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ARTICLE

Solid-state nuclear magnetic resonance investigation of neurosteroid compounds and magnesium interactions

Danny Wu[1] | Kathleen Joyce D. Carillo[2,3,4] | Shen-Long Tsai[1] | Jiun-Jie Shie[2,4] | Der-Lii M. Tzou[4,5]

[1]Chemical Engineering Department, National Taiwan University of Science and Technology, Taipei, Taiwan

[2]Taiwan International Graduate Program of Sustainable Chemical Science and Technology, Taipei, Taiwan

[3]Department of Applied Chemistry, National Chiao Tung University, Hsinchu, Taiwan

[4]Institute of Chemistry, Academia Sinica, Nankang, Taipei, Taiwan

[5]Department of Applied Chemistry, National Chia-Yi University, Chia-Yi, Taiwan

Correspondence
Der-Lii M. Tzou, Institute of Chemistry, Academia Sinica, Taipei, Taiwan.
Email: tzougate@gate.sinica.edu.tw

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The neurosteroid *trans*-dehydroandrosterone (DHEA) and its analogs with slightly different modifications in the side chain attached to C17, that is, (3*S*)-acetoxypregn-5-en-20-one (**1**) and (3*S*,20*R*)-acetoxypregn-5-en-20-ol (**2**), have been synthesized to investigate DHEA–cation interactions. In this study, we applied solid-state $^{1}H/^{13}C$ cross-polarization/magic-angle spinning (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy to a series of DHEA analog/Mg^{2+} mixtures at different Mg^{2+} concentrations. The high-resolution ^{13}C NMR spectra of **1**/Mg^{2+} mixtures exhibit two distinct ^{13}C spectral patterns, one attributable to **1** free from Mg^{2+}, and the other attributable to **1** with bound Mg^{2+}. For **2**, the ^{13}C NMR spectra exhibit three distinct spectral patterns; besides that of the free form, the other two can be assigned to Mg^{2+}-bound forms. Based on the analysis of the chemical shift deviations (CSDs), we conclude that both **1** and **2** might be subject to a cation–π interaction via the C5–C6 double bond, in contrast to that observed previously for DHEA. As demonstrated, DHEA possesses two Mg^{2+} binding sites, that is, C17–O and C5–C6 double bond, in which the binding affinity of the former is at least three times stronger than that of the latter. The solid-state ^{13}C NMR investigation allows better understanding of the underlying cation binding effects of neurosteroid molecules *in vitro*.

KEYWORDS

neurosteroid, *trans*-dehydroandrosterone (DHEA), solid-state NMR, conformational change, magnesium

1 | INTRODUCTION

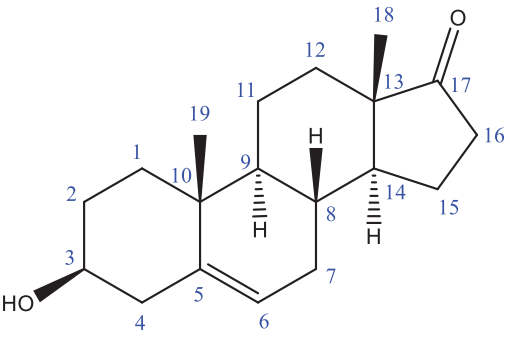
Neurosteroids are important modulators of brain activity and behavior and participate in the regulation of mood and memory.[1–4] Neurosteroids derived from cholesterol or steroidal precursors imported from peripheral sources are also present in neurons.[5–9] They act as modulators of neurotransmitter receptors such as $GABA_A$, NMDA, and sigma 1 receptors.[10–13] As reported in the literature, a deficiency of active neurosteroids may be associated with aging-related impairments.[6,14] Synthetic methodologies have been developed to produce neurosteroid molecules, such as pregnenolone (Preg), for the treatment of depressive disorders and chronic Alzheimer's disease. For example, it has been shown that Preg molecules display antidepressant efficacy in rats.[15] Upon inducing spinal cord injury, Preg molecules are able to reduce histopathological changes *in vivo*.[16] This neuroprotective effect could result from the direct action by Preg on spinal cord neurons, whereby it modulates the neuronal cytoskeleton dynamics through interaction with MAP2.[17] In association with microtubulin in the brain, the actions of Preg indicate its potential roles in brain development, plasticity, aging, and depression, as it has been suggested that hippocampal MAP2 expression may

be involved in the pathogenesis and pharmacology of depression.[18–21]

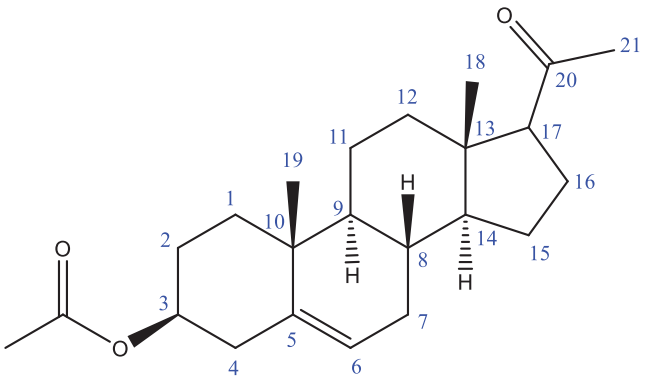
It is well known that the first step in the cascade of the biosynthesis process of steroidal hormones is to convert cholesterol into Preg, which is mediated by mitochondrial P450scc. Furthermore, Preg is transformed into progesterone, dehydroepiandrosterone (DHEA), and testosterone.[22] The synthesis normally takes place in steroidogenic tissues, such as gonads, adrenal glands, and placenta.[23] Besides these tissues, Preg can also be synthesized in the brain, which is well equipped with the enzyme machinery of steroidogenesis. The enzymes involved in the synthesis/metabolism pathways of steroids mainly target the C3-position of progesterone and its hydroxyl metabolites and the C17-position of androgens and estrogens. Researchers have reported that the 3α-hydroxyl configuration is required for binding and activity.[24,25] Therefore, synthetic strategies have been developed to prepare DHEA analogs with modification of C3–C17 positions, to be used as antagonists.

Magnesium ions (Mg^{2+}) have many diverse functions within the central nervous system, including voltage-dependent blocking of NMDA receptors.[26] In humans, Mg^{2+} deficiency is well documented to be correlated with a series of neuropsychiatric disorders.[27] Mg^{2+} serves as a cofactor in more than 300 enzymes, such as tyrosine and tryptophan hydroxylases,[28] as well as enzymes ubiquitous in energy metabolism.[26] Given that most of these enzymes are brain-related, Mg^{2+} deficiency is likely to be involved in a variety of neuroses. In this work, we aimed to exploit DHEA and the interactions of its analogs with Mg^{2+} by solid-state nuclear magnetic resonance (NMR) spectroscopy.

Solid-state NMR has been developed to acquire structural information on pharmaceutical and biomedical materials.[29] For example, it can be used to determine the average orientation and dynamics of ergosterol[30–32] and cholesterol[33] in model membranes and for examining steroid–lipid interactions.[34–36] In ^{13}C NMR solid-state spectroscopy, cross-polarization/magic-angle spinning (CP/MAS) techniques provide high-resolution spectra comparable to those observed in the solution phase, so that conformational information can be directly obtained.[33] High-resolution ^{1}H–^{13}C CP/MAS spectra of a variety of steroids, including testosterone, hydrocortisone, DHEA, spironolactone, vitamin D, deflazacort, and Prd *tert*-butyl acetate, show multiplet patterns with splittings of 0.2–2.1 ppm, which are indicative of different steroidal conformations.[37,38] In our previous study of steroid–cation interaction,[39] we demonstrated by solid-state NMR spectroscopy that the DHEA molecule is able to interact with Mg^{2+} and Ca^{2+}. The cation interaction induces different conformational changes in DHEA. And the most perturbed ^{13}C chemical shifts were resolved from the rings A and D, suggesting that Ca^{2+} and Mg^{2+} might interact with DHEA through the oxygen atom attached to C17 involving an interaction of the cation and lone pair of electrons. To validate this, we synthesized two DHEA analogs (compounds **1** and **2**) to further investigate the underlying binding sites. In terms of molecular structure, **1** and **2** have a skeleton identical to that of DHEA, but differ in the side chain attached to C17 as well as the substituent attached to C3 (Scheme 1). For **1**, high-resolution ^{13}C NMR spectra of **1**/Mg^{2+} mixtures exhibit two distinct ^{13}C spectral patterns, one attributable to **1** free from Mg^{2+}, and the other attributable **1** with bound Mg^{2+}, similar to that observed from DHEA. For **2**, however, the ^{13}C NMR spectra exhibit three distinct spectral patterns; besides that of the free form, the other two can be assigned to Mg^{2+}-bound forms. As demonstrated, on the basis



DHEA



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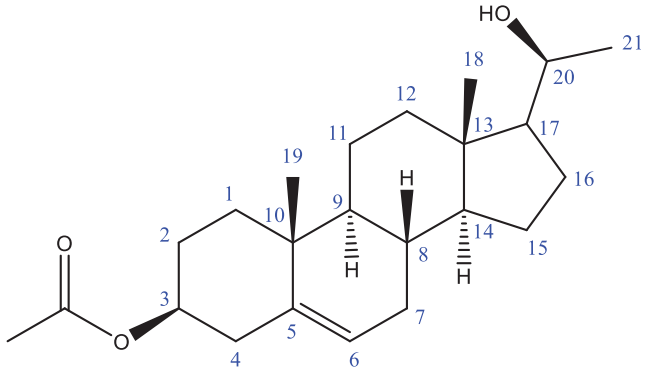

2

SCHEME 1 Molecular structures of dehydroepiandrosterone (DHEA), (3*S*)-acetoxypregn-5-en-20-one (**1**), and (3*S*,20*R*)-acetoxypregn-5-en-20-ol (**2**)

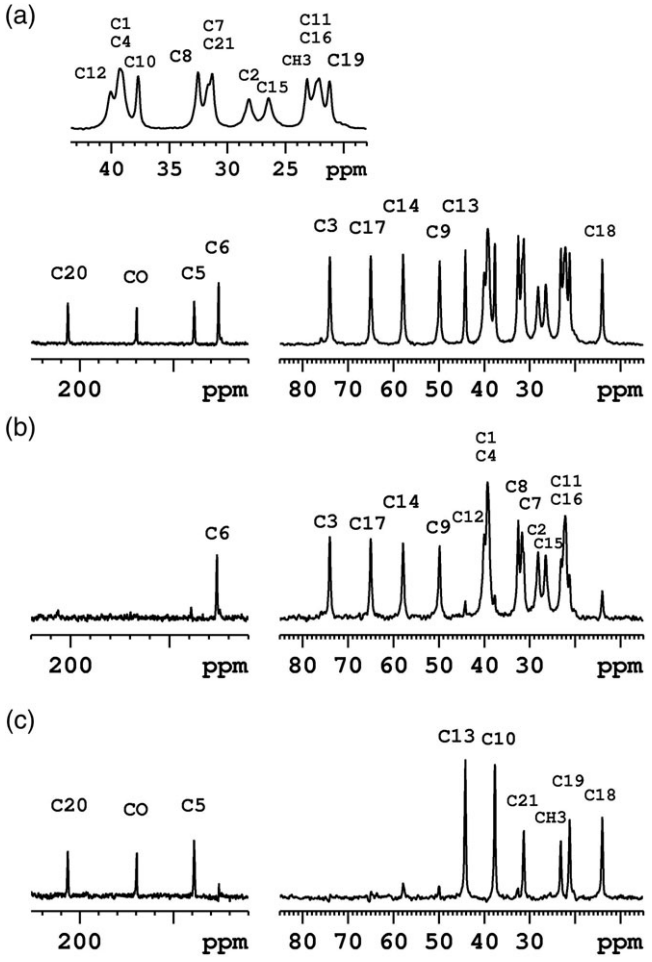

FIGURE 1 ^{13}C NMR signals of compound **1**: (a) Normal ^{1}H–^{13}C CP/MAS spectrum, (b) ^{1}H-filtered ^{13}C CP/MAS spectrum, and (c) short-contact-time ^{1}H–^{13}C CP/MAS spectrum. The ^{1}H-filtered ^{13}C and short-contact-time ^{1}H–^{13}C CP/MAS served as complementary experiments for acquiring the signals of nonprotonated and protonated C atoms, respectively. Superposition of the signals of the nonprotonated and protonated C atoms yields the normal CP/MAS signals. As shown, improved spectral resolution facilitated the assignment of ^{13}C resonances. The inset shows the same spectrum in the overcrowded region of 20–40 ppm. Signals for the methylated carbon atoms, C18 and C19, are not completely suppressed in the "nonprotonated" spectra

of analysis of the chemical shift deviations (CSDs), the double-bond π electron arising from C5–C6 at ring B contributes to the cation interaction as well. The solid-state NMR study of the DHEA analog/Mg^{2+} interaction can further advance our understanding of the cation binding effect in the neurosteroid molecule DHEA.

2 | RESULTS AND DISCUSSION

2.1 | Chemical shift assignments

In this solid-state NMR approach, we aimed to unravel the neurosteroid DHEA/Mg^{2+} binding sites by the two DHEA analogs **1** and **2** in the solid phase. Prior to the analysis of the DHEA analog/Mg^{2+} mixtures, we prepared Mg^{2+}-free samples following the same preparation process as described for the DHEA analog/Mg^{2+} mixtures (see Section 2). High-resolution ^{13}C CP/MAS NMR spectra of compounds **1** and **2** are displayed in Figures 1a and 2a, respectively, which represent fingerprints of the DHEA analogs. A typical spectral feature is that most of the ^{13}C resonances in the broad downfield region (45–210 ppm) are well dispersed, whereas the remaining ^{13}C signals in the narrow upfield region (20–45 ppm) are somewhat crowded and overlapped. This is similar to the ^{13}C spectra of other steroidal molecules, such as DHEA,[30–32] SPI,[29] hydrocortisone,[18] estradiol,[40] and prednisolone.[18]

To achieve better spectral resolution, we then performed ^{1}H-filtered ^{13}C NMR experiments and normal

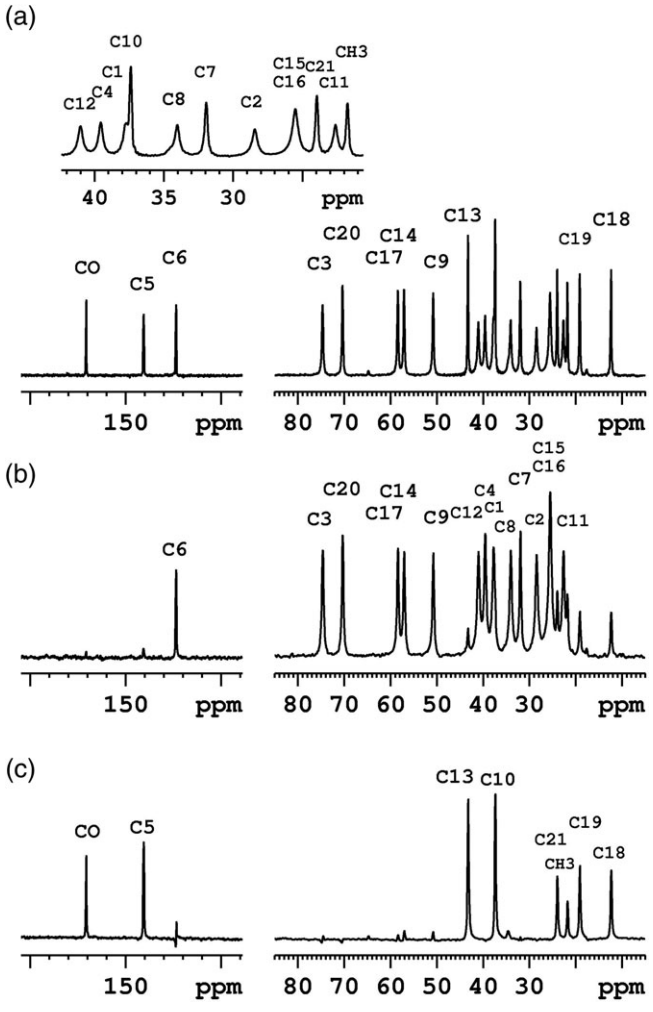

FIGURE 2 ^{13}C NMR signals of compound **2**: (a) Normal ^{1}H–^{13}C CP/MAS spectrum, (b) ^{1}H-filtered ^{13}C CP/MAS spectrum, and (c) short-contact-time CP/MAS spectrum. The ^{1}H-filtered ^{13}C and short-contact-time ^{1}H–^{13}C CP/MAS served as complementary experiments for acquiring the signals of nonprotonated and protonated C atoms, respectively. Superposition of the signals of the nonprotonated and protonated C atoms yields the normal CP/MAS signals. As shown, improved spectral resolution facilitated the assignment of ^{13}C resonances. The inset shows the same spectrum in the overcrowded region of 20–42 ppm. Signals for the methylated carbon atoms C18 and C19 are not completely suppressed in the "nonprotonated" spectra

TABLE 1 ^{13}C chemical shifts of the free forms and Mg^{2+}-bound forms of DHEA, **1** (3β-acetoxypregna-5-en-20-one), and **2** (3β-acetyloxypregna-5-ene) and their chemical shift deviations (CSDs)[a]

C atom	DHEA[b] [1]	DHEA/Mg[b] [2]	CSD[b,c] [2] – [1]	1 [3]	1/Mg [4]	CSD[c] [4] – [3]	2 [5]	2/Mg [6]	CSD[c] [6] – [5]
C1	36.63	37.96	1.33 (25%)	39.04	39.25	0.21 (5%)	37.72	38.63	0.91 (30%)
C2	33.44	34.37	0.93 (18%)	28.16	27.9	−0.26 (6%)	28.44	26.82	−1.62 (53%)
C3	71.30	71.85	0.55 (11%)	74.06	73.24	−0.82 (20%)	75.65	74.01/73.09	−1.64/−2.56 (54/20%)
C4	42.07	42.07	0.00 (0%)	39.3	40.74	1.44 (35%)	39.53	38.63	−0.90 (30%)
C5	143.16	143.84	0.68 (13%)	138.76	140.92	2.16 (53%)	140.56	142.20	1.64 (54%)
C6	119.71	119.09	−0.62 (12%)	125.77	121.71	−4.06 (100%)	123.51	121.51/120.48	−2.00/−3.03 (66/100%)
C7	33.03	32.11	−0.92 (18%)	31.65	32.06	0.41 (10%)	31.93	32.32	0.39 (13%)
C8	32.21	30.36	−1.85 (35%)	32.52	34.06	1.54 (38%)	34.01	33.96/33.09	−0.05/−0.92 (2/30%)
C9	51.42	50.08	−1.34 (26%)	49.83	52.19	2.36 (58%)	50.75	51.31/50.96	0.56/0.21 (18/7%)
C10	37.45	37.96	0.51 (10%)	37.66	37.04	−0.62 (15%)	37.37	37.91	0.54 (18%)
C11	20.50	19.68	−0.82 (16%)	22.1	22.87	0.77 (19%)	22.61	22.56	−0.05 (2%)
C12	32.21	31.70	−0.51 (10%)	40.02	40.74	0.72 (18%)	40.99	39.04	−1.95 (64%)
C13	48.34	47.62	−0.72 (14%)	44.18	43.3	−0.88 (22%)	43.23	43.56/43.0/42.59	0.33/−0.23/−0.64 (11/8/21%)
C14	54.39	51.21	−3.18 (61%)	57.89	57.99	0.10 (2%)	57.04	56.81	−0.23 (8%)
C15	22.76	22.97	0.21 (4%)	26.46	24.51	−1.95 (48%)	25.49	25.85	0.36 (12%)
C16	35.81	37.96	2.15 (41%)	22.35	22.87	0.52 (13%)	25.54	26.82	1.28 (42%)
C17	224.15	218.92	−5.23 (100%)	65.02	63.74	−1.28 (32%)	58.4	59.53	1.13 (37%)
C18	13.01	13.42	0.41 (8%)	13.98	15.22	1.24 (31%)	12.32	13.42/11.06	1.10/−1.26 (36/42%)
C19	21.84	22.56	0.72 (14%)	21.22	21.28	0.06 (1%)	19.09	19.94/19.07	0.85/−0.02 (28/1%)
C20				206.44	207.98	1.54 (38%)	70.34	71.39	1.05 (35%)
C21				31.29	31.19	−0.10 (2%)	23.95	24.66/23.84	0.71/−0.11 (23/4%)
CO[d]				169.52	170.7	1.18 (29%)	170.60	171.88/169.52	1.28/−1.08 (42/36%)
CH_3[d]				23.12	21.64	−1.48 (36%)	21.74	21.79/21.12	0.05/−0.62 (2/20%)
Ave	—	—	1.19	—	—	1.12	—	—	0.92

Note. Ave: average of absolute values of all the chemical shift differences.
[a]Chemical shifts are in units of ppm referenced to the glycine carboxyl resonance at 176.4 ppm, within an uncertainty of ±0.1 ppm.
[b]Solid-state ^{13}C NMR data were previously determined for DHEA reported elsewhere.[39]
[c]CSD: ^{13}C chemical shift differences determined by subtracting the chemical shift of the Mg^{2+}-bound form of DHEA (**1** or **2**) from that of free form. CSD values greater than the average are highlighted in bold type. The variations of CSDs in percentage are normalized according to the highest variation as 100% shown in bracket.
[d]Carbonyl or methyl carbon of the acetyl group attached to C3.

^{1}H–^{13}C CP/MAS experiment using a short contact time of 50 μs. These two experiments were complementary: The ^{1}H-filtered ^{13}C NMR spectroscopy targeted "non-protonated" C atoms, and the short CP/MAS approach targeted "protonated" C atoms. The ^{13}C NMR spectra of **1** and **2** are shown in Figures 1b and 2b, respectively. By virtue of the higher spectral resolution compared to normal CP/MAS spectra, the nonprotonated C signals of **1**, in particular, those of C10, C19, C21, and CH_3, are distinct without any overlap (Figure 1a–c). Thus, selective observation of nonprotonated and protonated ^{13}C signals in **1** and **2** was achieved. Because the ^{13}C chemical shifts observed in the solid phase are highly correlated with those in the solution phase (see Supporting Information, Figures S1 and S2), the solid-state ^{13}C chemical shift assignments can be inferred from the assignments determined from solution NMR experiments without ambiguity. The full ^{13}C chemical shift assignments of **1** and **2** are listed in Table 1.

2.2 | Chemical shift deviations

In our previous solid-state NMR study, we demonstrated that Mg^{2+} is capable of inducing a conformational change in DHEA.[39] It was suggested that Mg^{2+} may associate with the oxygen atom attached to C17 through a lone pair of electrons. To further exploit the underlying cation interaction, in the current work we have investigated the effect of Mg^{2+} upon the steroid ring conformation of DHEA analogs, that is, compounds **1** and **2**, which differ slightly in the substituent attached at C17 (see Scheme 1). The ^{13}C NMR spectra of compounds **1** and **2** incubated with Mg^{2+} at different Mg^{2+} concentrations are displayed in Figures 3a–e and 4a–e, respectively. For compound **1**, the NMR spectra of the mixtures with Mg^{2+} showed two sets of ^{13}C spectral signals. For clarity, these two sets will be hereafter referred to as pertaining to the free form and the Mg^{2+}-associated bound form. For the free form, the spectral pattern is exactly identical to that seen for **1** free from Mg^{2+} (Figure 1a). For the bound form, however,

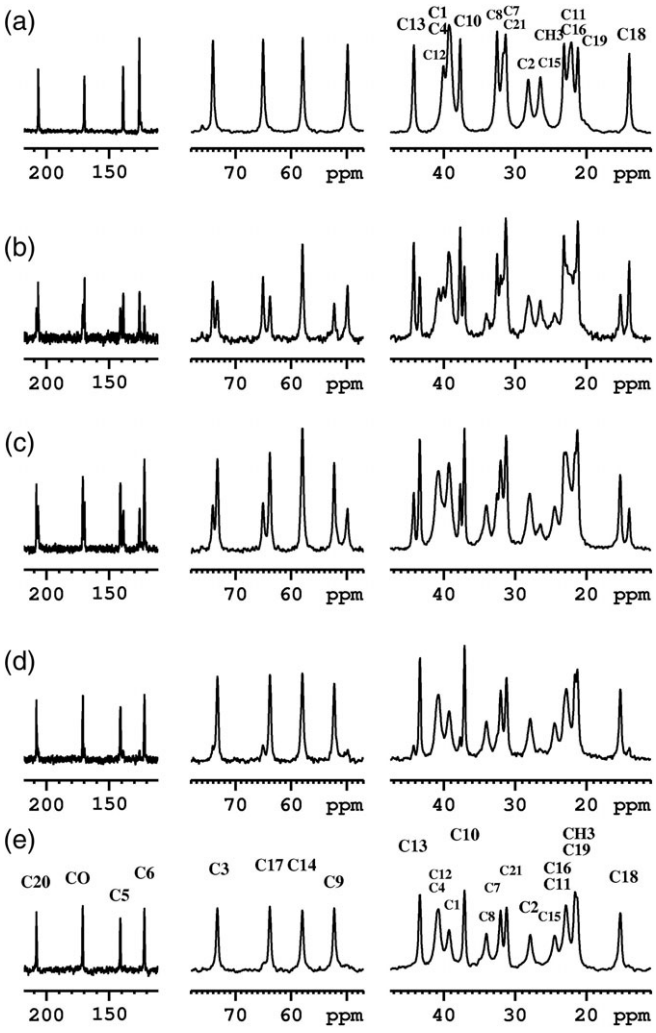

FIGURE 3 Solid-state ^{13}C NMR analysis of mixtures of compound **1** and Mg^{2+}. ^{1}H–^{13}C CP/MAS spectra of **1** (0.124 M) mixed with Mg^{2+} at concentrations of (a) 0, (b) 0.076, (c) 0.124, (d) 0.294, (e) 0.434 M. In comparison with that of compound **1** alone (a), the spectra of **1**/Mg^{2+} feature an additional set of ^{13}C signals. The intensities of the additional ^{13}C signals are dependent on the Mg^{2+} concentration, suggesting that they arise from the Mg^{2+}-bound form; for details, see text. For clarity, the signals in the regions 10–50, 50–80, and 110–220 ppm are shown in different scales. The ^{13}C chemical shift assignments of the **1**/Mg^{2+} mixture are indicated (see also Table 1)

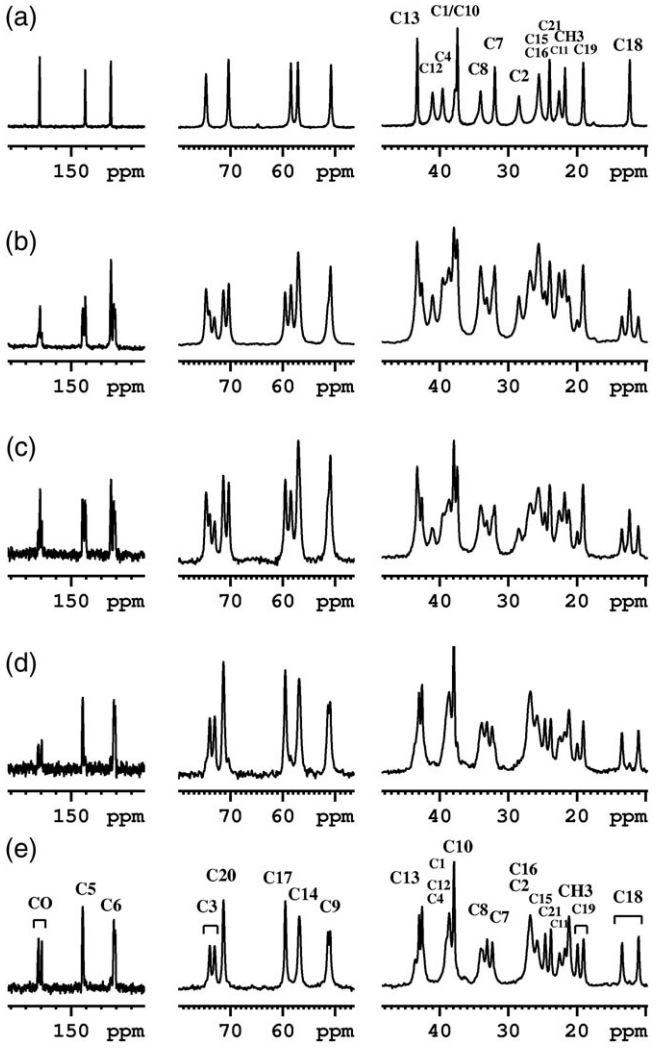

FIGURE 4 Solid-state ^{13}C NMR analysis of mixtures of compound **2** and Mg^{2+}. ^{1}H–^{13}C CP/MAS spectra of **2** (0.174 M) mixed with Mg^{2+} at concentrations of (a) 0, (b) 0.13, (c) 0.174, (d) 0.324, (e) 0.466 M. In comparison with that of compound **2** alone (a), the spectra of **2**/Mg^{2+} feature two additional sets of ^{13}C signals. The signal intensities of these sets are comparable and are dependent on the Mg^{2+} concentrations, suggesting that **2**/Mg^{2+} might adopt different steroidal ring conformations; for details, see text. For clarity, the signals in the regions 10–50, 50–80, and 110–190 ppm are shown in different scales. The ^{13}C chemical shift assignments of the **2**/Mg^{2+} mixture are indicated (see also Table 1)

the spectral pattern is highly distinct, with the ^{13}C signals being shifted either upfield or downfield by 0.1–4.0 ppm.

It is possible that due to co-crystallization or polymorphism, the molecular conformation might not be unique, in which the signals of relevant carbon atoms are split. As shown in Figures 3a and 4a, the ^{13}C NMR spectra of **1** and **2** in the absence of Mg^{2+} (same sample preparation as that in the presence of Mg^{2+}) revealed singlet patterns, indicating a single conformation. That basically ruled out such possibilities. The ^{13}C spectra of compounds **1** and **2** in the presence of Mg^{2+} feature additional sets of ^{13}C spectral signals, which were therefore attributable to the Mg^{2+}-associated bound forms (Figures 3b–e and 4b–e). It is noteworthy that the spectral patterns of the bound forms remained invariant in the DHEA analog/Mg^{2+} mixtures. To confirm whether the multiple splitting patterns detected from **2** are due to molecular packing effects or to different molecular conformations, we further measured the resonance line widths (i.e., C3, C17, and C18) at half-maximum, which were well resolved in these spectra (Figure 3a–e) and free of any overlapping signals. Similar to other signals, these signals gave rise to line widths in the range of 20–35 Hz, independent of the cation concentration. The line broadening of the resonances may be due to the homogeneous broadening arising from heteronuclear dipolar interactions or to inhomogeneous broadening arising from different molecular packing effects. If the packing inhomogeneity occurred because of the presence of cations, the resulting ^{13}C signals would be expected to have higher line widths compared to the signals in the

absence of cations. However, our data indicated that the line widths were unaffected by increasing cation concentration, suggesting that line broadening is primarily due to homogeneous broadening. We therefore interpret the multiplet patterns as an indication of different steroidal conformations.

In view of the fact that ^{13}C chemical shifts have been routinely used to obtain information about molecular structure in various biological systems,[41,42] in the present study we used CSD values in an attempt to delineate Mg^{2+} binding sites in the DHEA analogs. We deduced ^{13}C CSDs by subtracting the chemical shifts of the Mg^{2+}-bound form from those of the free form. The CSDs induced in these molecules by Mg^{2+} are listed in Table 1. For compound **1**, a number of C atoms, namely C4, C5, C6, C8, C9, C15, C17, C18, C20, CO, and CH_3, showed CSDs greater than the average perturbation value (1.12 ppm). Within these residues, the most perturbed were C6 (4.06 ppm), C9 (2.36 ppm), and C5 (2.16 ppm) in ring B, with CSDs reaching at most three times the average value. For compound **2**, several C atoms, namely C2, C3, C5, C6, C12, C16, C17, C18, C20, and CO, showed CSDs greater than the average perturbation value (0.92 ppm). Among them, the most perturbed were C6 (2.0 and 3.03 ppm), C3 (1.64 and 2.56 ppm), and C12 (1.95 ppm) in rings B, A, and C, with CSDs twice as large as the average value. Thus, in both **1** and **2**, C6 showed the greatest perturbation in terms of the CSD value, which is associated with the C5–C6 double bond. We therefore presume that this double bond is involved in binding to Mg^{2+}. Unlike DHEA, the greatest perturbation was detected from the C17–O double bond (see Scheme 1 and Table 1). On this basis, we used the C6 signal variations in the Mg^{2+} titration experiment to extract the binding affinity, as elaborated below.

In this solid-state NMR study, we find that the ^{13}C NMR spectra of DHEA and its analogs show both positive and negative CSDs on incubating with $MgCl_2$. It is thus possible that the negative CSDs were caused by the negatively charged chloride anions. And the chloride anions might be associated with the ion–dipole interaction regions where the Mg^{2+} and steroidal molecules are interacting, such as the D ring of DHEA. If indeed this is the case, the electrons surrounding C17–O are to be pulled toward the Mg^{2+}, making the C17 partially electron-positive. And the chloride anions are drawn to the C17 as well as the neighboring atoms of C17–O, giving rise to negative CSDs. As indicated in Table 1, negative CSD values were observed from the C17 of DHEA and **1** as well as those in the neighborhood of C17, such as C13, C18, and C19 of DHEA, **1**, and **2**, suggesting this might be the case.

2.3 | Mg^{2+} binding affinity

In order to determine Mg^{2+} binding affinities, we further monitored the signal intensities of C5, C6, C20, and CO as a function of Mg^{2+} concentration. We have plotted the relative signal intensity of C6, representing the Mg^{2+} binding site, as a function of Mg^{2+} concentration in Figure 5. It is seen that the signals reveal a nonlinear dependence on the Mg^{2+} concentration. In the simulations, we utilized a nonlinear correlation function for experimental curve-fitting, in which the relative intensity arising from the Mg^{2+}-bound form was correlated with the Mg^{2+} concentration by an exponential relationship $[1 - \exp(-\kappa\mu)]$, where κ represents the binding affinity and μ the Mg^{2+} concentration. The binding affinity κ was then deduced from experimental-curve fitting as a measure of metal cation binding affinity: that is, the higher the value, the stronger the binding affinity. As can be seen, compounds **1** and **2** showed similar responses with respect to Mg^{2+}, although compound **1** gave a slightly steeper curve than compound **2**, suggesting that the former has a somewhat higher affinity. In these analyses, a binding affinity κ of 8.3 M^{-1} was extracted from the best curve-fit of the relative intensities of C6 of compound **1**, whereas a lower value of 5.0 M^{-1} (0.75) was derived from the same C atom of **2**. Based on the higher κ value, we surmise that compound **1** has a moderately stronger Mg^{2+} binding affinity than compound **2**. As illustrated in Figure 5, DHEA got converted into the Mg^{2+}-bound form at a higher rate, in which the complete conversion was observed at a lower Mg^{2+} concentration of 0.12 M for DHEA/Mg^{2+}, as compared to 0.6 M for DHEA analog/Mg^{2+}, revealing a fivefold difference. And a binding affinity κ of 25 M^{-1} (3.3) was deduced from DHEA/Mg^{2+} (Figure 5), which is three times greater than that for **1** and five times greater than that for **2**. Thus, DHEA indeed has a higher Mg^{2+} binding affinity as compared to

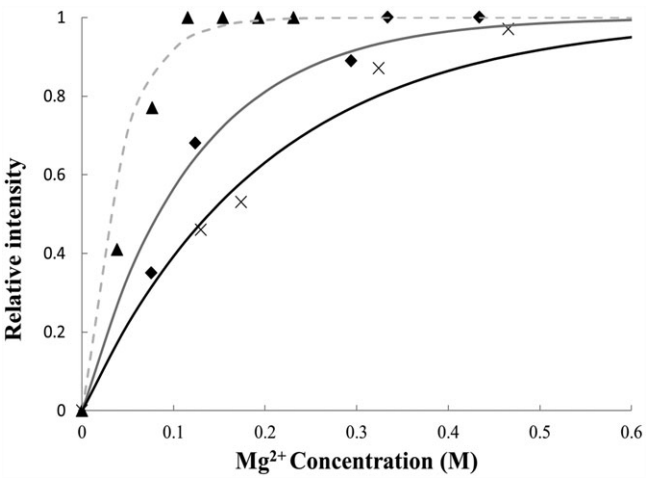

FIGURE 5 Investigation of DHEA analogs/Mg^{2+} interaction by solid-state NMR spectroscopy. The ^{13}C intensity ratios of the C17 signals of DHEA [39] (▲) and C6 signals of compounds **1** (♦) and **2** (×) are plotted as a function of Mg^{2+} concentration. The signal intensities were calculated with reference to that of the C14 signal. The Mg^{2+} binding effect was analyzed by nonlinear exponential curve-fitting simulation for the two compounds. Note that the two compounds gave rise to two slightly different nonexponential growth curves for the ^{13}C signals arising from their Mg^{2+}-bound forms; for details, see text. In the simulations, a nonexponential curve was used to optimize the best fit, and Mg^{2+} binding affinities (κ of 25, 8.3, and 5.0 M^{-1}) were deduced for compounds DHEA, **1**, and **2**, respectively

the DHEA analogs. Based on these binding affinities, we conclude that the Mg^{2+} binding affinity of DHEA is much stronger than that of the DHEA analogs, following a decreasing order of DHEA >> **1** > **2**.

Compared to the alkyl ketones (C–O) of **1** and the ester group of **2**, the cyclic ketone group (C17–O) of DHEA is capable of inducing ring strain and increasing the S-character in the C–C bonds of ring D, resulting in a higher degree of acidity. Because of this, the C17–O binding to basic Mg^{2+} is stronger than that of C–O. similarly, for **1** and **2**, the alkene group (C5–C6) has a higher acidity than the ketone (C–O) and ester group; this explains why the C5–C6 binding to basic Mg^{2+} is stronger than the C–O binding.

We here show that the Mg^{2+} binding sites of DHEA analogs are different from those previously observed for DHEA.[39] In the case of DHEA, the most perturbed CSD value was detected at C17, indicating that C17–O is involved in the Mg^{2+} interaction, whereas in the case of **1** and **2** the greatest perturbations in terms of CSD value were detected at C5 and C6 (see Table 1), suggesting that Mg^{2+} prefers to interact with C5–C6, albeit weakly, mediated by a π-electron pair. Here we propose that DHEA possesses two Mg^{2+} binding sites, that is, the C17–O and C5–6 double bonds, and the two binding sites function in a competitive manner. Since the binding affinity of the former is at least threefold stronger than the latter, one is normally expected to have the C17–O for Mg^{2+} binding while having the C5–C6 silent due to its lower affinity. However, in the absence of C17–O, for example, in the cases of **1** and **2**, one is then able to have the C5–C6 binding site observable for the detection.

Even though both **1** and **2** share a high degree of similarity in the steroidal ring skeleton, as mentioned in the introduction, it is possible that both compounds bind Mg^{2+} but mediated by different mechanisms. In the presence of Mg^{2+}, **1** showed a single conformation while **2** gave rise to two conformations, suggesting that 2 might have a higher degree of molecular plasticity. Aside from this, in the case of **1**, the most perturbed chemical shifts were found at ring B, such as C5, C6, C8, and C9, whereas in the case of the two most perturbed chemical shifts were found to a larger extent including rings A, B and C, for instance, C2, C3, C5, C6, and C12. Based on our solid-state NMR observations, we therefore interpret that though both **1** and **2** bind Mg^{2+}, and yet their underlying binding mechanisms are somewhat dissimilar.

3 | EXPERIMENTAL

3.1 | General

All reagents used were commercially available and used without further purification unless indicated otherwise. All solvents were anhydrous grade unless indicated otherwise. All non-aqueous reactions were carried out in oven-dried glassware under a slightly positive pressure of argon unless otherwise noted. Reactions were magnetically stirred and monitored by thin-layer chromatography on silica gel. Column chromatography was performed on silica gel of 40–63 μm particle size. Yields are reported for spectroscopically pure compounds. Melting points were recorded on an Electrothermal Mel-Temp 1101D melting point apparatus and were not corrected.

3.2 | NMR measurements

The ^{1}H–^{13}C CP/MAS NMR spectra were acquired on a Bruker Avance 300 MHz NMR spectrometer (Bruker Spectrospin, Rheinstetten, Germany), equipped with a 4-mm double-resonance probe operating at ^{1}H and ^{13}C Larmor frequencies of 300.13 and 75.47 MHz, respectively. The ^{1}H-to-^{13}C polarization transfer was optimized to fulfill the Hartman–Hahn matching condition.[43] For normal CP experiments, the contact time was set to 1 ms, and an rf field strength of 41.0 kHz was chosen for both the ^{1}H and ^{13}C channels. In the ^{1}H-filtered experiments, a 41.5 μs delay was applied immediately before data acquisition, allowing ^{1}H-coupled ^{13}C signals to be attenuated under strong ^{1}H–^{13}C dipolar coupling and resulting in acquisition of only the ^{13}C signals of nonprotonated carbon atoms. A complementary short CP experiment was performed, in which a contact time of 50 μs was used to detect only the ^{13}C signals of protonated carbon atoms. During data acquisition, ^{1}H decoupling by two-pulse phase modulation[44] was applied, with an rf field strength of 79.3 kHz. Unless otherwise specified, the ^{13}C spectra were acquired with a sample spinning frequency of 8 kHz, regulated by a spinning controller to within about 1 Hz. All ^{1}H–^{13}C CP/MAS experiments were conducted at ambient temperature. The ^{13}C chemical shifts were referenced to the glycine carboxyl carbon signal at 176.4 ppm.

3.3 | Sample preparations

To prepare powdered samples, compounds **1** and **2** were dissolved in anhydrous methanol and combined with $MgCl_2$ solution to prepare DHEA analog/Mg^{2+} mixtures, in which **1** (0.124 M) was mixed with Mg^{2+} at six different concentrations (0, 0.076, 0.124, 0.294, 0.334, and 0.434 M), and **2** (0.174 M) was mixed with Mg^{2+} at five different concentrations (0, 0.13, 0.174, 0.324, and 0.466 M), respectively. After lyophilization, the DHEA analog/Mg^{2+} mixtures in powdered form were packed into a 4-mm zirconium MAS rotor and analyzed by high-resolution ^{1}H–^{13}C CP/MAS solid-state NMR spectroscopy.

3.4 | (3S)-Acetoxypregn-5-en-20-one (1)

Acetic anhydride (13 mL, 134 mmol, 14.1 equiv) was added to a solution of pregnenolone (3.0 g, 9.48 mmol, 1 equiv) in

pyridine (26 mL, 315 mmol, 33.2 equiv) over a period of 10 min. After stirring at room temperature for 32 h, the reaction mixture was concentrated to dryness under reduced pressure. The residue was redissolved in CH_2Cl_2, and the solution was washed with saturated aqueous $NaHCO_3$ solution (2 × 50 mL) and 1 N HCl (2 × 50 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated to afford the desired compound **1** (3.33 g, 98%) as a white solid. M.p. 145–148°C. ^1H and ^{13}C NMR spectra and 2D COSY and HSQC spectra were acquired, see Figures S1–S4. ^1H and ^{13}C chemical shifts (see Table S1) and the melting points are consistent with those reported in the literature. [45,46]

3.5 | (3S,20R)-Acetoxypregn-5-en-20-ol (2)

Sodium borohydride (0.11 g, 2.79 mmol, 1 equiv) was slowly added to a cold (0°C) solution of (3*s*)-acetoxypregn-5-en-20-one (**1**) (1.0 g, 2.79 mmol, 1 equiv) in methanol (30 mL) and CH_2Cl_2 (5 mL). After stirring for 1.25 h in an ice bath, the reaction was quenched with saturated aqueous NH_4Cl solution and extracted with CH_2Cl_2 (2 × 50 ml). The combined organic extracts were washed with saturated aqueous $NaHCO_3$ solution and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (hexane/EtOAc, 3:1) to afford the desired compound **2** (0.82 g, 82%) as a white solid. Melting point 152–155°C. ^1H and ^{13}C NMR spectra and the 2D COSY and HSQC spectra were determined, see Figures S5–S8. ^1H and ^{13}C chemical shifts (see Table S2) and the m.p. were consistent with that of the 20(*R*) form reported in the literature.[47]

4 | CONCLUSIONS

To unravel the Mg^{2+} binding sites of the neurosteroid, we have applied high-resolution ^{13}C CP/MAS NMR spectroscopy to DHEA and its analogs **1** and **2**. The side chain attached to C17 and the substituent attached to C3 were modified in **1** and **2**. In DHEA, the most perturbed C atom was found to be C17, suggesting the C17–O double bond to be responsible for the interaction with Mg^{2+}. In these DHEA analogs, unlike DHEA, the most perturbed C atom was found to be C6 in ring B, suggesting that the C5–C6 double bond of distinct π-electron character is responsible for the interaction. In this solid-state NMR study, we demonstrated that DHEA possesses two Mg^{2+} binding sites, that is, C17–O and C5–C6 double bonds, acting in a competitive manner. The Mg^{2+} binding of C17–O is mediated by the cation/lone-pair electron interaction, whereas the binding of C5–C6 is through cation/π interaction, in which the former has a higher binding affinity (at least three times stronger) than the latter.

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