

## PHENOLIC COMPOUNDS FROM *Sidastrum micranthum* (A. St.-Hil.) FRYXELL AND EVALUATION OF ACACETIN AND 7,4'-DI-O-METHYLISOSCUTELLAREIN AS MODULATOR OF BACTERIAL DRUG RESISTENCE

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From the aerial parts of *Sidastrum micranthum* (A. St.-Hil.) Fryxell (Malvaceae) were isolated *m*-methoxy-*p*-hydroxy-benzaldehyde, *o*-hydroxy-benzoic acid, acacetin, quercetin, 7,4'-Di-O-methylisoscuteollarein, genkwanin and tiliroside. These compounds were identified by data analyses of spectroscopic methods. Although acacetin and 7,4'-Di-O-methylisoscuteollarein did not display relevant antibacterial activity (MIC = 256 µg/mL), they modulated the activity of antibiotics, i.e. in combination with antibiotics at 64 µg/mL (¼ MIC), a two-fold reduction in the MIC was observed for norfloxacin and ethidium bromide; regarding tetracycline and erythromycin a two-fold reduction in the MIC was observed only with 7,4'-Di-O-methylisoscuteollarein.

Keywords: *Sidastrum micranthum*; Malvaceae; modulation of drug resistance.

### INTRODUCTION

Malvaceae is a widespread family with about 243 genera and 4225 species, particularly distributed in tropical areas.<sup>1</sup> Natural products extracted from plants that belong to the Malvaceae family are used in the treatment of many diseases worldwide.<sup>2</sup> Some of these uses include snakebites, asthma, diuretic, treatment of rheumatism and gastrointestinal disorders, among others.<sup>3</sup>

*Sidastrum micranthum* (A. St.-Hil.) Fryxell, commonly known in Brazil as "malva-preta", consists of a small shrub belonging to the Malvaceae family. It is a neotropical species, occurring commonly in Cuba, Costa Rica, Venezuela, Guyana and Brazil.<sup>4</sup> Furthermore, a nutraceutical (tea or infusion) made from leaves is used traditionally against bronchitis, coughs and asthma. The leaves are used in cataplasms, with hot butter and olive oil as a moisturizing agent.<sup>5</sup>

In recent years, bacterial resistance to antibiotics has become a serious public health problem that concerns almost all antibacterial agents. Efflux pumps are integral proteins of the bacterial membrane and are recognized as a major cause of bacterial resistance since they extract antibiotics from the cell.<sup>6</sup>

One option for reversing or reducing the efflux-mediated resistance is the development of modulators of drug resistance such as efflux pump inhibitors (EPI), which can potentiate antibiotic activity. Plants provide a rich source of EPIs and several compounds have already been identified as potent inhibitors.<sup>7</sup>

In our ongoing project to evaluate natural compounds isolated from plants of the Brazilian biodiversity, mainly of the Malvaceae family, as modulators of antibiotic resistance, we have already demonstrated the modulatory activity of a pentamethoxyflavone and of a glycosylated kaempferol derivative (tiliroside) isolated from *Herissantia tiubae* (K. Schum) Brizicky.<sup>8</sup> In the present work, we evaluate the flavonoids ac-

acetin and 7,4'-Di-O-methylisoscuteollarein as modulators of antibiotic resistance using effluxing strains of *Staphylococcus aureus*.

### EXPERIMENTAL

#### Instruments and reagents

Melting points were recorded in a Model MQAPF-302 (Microquímica Equipamentos Ltda). Isolated compounds were identified by IR (Perkin-Elmer, FT-IR-1750 and Shimadzu – IR prestige 21) and extensive 1D and 2D NMR analysis (<sup>1</sup>H 200 MHz, <sup>13</sup>C 50 MHz - Varian-Mercury and <sup>1</sup>H 500 MHz, <sup>13</sup>C 125 MHz - Bruker-AC, COSY, HSQC, HMBC, NOESY) using deuterated chloroform (CDCl<sub>3</sub>), dimethyl sulfoxide (DMSO-d<sub>6</sub>) or methanol (CD<sub>3</sub>OD) as solvents.

Chromatography columns were carried out on silica gel (ASTM, 230-400 mesh, Merck) and Sephadex LH-20 (GE Healthcare Bio-Sciences AB). TLC were performed on silica gel PF<sub>254</sub> plates and the spots were visualized under UV light (244 and 366 nm) and by spraying with 5% FeCl<sub>3</sub> reagent.

#### Plant material

The aerial parts of *Sidastrum micranthum* were collected in June 2006 in the city of Santa Rita at the State of Paraíba, Brazil. A voucher specimen (Agra 6865) is deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), Universidade Federal da Paraíba, Paraíba – Brazil.

#### Extraction and isolation

The dried aerial parts of *Sidastrum micranthum* were powdered (6000 g) and subjected to maceration with ethanol 95%. Ethanol solution was concentrated under reduced pressure to afford 200 g of crude ethanol extract. The material was solubilized with ethanol:water (8:2)

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and posteriorly partitioned with hexane, chloroform, ethyl acetate and *n*-butanol, resulting in 46 g of hexane phase, 7 g of chloroform phase, 6 g of ethyl acetate phase and 8 g of *n*-butanol phase.

An aliquot of the hexane phase (6 g) was subjected to silica gel column chromatography and eluted with hexane, ethyl acetate and then methanol, gradually increasing the polarity, yielding 239 fractions which were combined after TLC analysis. The sub-fraction 144-151 (0.204 g) was also submitted to chromatography column over silica gel, using hexane, dichloromethane and methanol, resulting in 37 fractions, also combined by TLC. The Sub-fraction 34 led to isolation of compound **1** (8.0 mg).

The chloroform phase (7.0 g) was subjected to filtration under reduced pressure using silica gel 60 as stationary phase into a funnel of porous plate and was eluted with: hexane, ethyl acetate and methanol pure or mixture binaries. The fraction hexane-ethyl acetate (50:50) (0.823 mg) from this procedure was flash chromatographed on silica gel column, eluting with hexane, ethyl acetate and methanol, with increasing polarity to yield 119 sub-fractions. Sub-fraction 10-13 led to isolation of compound **2** (4.5 mg). Fraction ethyl acetate-methanol (50:50) was further purified by repeated CC on Sephadex LH-20 (MeOH) yielding compound **3** (15 mg).

The ethyl acetate phase (6 g) was subjected to filtration under reduced pressure using silica gel 60 as stationary phase into a funnel of porous plate, being eluted with: hexane/ethyl acetate (30:70), (50:50), (30:70), ethyl acetate, ethyl acetate/methanol (90:10), (70:30) and (50:50), yielding the respective fractions. The fraction hexane/ethyl acetate (50:50) (218 mg) which was purified after recrystallization using hexane with drops of ethyl acetate led to isolation of compound **4** (42 mg). The fraction hexane/ethyl acetate (30:70) (160 mg) from vacuum liquid chromatography of ethyl acetate phase was submitted to chromatography over flash silica gel, eluting with hexane, ethyl acetate and methanol, yielding 74 fractions. After TLC analysis, fractions 45-58 yielded compound **5** (30 mg). Fraction ethyl acetate (1.21 g) from the ethyl acetate phase was subjected to the same previous chromatographic procedure resulting in 100 fractions. Subfractions 09-11 and 66-67 were gathered and then recrystallized with acetone, yielding compound **6** (19 mg) and compound **7** (120 mg) respectively.

#### *Quercetin (3,7,3',4'-tetrahydroxy-flavone) (3)*

<sup>1</sup>H NMR ( $\delta$ , CD<sub>3</sub>OD, 500 MHz): 7.72 (*d*, *J* = 2.5 Hz, H-2'), 7.61 (*dd*, *J* = 8.5 Hz, H-6''), 6.87 (*d*, *J* = 8.0 Hz, H-5'), 6.37 (*d*, *J* = 2.0 Hz, H-8), 6.17 (*d*, *J* = 2.0 Hz, H-6). <sup>13</sup>C NMR (125 MHz): 177.3 (C-4), 165.5 (C-7), 162.4 (C-5), 158.2 (C-9), 148.7 (C-4'), 148.0 (C-2), 146.2 (C-3'), 137.2 (C-3), 124.1 (C-1'), 121.6 (C-6'), 116.2 (C-5'), 116.1 (C-2'), 104.5 (C-10), 99.2 (C-6), 99.4 (C-8).

#### *Acacetin (5,7-dihydroxy-4'-methoxy-flavone) (4)*

<sup>1</sup>H NMR ( $\delta$ , CDCl<sub>3</sub> with drops of CD<sub>3</sub>OD, 500 MHz): 7.72 (*d*, *J* = 9.0 Hz, H-2'/6'), 6.89 (*d*, *J* = 9.0 Hz, H-3'/5'), 6.43 (*s*, H-3), 6.32 (*d*, *J* = 2 Hz, H-8), 6.15 (*d*, *J* = 2.0 Hz, H-6), 3.76 (*s*, OCH<sub>3</sub>-4'). <sup>13</sup>C NMR (125 MHz): 182.3 (C-4), 164.0 (C-2), 163.9 (C-7), 162.5 (C-4'), 161.4 (C-5), 157.8 (C-9), 127.9 (C-2'/6'), 123.3 (C-1'), 114.3 (C-3'/5'), 104.4 (C-10), 103.6 (C-3), 99.13 (C-6), 94.1 (C-8), 55.3 (OCH<sub>3</sub>-4').

#### *7,4'-Di-O-methylisoscuteallarein (5,8-dihydroxy-7,4'-dimethoxy-flavone) (5)*

<sup>1</sup>H NMR ( $\delta$ , DMSO-*d*<sub>6</sub>, 500 MHz): 8.09 (*dd*, *J* = 7.0 Hz and *J* = 2.0 Hz, H-2'/6'), 7.12 (*dd*, *J* = 7.0 Hz and *J* = 2.0 Hz, H-3'/5'), 6.84 (*s*, H-3), 6.53 (*s*, H-6), 3.88 (*s*, OCH<sub>3</sub>-7), 3.85 (*s*, OCH<sub>3</sub>-4'). <sup>13</sup>C NMR (125 MHz): 182.4 (C-4), 163.5 (C-2), 162.4 (C-4'), 154.3 (C-7), 153.0 (C-5), 144.4 (C-9), 128.5 (C-2'/6'), 126.2 (C-9), 123.0 (C-1'), 114.5 (C-3'/5'), 103.9 (C-10), 103.0 (C-3), 95.7 (C-6), 56.3 (OCH<sub>3</sub>-7), 55.6 (OCH<sub>3</sub>-4').

#### *Genkwanin (5,4'-dihydroxy-7-methoxy-flavone) (6)*

<sup>1</sup>H NMR ( $\delta$ , DMSO-*d*<sub>6</sub>, 500 MHz): 7.94 (*d*, *J* = 9.0 Hz, H-2'/6'), 7.08 (*d*, *J* = 9.0 Hz, H-3'/5'), 6.63 (*s*, H-3), 6.46 (*d*, *J* = 2.0 Hz, H-8), 6.21 (*d*, *J* = 2.0 Hz, H-6), 3.88 (*s*, OCH<sub>3</sub>-7). <sup>13</sup>C NMR (125 MHz): 181.9 (C-4), 165.1 (C-7), 164.11 (C-2), 161.3 (C-4'), 161.2 (C-5), 157.2 (C-9), 116.0 (C-3'/5'), 128.5 (C-2'/6'), 121.1 (C-1'), 104.7 (C-10), 103.0 (C-3), 97.9 (C-6), 92.7 (C-8), 56.61 (OCH<sub>3</sub>-7).

#### *Tiliroside [Kampherol-3-O- $\beta$ -D-(6''-E-p-coumaroil) glycosídeo] (7)*

<sup>1</sup>H NMR ( $\delta$ , CD<sub>3</sub>OD, 200 MHz): 7.96 (*d*, *J* = 9.0 Hz, H-2'/6'), 7.38 (*d*, *J* = 15.9 Hz, H- $\beta$ ), 7.25 (*d*, *J* = 8.6 Hz, H-2'''/6'''), 6.79 (*d*, *J* = 9.0 Hz, H-3'/5'), 6.77 (*d*, *J* = 8.6 Hz, H-3'''/5'''), 6.27 (*d*, *J* = 2.0 Hz, H-8), 6.11 (*d*, *J* = 2.0 Hz, H-6), 6.05 (*d*, *J* = 15.9 Hz, H- $\alpha$ ), 5.23 (*d*, *J* = 7.6 Hz, H-1''), 4.19 (*dd*, *J* = 11.8 and 2.2 Hz, H-6''), 4.06 (*dd*, *J* = 11.6 and *J* = 6.4 Hz, H-6''), 3.38-3.34 (*m*, H-2'', 3'', 4''), 3.25-3.16 (*m*, H-5''). RMN <sup>13</sup>C (50 MHz): 179.3 (C-4), 168.8 (COO), 165.7 (C-7), 162.8 (C-5), 161.4 (C-4'), 161.0 (C-4''), 159.2 (C-2), 158.2 (C-9), 146.5 (CH- $\beta$ ) 135.2 (C-3), 132.1 (CH-2'/CH-6'), 131.1 (CH-2'''/6'''), 127.0 (C-1'''), 122.6 (C-1'), 116.7 (CH-3'''/5'''), 115.9 (CH-3'/5'), 114.7 (CH- $\alpha$ ), 105.5 (C-10), 104.0 (CH-1''), 99.9 (CH-6), 94.8 (CH-8), 77.9 (CH-3''), 75.7 (CH-2'', CH-5''), 71.6 (CH-4''), 64.3 (CH2-6'').

#### Bacterial strains

Three strains of *S. aureus* were used: SA-1199B, which overexpresses the *norA* gene encoding the NorA efflux protein which extrudes hydrophilic fluorquinolones and other drugs such as DNA-intercalating dyes;<sup>9</sup> RN4220 harboring plasmid pUL5054, which carries the gene encoding the MsrA macrolide efflux protein;<sup>10</sup> and IS-58, which possesses the TetK tetracycline efflux protein.<sup>11</sup> The strains, kindly provided by Prof. S. Gibbons (University of London), were maintained on blood agar base (Laboratórios Difco Ltda., Brazil) slants and, prior to use, the cells were grown overnight at 37 °C in brain heart infusion broth (BHI – Laboratórios Difco Ltda., Brazil).

#### Antibiotics, ethidium bromide and flavonoids

Norfloxacin, erythromycin, tetracycline and ethidium bromide were obtained from Sigma Aldrich Co. Ltd. The stock solutions of the antibiotics were prepared according to CLSI guidelines,<sup>12</sup> and the ethidium bromide in sterile distilled water. The stock solution of acetamin and 7,4'-di-O-methylisoscuteallarein were prepared in DMSO, and its highest concentration remaining after dilution into broth (4%) caused no inhibition of bacterial growth.

#### Drug susceptibility testing and modulation assay

The minimum inhibitory concentrations (MICs) of the antibiotics, ethidium bromide and flavonoids were determined in BHI by the microdilution assay using a suspension of *ca.* 10<sup>5</sup> cfu/mL and a drug concentration range of 256 to 0.5  $\mu$ g/mL (two-fold serial dilutions). The MIC is defined as the lowest concentration at which no growth is observed. A solution of resazurin (0.01% w/v in sterile distilled water) was used to detect bacterial growth by a color change from blue to pink. For the evaluation of flavonoids as modulators of drug resistance, the 'modulation assay' was used, a method that has been widely applied to identify potential EPIs,<sup>13</sup> i.e. the MICs of the antibiotics were determined in the presence of the flavonoids (in the BHI) at a sub-inhibitory concentration.

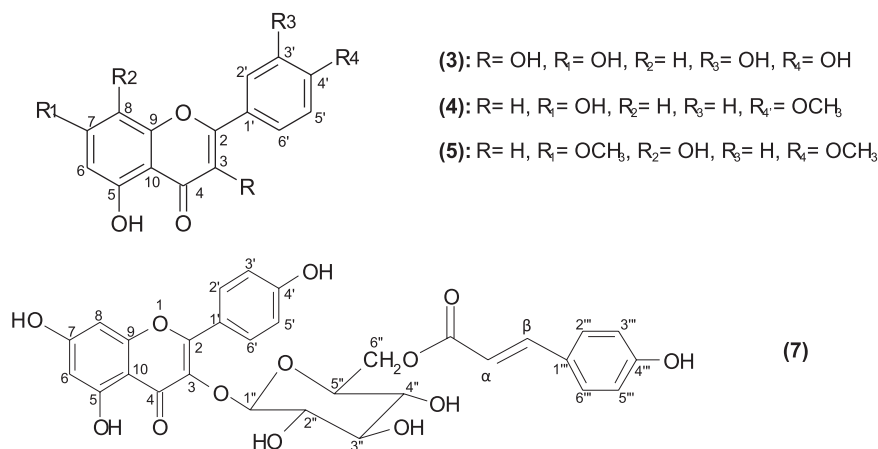


Figure 1. Compounds isolated from *Sidastrum micranthum*

## RESULTS AND DISCUSSION

The structural assignments of compounds **1** and **2** were made based on the spectral analysis and are in good agreement with those reported in the literature. Thus, their structures were identified as Vanillin<sup>14</sup> and salicylic acid.<sup>15</sup> The structural identifications of the compounds **3-7** were based on spectral analysis and also by comparison with literature data, which allowed their assignments as quercetin (**3**),<sup>16</sup> acacetin (**4**),<sup>17</sup> 7,4'-Di-O-methylisoscuteallarein (**5**),<sup>18</sup> Genkwainin (**6**),<sup>17</sup> and tiliroside (**7**).<sup>19</sup> This is the first study reporting the presence of these compounds in *Sidastrum micranthum* and the first report of the compounds **4**, **5**, and **6** from the Malvaceae family.

The IR spectrum of compound **5** showed a hydroxyl signal at 3458 cm<sup>-1</sup>, a conjugated carbonyl hydroxyl absorbance at 1665 cm<sup>-1</sup> and aromatic C=C stretching absorptions at 1609 and 1510 cm<sup>-1</sup>. The <sup>1</sup>H NMR spectra showed a pair of double doublets at δ<sub>H</sub> 8.09 (*J* = 7.0 Hz and *J* = 2.0 Hz) and δ<sub>H</sub> 7.12 (*J* = 7.0 Hz and *J* = 2.0 Hz), which suggests an AA'BB'-type system of hydrogens bonded to aromatic carbon. A methoxyl group in one of the aromatic nuclei was inferred by the signal at δ<sub>H</sub> 3.88.

The <sup>13</sup>C NMR spectral data strengthened the information provided by the <sup>1</sup>H NMR spectra, emphasizing the presence of the AA'BB' system due to signal at δ<sub>C</sub> 128.5 and δ<sub>C</sub> 114.5.

The positions of methoxyl groups were established by HMBC spectrum, which showed interactions between the absorptions at δ<sub>H</sub> 3.85 (*s*, OCH<sub>3</sub>-4') and δ<sub>C</sub> 162.4 (C-4'). In addition to interactions between the absorptions at δ<sub>H</sub> 3.88 (*s*, OCH<sub>3</sub>-7) δ<sub>C</sub> 154.3 (C-7). Through the analysis of NOESY spectrum was possible to strengthen the positions of methoxyl groups by correlations between the absorptions δ<sub>H</sub> 3.88 (*s*, OCH<sub>3</sub>-7) and δ<sub>H</sub> 6.53 (*s*, H-6). In addition to the interactions between absorptions at δ<sub>H</sub> 3.85 (*s*, OCH<sub>3</sub>-4') and δ<sub>H</sub> 7.12 (*d*, *J* = 9.0 Hz, H-3'/5'). Some others assignments for the two-dimensional spectra (HMBC and NOESY) are shown in Figure 2.

The combination of spectral analysis of IR spectroscopy, <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance, with aid of two dimensional techniques, and by the comparisons with literature data, have ratified the structure **5** as being 7,4'-di-O-methylisoscuteallarein, flavonoid unpublished in the Malvaceae family.

### Modulation of antibiotic activity

Acacetin and 7,4'-Di-O-methylisoscuteallarein showed no antibacterial activity at 256 μg/mL against any strains of *S. aureus* used. When the compounds were incorporated into the growing medium at 64 μg/mL (1/4 MIC), a two-fold reduction in the MIC was observed

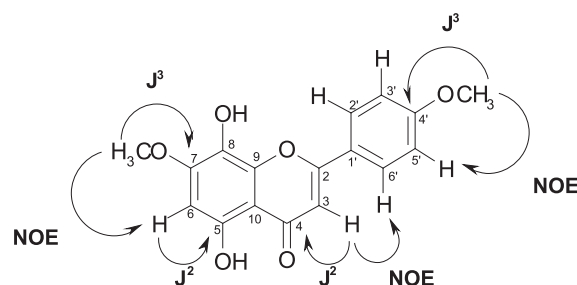


Figure 2. Some correlations observed at the HMBC (*J*<sup>a</sup>) and NOESY (NOE) spectra of **5**

for norfloxacin and ethidium bromide. Regarding tetracycline and erythromycin a two-fold reduction in the MIC was observed only with 7,4'-Di-O-methylisoscuteallarein. All experiments were carried out at least twice with similar results (Table 1).

Table 1. Minimum inhibitory concentrations (MICs) of antibiotics and ethidium bromide in the absence and presence of acacetin or 7,4'-Di-O-methylisoscuteallarein against effluxing strains of *Staphylococcus aureus* strain

Strain (drug)	MIC (μg/mL)		
	Alone	+ ACA (64 μg/mL)	+ DMIS (64 μg/mL)
1199B (norfloxacin)	64	32 (2) <sup>a</sup>	32 (2)
1199B (ethidium bromide)	32	16 (2)	16 (2)
IS-58 (tetracycline)	32	32(0)	16 (2)
RN-4220 (erythromycin)	256	256 (0)	128 (2)

ACA, acacetin; DMIS, 7,4'-Di-O-methylisoscuteallarein. <sup>a</sup> (fold reduction in MIC).

The flavonoids modulated activities of the antibiotics by reducing the concentration of antibiotics needed to inhibit the growth of the drug resistant effluxing bacteria. At least regarding tetracycline and erythromycin, 7,4'-Di-O-methylisoscuteallarein can be favorably compared with acacetin, which may be related to its higher lipophilicity due to an additional methoxyl group at C-7, since lipophilicity is a common feature of several EPIs and may be a key factor for the inhibition in Gram-positive bacteria.<sup>13</sup> Ethidium bromide is a well known substrate for NorA efflux protein, and active efflux is the only

known mechanism of resistance to this DNA-intercalating dyes.<sup>20</sup> Therefore, the use of ethidium bromide against the strain SA-1199B is of worth to demonstrate that the flavonoids evaluated in this case modulated the resistance to norfloxacin by inhibition of efflux pump.

Some methoxylated flavones that potentiate the activity of antimicrobial drugs have been described.<sup>21</sup> However, as far as we know, neither acacetin nor 7,4'-Di-O-methylisoscuteallarein had been previously evaluated. The present finding indicates that plants of the Malvaceae family could be useful as a source of potential adjuvant of antibiotics.

#### SUPPLEMENTARY MATERIAL

Available at <http://quimicanova.sbq.org.br>, in PDF file, with free access.

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Supplementary Material

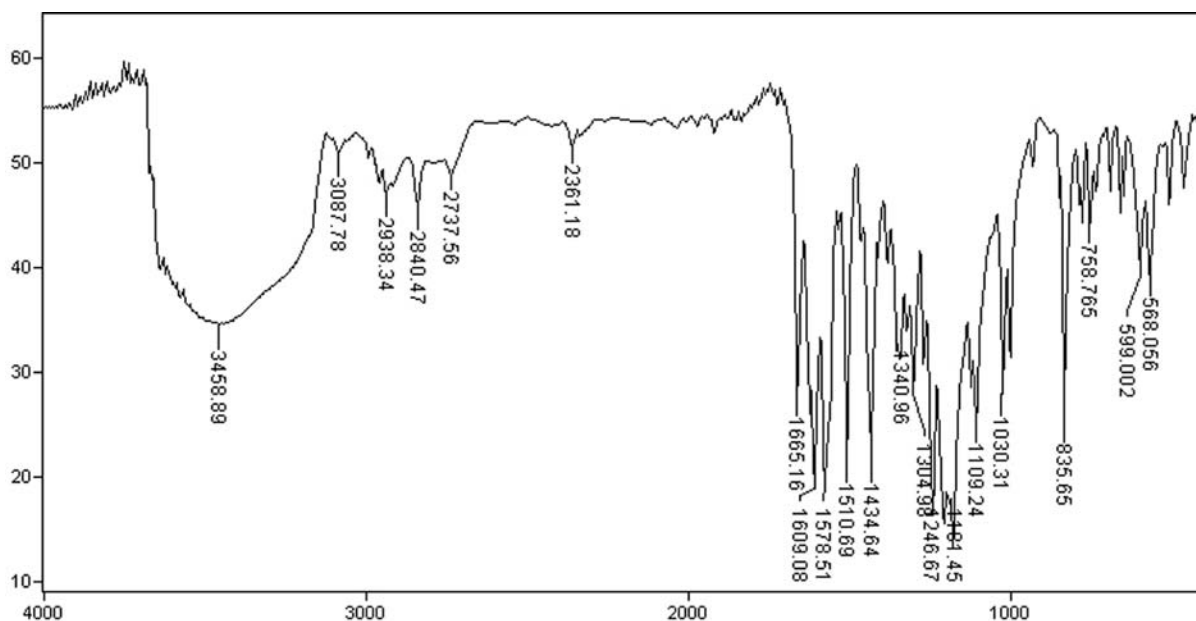


Figure 1S. IR spectrum of 5 (KBr,  $\text{cm}^{-1}$ )

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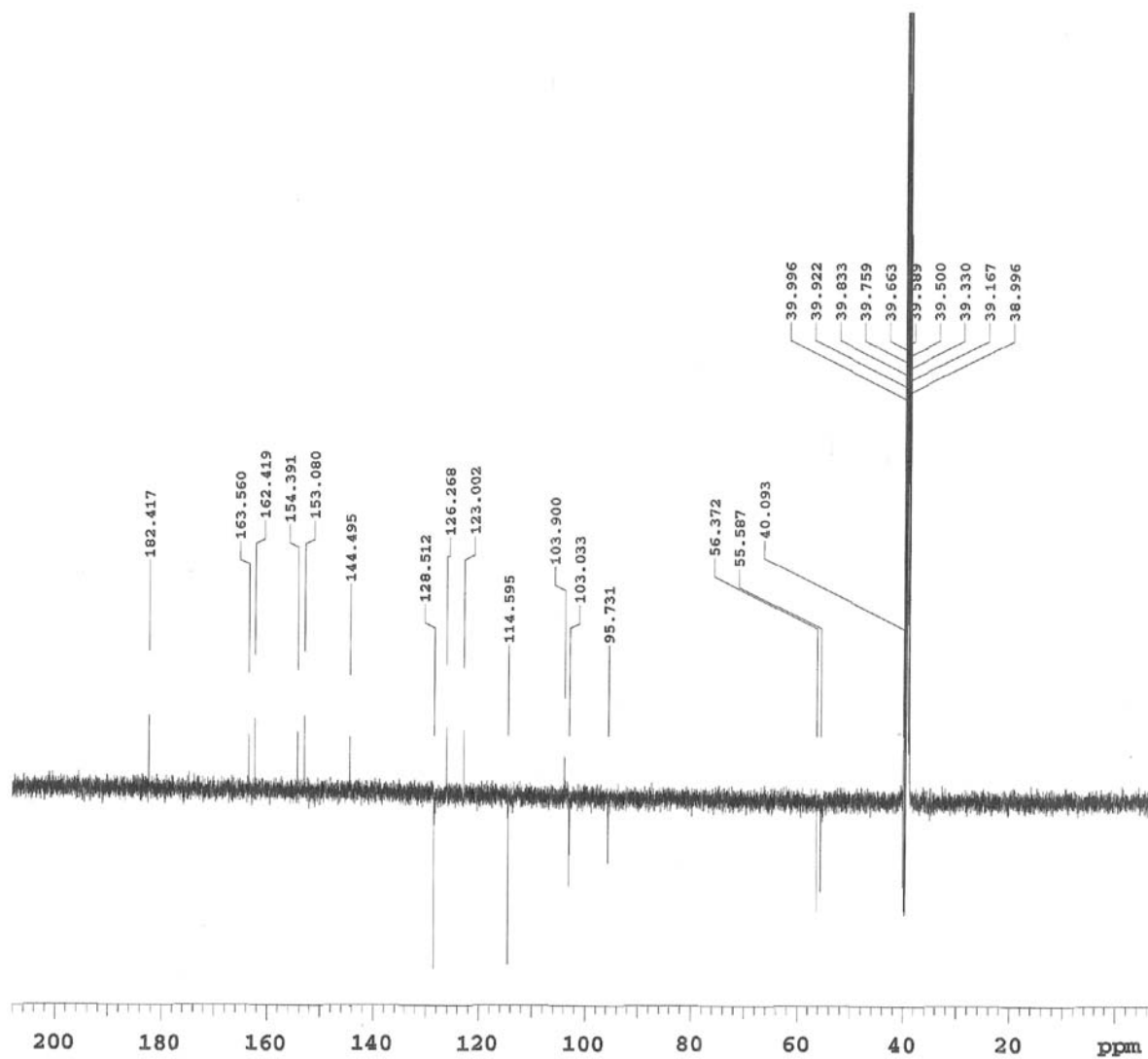


Figure 2S.  $^{13}\text{C}$  NMR spectrum of 5 ( $\delta$ ,  $\text{DMSO-}d_6$ , 125 MHz)

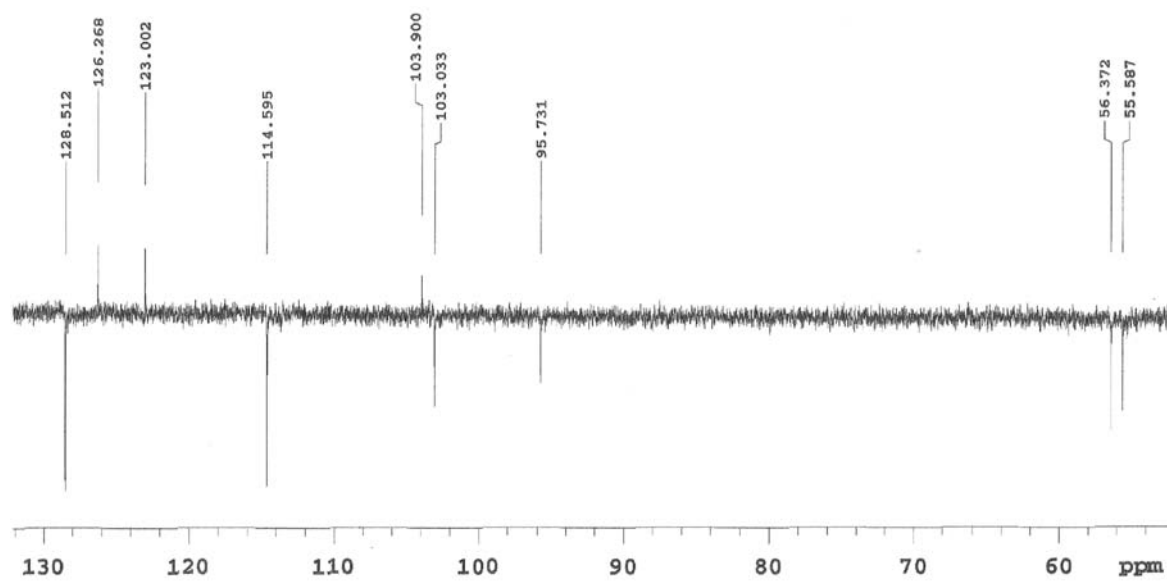


Figure 3S. Expansion of the  $^{13}\text{C}$  NMR spectrum of 5 ( $\delta$ ,  $\text{DMSO-}d_6$ , 125 MHz)

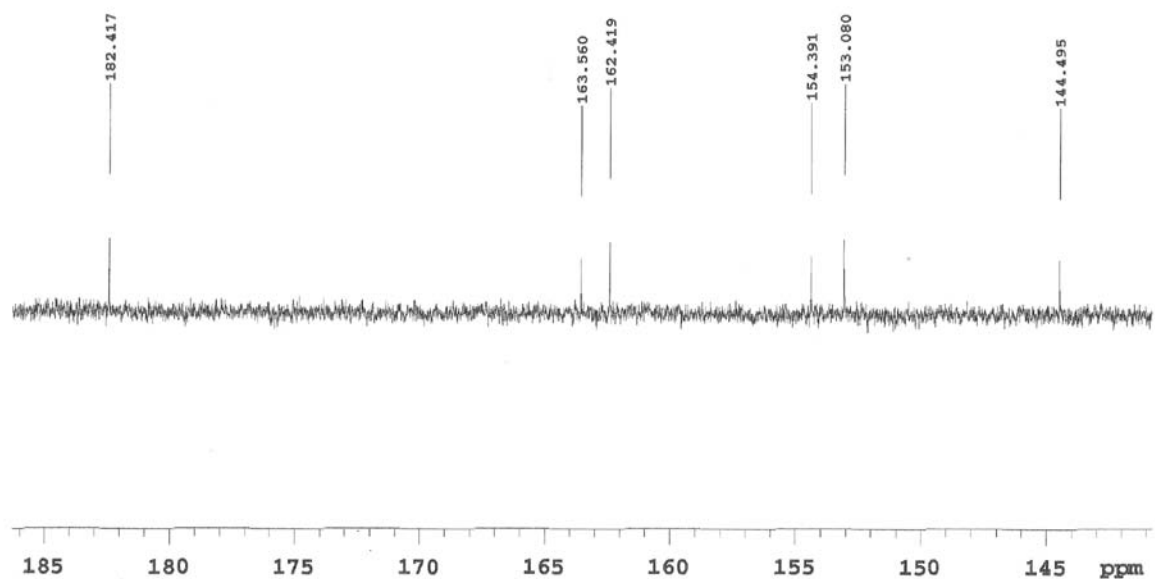


Figure 4S. Expansion of the  $^{13}\text{C}$  NMR spectrum of **5** ( $\delta$ ,  $\text{DMSO-}d_6$ , 125 MHz)

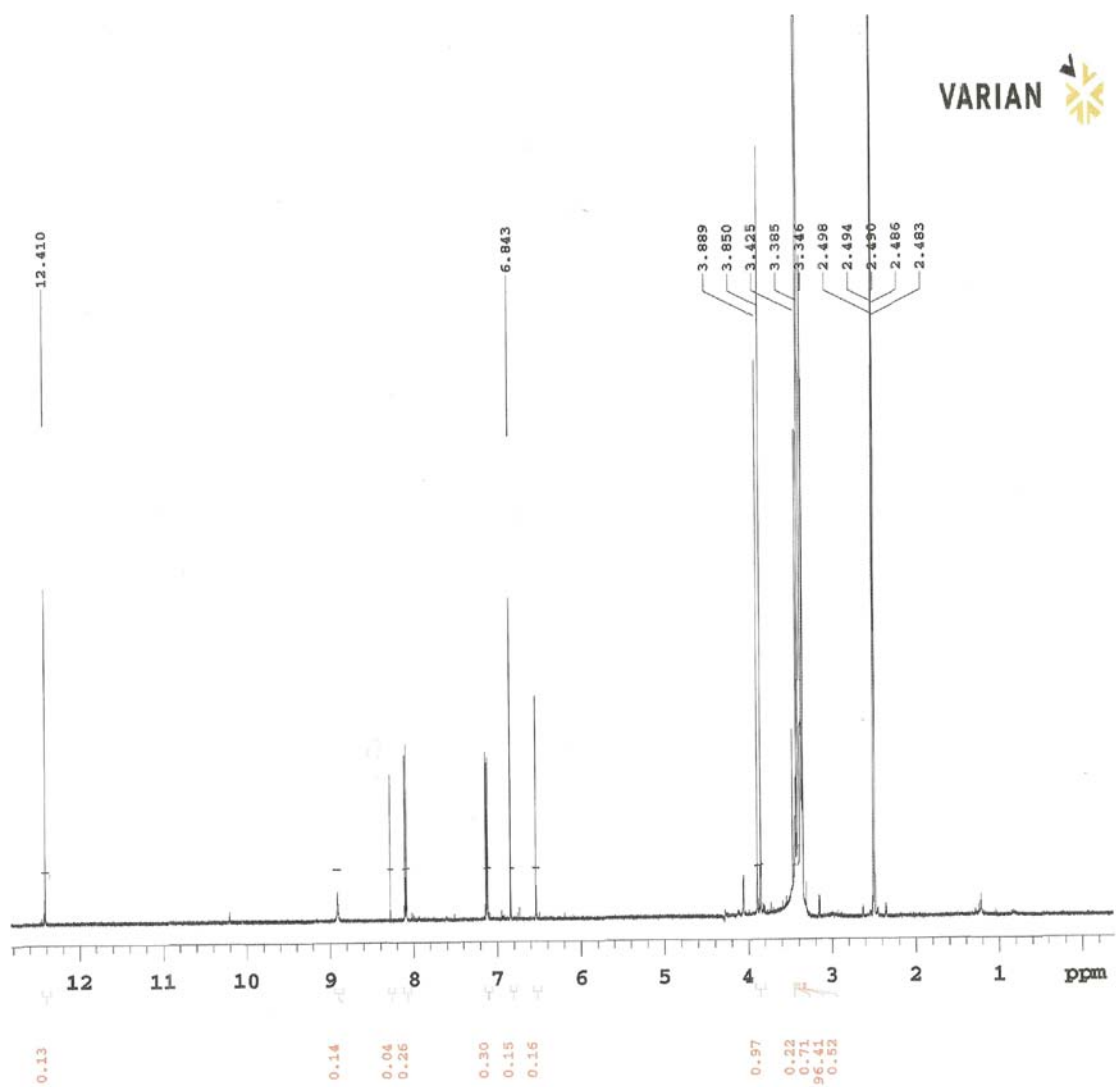


Figure 5S.  $^1\text{H}$  NMR spectrum of **5** ( $\delta$ ,  $\text{DMSO-}d_6$ , 500 MHz)

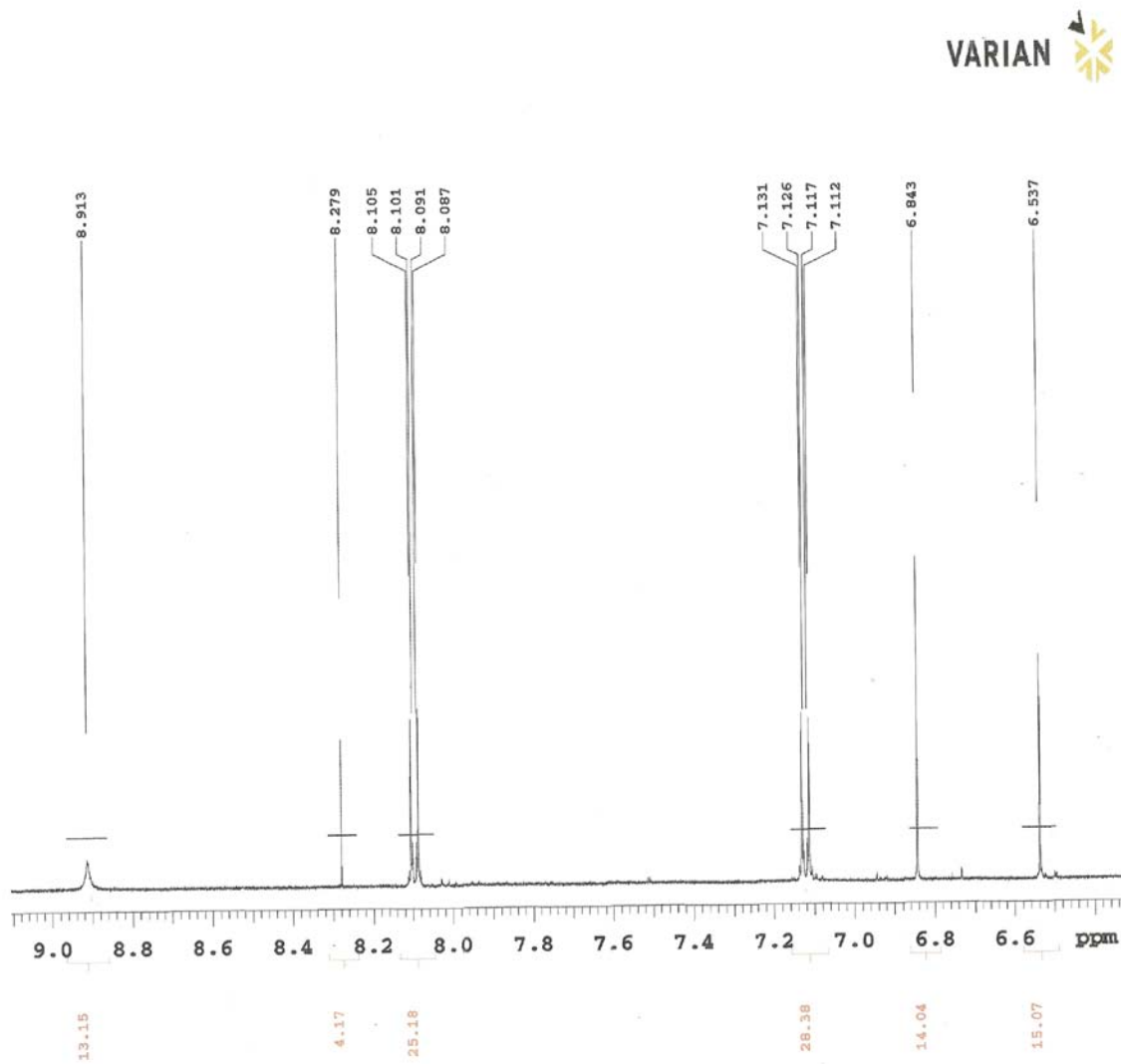


Figure 6S. Expansion of the  $^1\text{H}$  NMR spectrum of **5** ( $\delta$ ,  $\text{DMSO-d}_6$ , 500 MHz)



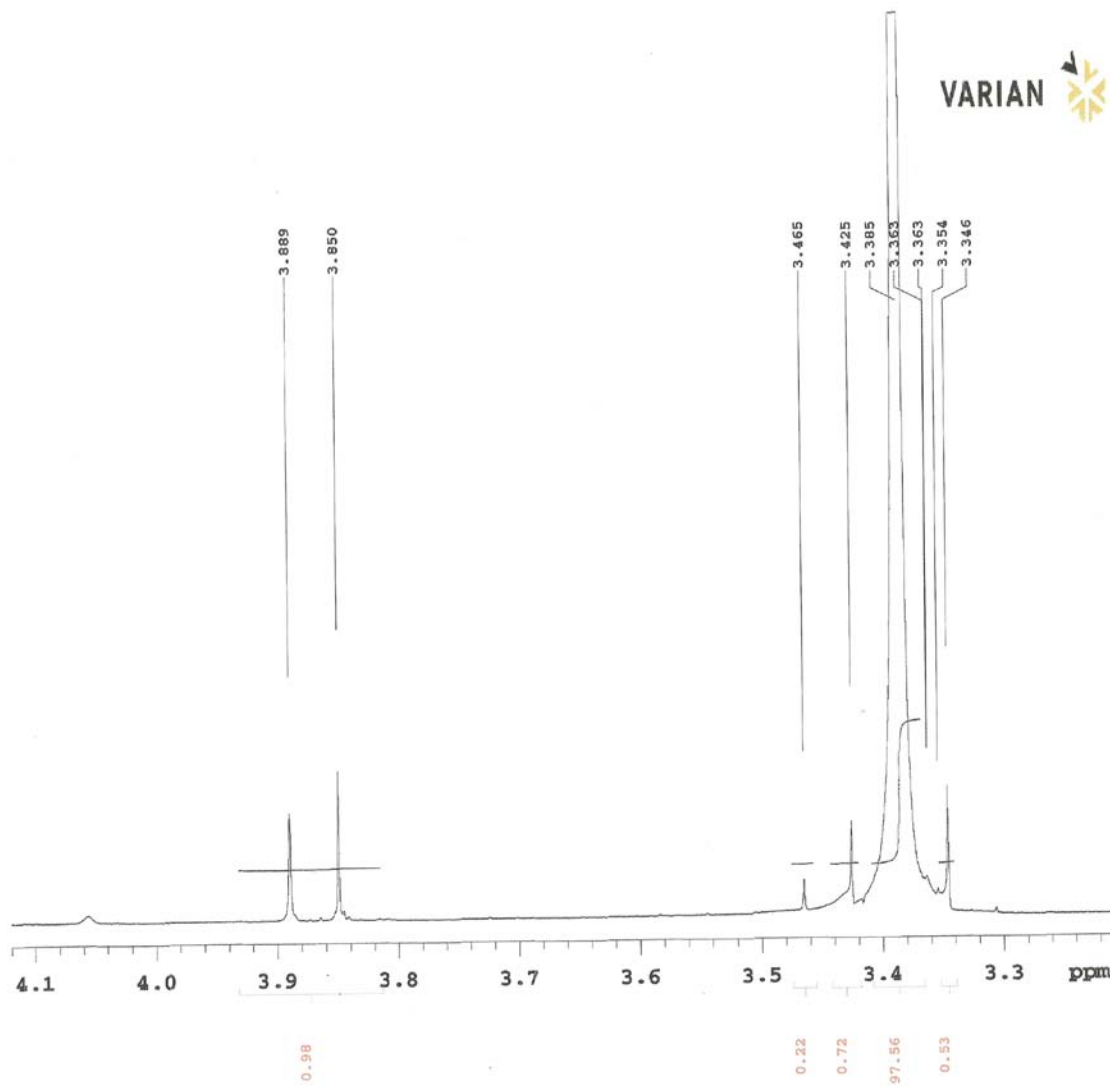


Figure 7S. Expansion of the  $^1\text{H}$  NMR spectrum of **5** ( $\delta$ ,  $\text{DMSO-}d_6$ , 500 MHz)

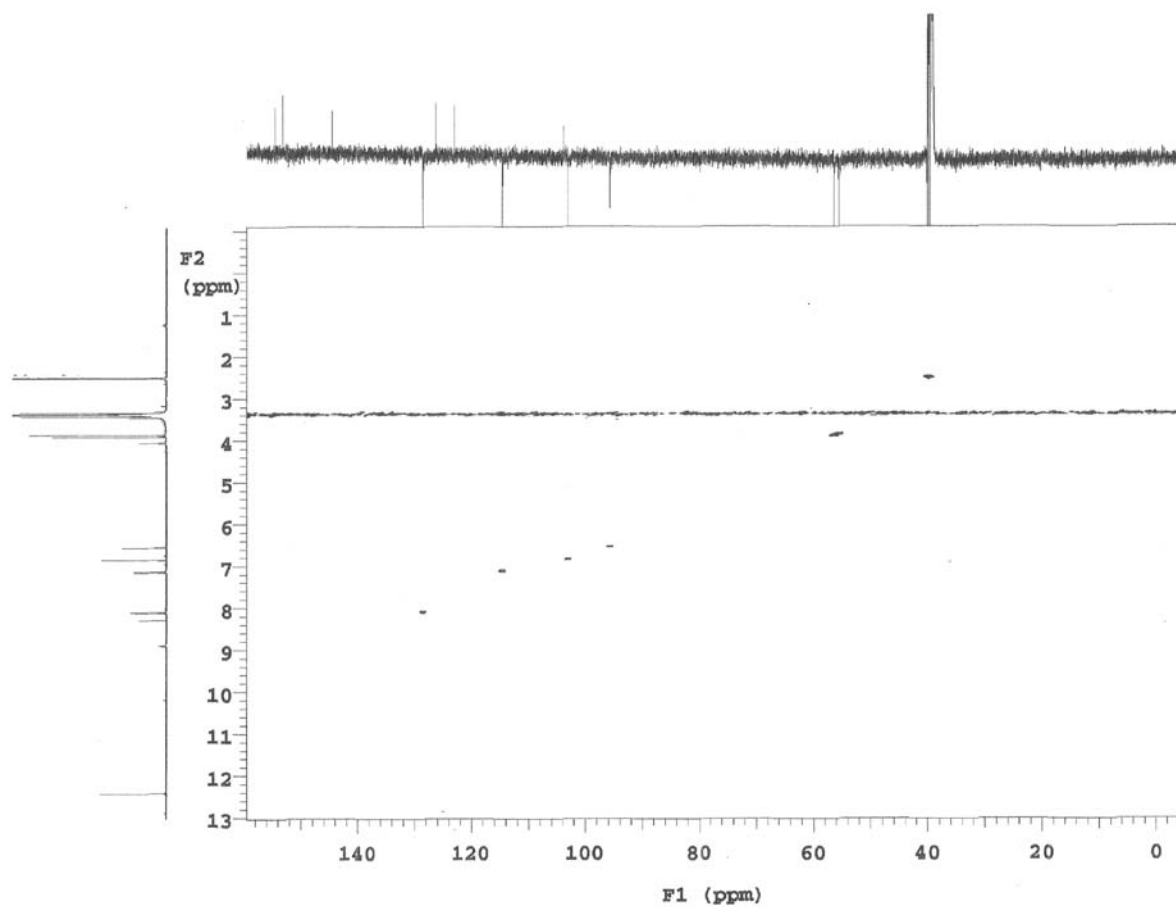


Figure 8S.  $^1\text{H} \times ^{13}\text{C}$ -HMQC NMR spectrum of **5** ( $\delta$ ,  $\text{DMSO-}d_6$ , 500 MHz)

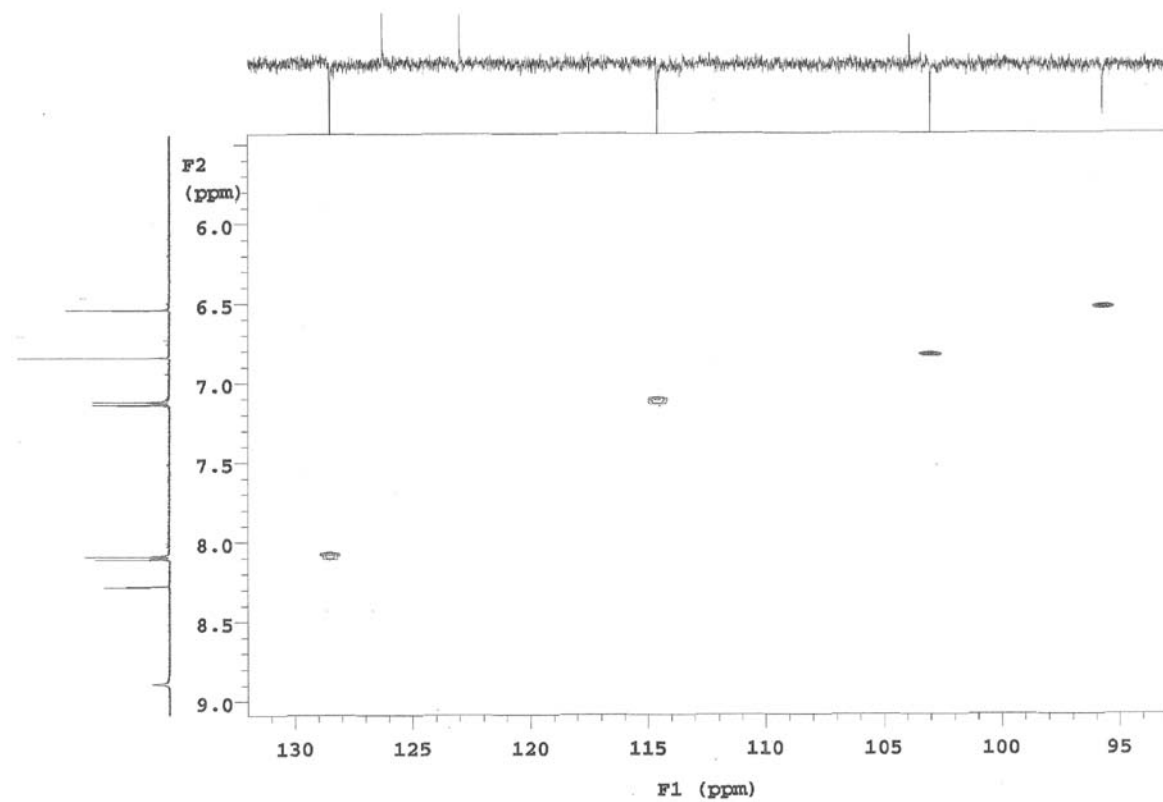


Figure 9S. Expansion of the  $^1\text{H} \times ^{13}\text{C}$ -HMQC NMR spectrum of **5** ( $\delta$ ,  $\text{DMSO-}d_6$ , 500 MHz)

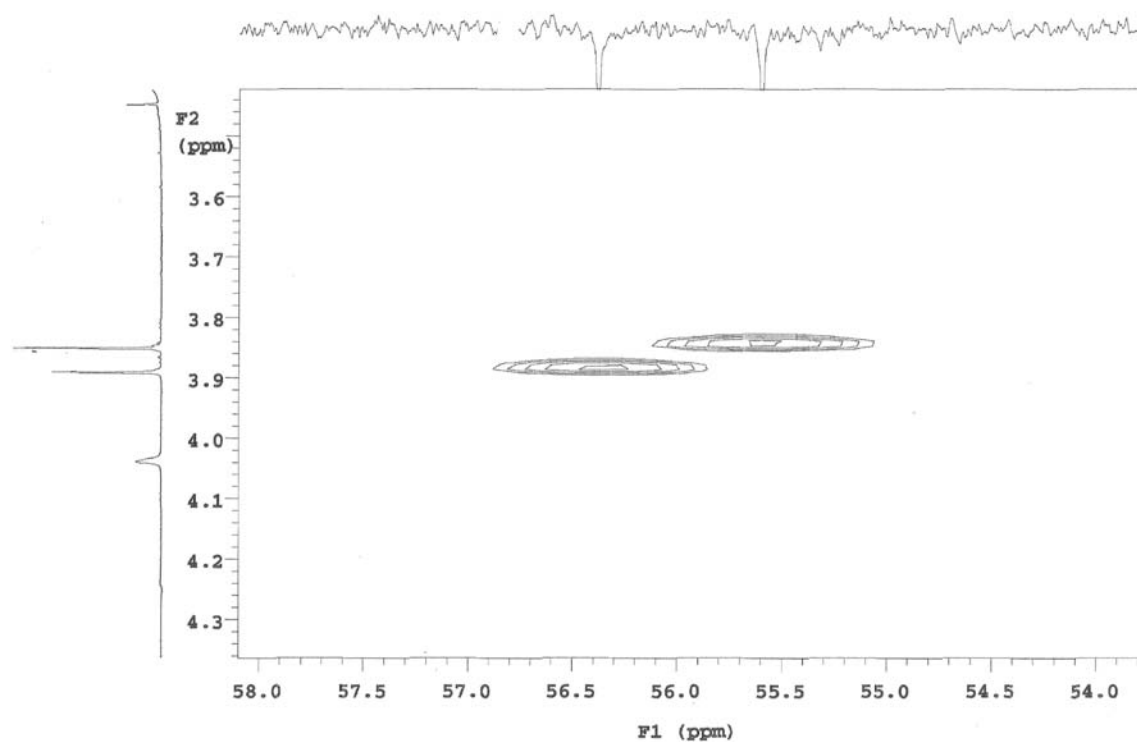


Figure 10S. Expansion of the  $^1\text{H} \times ^{13}\text{C}$ -HMQC NMR spectrum of 5 ( $\delta$ ,  $\text{DMSO}-d_6$ , 500 MHz)

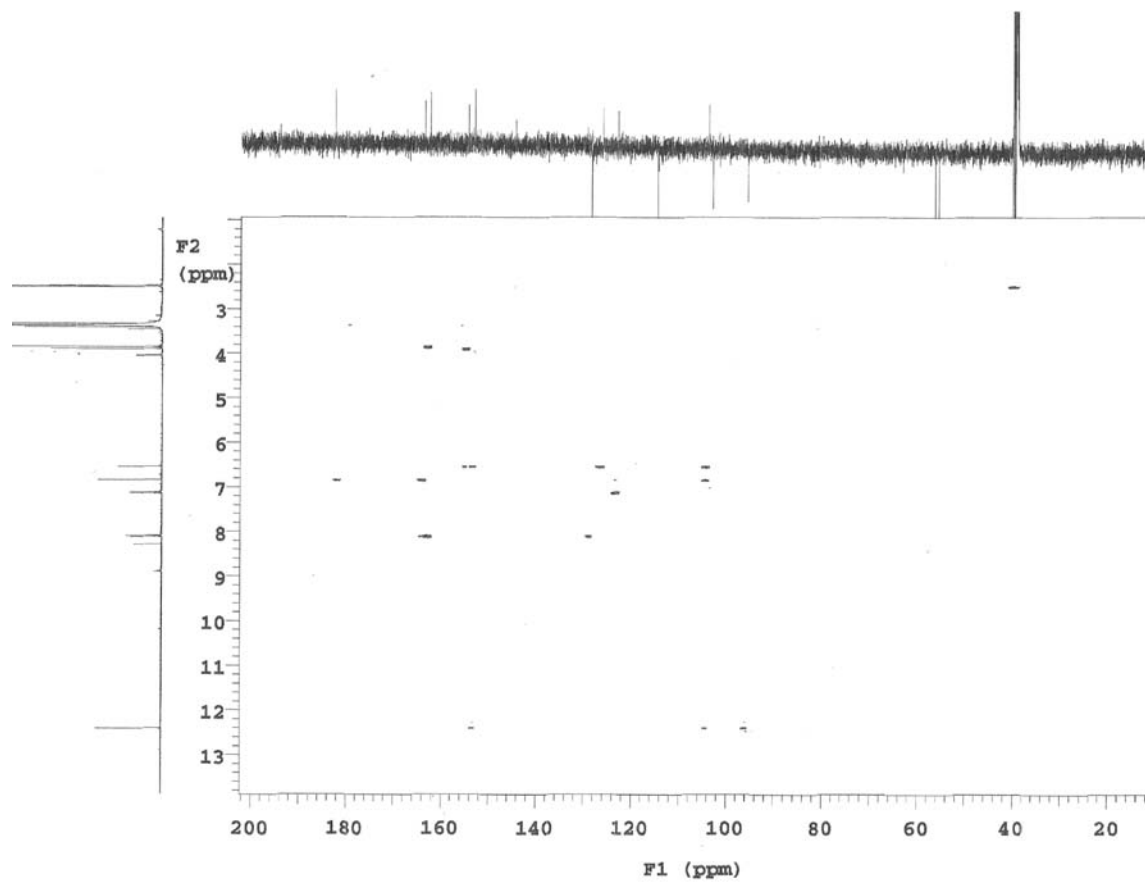


Figure 11S.  $^1\text{H} \times ^{13}\text{C}$ -HMBC NMR spectrum of 5 ( $\delta$ ,  $\text{DMSO}-d_6$ , 500 MHz)

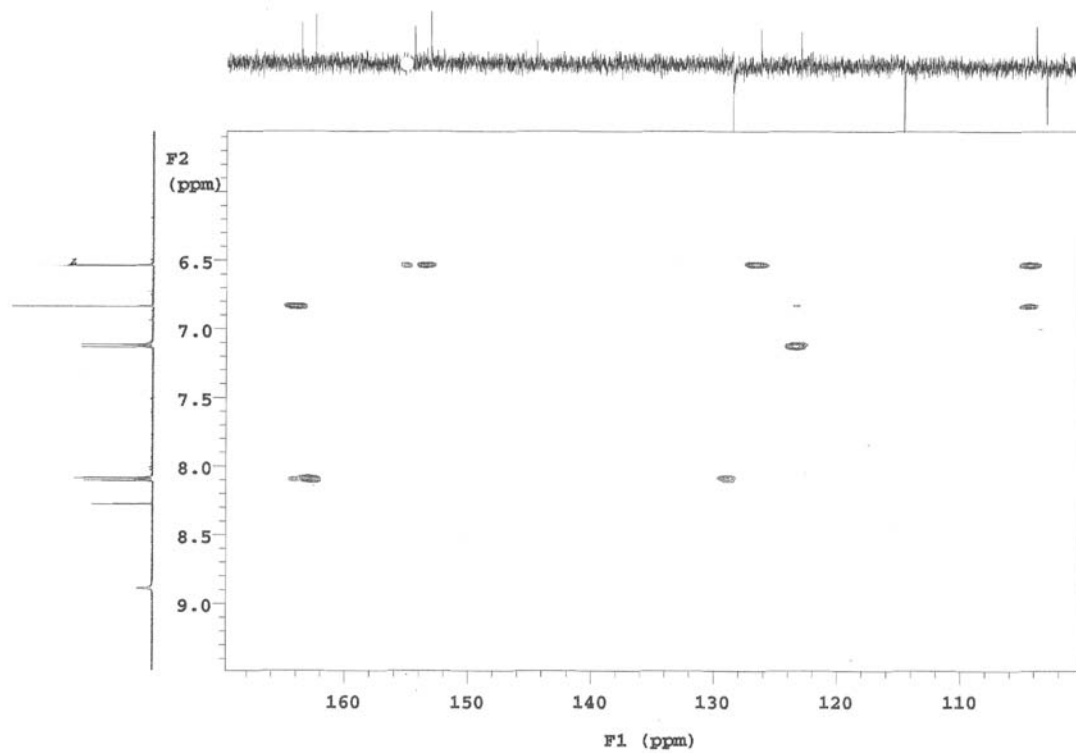


Figure 12S. Expansion of the  $^1\text{H} \times ^{13}\text{C}$ -HMBC NMR spectrum of 5 ( $\delta$ ,  $\text{DMSO}-d_6$ , 500 MHz)

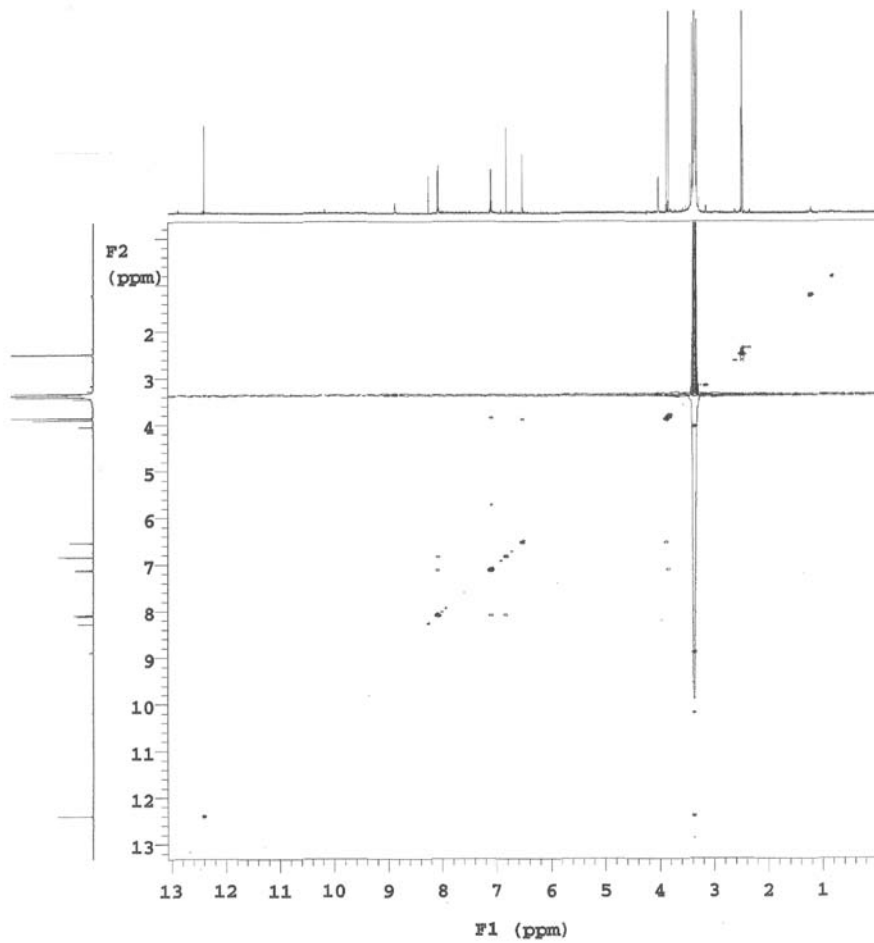


Figure 13S.  $^1\text{H} \times ^1\text{H}$ -NOESY NMR spectrum of 5 ( $\delta$ ,  $\text{DMSO}-d_6$ , 500 MHz)

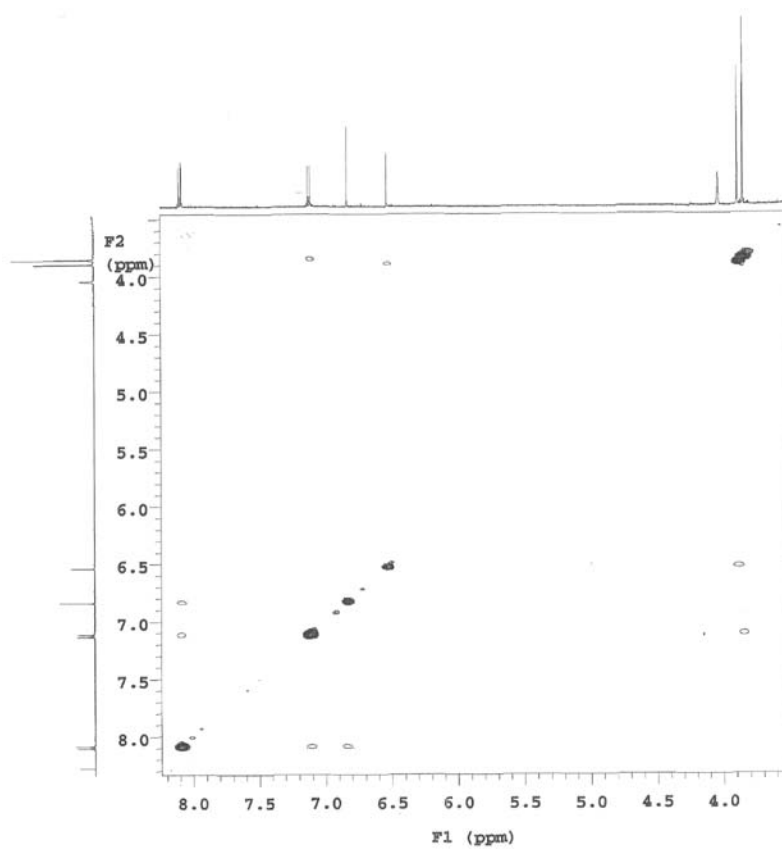


Figure 14S. Expansion of the  $^1\text{H} \times ^1\text{H}$ -NOESY NMR spectrum of 5 ( $\delta$ ,  $\text{DMSO}-d_6$ , 500 MHz)

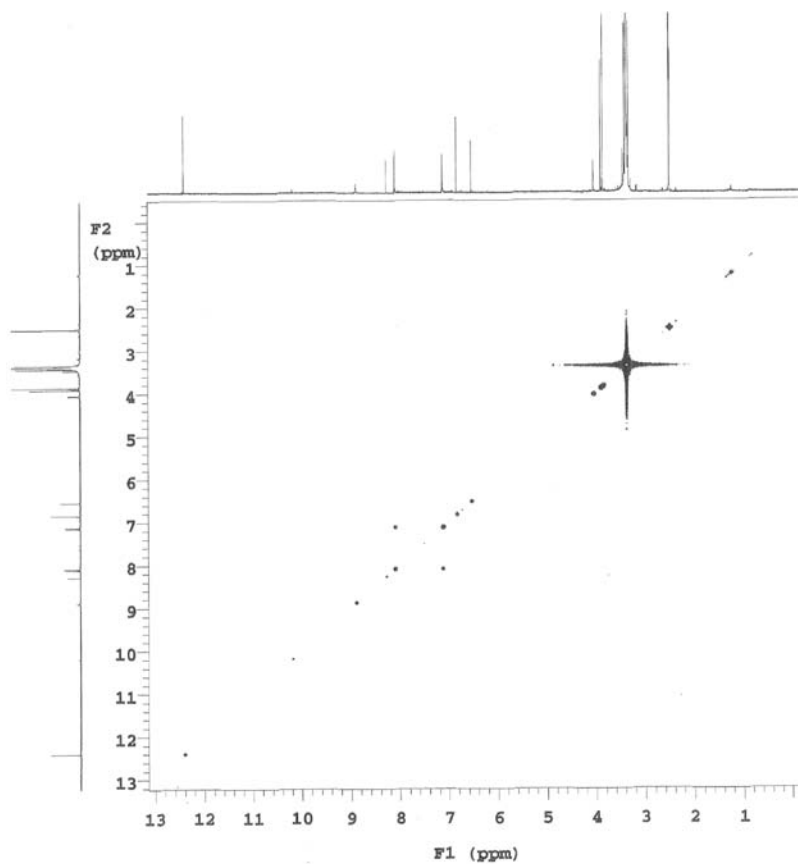
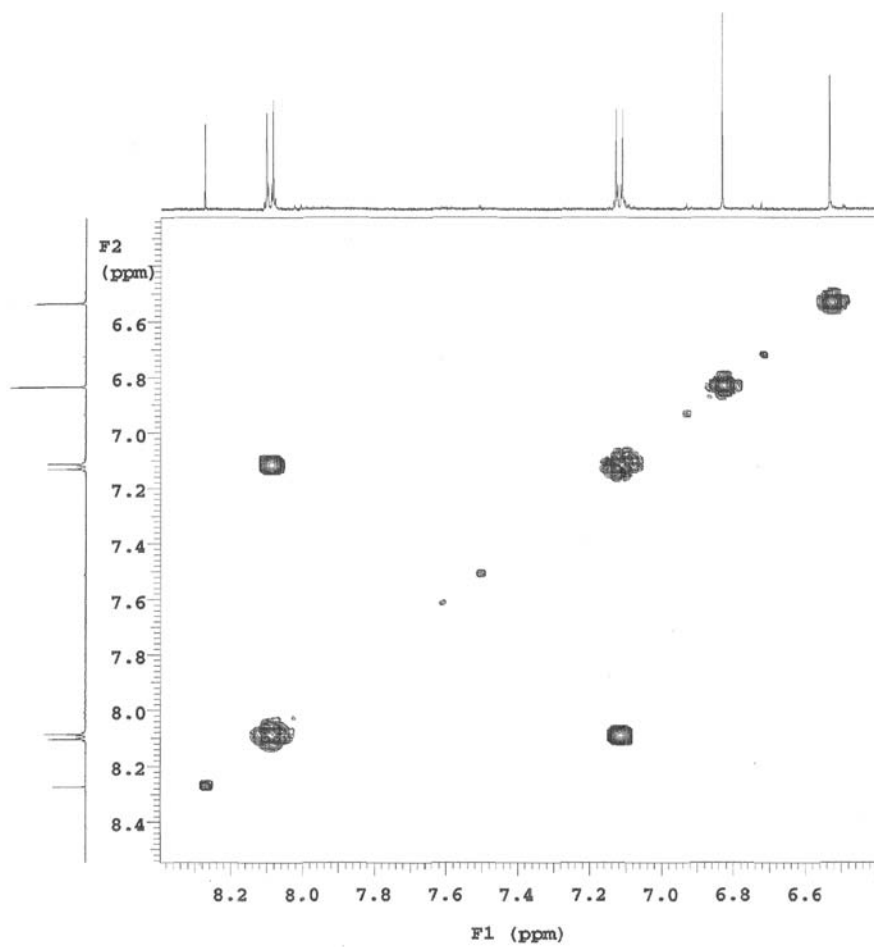


Figure 15S.  $^1\text{H} \times ^1\text{H}$ -COSY NMR spectrum of 5 ( $\delta$ ,  $\text{DMSO}-d_6$ , 500 MHz)



**Figure 16S.** Expansion of the  $^1\text{H} \times ^1\text{H}$ -COSY NMR spectrum of **5** ( $\delta$ ,  $\text{DMSO-}d_6$ , 500 MHz)