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New minor phytoecdysteroids from the juice of *Serratula coronata* L. (Asteraceae)

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Abstract
Minor phytoecdysteroids - ajugasterone C 2-, 3-, and 11-acetates and calonysterone were isolated for the first time from the Serratula coronata plant. Out of these, ajugasterone 11-acetate is new ecdysteroid.

Keywords: Phytoecdysteroids, isolation, identification, ajugasterone C acetates, calonysterone

1. Introduction
Phytoecdysteroids are isolated for the first time from plants of *Podocarpus nakaii* (Nakanishi *et al.* 1966) [13] and *Taxus cuspidata* (Takemoto *et al.* 1968) [19]. Currently they are abundant in many plant species, being analogues of insect steroid hormones (Lafont, 1998) [11] and they are produced in plants for protection from phytophagous insects (Dinan, 1998) [7]. Phytoecdysteroids are non-toxic for mammals, they possess various pharmacological effects and are of interest for medicine (Bathori 2008, Dinan, 2001, 2006; *et al.*) [1, 6, 8].
Serratula coronata L. is one of the most promising sources of phytoecdysteroids (Volodin, *et al.* 1998) [20]. We previously reported the isolation of eighteen phytoecdysteroids from the juice of *Serratula coronata* (Odinokov *et al.* 2002, 2005) [14, 15]. We now report the isolation and identification of four minor phytoecdysteroids from the low polar fraction of *Serratula coronata* juice. One of them is new phytoecdysteroid, ajugasterone C 11-acetate (3). Ajugasterone C 2-acetate (1), ajugasterone C 3-acetate (2) and calonysterone (4) were isolated for the first time from *Serratula coronata.*

2. Experimental
2.1. Plant material
The *Serratula coronata* plants were gathered in June 2013 in the Karmaskalinsky region (Republic of Bashkortostan, Russian Federation). The plants were classified by A. A. Muldashev, Candidate of Biology, senior researcher of the Institute of Biology, Ufa Scientific Center, Russian Academy of Sciences.

2.2. Extraction and isolation
From 250 kg of fresh *Serratula coronata* plants (32 kg of air dried material) 112 L of juice was obtained by a juice squeezer. The juice was filtered through a folded filter, and the filtrate was concentrated to 5 L on a rotary evaporator at a residual pressure of 30-40 Torr and bath temperature not exceeding 80ºC. The concentrate was cooled down to room temperature and extracted into ethyl acetate (10 × 30 L), and the extract was concentrated (60ºC, 30-40 Torr) to a 6 L volume and cooled down to room temperature. The precipitated crystals of 20-hydroxyecdysone were filtered off [(270 g, m.p. 240-241°C, the ^{1}H and ^{13}C NMR spectra are identical to those reported previously (Odinokov *et al.* 2002) [14]]. The filtrate was evaporated to dryness, which gave 112 g of a light-yellow solid residue. Column chromatography of the residue (SiO_2, elution with MeOH-$CHCl_3$, 1:20) gave fractions A (TLC: R_f 0.6, *Silufol*, MeOH-$CHCl_3$, 1:5), B (TLC: R_f 0.5, *Silufol*, MeOH-$CHCl_3$, 1:5), and C (TLC: R_f 0.4, *Silufol*, MeOH-$CHCl_3$, 1:5). Evaporation of fraction A resulted in 0.1 g of a crystalline residue, HPLC of which gave 20-hydroxyecdysone 2-acetate [(τ 8.48 min, $14 \cdot 10^{-3}$ g, $4 \cdot 10^{-5}$%, mp 222-223 °C, $[\alpha]_D^{20}$ +55.6 ° (c 0.96; MeOH); lit.: mp 219-220 °C, $[\alpha]_D^{20}$ +54.0 ° (c 0.98, MeOH), with the ^{1}H and ^{13}C NMR spectra identical to the reported data (Odinokov, *et al.* 2005) [15],

3-acetate 2 (9.55 min, $14 \cdot 10^{-3}$ g, $4 \cdot 10^{-5}$%), 26(*S*)-inokosterone acetate [(τ=11.34 min, $11 \cdot 10^{-3}$ g $3 \cdot 10^{-5}$%, mp 250-251 °C, $[\alpha]_D^{20}$ +57.4° (c 0.78, MeOH), with the ^{1}H and ^{13}C NMR spectra identical to the reported data (Odinokov, *et al*., 2005) [15]], and 11-acetate 3 (16.88 min, $10 \cdot 10^{-3}$ g, $3 \cdot 10^{-5}$%). Separation of fraction B resulted in 0.083 g of a crystalline residue, HPLC of which gave hydroquinone [(τ=6.18 min, $8.3 \cdot 10^{-3}$ g, $4 \cdot 10^{-5}$%, mp 171-172 °C, $[\alpha]_D^{20}$ +57.4° (c 0.76, MeOH); lit.: mp 174.5-175 °C, $[\alpha]_D^{20}$ +54.0° (c 0.82, MeOH), with the ^{1}H and ^{13}C NMR spectra identical to the reported data (Odinokov *et al*., 2014) [16] and calonysterone 4 (τ 8.48 min, $15 \cdot 10^{-3}$ g, $5 \cdot 10^{-5}$%). Evaporation of fraction C resulted in 0.122 g of a crystalline residue, HPLC of which gave 2-acetate 1 (τ 17.00 min, $12.6 \cdot 10^{-3}$ g, $4 \cdot 10^{-5}$%) and ecdysone 22-acetate [(τ=22.43 min, $12 \cdot 10^{-3}$ g, $4 \cdot 10^{-5}$%, mp 144-145 °C, $[\alpha]_D^{21}$ +51.0 ° (c 1.00, EtOH); lit.: mp 147-148 °C, $[\alpha]_D^{20}$ +49.5 ° (c 0.96, MeOH), with the ^{1}H and ^{13}C NMR spectra identical to the reported data (Odinokov, *et al*., 2005) [15].

2-O-Acetylajugasterone C (1): white crystals, mp 249-250 °C, $[\alpha]_D^{21}$+65.0 ° (c 0.48, EtOH); MS MALDI-TOF 545.3093 $[M+Na]^+$, calcd for $C_{29}H_{46}O_8$+Na 545.3090; for ^{1}H and ^{13}C spectroscopic data, see Tables 1 and 2. Elemental analysis results for compound (1): found C, 66.58; H, 8.80, calcd for $C_{29}H_{46}O_8$: C, 66.63; H, 8.81%.

3-O-Acetylajugasterone C (2): white crystals, mp 242-243 °C, $[\alpha]_D^{21}$ +62.0 ° (c 0.65, EtOH); lit.: mp 234-235 °C, $[\alpha]_D^{20}$ +68.5 ° (c 0.92, MeOH); NMR data (D_2O) were identical to those reported previously (Crouzet *et al*., 2009) [4]. MS MALDI-TOF 545.3088 $[M+Na]^+$, calcd for $C_{29}H_{46}O_8$+Na 545.3090; NMR data (CD_3OD and C_5D_5N) see Tables 1 and 2. Elemental analysis results for compound (**2**): found C, 66.67; H, 8.85, calcd for $C_{29}H_{46}O_8$: C, 66.63; H, 8.81%.

11-O-Acetylajugasterone C (3): white crystals, mp 247-248 °C, $[\alpha]_D^{21}$ +58.0 ° (c 0.68, EtOH), MS MALDI-TOF 545.3089 $[M+Na]^+$, calcd for $C_{29}H_{46}O_8$+Na 545.3090; NMR data see Tables 1 and 2. Elemental analysis results for compound (3): found C, 66.68; H, 8.87, calc. for $C_{29}H_{46}O_8$: C, 66.63; H, 8.81%.

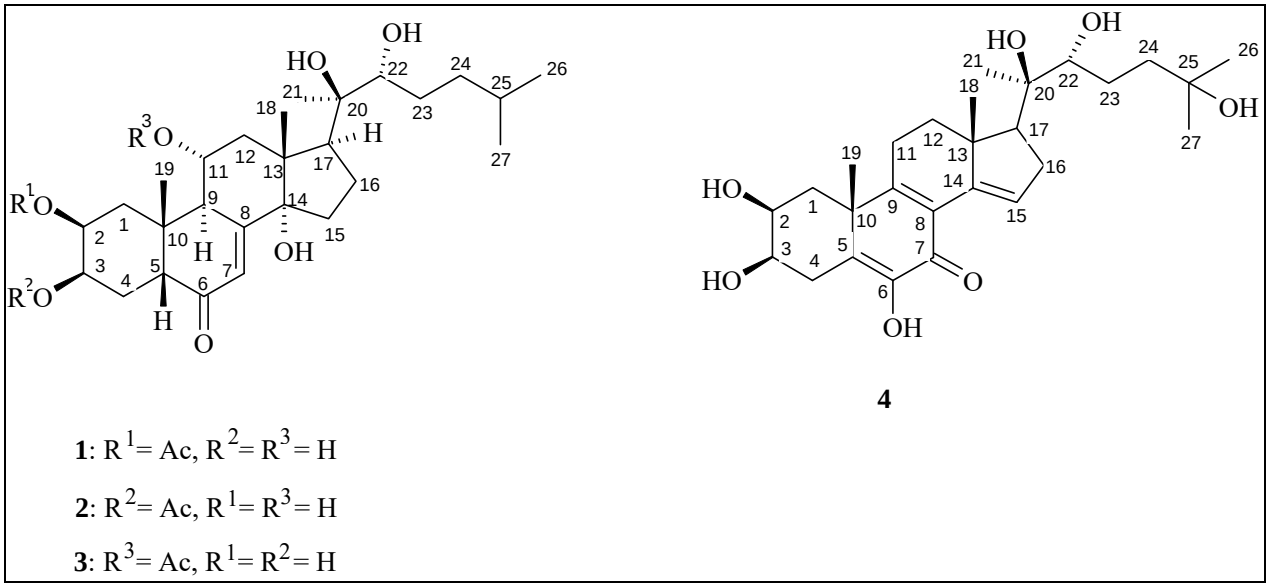
Calonysterone (4): white crystals, mp 252-253 °C, $[\alpha]_D^{21}$ +45.0 ° (c 0.72, EtOH), lit.: mp 234-235 °C, $[\alpha]_D^{20}$ +76.8 ° (c 1.00, MeOH) (Canonica, *et al*.,1975) [3]; MALDI-TOF 499.2672 $[M+Na]^+$, calcd $C_{27}H_{40}O_7$+Na 499.2669; NMR data (C_5D_5N) see Tables 1 and 2. Elemental analysis results for compound (4): found C, 68.02; H, 8.46, calc. for $C_{27}H_{40}O_7$: C, 67.98; H, 8.39%.

2.3. General experimental procedure

One-dimensional (^{1}H and ^{13}C) and two-dimensional (COSY, NOESY, HSQC, and HMBC) NMR spectra were recorded on a Bruker Avance 500 spectrometer (500.17 MHz for ^{1}H and 125.78 MHz for ^{13}C) and NMR spectra were recorded on a Bruker Ascend 400 spectrometer (400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C). Mass spectra were measured by MALDI-TOF methods on a Bruker Autoflex III spectrometer with registration of positive ions. Elemental analysis was performed on a 1106 Carlo Erba apparatus. Melting points were determined on Boetius hot stage. Column chromatography and TLC were performed using silica gel (≤0.06 mm) and pre-coated silica gel (Silufol plates), respectively; spots were processed by treatment with a solution of 4-hydroxy-3-methoxybenzaldehyde in ethanol, acidified with sulfuric acid. HPLC was performed on a liquid chromatography Shimadzu LC-20 Prominence (column – Reprosil - Pur C_{18}-AQ, 5μ, 250×10 mm, the eluent CH_3CN – H_2O, 40:60, flow rate 2 ml/min, λ 242 Nm).

3. Results and discussion

The structures of compounds 1-4 were established by homo- and heteronuclear correlation ^{13}C and ^{1}H NMR spectroscopy and MALDI-TOF/TOF mass spectrometry. The MALDI-TOF/TOF mass spectra of compounds 1-3 exhibit $[M+Na]^+$ molecular ions (m/z 545.3093, 545.3088, and 545.3089, respectively) corresponding to ajugasterone C monoacetate (calc. m/z 545.3090). The presence of acetate group in molecules 1-3 is indicated by signals at δ_c 172.1-172.8 and 21.3-21.8 ppm and $\delta_н$ 2.07-2.13 ppm (^{13}C and ^{1}H NMR spectra in CD_3OD) (Tables 1 and 2)



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1: R^1= Ac, R^2= R^3= H

2: R^2= Ac, R^1= R^3= H

3: R^3= Ac, R^1= R^2= H

Fig 1

The position of the acetate group was derived from the HSQC and HMBC spectra. The HMBC spectrum of compound 1 (CD_3OD) shows a cross-peak for the coupling between the C5 carbon (δ 52.7 ppm) and the H-3 proton (δ 4.20 ppm), which is linked to C3 (δ 65.9 ppm) (HSQC spectrum). The cross-peak between the H-9 proton (δ 3.21 ppm) and the δ 69.7 ppm

signal (HMBC spectrum) indicates that the latter is due to C11. Since the ^{13}C NMR chemical shifts of the signals for C3 and C11, like that for C22 (δ 78.1 ppm), are the same for compound 1 and ajugasterone C (Odinokov *et al*. 2002) [14], these atoms are not bonded to the acetate group, while the signal that shifts downfield (δ 73.4 ppm) belongs to C2. Thus, compound 1 is ajugasterone C 2-acetate, mentioned in the patent (Meybeck *et al*., 1993) [12], as one of ecdysteroids used in cosmetic compositions and dermatological compositions, but it spectra were not given. NMR ^{1}H and ^{13}C spectra were recorded in CD_3OD, C_5D_5N and D_2O and are classification are all signals ajugasterone C 2-acetate (Tables 1 and 2).

Unlike 2-acetate 1, in compound 2, the signal for C2, which was assigned relying on the cross-peak with the axial H-1β proton (δ 1.40 ppm) in the HMBC spectrum recorded in CD_3OD, occurred at δ 67.4 ppm. Meanwhile, the signal for C3 (δ 72.1 ppm) of 2 shifted downfield ($\Delta\delta_c$ 3.5 ppm), which supports the identification of this compound as ajugasterone C 3-acetate. The ^{1}H and ^{13}C NMR spectra in C_5D_5N and D_2O were also recorded for 2. The ^{1}H and ^{13}C NMR spectra of 2 in D_2O are identical to the spectra of ajugasterone C 3-acetate isolated recently from *Cyanotis longifolia* (Crouzet *et al.* 2009) [4].

The HMBC spectrum of compound 3 shows a cross-peak between the H-9 proton signals (δ 3.47 ppm) and the carbon signal at δ 72.9 ppm belonging to C11, which has shifted downfield ($\Delta\delta_c$ 3.4 ppm) relative to the corresponding ^{13}C NMR signal of ajugasterone C (Odinokov *et al.* 2002) [14]. This is indicative of C11-position of the acetoxy group in compound 3, a previously unknown ecdysteroid, ajugasterone C 11-acetate. The cross-peak between the H-11 proton (δ 5.34 ppm) and β-$CH_3$18 protons (δ 0.95 ppm) and β-$CH_3$19 protons (δ 1.04 ppm) in the NOESY spectrum shows its β-configuration and the α-orientation of the 11-methoxy group in compound 3, accordingly.

Calonysterone (4) is a rare phytoecdysteroid of unusual structure isolated previously from *Ipomoea calonyction* (Canonica *et al.* 1973, 1975) [2, 3], *Vitex canescens* (Suksamrarn *et al*. 1997) [18], and *Asparagus dumosus* (Khaliq-uz-Zaman *et al*. 2000) [9]. Calonysterone was also reported to form upon autooxidation of 20-hydroxyecdysone in aqueous methanol in the presence of NaOH (Suksamrarn *et al.* 1994; Csabi *et al*. 2015) [17, 5].

Earlier, the calonysterone structure was determined for the first time by IR, UV, ^{1}H NMR spectroscopy and mass spectrometry (Canonica *et al.* 1973) [2]. For the ^{1}H and ^{13}C NMR spectra of calonysterone recorded in C_5D_5N or $(CD_3)_2SO$, no full signal assignment was given (Lafont *et al.* 2015) [10] or the assignment was ambiguous (Khaliq-uz-Zaman *et al*. 2000) [9]. Recently (Csabi *et al.* 2015) [5], the ^{1}H and ^{13}C NMR spectra (in CD_3OD) of semisynthetic calonysterone (Suksamrarn *et al.* 1994) [17] were recorded, and the signals were assigned resorting to 1D and 2D HSQC, HMBC, and ROESY experiments.

We isolated calonysterone for the first time from the *Serratula coronata* juice and confirmed its structure by 1D and 2D NMR experiments (COSY, HSQC, HMBC). All of the C and H signals in the spectra of 4 recorded in C_5D_5N were assigned (Tables 1 and 2).

The NOESY correlation between the H-4β (δ 3.13 ppm) and H_3-19 (δ 1.77 ppm) protons (Fig. 1) identifies a chair conformation ($_3C^{10}$) of ring A. In this conformation, H-2 is in the equatorial position and H-3 is in the axial position, whereas for most ecdysteroids, H-2 is axial and H3 is equatorial. As a result, inversion of H-2 and H-3 signal positions is observed in the ^{1}H NMR spectrum of calonysterone: the H-2 signal is located in a lower field (δ 4.46 ppm) and appears as a broadened singlet, while the H-3 signal is in a higher field (δ 4.07 ppm) and is a doublet (J 12 Hz). The same constant (J 12 Hz) is observed for coupling of the axial H-4β proton with the transoid H-3 proton and the geminal H-4α proton (δ 3.87 ppm), which is consistent with the chair conformation ($_3C^{10}$) of ring A, and the expected doublet-doublet H-4β signal becomes a broadened triplet (J 12 Hz). The NOESY spectrum of compound 4 also shows H_3-18 / H-11β, H_3-19 / H-11β, H_3-21 / H-12β, and H-17 / H-12α correlations (Fig.1).

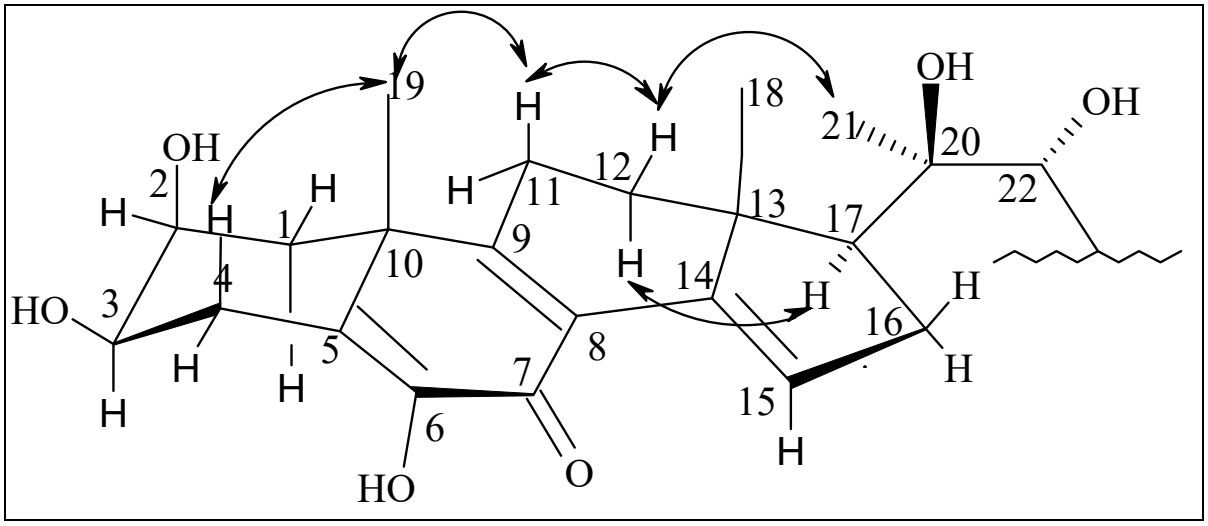

Fig 2: Key NOESY correlations in structure 4.

Thus, we isolated ajugasterone C 2-, 3-, and 11-acetates and calonysterone for the first time from the *Serratula coronata* plants. One of them compounds (ajugasterone C 11- acetate) are novel ecdysteroid. According to the NOESY spectrum, ring A of calonysterone has a chair conformation ($_3C^{10}$) with axial position of the H-3 proton and equatorial position of the H-2 proton, unusual for ecdysterones. Each of the protons has α-configuration, which corresponds to the β-orientation of the 2- and 3-hydroxyl groups in ecdysteroids.

Table 1: ^{1}H NMR spectroscopic data of compounds 1-4

Proton	1 (500 MHz)			2 (500 MHz)		3 (400 MHz)	4 (400 MHz)
	CD_3OD	C_5D_5N	D_2O	CD_3OD	C_5D_5N	CD_3OD	C_5D_5N
1 α	2.58 dd (4.0, 13.0)	3.35 dd (4.0, 12.5)	2.53 dd (4.5, 13.5)	2.76 dd (4.0, 12.5)	3.59 dd (4.0, 13.0)	1.65 m	2.33 m
1 β	1.64 m	2.29 m	1.51 m	1.40 t (12.5)	1.95 m	1.49 m	2.69 m
2	5.15 dt (3.0, 12.0)	5.65 dt (3.5, 12.5)	5.21 m	4.15 m	4.67 m	4.00 m	4.46 br s
3	4.20 br s	4.52 m	4.23 m	5.18 d (2.5)	5.53 br s	4.00 br s	4.07 br d (12.0)
4 α	1.69 m	1.79 m	1.77 m	1.78 m	1.97 m	1.74 m	3.87 dd (12.0, 4.0)
4 β	1.86 m	1.98 m	1.84 m	1.85 m	2.25 m	1.81 m	3.13 br t (12.0)
5	2.40 m	3.10 dd (3.5,13.0)	2.32 m	2.20 m	2.74 m	2.37 m	-
6	-	-	-	-	-	-	-
7	5.84 d (1.5)	6.32 d (2.0)	6.02 d (2.0)	5.84 d (2.5)	6.32 m	5.89 d (2.0)	-
8	-	-	-	-	-	-	-
9	3.21 d (7.0)	3.93 dd (2.0, 8.5)	3.19 dd (2.5, 8.5)	3.18 dd (2.0, 8.5)	3.84 d (8.5)	3.47 dd (2.0; 8.5)	-
10	-	-	-	-	-	-	-
11	4.10 m	4.60 m	4.26 m	4.16 m	4.67 m	5.34 dd (8.5;16.0)	2.66 m
12 α	2.18 m	2.71 dd (5.5, 12.0)	2.07 m	2.22 m	2.75 m	2.20 m	1.49 m
12 β	2.24 m	3.08 m	2.29 m	2.26 m	3.06 m	2.40 m	2.31 m
13	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-
15 α	1.60 m	1.99 m	1.65 m	1.60 m	1.53 m	1.66 m	7.41 br s
15 β	1.98 m	2.29 m	2.05 m	1.99 m	1.77 m	2.01 m	-
16 α	1.74 m	1.56 m	1.81 m	1.74 m	1.59 m	1.73 m	2.52 m
16 β	2.00 m	1.69 m	1.89 m	2.01 m	2.01 m	2.02 m	3.23 dd (15.6, 11.6)
17	2.44 m	3.05 m	2.37 m	2.44 m	3.04 m	2.39 m	2.29 m
18	0.89 s	1.30 s	0.88 s	0.88 s	1.31 s	0.95 s	1.47 s
19	1.11 s	1.39 s	1.14 s	1.10 s	1.36 s	1.04 s	1.77 s
20	-	-	-	-	-	-	-
21	1.21 s	1.58 s	1.26 s	1.22 s	1.59 s	1.17 s	1.61 s
22	3.33 m	3.81 d (10.5)	3.45 d (9.0)	3.32 m	3.81 d (10.0)	3.33 m	3.93 d (8.4)
23 α	1.24 m	1.54 m	1.28 m	1.24 m	1.53 m	1.23 m	1.87 m
23 β	1.58 m	1.79 m	1.59 m	1.58 m	1.97 m	1.53 m	2.11 m
24 α	1.24 m	1.45 m	1.28 m	1.24 m	1.42 m	1.25 m	1.89 m
24 β	1.49 m	1.72 m	1.38 m	1.49 m	1.74 m	1.48 m	1.51 m
25	1.60 m	1.50 m	1.57 m	1.58 m	1.49 m	1.58 m	-
26	0.93 d (6.5)	0.84 d (3.0)	0.91 d (6.5)	0.92 d (5.0)	0.83 d (6.0)	0.94 d (4.4)	1.45 s
27	0.95 d (6.5)	0.85 d (3.0)	0.93 d (6.5)	0.95 d (5.0)	0.84 d (6.0)	0.92 d (4.4)	1.45 s
CH3CO	2.07 s	1.82 s	2.14 s	2.13 s	2.01 s	2.13 s	-

Table 2: ^{13} C NMR spectroscopic data of compounds 1-4

Carbon	1 (125 MHz)			2 (125 MHz)		3 (100 MHz)	4 (100 MHz)
	CD_3OD	C_5D_5N	D_2O	CD_3OD	C_5D_5N	CD_3OD	C_5D_5N
1	36.1	36.4	36.6	40.3	40.5	39.0	43.1
2	73.4	73.8	73.9	67.4	66.3	68.9	70.3
3	65.9	65.2	67.0	72.1	71.2	68.8	73.9
4	33.4	33.6	33.6	30.9	31.7	32.9	28.6
5	52.7	52.5	53.2	53.7	52.8	52.6	133.3
6	206.1	203.8	209.8	205.8	202.5	205.5	144.9
7	122.9	122.7	124.0	122.7	121.8	123.7	181.4
8	165.7	164.6	167.3	166.3	164.5	164.3	124.7
9	43.1	43.3	43.6	43.1	42.6	39.8	164.2
10	40.1	39.9	40.9	39.9	39.2	40.4	42.2
11	69.7	69.5	70.3	69.7	68.6	72.9	25.1
12	43.8	44.4	43.8	43.9	43.8	39.1	37.3
13	48.6	48.7	49.2	49.9	47.9	47.6	47.7
14	85.0	84.7	86.7	84.9	83.9	84.6	142.7
15	31.9	32.4	32.5	31.9	30.1	31.9	127.9
16	21.7	21.9	22.9	21.7	21.3	21.7	32.9
17	50.4	50.4	50.9	50.4	49.7	50.5	56.4
18	19.0	19.4	19.8	19.0	17.8	18.7	18.9
19	24.7	25.3	25.3	24.7	24.5	24.6	27.7
20	77.9	77.2	79.9	77.9	76.5	77.7	76.5
21	21.1	21.9	21.5	21.1	21.3	21.0	21.2
22	78.1	77.2	79.0	78.1	76.5	78.1	78.2
23	30.6	30.8	30.8	30.6	30.0	30.6	27.8
24	37.8	37.6	37.9	37.8	36.9	37.8	43.1
25	29.4	28.7	29.6	29.4	27.9	29.4	70.0
26	22.9	22.9	23.7	22.9	22.1	23.6	30.4
27	23.6	23.8	24.5	23.6	23.1	22.9	30.9
CH3CO	172.3	172.8	175.6	172.8	170.4	172.4	-
CH3CO	21.3	21.6	22.1	21.3	20.9	21.8	-

4. Acknowledgements
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