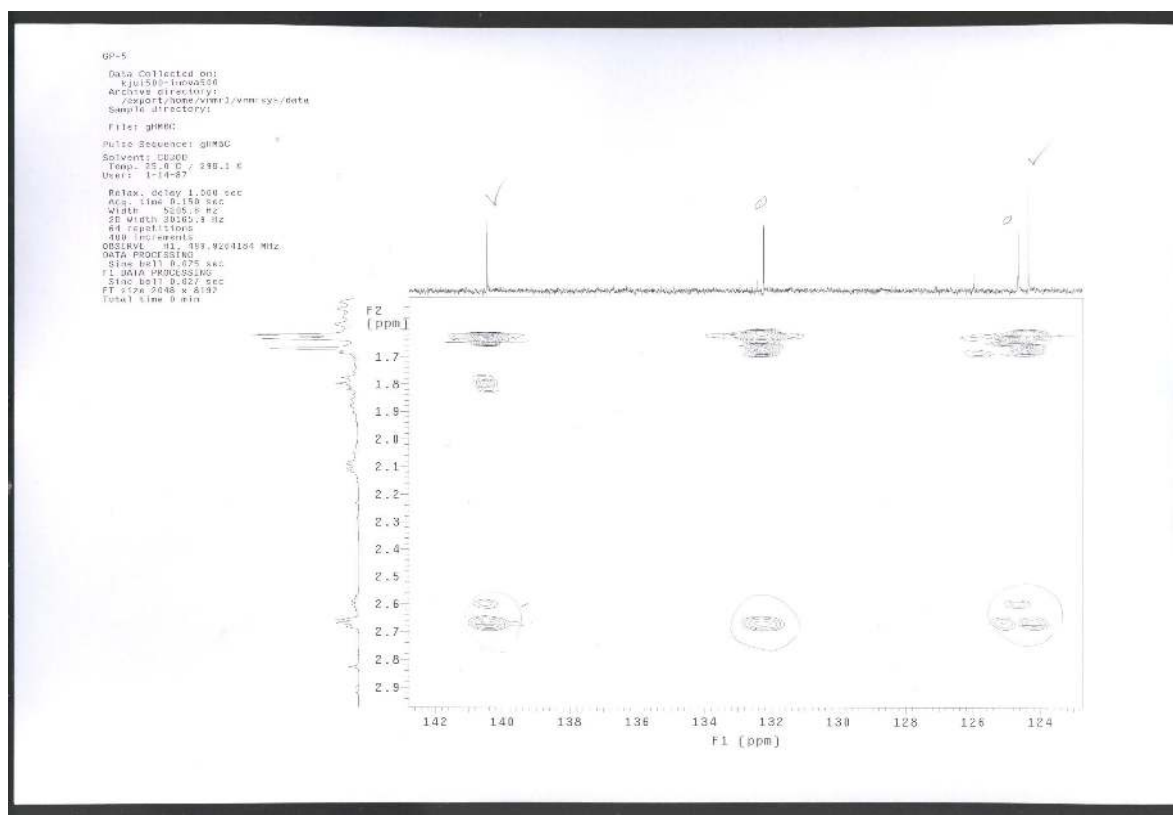


Fig. S1.9. HMBC NMR spectrum of damulin A (1)

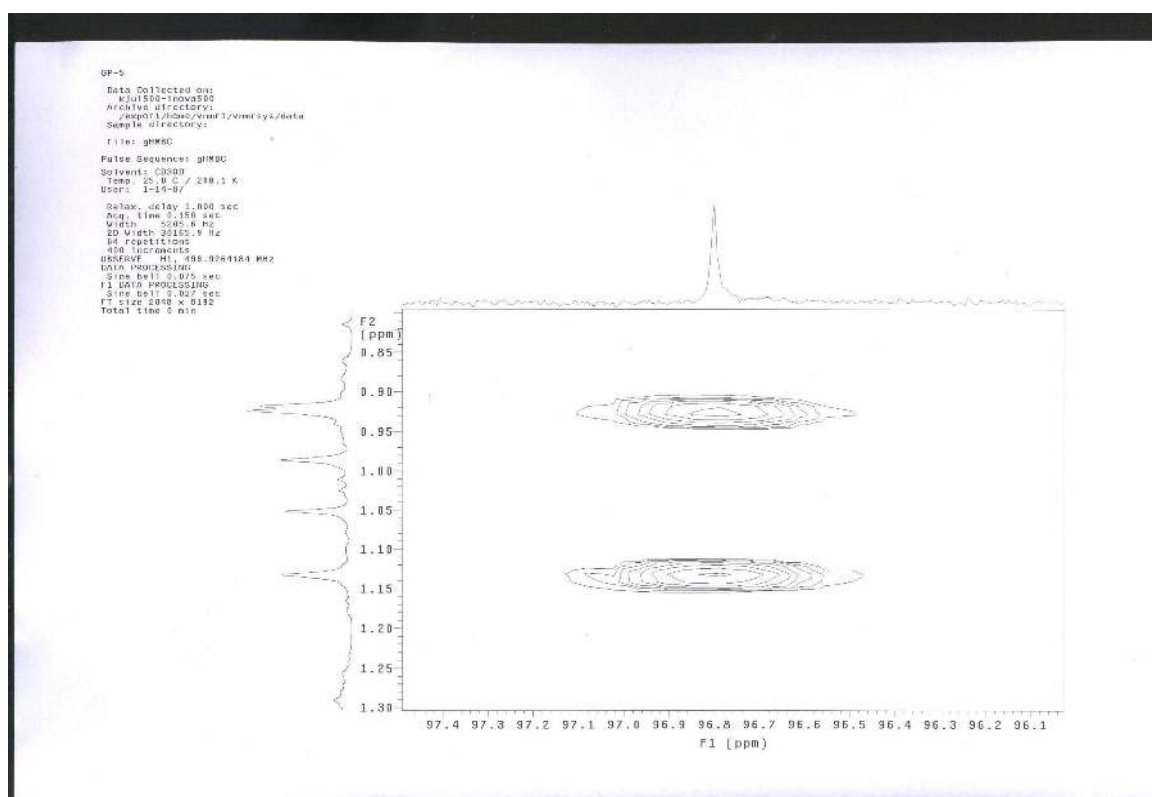


Fig. S1.10. HMBC NMR spectrum of damulin A (1)

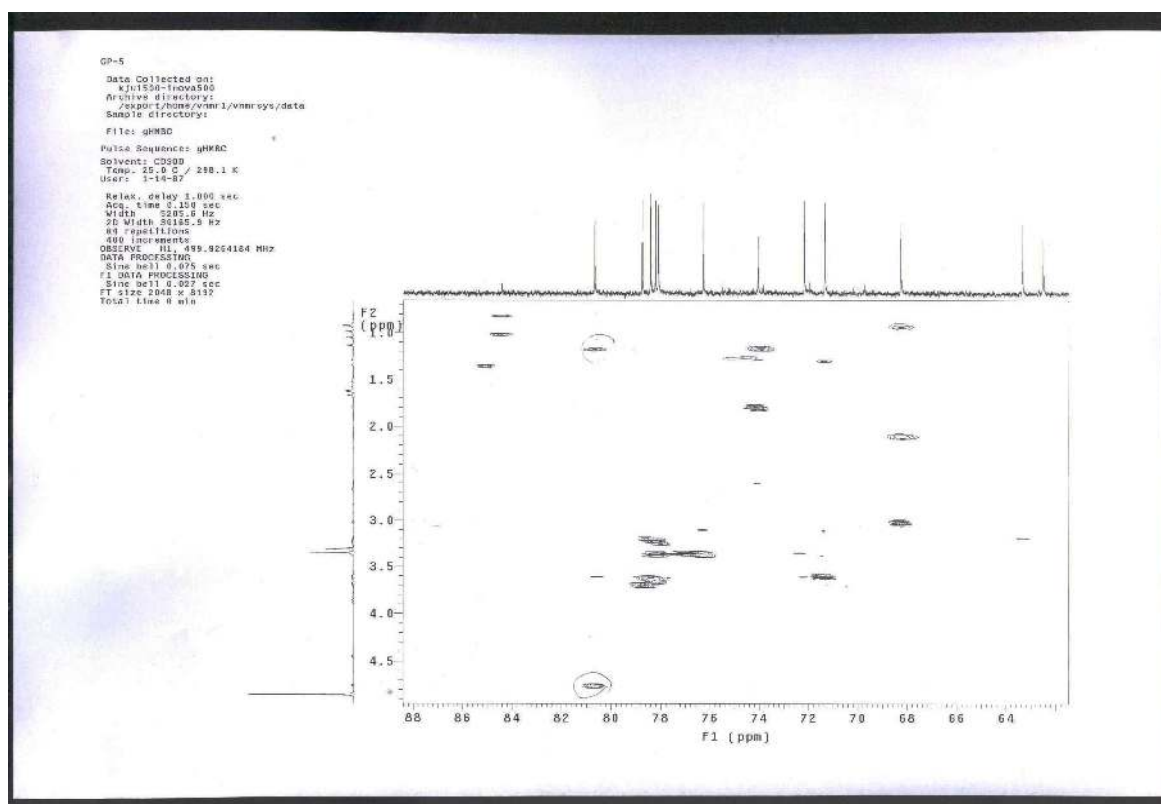


Fig. S1.11. HMBC NMR spectrum of damulin A (1)

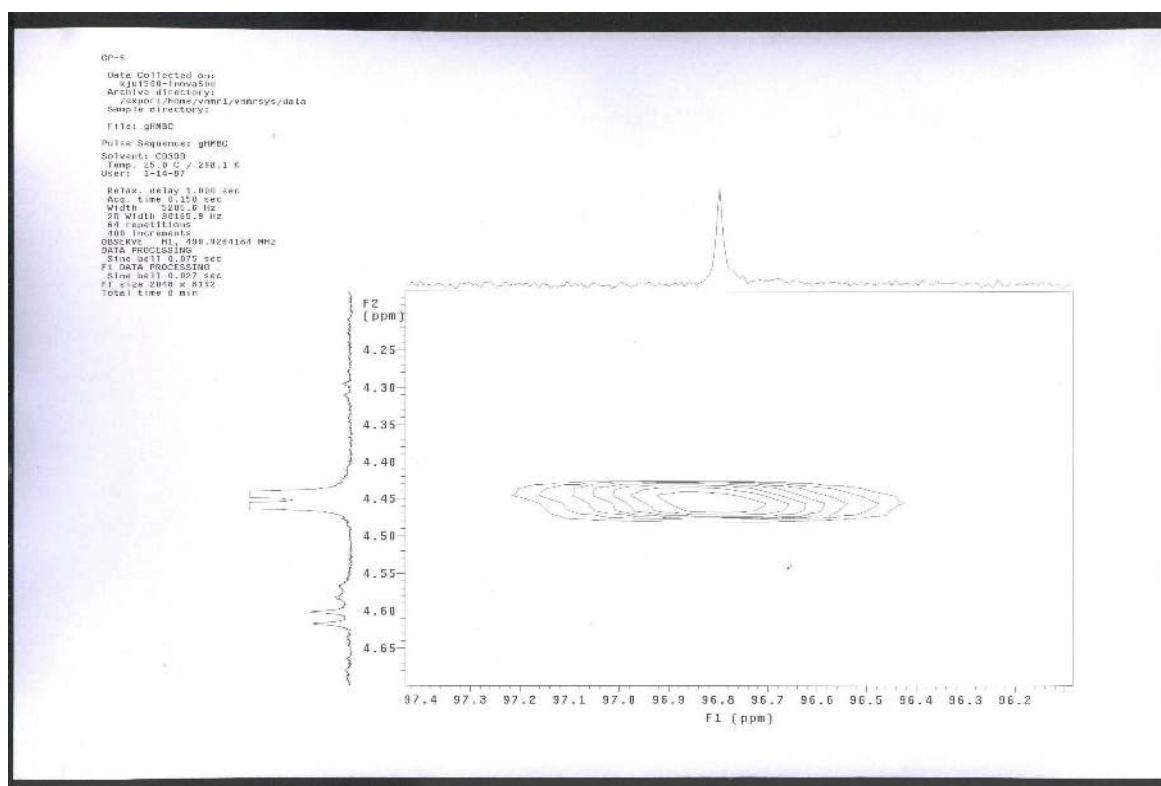


Fig. S1.12. HMBC NMR spectrum of damulin A (1)

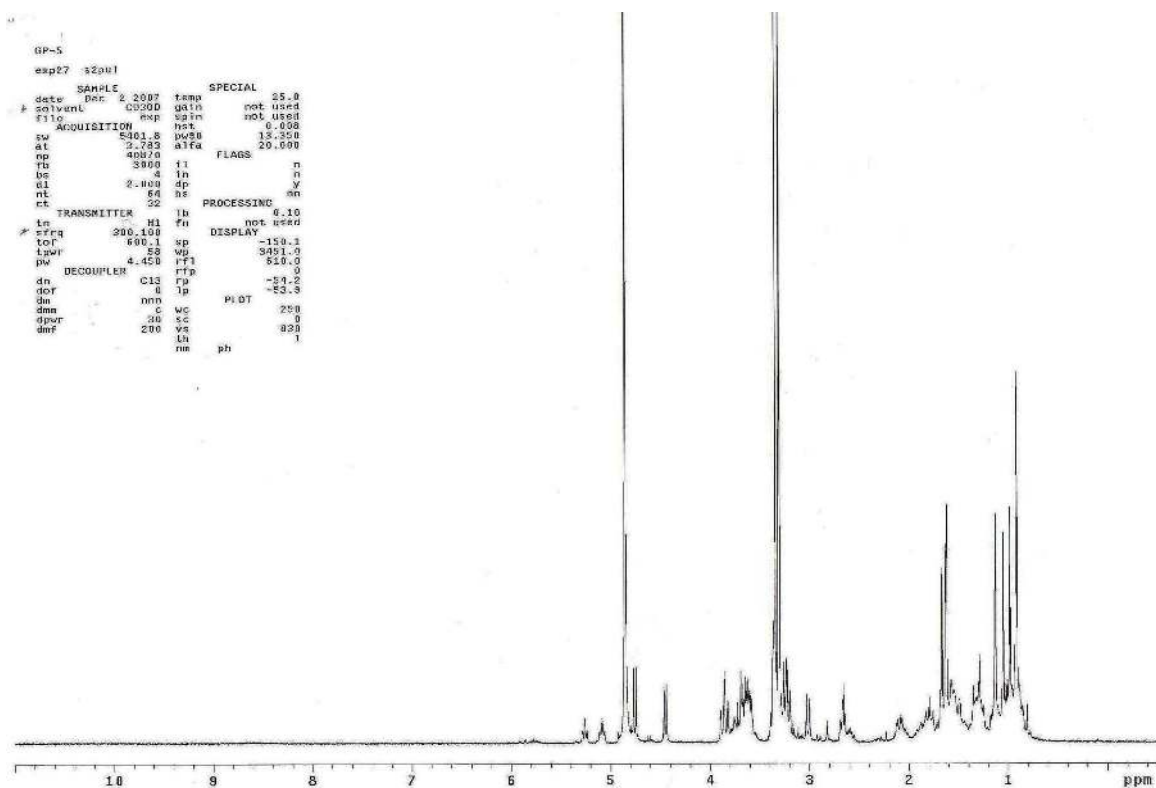


Fig. S1.1. ^1H NMR spectrum of damulin A (1)

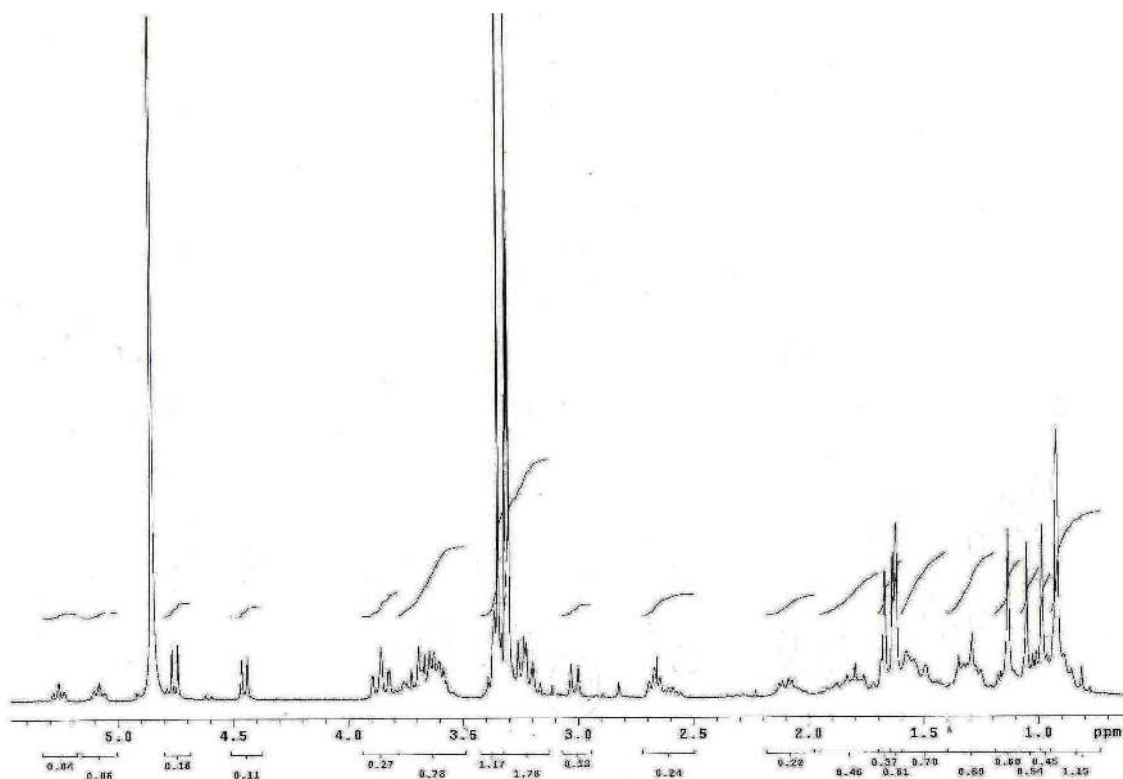


Fig. S1.2. ^1H NMR spectrum of damulin A (1)

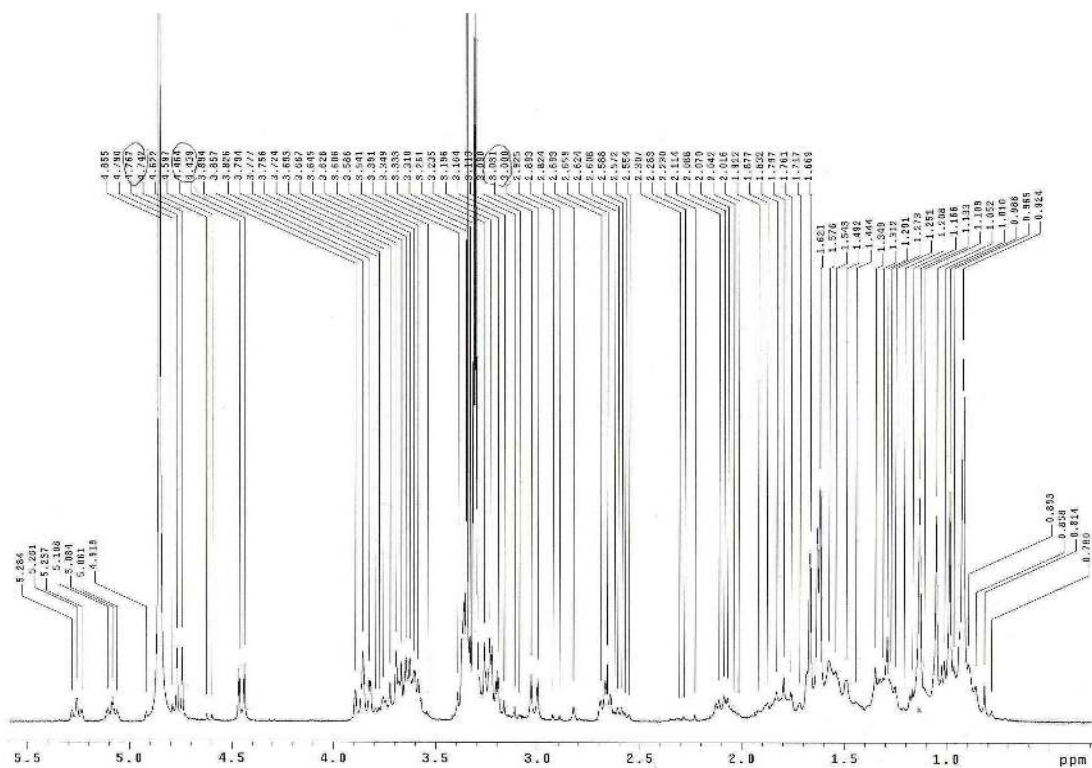


Fig. S1.3. ^1H NMR spectrum of damulin A (1)

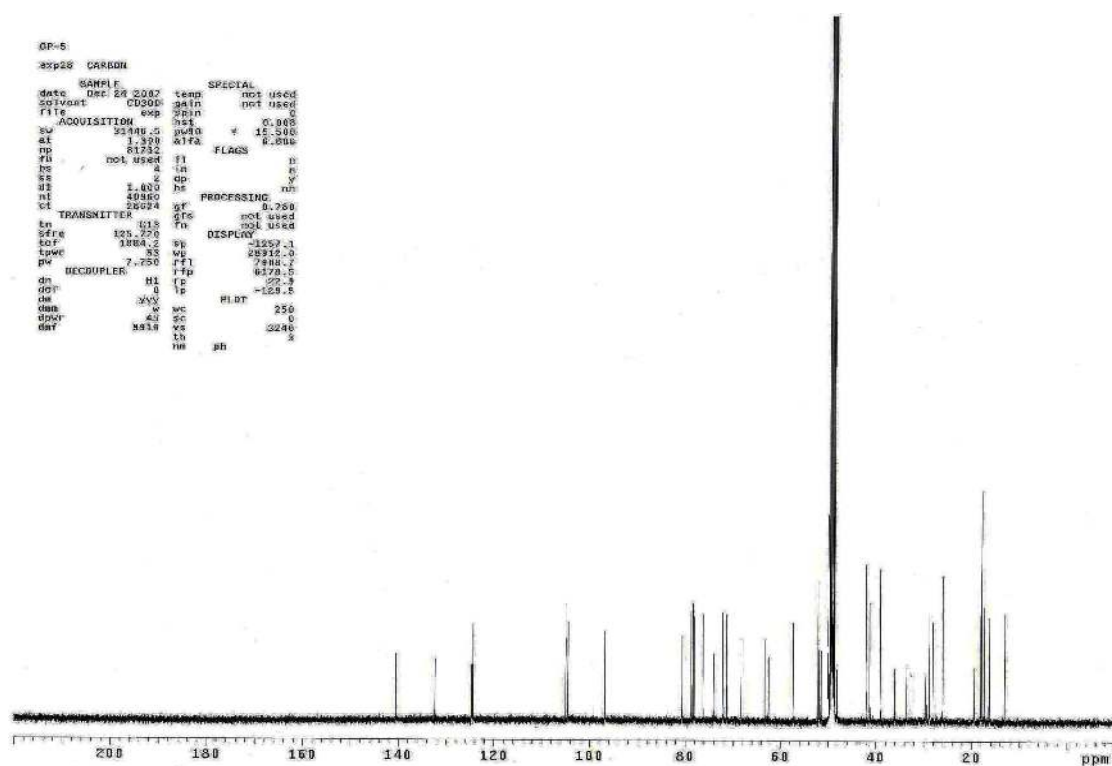


Fig. S1.4. ^{13}C NMR spectrum of damulin A (1)

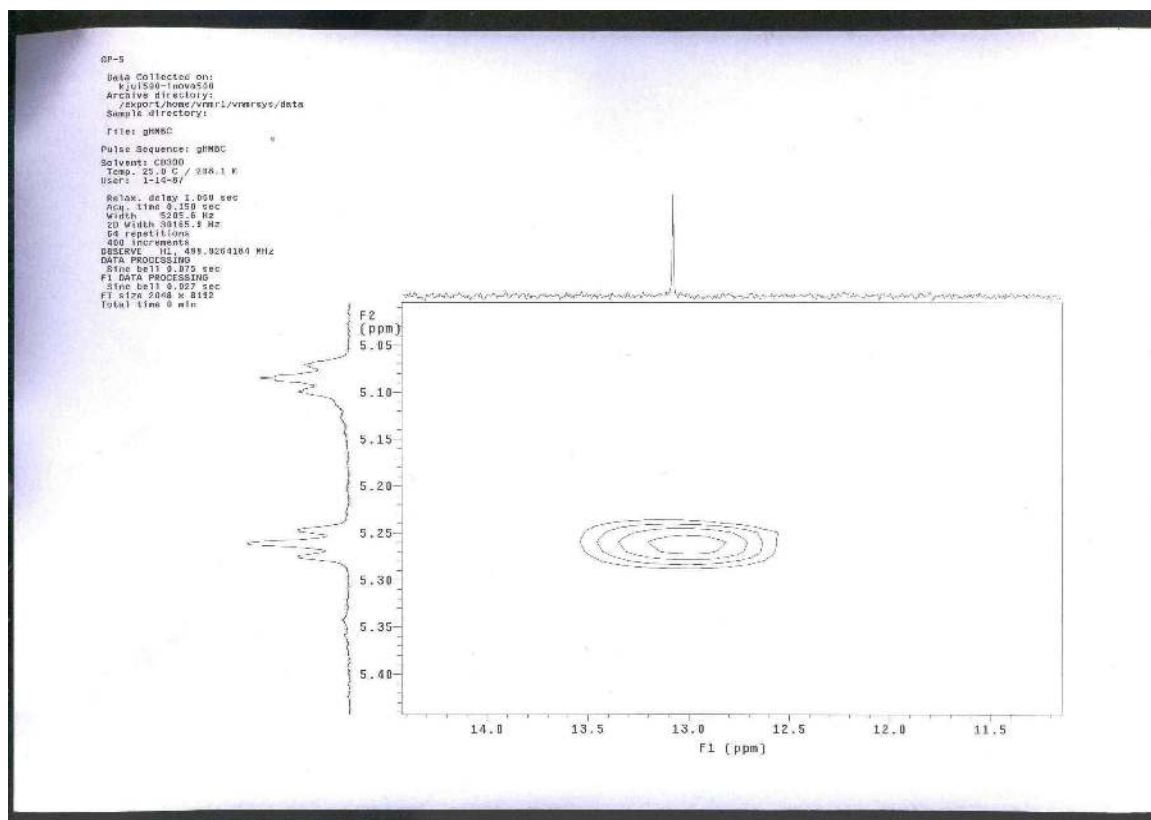


Fig. S1.17. HMBC NMR spectrum of damulin A (1)

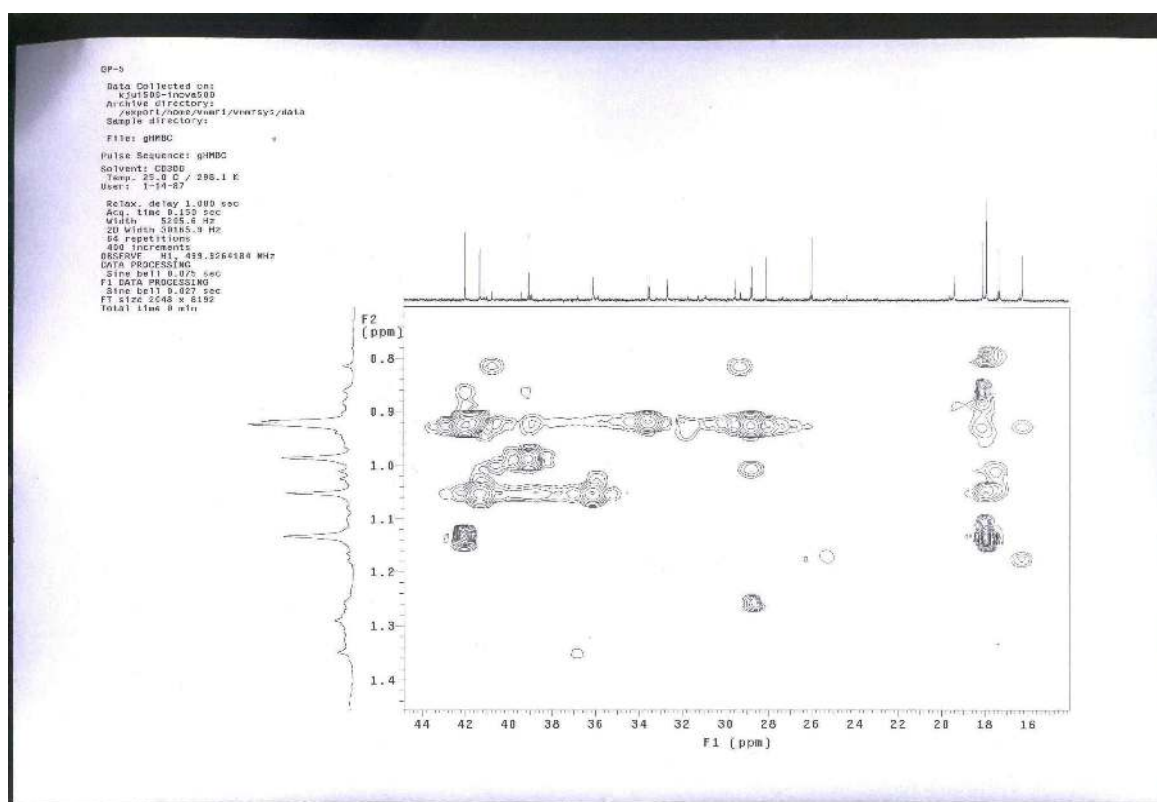


Fig. S1.18. HMBC NMR spectrum of damulin A (1)

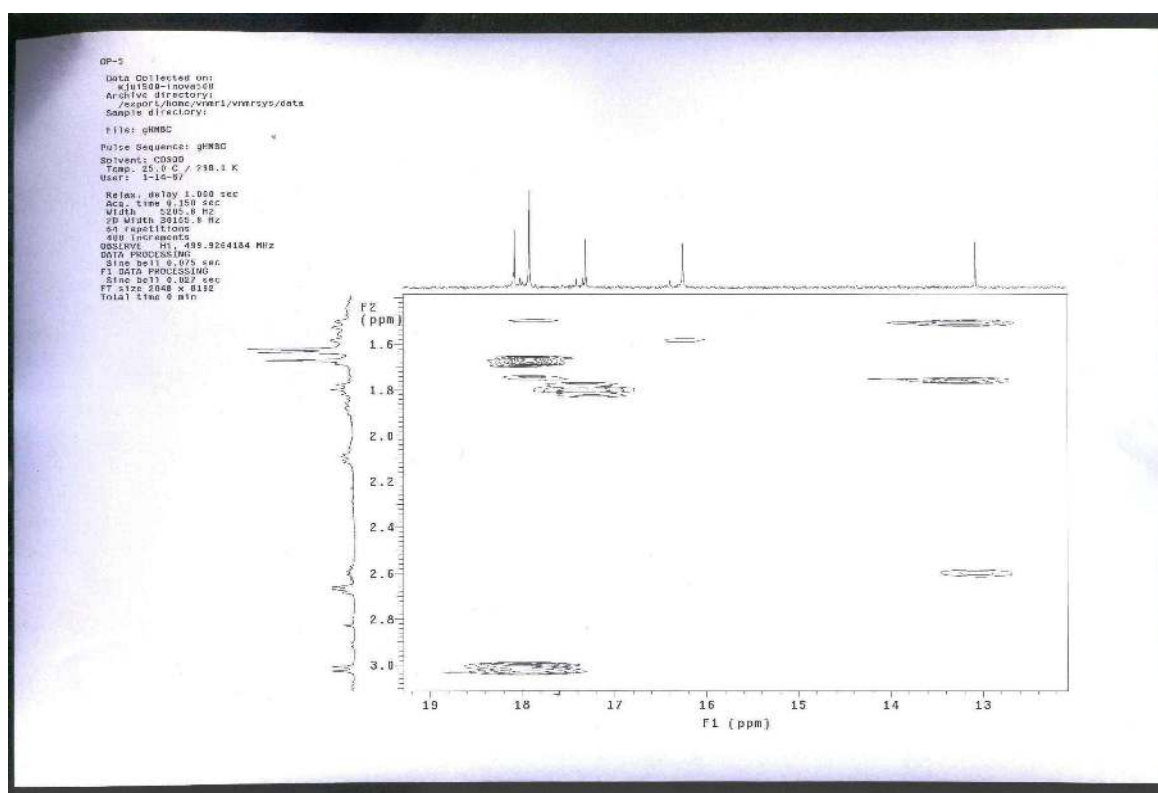


Fig. S1.19. HMBC NMR spectrum of damulin A (1)

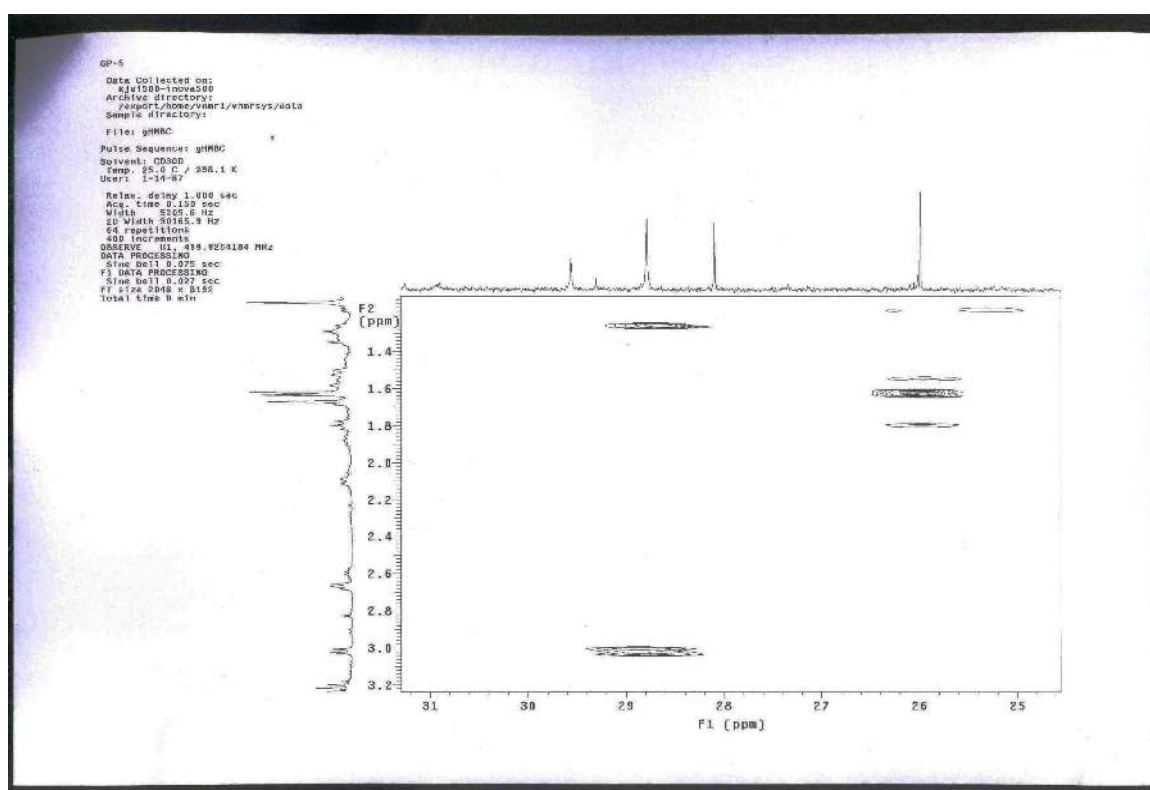


Fig. S1.20. HMBC NMR spectrum of damulin A (1)

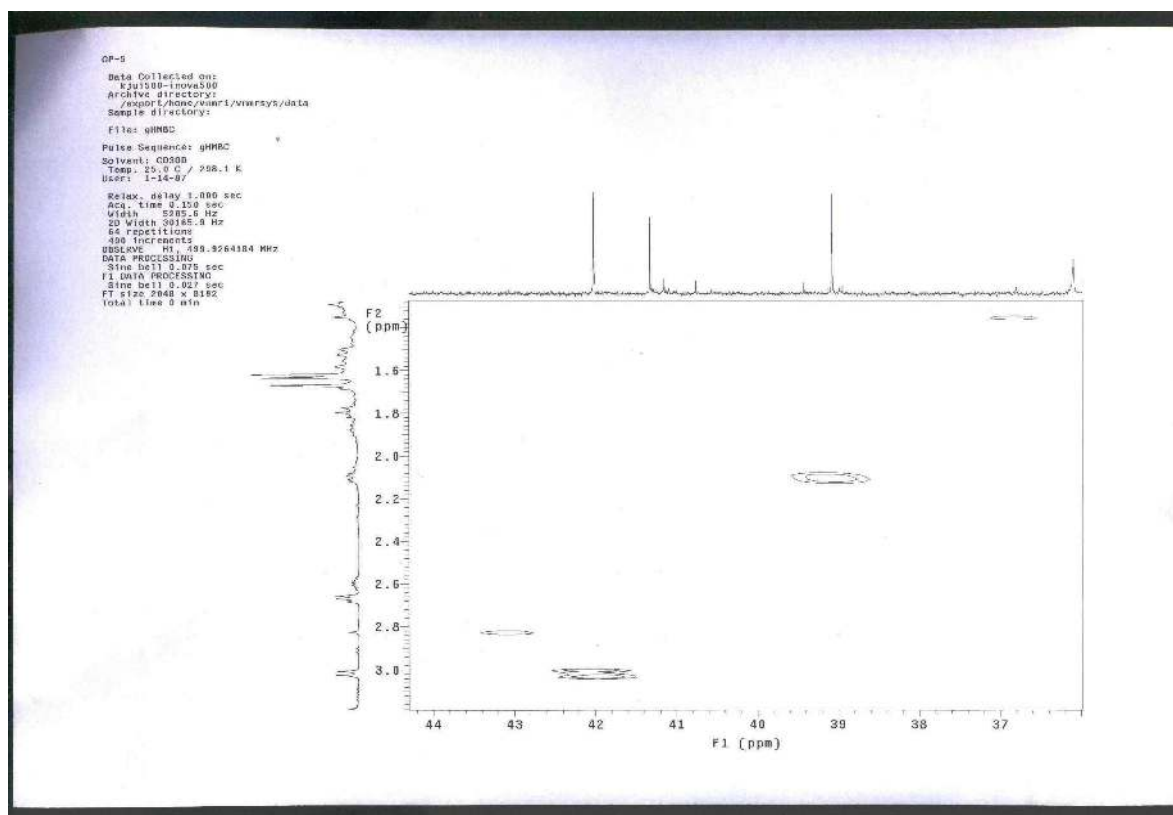


Fig. S1.21. HMBC NMR spectrum of damulin A (1)

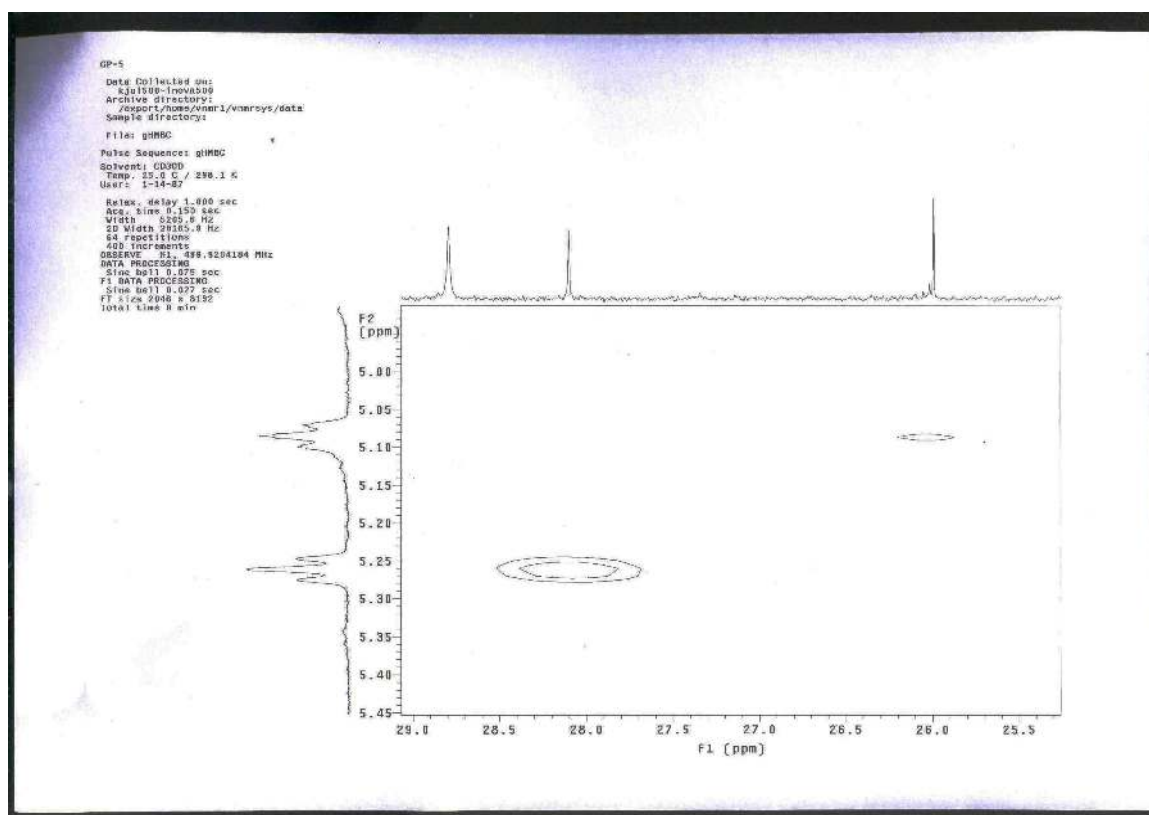


Fig. S1.22. HMBC NMR spectrum of damulin A (1)

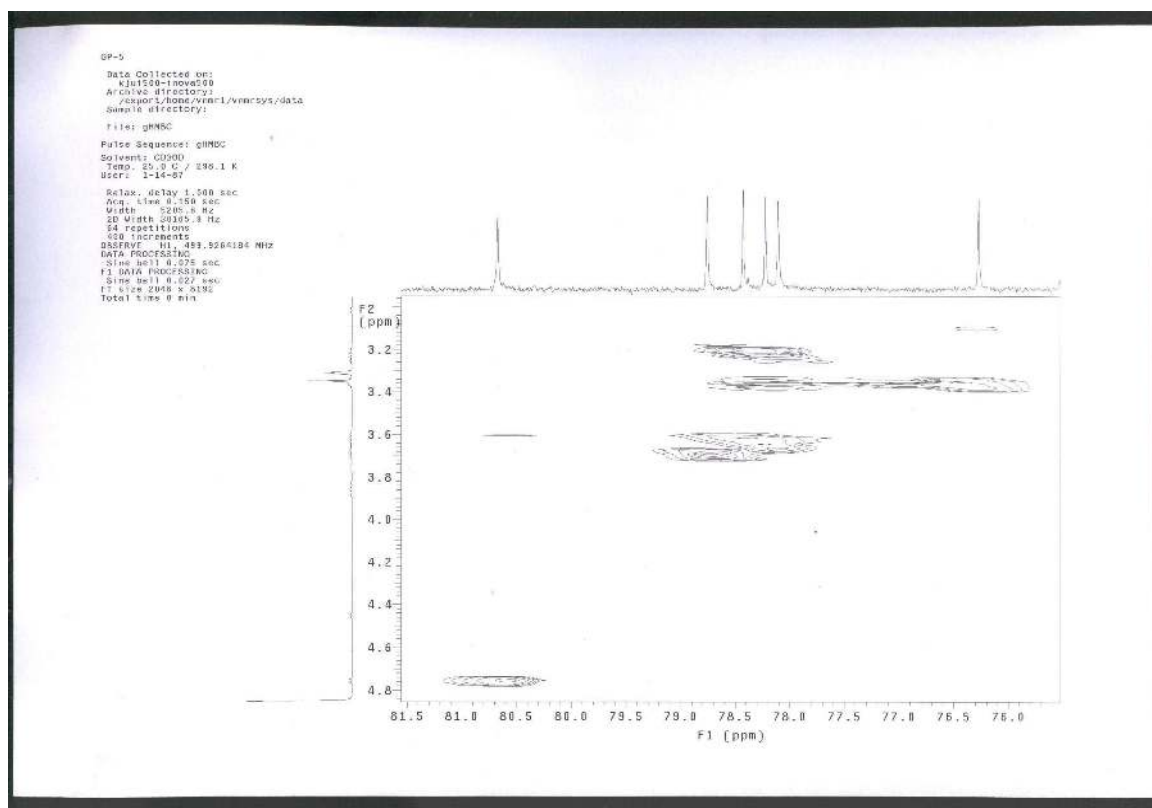


Fig. S1.13. HMBC NMR spectrum of damulin A (1)

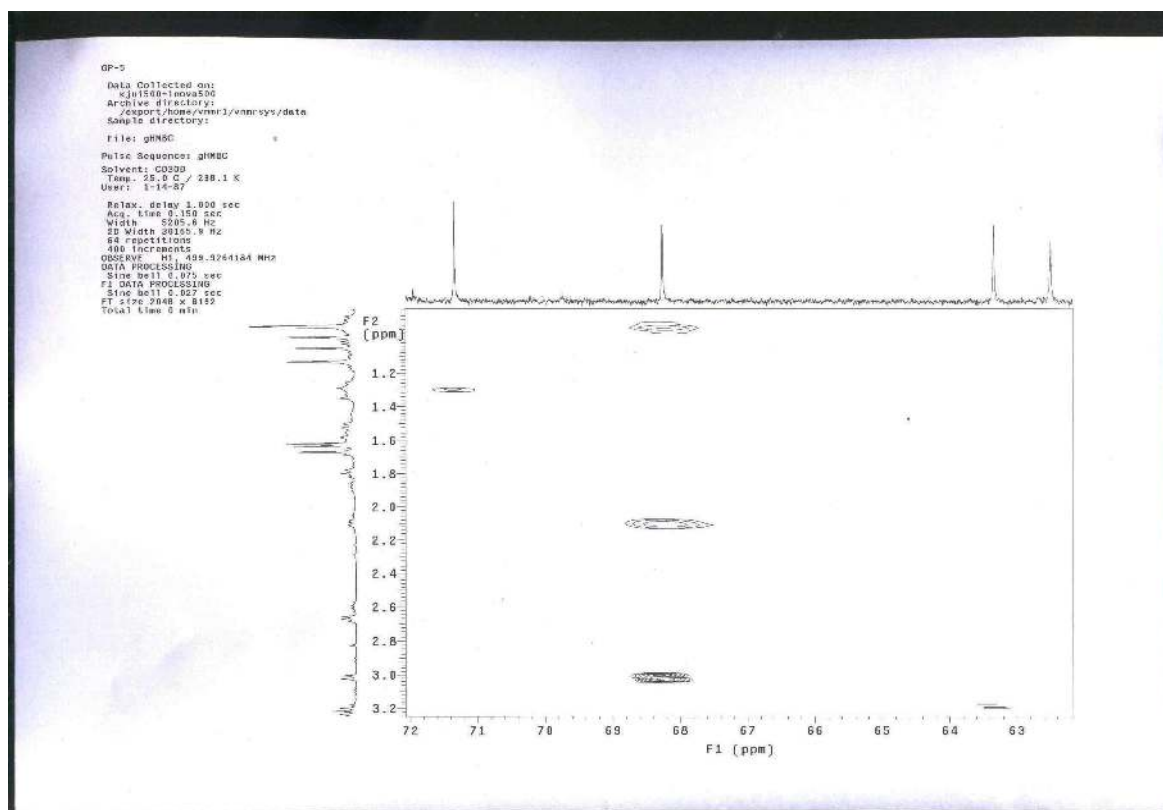


Fig. S1.14. HMBC NMR spectrum of damulin A (1)

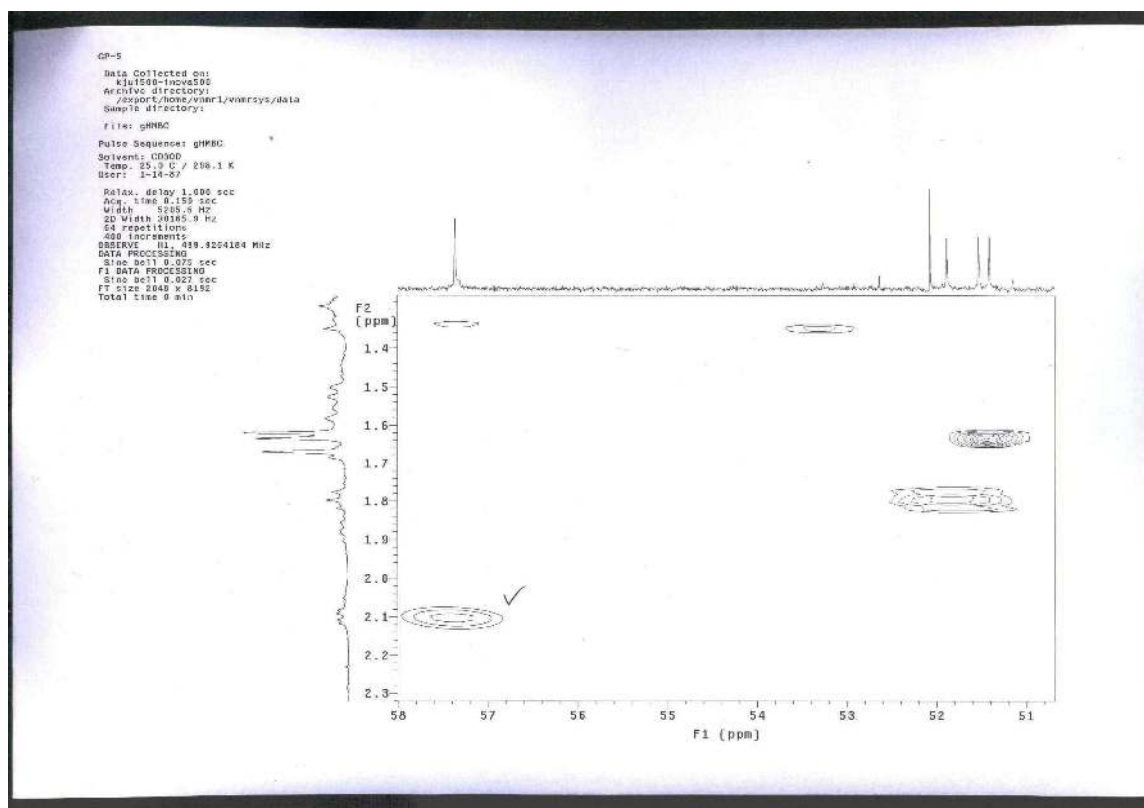


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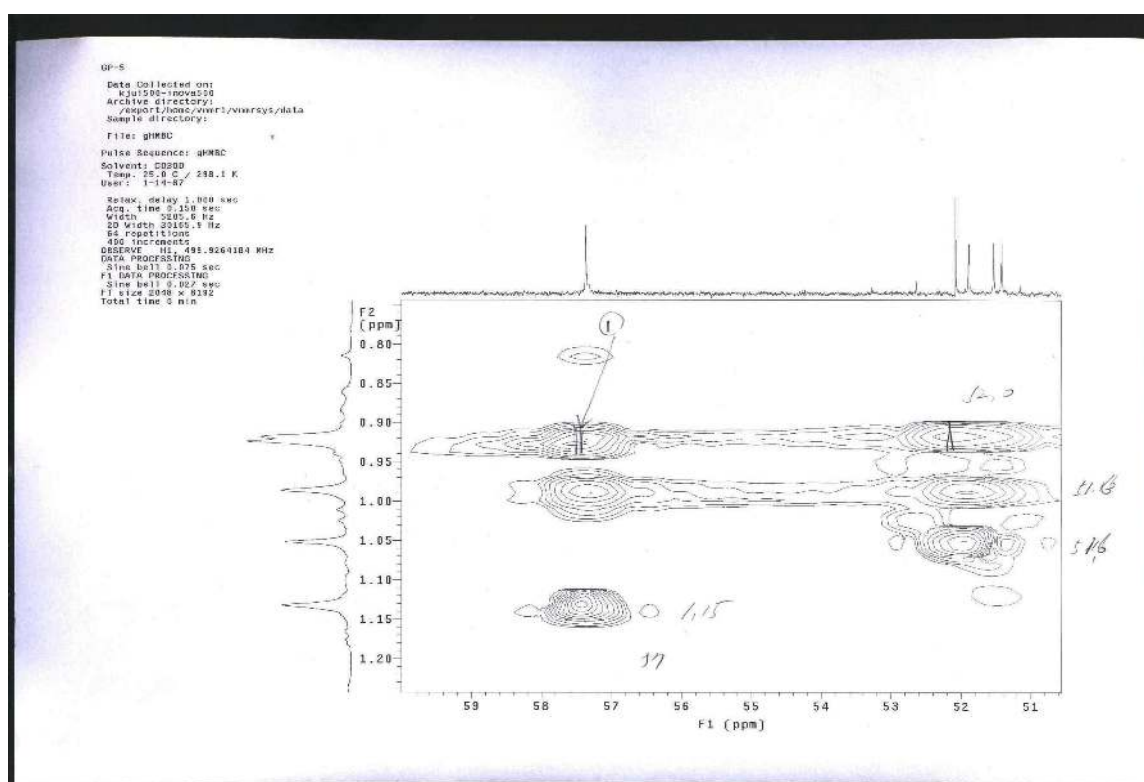


Fig. S1.16. HMBC NMR spectrum of damulin A (1)

024-072
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 Pulse Sequence: ghs90
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 Date: 1-14-07
 Relax. delay: 1.000 sec
 Acq. time: 0.150 sec
 Width: 2001.6 Hz
 20 width: 22297.5 Hz
 18 repetitions
 2 x 900 increments
 OBSFREQ: 41. 400.4254185 MHz
 DECUPLE: 033. 125.7100117 MHz
 Power: 57.00
 On during acquisition
 Off during delay
 400. Lipt's modulation
 Data PROCESSING
 Gauss apodization 0.003 sec
 F1 data PROCESSING
 Gauss apodization 0.011 sec
 FT size: 1024 x 2048
 Total time: 5 min

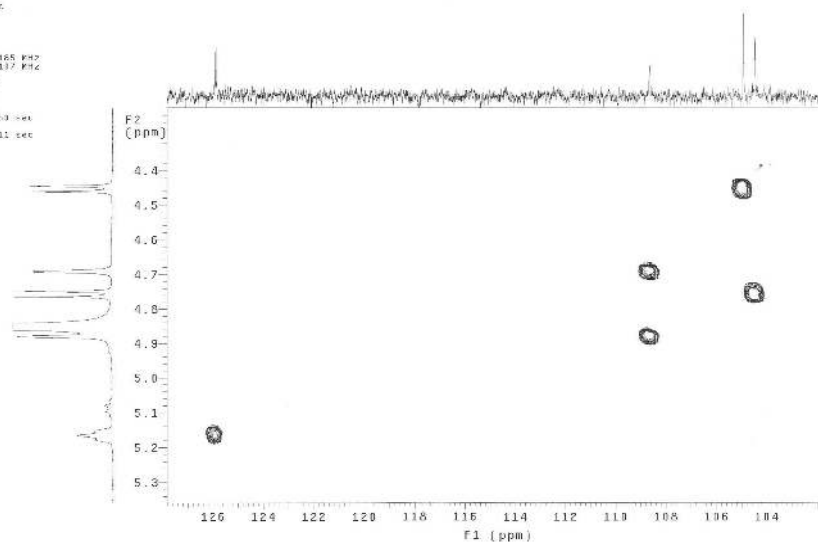


Fig. S2.11. HSQC NMR spectrum of damulin B (2)

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 Date: 1-14-07
 Relax. delay: 1.000 sec
 Acq. time: 0.150 sec
 Width: 2001.6 Hz
 20 width: 22297.5 Hz
 18 repetitions
 2 x 900 increments
 OBSFREQ: 41. 400.4254185 MHz
 DECUPLE: 033. 125.7100117 MHz
 Power: 57.00
 On during acquisition
 Off during delay
 400. Lipt's modulation
 Data PROCESSING
 Gauss apodization 0.003 sec
 F1 data PROCESSING
 Gauss apodization 0.011 sec
 FT size: 1024 x 2048
 Total time: 5 min

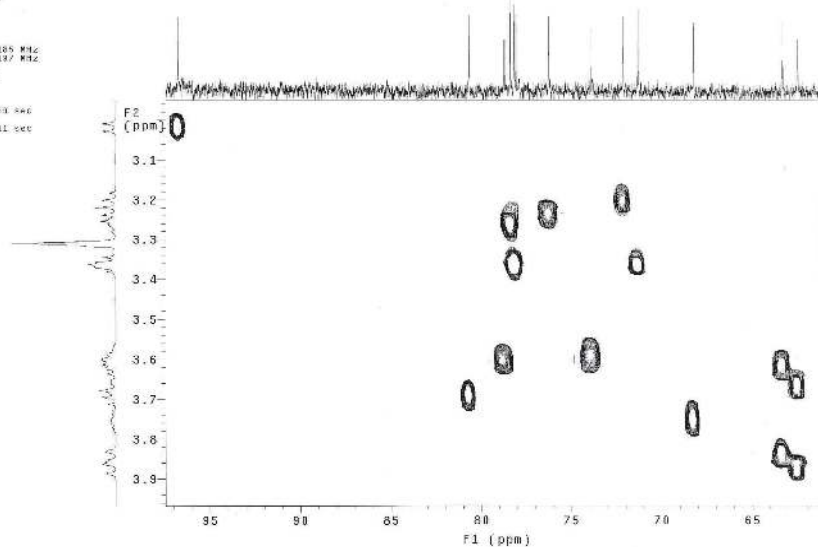


Fig. S2.12. HSQC NMR spectrum of damulin B (2)

B31-472
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 Sample directory:
 Files: gCOSY
 Pulse Sequence: gCOSY
 Solvent: CD3OD
 Temp: 25.0 C / 298.1 K
 Relax: delay 1.000 sec
 Acq: time 0.150 sec
 Width: 4001.6 Hz
 F2 width 4001.6 Hz
 16 repetitions
 160000000
 OBSERVE: H1: 400.15064185 MHz
 DATA PROCESSING:
 Acq: time 0.011 0.014 sec
 F2: 400.15064185 MHz
 Acq: time 0.011 0.014 sec
 F1: 100.61824 X 1025
 Total time 0 min

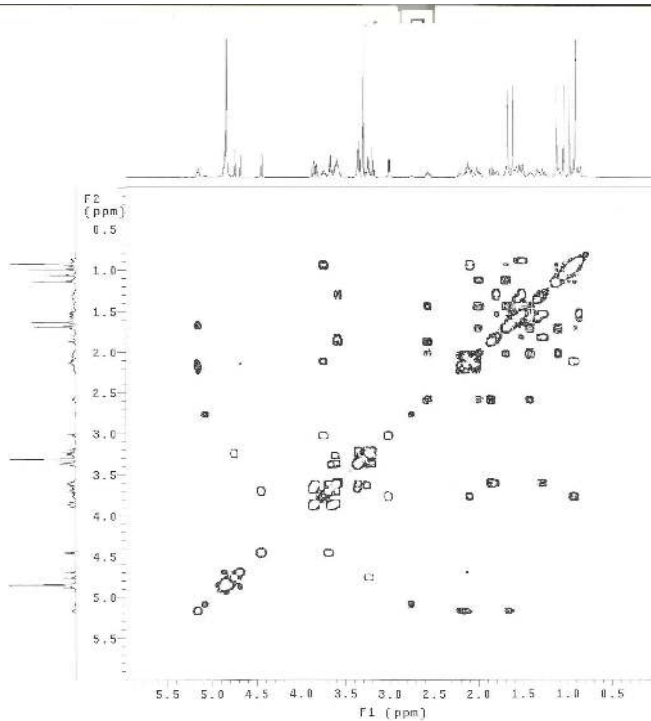


Fig. S2.13. COSY NMR spectrum of damulin B (2)

B34-572
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 Sample directory:
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 Pulse Sequence: gCOSY
 Solvent: CD3OD
 Temp: 25.0 C / 298.1 K
 Relax: delay 1.000 sec
 Acq: time 0.150 sec
 Width: 4001.6 Hz
 F2 width 4001.6 Hz
 16 repetitions
 160000000
 OBSERVE: H1: 400.15064185 MHz
 DATA PROCESSING:
 Acq: time 0.011 0.014 sec
 F2: 400.15064185 MHz
 Acq: time 0.011 0.014 sec
 F1: 100.61824 X 1025
 Total time 0 min

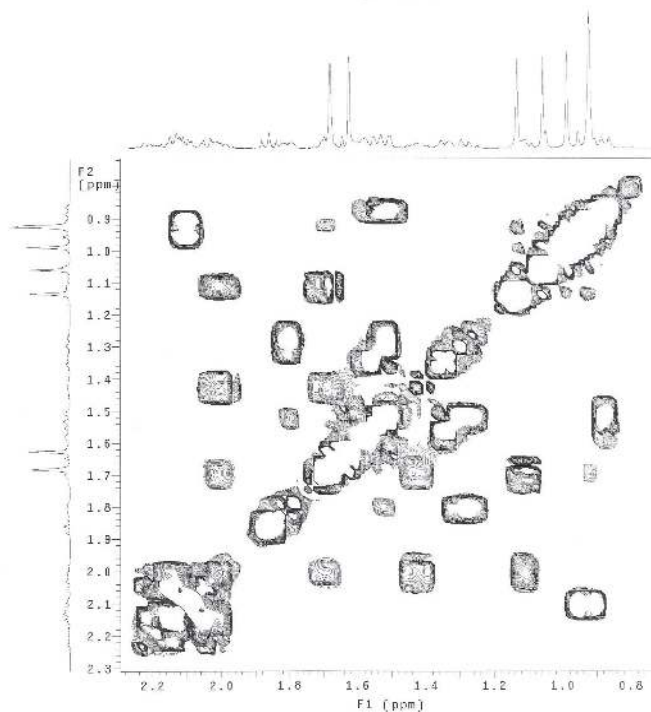


Fig. S2.14. COSY NMR spectrum of damulin B (2)

B34-572
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 Relax delay: 1.000 sec
 Acq. time: 0.150 sec
 Width: 4001.6 Hz
 2D Width: 4001.6 Hz
 16 F2 acq. files
 240 F2 increments
 OBSERVE: F1, 400.1251100 MHz
 NUC1: 13C-1013100
 N2: 13C-1013100
 F2 DATA PROCESSING
 S0: 13C-1013100
 F1 size: 1024 x 1024
 Total time: 0 min

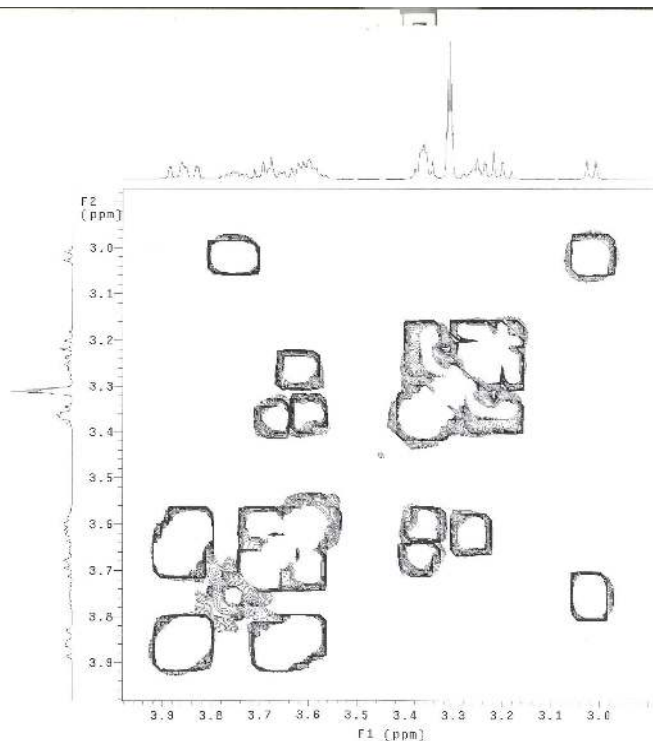


Fig. S2.15. COSY NMR spectrum of damulin B (2)

B34-572
 Date Collected on: 01/12/08
 Archive directory: /export/home/vnmr1/vnmrsys/data
 Sample directory: /
 File: gHMBC
 Pulse Sequence: gHMBC
 Solvent: DMSO
 Temp: 25.0 C / 298.1 K
 User: 1-14-87
 Relax delay: 1.000 sec
 Acq. time: 0.150 sec
 Width: 4001.6 Hz
 2D Width: 4001.6 Hz
 16 F2 acq. files
 240 F2 increments
 OBSERVE: F1, 400.1251100 MHz
 NUC1: 13C-1013100
 F1 DATA PROCESSING
 S0: 13C-1013100
 F1 size: 1024 x 1024
 Total time: 0 min

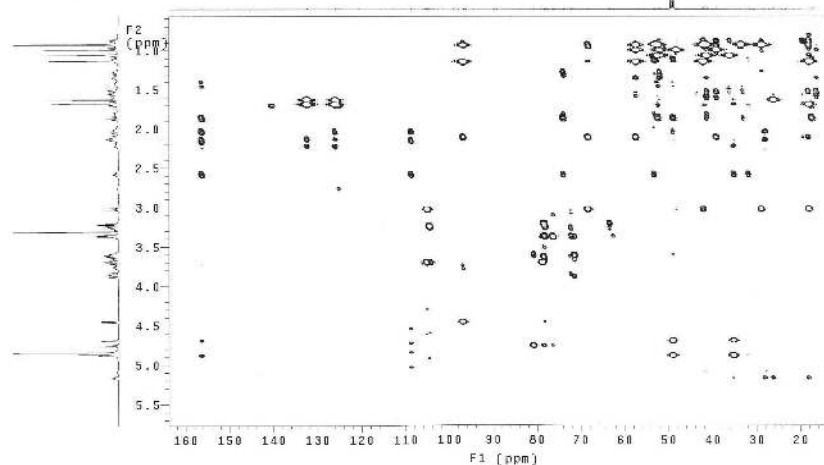


Fig. S2.16. HMBC NMR spectrum of damulin B (2)

554-572
 Data Collected on:
 5/10/00-1000000
 Archive directory:
 /export/home/vmr/vmr/sys/data
 Sample directory:
 /
 Title: gnmbo
 Pulse Sequence: gnmbo
 Solvent: CDCl3
 Temp: 25.0 C / 298.1 K
 User: 1-16-97
 Relax delay 1.000 sec
 Acq. time 0.150 sec
 FID 400.0 Hz
 2D width 20165.0 Hz
 24 repetitions
 2D increments
 OBSERVE F1: 400.0260180 MHz
 DATA PROCESSING
 Size 1024 x 2048
 F1 DATA PROCESSING
 Size 1024 x 2048
 F2 size 1024 x 2048
 Total time 0 min

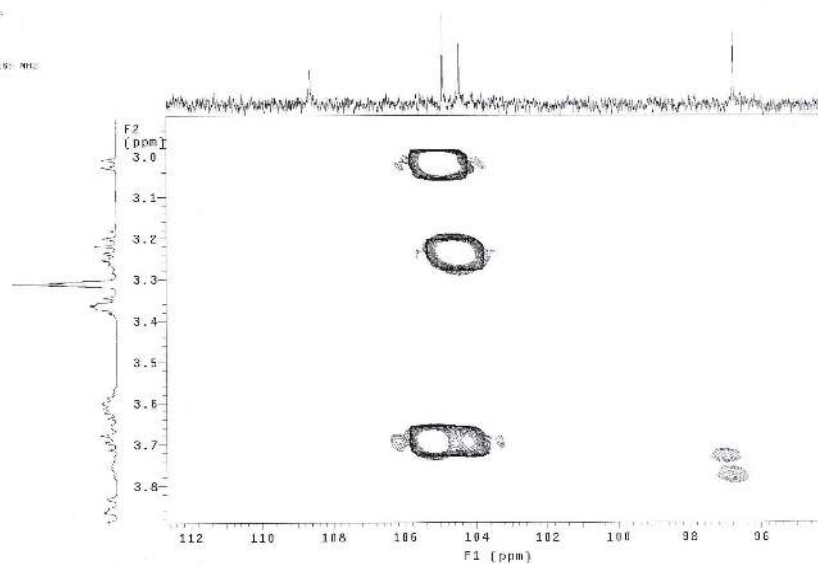


Fig. S2.17. HMBC NMR spectrum of damulin B (2)

554-572
 Data Collected on:
 5/10/00-1000000
 Archive directory:
 /export/home/vmr/vmr/sys/data
 Sample directory:
 /
 Title: gnmbo
 Pulse Sequence: gnmbo
 Solvent: CDCl3
 Temp: 25.0 C / 298.1 K
 User: 1-16-97
 Relax delay 1.000 sec
 Acq. time 0.150 sec
 FID 400.0 Hz
 2D width 20165.0 Hz
 24 repetitions
 2D increments
 OBSERVE F1: 400.0260180 MHz
 DATA PROCESSING
 Size 1024 x 2048
 F1 DATA PROCESSING
 Size 1024 x 2048
 F2 size 1024 x 2048
 Total time 0 min

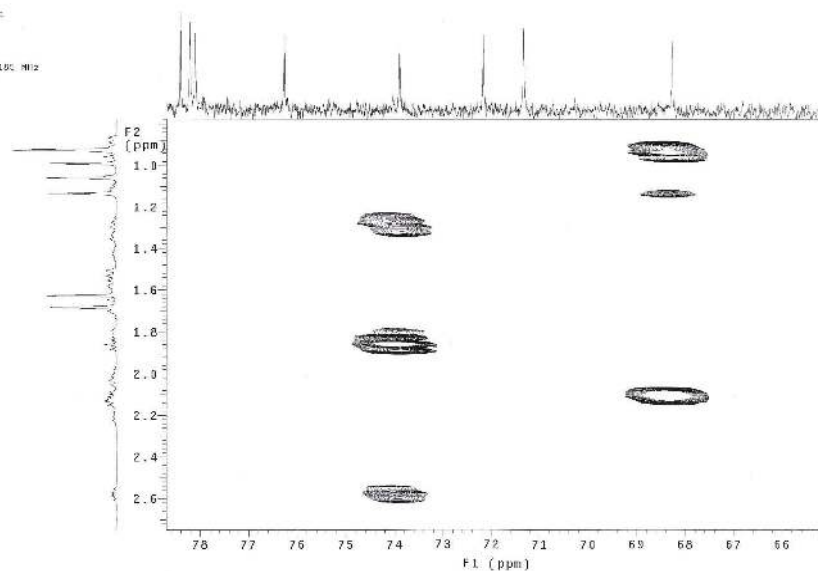


Fig. S2.18. HMBC NMR spectrum of damulin B (2)

554-572
 Data Collected on:
 12/15/00-invest09
 Archive directory:
 /export/home/vinn/1/vinn/sys/data
 Sample directory:
 Files: gnmbr
 Pulse Sequence: gnmbr
 Solvent: CD3OD
 Temp: 25.0 C / 298.1 K
 User: j-14-87
 Relax, delay 1.000 sec
 Acq. time 0.150 sec
 Width 800.0 Hz
 ZF Width 20100.9 Hz
 F1 Freq: 513.000
 400 Increments
 RESOLVE F1, 499.9264180 MHz
 D0.0 PROULSING
 Sine bell 0.000 sec
 F1 Data PROCESSING
 Sine bell 0.000 sec
 F1 size 1024 x 1024
 Total time 0 min

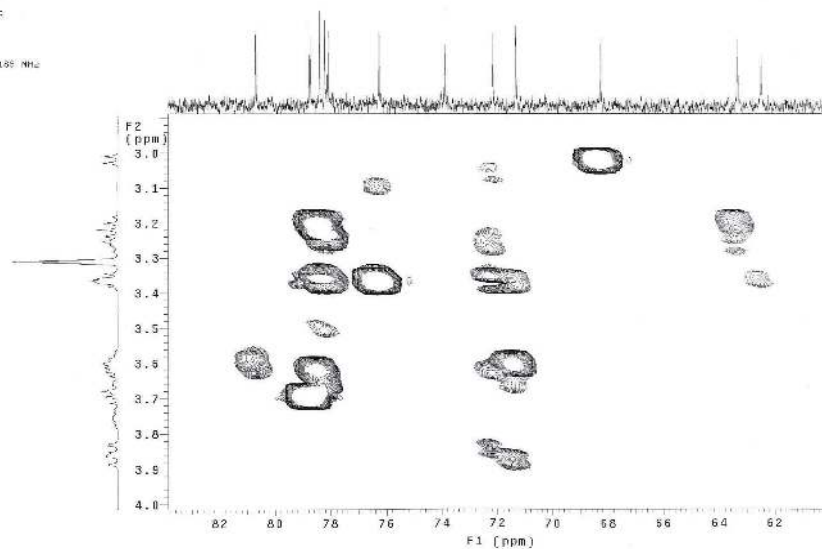


Fig. S2.19. HMBC NMR spectrum of damulin B (2)

554-572
 Data Collected on:
 12/15/00-invest09
 Archive directory:
 /export/home/vinn/1/vinn/sys/data
 Sample directory:
 Files: gnmbr
 Pulse Sequence: gnmbr
 Solvent: CD3OD
 Temp: 25.0 C / 298.1 K
 User: j-14-87
 Relax, delay 1.000 sec
 Acq. time 0.150 sec
 Width 800.0 Hz
 ZF Width 20100.9 Hz
 F1 Freq: 513.000
 400 Increments
 RESOLVE F1, 499.9264180 MHz
 D0.0 PROULSING
 Sine bell 0.000 sec
 F1 Data PROCESSING
 Sine bell 0.000 sec
 F1 size 1024 x 1024
 Total time 0 min

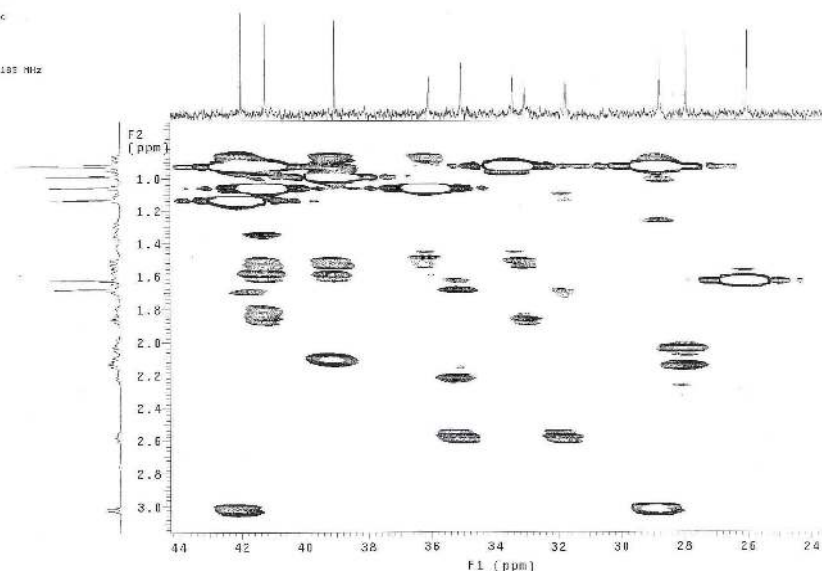


Fig. S2.20. HMBC NMR spectrum of damulin B (2)

B25-072
 Data Collected on:
 F20100-100000
 Archive directory:
 /export/home/vmr1/vmr000/data
 Sample directory:
 Files: gnm00
 Pulse Sequence: gnm00
 Solvent: CDCl3
 Temp: 25.0 C / 298.1 K
 User: j-14-07
 Relax: delay 1.500 sec
 Acq: time 0.150 sec
 Width 4001.0 Hz
 2D Width 20105.9 Hz
 2D repetitions
 200 increments
 Data File: F1_001.0264195 MHz
 Data Processed:
 Size 1024 x 1024
 F1 Data Processed:
 Size 1024 x 1024
 F2 Data Processed:
 Size 1024 x 1024
 Total time 0 min

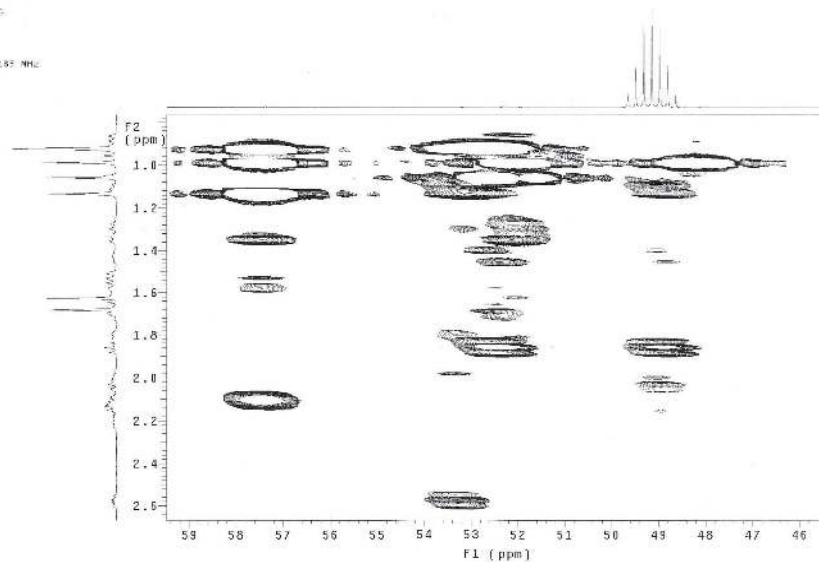


Fig. S2.21. HMBC NMR spectrum of damulin B (2)

B24-070
 Data Collected on:
 F20100-100000
 Archive directory:
 /export/home/vmr1/vmr000/data
 Sample directory:
 Files: gnm00
 Pulse Sequence: gnm00
 Solvent: CDCl3
 Temp: 25.0 C / 298.1 K
 User: j-14-07
 Relax: delay 1.500 sec
 Acq: time 0.150 sec
 Width 4001.0 Hz
 2D Width 20105.9 Hz
 2D repetitions
 200 increments
 Data File: F1_001.0264195 MHz
 Data Processed:
 Size 1024 x 1024
 F1 Data Processed:
 Size 1024 x 1024
 F2 Data Processed:
 Size 1024 x 1024
 Total time 0 min

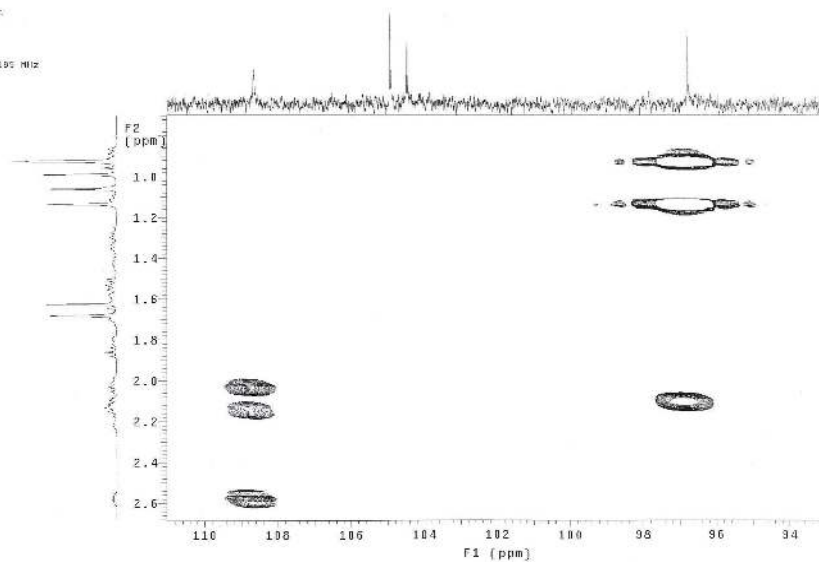


Fig. S2.22. HMBC NMR spectrum of damulin B (2)

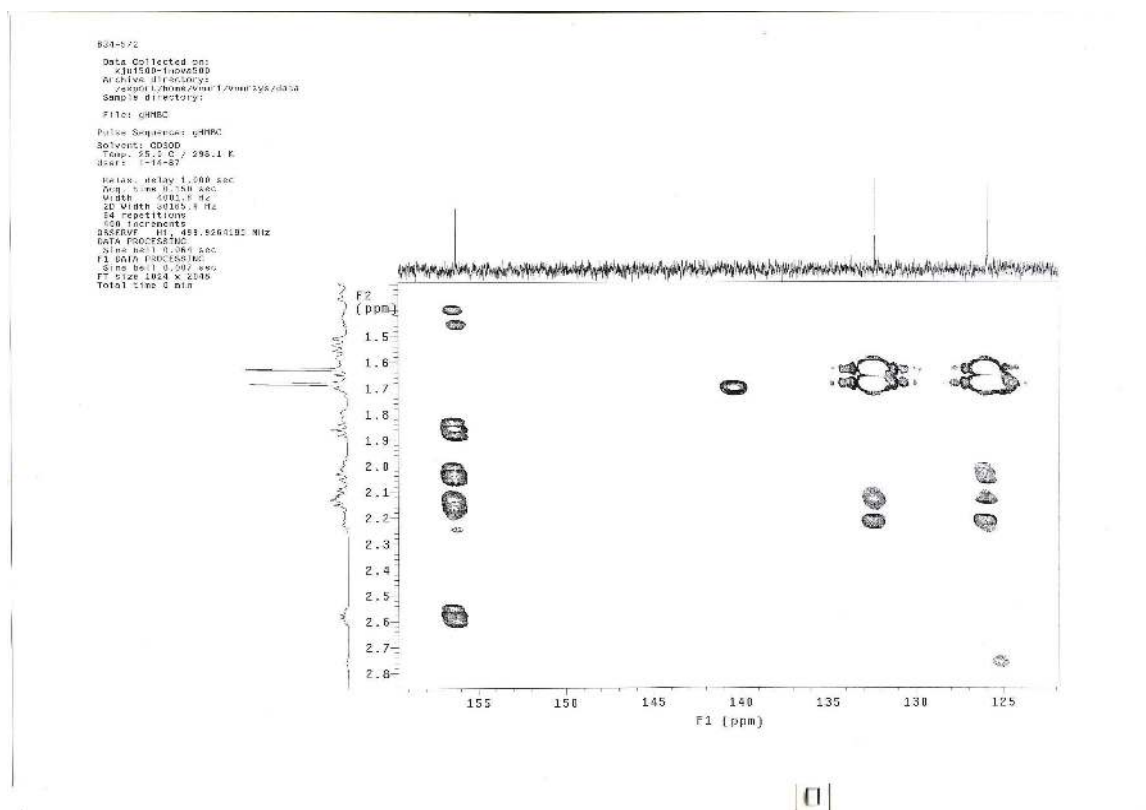


Fig. S2.23. HMBC NMR spectrum of damulin B (2)

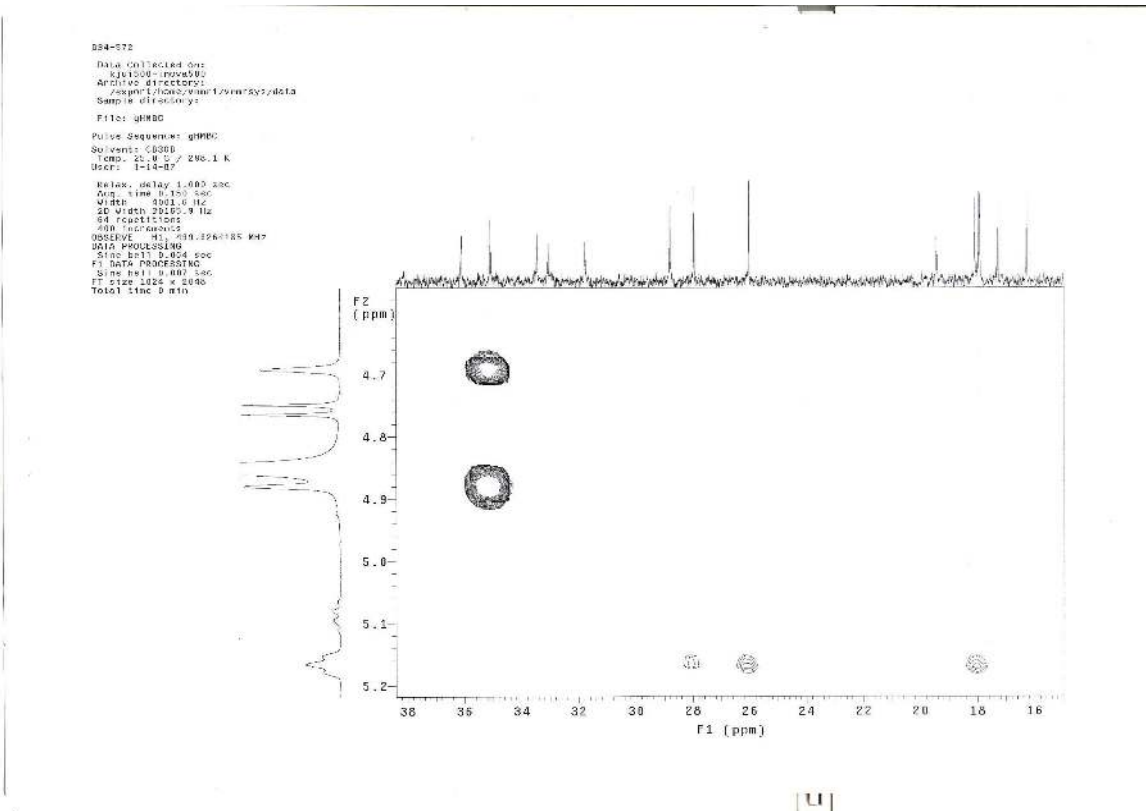


Fig. S2.24. HMBC NMR spectrum of damulin B (2)

B30-572
 Data collected on:
 kji1001-nova500
 Archive directory:
 /export/home/vmr1/vmrsys/data
 Sample directory:
 F11a: gHMBC
 Pulse Sequence: gHMBC
 Solvent: CD3OD
 Temp: 25.0 C / 298.1 K
 User: 1-14-07
 Relax delay 1.000 sec
 Acq. time 0.150 sec
 Width 3001.8 Hz
 SQ width 20305.1 Hz
 32 repetitions
 320 increments
 OBSERVE H1 401.264185 MHz
 DATA PROCESSING
 F2 size 1024 x 2048
 F1 size 1024 x 2048
 Total time 0 min

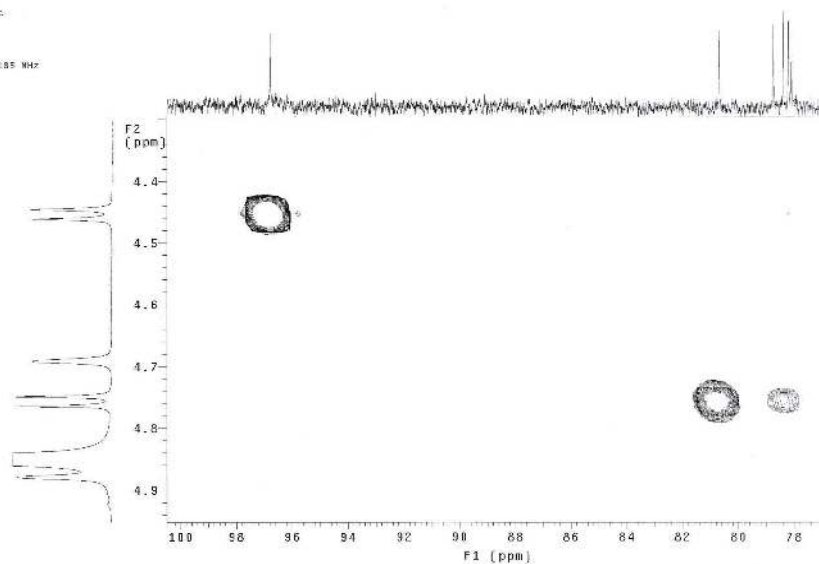


Fig. S2.25. HMBC NMR spectrum of damulin B (2)

B34-579
 Data collected on:
 kji1001-nova500
 Archive directory:
 /export/home/vmr1/vmrsys/data
 Sample directory:
 F11a: gHMBC
 Pulse Sequence: gHMBC
 Solvent: CD3OD
 Temp: 25.0 C / 298.1 K
 User: 1-14-07
 Relax delay 1.000 sec
 Acq. time 0.150 sec
 Width 3001.8 Hz
 SQ width 20305.1 Hz
 32 repetitions
 320 increments
 OBSERVE H1 401.264185 MHz
 DATA PROCESSING
 F2 size 1024 x 2048
 F1 size 1024 x 2048
 Total time 0 min

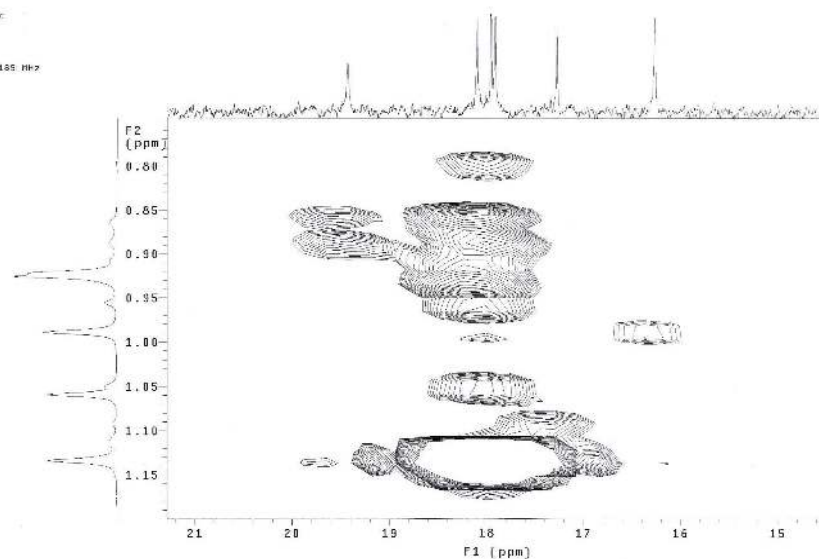


Fig. S2.26. HMBC NMR spectrum of damulin B (2)

B24-072
 Data Collection on:
 KJULSON-000000
 Archive directory:
 /workspace/vnmr1/vnmrsys/data
 Sample directory:
 File: ghs00
 Pulse Sequence: ghs00
 Solvent: CDCl3
 Temp: 25.0 C / 298.1 K
 User: 1-10-97
 Relax, delay 1.000 sec
 Acq. time 0.150 sec
 Q H₂O 2001.8 MHz
 2D width 21267.5 Hz
 16 repetitions
 2 x 200 increments
 DECOUPLE C13, 125.7180137 MHz
 Power 12 dB
 on during acquisition
 off during delay
 640 H₂O 125.7180137 MHz
 Data Processing
 Gauss apodization 0.659 sec
 F1 DATA PROCESSING
 Gauss apodization 0.011 sec
 FT size 1024 x 2048
 Total time 6 min

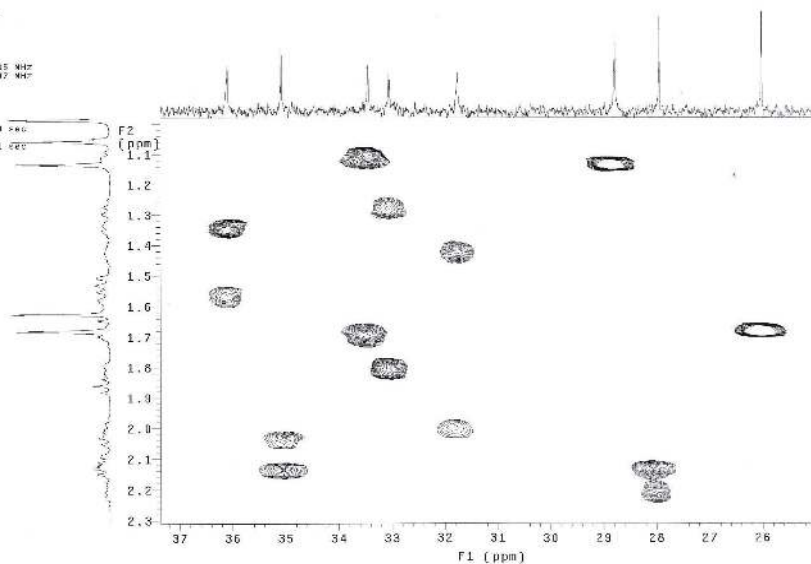


Fig. S2.9. HSQC NMR spectrum of damulin B (2)

B24-072
 Data Collection on:
 KJULSON-000000
 Archive directory:
 /workspace/vnmr1/vnmrsys/data
 Sample directory:
 File: ghs00
 Pulse Sequence: ghs00
 Solvent: CDCl3
 Temp: 25.0 C / 298.1 K
 User: 1-10-97
 Relax, delay 1.000 sec
 Acq. time 0.150 sec
 Q H₂O 2001.8 MHz
 2D width 21267.5 Hz
 16 repetitions
 2 x 200 increments
 DECOUPLE C13, 125.7180137 MHz
 Power 12 dB
 on during acquisition
 off during delay
 640 H₂O 125.7180137 MHz
 Data Processing
 Gauss apodization 0.659 sec
 F1 DATA PROCESSING
 Gauss apodization 0.011 sec
 FT size 1024 x 2048
 Total time 6 min

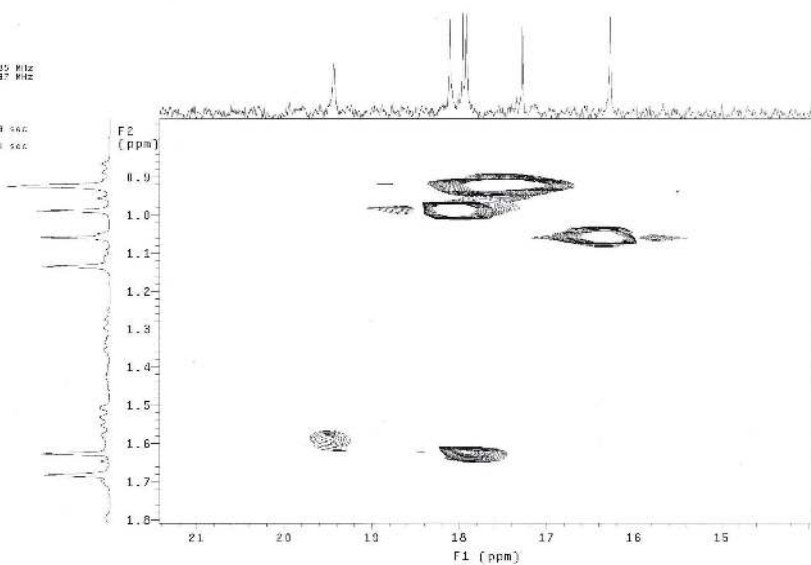


Fig. S2.10. HSQC NMR spectrum of damulin B (2)