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Narcissistic aggregation of steroid compounds in diluted solution elucidated by CSI-MS, PFG NMR and X-ray analysis

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Abstract—Large-scale aggregated chain structures of progesterone, estrone, cortisone, hydrocortisone and cholic acid were observed in diluted solution by means of cold-spray ionization mass spectrometry (CSI-MS) and pulsed field gradient (PFG) NMR. The crystal structures were determined by X-ray crystallography, and the relationship between the crystal and solution structures is discussed. It is suggested that the intermolecular hydrogen bondings observed in the crystal might be partly retained in diluted solution.

1. Introduction

We recently developed a direct solution analysis method, cold-spray ionization (CSI)-MS,[1,2] a variant of electrospray ionization (ESI)-MS[3] operating at low temperature. The optimum spray temperature is estimated to be around −20 °C. Generally, solvents show a higher dielectric constant at low temperature. This fact, as well as the solvation of the molecule, promotes electrolytic dissociation to form the molecular ion in solution, because desolvation should not occur readily at such a low temperature. Therefore, it should be possible to detect extremely labile complexes without decomposition. The CSI apparatus has been refined and applied to investigations of the solution structures of primary biomolecules, labile organic species including Grignard reagents, asymmetric catalysts and supramolecules.[4,5] This method allows simple and precise characterization of labile non-covalent complexes, which are difficult or impossible to observe by conventional MS techniques.

In the course of our studies of biomolecular solution structures by using CSI-MS, we have found large-scale hydrogen bonding aggregates, or chain clusters, of nucleosides, amino acids and monosaccharides in diluted solution.[6,7] Interestingly, the crystal structures of these compounds exhibit strong intermolecular hydrogen bonding to form chain structures, apparently identical with the large-scale aggregates observed in solution by CSI-MS. Similar chain structures were also observed in lipids and micelles including steroid compounds. In this report, we present a detailed behavior of the large-scale aggregated chain structures (chain clusters) of steroid compounds observed in solution by means of CSI-MS and PFG NMR. The solution structures are compared with those observed in the crystalline state by single-crystal X-ray analyses.

2. Results

2.1. Cold-spray ionization mass spectrometry

The CSI mass spectra of progesterone **1**, estrone **2**, cortisone **3**, hydrocortisone **4** and cholic acid **5** are shown in Figure 1.

Characteristic chain structures based on strong intermolecular hydrogen bonding in the cases of **3**, **4** and **5** were clearly present in solution, yielding a series of clusters. Ion peaks assigned as $[n\mathrm{M}+\mathrm{Na}]^+$ (n=1 to 12 or 13), were seen in the range of m/z 0–5000 for these compounds. In the case of **2**, intermolecular interaction appeared to be weaker, based the CSI mass spectrum (Fig. 1(b)). The strength of the intermolecular interaction for each compound might depend on the number of hydroxyl or carboxyl groups attached. As expected, no chain cluster was observed in the CSI mass spectrum of **1**, which has neither hydroxyl nor

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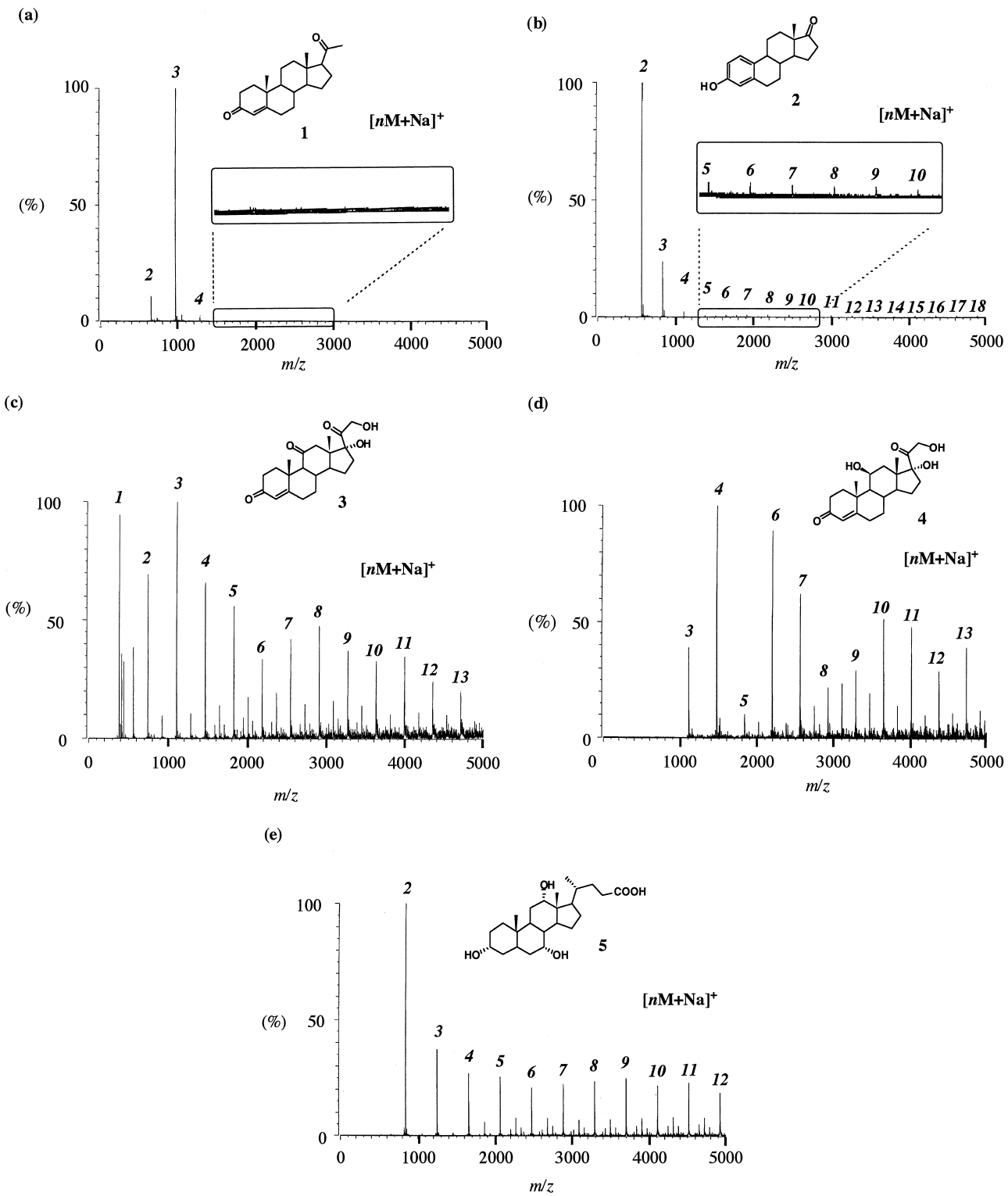


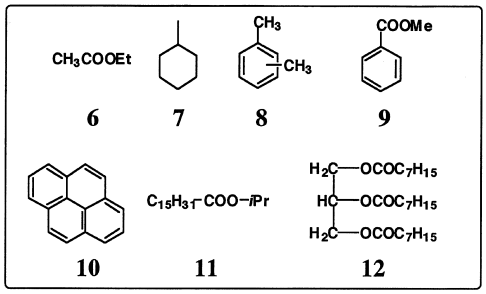
Figure 1. Cold-spray ionization mass spectra of steroid compounds: (a) **1**; (b) **2**; (c) **3**; (d) **4**; (e) **5**.

carboxyl groups. This result is consistent with the X-ray analysis findings, which will be discussed later.

2.2. PFG NMR diffusion studies

Recently, it was demonstrated that PFG NMR is a useful method for the characterization of a variety of interacting systems in solution.[8–10] First, we examined the diffusion coefficients of molecules practically having no hydrogen bonding for comparison with those of the steroid compounds. As shown in Figure 2(a), the compounds adopted for this calibration study were ethyl acetate **6**, methylcyclohexane **7**, xylenes **8**, methyl benzoate **9**, pyrene **10**, isopropyl palmitate **11** and tri-*n*-caprylin **12**. No hydrogen bonding of these compounds is expected, because they have neither hydroxyl nor carboxyl groups.

(a)



(b)

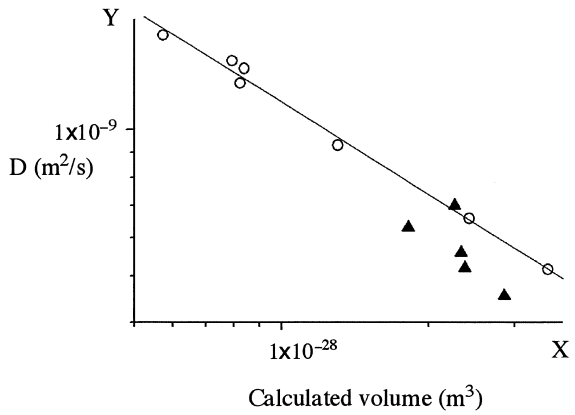


Figure 2. (a) The molecular structures adopted for calibration, and (b) relation of observed diffusion coefficients versus calculated molecular volume for compounds **6**-**12** (○) and the steroid compounds **1**-**5** (▲). The *X*- and *Y*-axes are logarithmic.

A calibration profile was obtained from the observed diffusion coefficients and molecular volumes calculated by using MOPAC[11] [Fig. 2(b), Table 1]. Diffusion coefficients were obtained as a function of molecular volume instead of molecular weight.[12,13]

The attenuation of signal intensities is given by Eq. 1.[14]

$$I = I_0 \exp(-kD) \quad (1)$$

Here $k=[(\gamma G\delta)^2(\Delta-\delta/3-\tau/2)]$, where γ is the gyromagnetic ratio of ^{1}H, G is the gradient strength, D is the diffusion coefficient, δ is the field gradient pulse duration, Δ is the diffusion time and τ is the gradient delay. Diffusion coefficients were calculated as a function of the value of k by using Eq. 1. The calculated diffusion coefficients obtained from the calibration curve in Figure 2(b) and the observed diffusion coefficients in PFG NMR experiments are shown in Table 2.

Table 1. The calculated molecular volumes of compounds **1**-**12**. These values were calculated by using MOPAC

	Compound	Volume ($\times10^{-29}$ m^3)
Steroid	1	22.5
	2	18.1
	3	23.2
	4	23.6
	5	28.4
Reference	6	5.73
	7	8.37
	8	7.91
	9	8.22
	10	13.0
	11	24.1
	12	34.9

Table 2. The observed and calculated diffusion coefficients of steroid compounds

Steroid	$D_{obs.}$ ($\times10^{-10}$ m^2/s)[a]	$D_{cal.}$ ($\times10^{-10}$ m^2/s)[b]
1	6.98	6.78
2	6.29	7.77
3	5.58	6.65
4	5.18	6.58
5	4.54	5.85

[a] Value obtained by NMR observation.
[b] Value obtained by calculation using the equation from the calibration plot ($y=2.53\times10^{-27}x^{-0.63}$) in Figure 2(b).

In the case of **1**, the observed diffusion coefficient is almost identical with the calculated value. This result indicates that **1** has no intermolecular interactions. On the other hand, the diffusion coefficients of **2**-**5** were slightly different from the calculated values, presumably because hydrogen bonding occurs to afford molecular clusters. These findings are consistent with the results of CSI-MS, which also indicated the existence of clusters based on hydrogen bonding in solution. It is suggested that PFG NMR data reflect the size of the molecular aggregates formed by non-covalent interactions such as hydrogen bonding.

2.3. X-ray analysis

The crystal structures of these compounds were then elucidated by means of X-ray diffraction. The crystal structures obtained are shown in Figure 3.

Although no intermolecular interaction based on hydrogen bonding was observed because of the absence of hydroxyl and/or carboxyl groups in **1**,[15] other species having these functional groups exhibited liner or two-dimensional chain structures in the crystal. The head-to-tail interaction of hydroxyl and ketone groups was seen in the case of **2**.[16] In the cases of **3**,[17] **4**[17] and **5**,[18] plural intermolecular hydrogen bonds were observed in the crystal. Solvent molecule(s), methanol, were included in **4** and **5**. Compounds **2**-**5**, exhibited two, four, seven and eight hydrogen bondings, respectively. Very strong hydrogen bonding, with a distance of 2.594(3) Å, was observed between the hydroxyl group at the 5-position and OH of the carboxyl group in **5**. Almost all the hydrogen-bonding distances ranged from 2.7 to 3.0 Å.

3. Discussion

We have elucidated the structures of five steroid compounds having different numbers of polar functional groups (OH and/or COOH) in solution by using CSI-MS. Clear differences of the aggregated structures, according to the number of polar groups, were found in methanol solution. Though the formation of large-scale aggregated clusters was not observed in the case of **1**, which has no polar functional groups, large clusters were observed for compounds with such functional group(s). The degree of the clustering

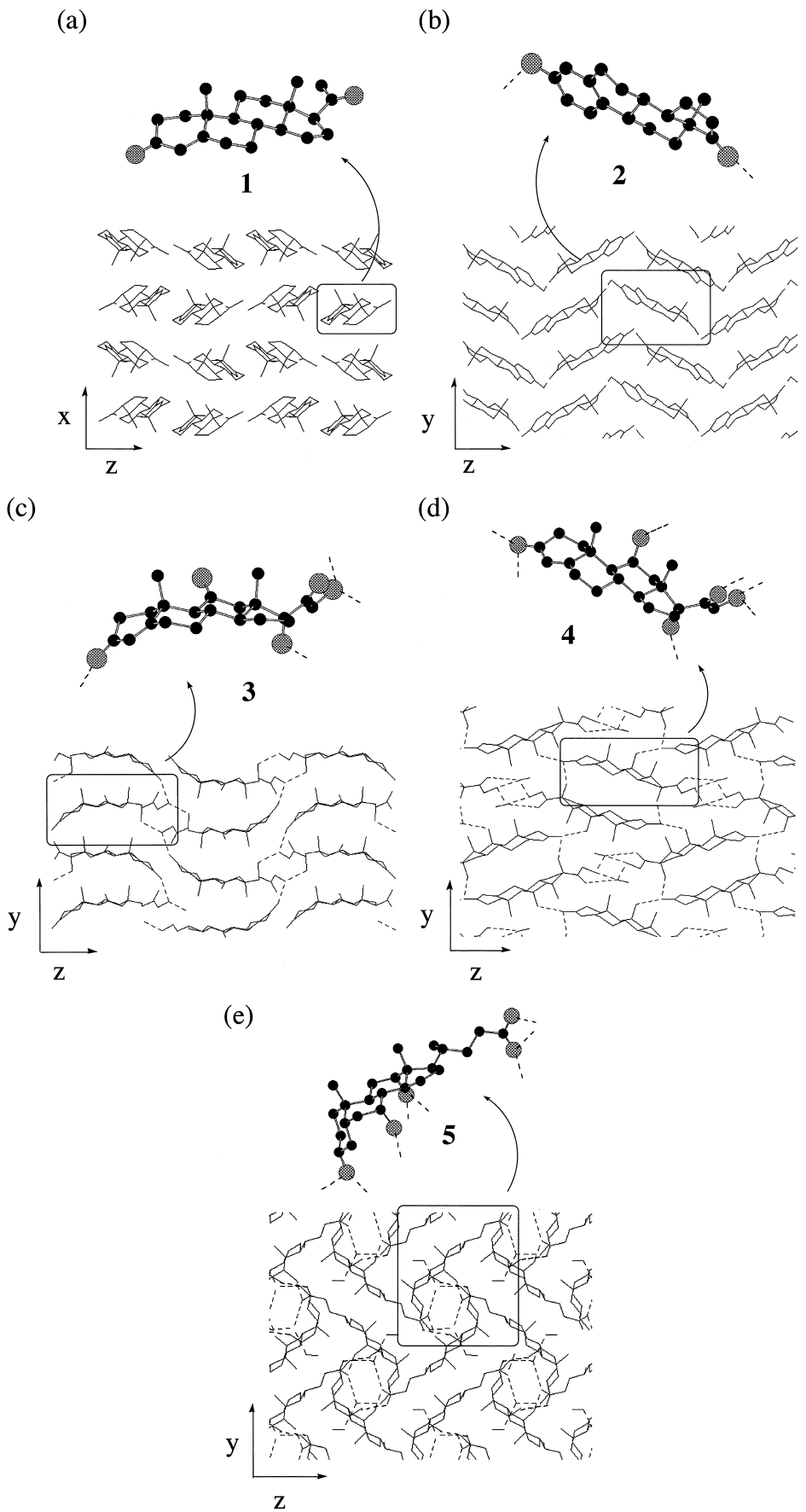


Figure 3. (a)–(e) present the crystal structure of **1-5**, respectively. Dotted lines indicate hydrogen bonding.

tended to increase with increased in the number of functional groups.

Moreover, PFG NMR measurements were also applied to the same solutions (CD_3OD:D_2O=98:2, 10 mM) as used for CSI-MS. A calibration profile was obtained based on molecular volume, instead of molecular weight, and intermolecular interactions based on hydrogen bonding in solution were confirmed by comparing the two series of compounds using the calibration profile. However, the measured diffusion coefficients could not be used to distinguish cluster-forming propensity.

In the case of CSI-MS measurement, large-scale aggregated clusters of the steroid compounds were easily detected in solution, because these clusters were stably ionized at low temperature. The CSI-MS findings were supported by the results of X-ray crystallography, which revealed strong hydrogen bonding in the crystal lattice. It is suggested that the structures of the large clusters observed by CSI-MS might be similar to those observed in the crystal. Further, no large cluster was detected in the case of **1**, which showed no hydrogen bonding in the crystal.

4. Conclusion

We identified large-scale-aggregated structures of steroid compounds in solution by using CSI-MS and PFG NMR and in the solid state by X-ray crystallography. The structures in solution and in the solid state were broadly similar, being based on hydrogen bonding. The average size of the clusters of each steroid compound was estimated in PFG NMR diffusion experiments. These compounds appear to maintain ordered clusters based on hydrogen bonding in diluted solution, at least in part, instead diffusing randomly as individual molecules, as has been generally accepted. We have observed this unprecedented phenomenon by CSI-MS, which can clearly confirm the existence of such clusters. This narcissistic aggregation might be strongly related to the biological activity of these compounds, and this will be examined in near future.

5. Experimental

Steroid compounds **1**, **2**, **3**, **4** and **5** were purchased from Tokyo Kasei Kogyo Co., Ltd. Compounds **6**-**12** were also commercial products. All reagents were used without purification. Deuterated solvents, 99.8% d_4-methanol and 99.9% deuterium oxide, were purchased from Merck Ltd and Cambridge Isotope Laboratories, Inc., respectively.

CSI-MS measurements were performed with a sector (BE) mass spectrometer (JMS-700, JEOL) equipped with a CSI source. Typical measurement conditions are as follows: ionization mode, positive CSI; acceleration voltage, 5.0 kV; needle voltage, 0 kV; orifice voltage, 40 kV; sample flow rate, 8 μl/min; solvent, CH_3OH:H_2O=98:2; sample concentration, 10 mM; spray temperature, −20 °C; resolution (10% valley definition), 1000.

NMR diffusion experiments were carried out in a JEOL JNM LA-600 spectrometer. The measurement conditions were as follows: pulse sequence, Bipolar-Pulse-Pair Stimulated-Echo (BPP-STE);[19] gradient length, 0.8–1.5 ms; diffusion time, 100–110 ms; solvent, CD_3OD:D_2O=98:2; temperature, 10 °C; sample concentration, 10 mM. The gradient strength was varied in 15 steps to 30 G/cm. Diffusion coefficients were measured after 1 h at 10 °C for stabilization, with 3 mm I.D. sample tubes to decrease the influence of thermal convection.

All NMR diffusion experiments employed the BPP-STE pulse sequence. We used the proton signal of the C-18 methyl group in the steroid backbone for determination of diffusion coefficients, because it is a singlet peak and this group is not involved in hydrogen bonding. The NMR signals showed single exponential decay. The diffusion coefficients were calculated by curve fitting of the peak intensities of the C-18 methyl proton and k in Eq. 1.

Single crystals were obtained by recrystallization from methanol at room temperature. A Bruker SMART 1000 CCD diffractometer with graphite-monochromated Mo K_α radiation was used. The structures were solved by the direct method and refined by the full-matrix least-squares method. All non-hydrogen atoms were refined anisotropically.

All calculations were performed using the teXsan crystal structure solution software package.

5.1. X-ray crystallography

5.1.1. Progesterone 1. $C_{21}H_{30}O_2$, M=314.47, orthorhombic, space group $P2_12_12_1$(#19), a=10.287(3), b=12.524(4), c=13.701(4), V=1765.318(8) Å^3, Z=4, D_{calc}=1.18 g cm^{-3}, T=173.2 K, μ=0.74 cm^{-1}, (Mo K_α=0.71069 Å), R=0.044 (R_w=0.055) for 10476 observed reflections [$I>2.0\sigma(I)$], GOF=1.344. (CCDC 228768).

5.1.2. Estrone 2. $C_{18}H_{22}O_2$, M=270.37, orthorhombic, space group $P2_12_12_1$(#19), a=7.773(10), b=9.983(13), c=18.412(2), V=1428.816(3) Å^3, Z=4, D_{calc}=1.26 g cm^{-3}, T=173.2 K, μ=0.80 cm^{-1}, (Mo K_α=0.71069 Å), R=0.034 (R_w=0.040) for 8517 observed reflections [$I>2.0\sigma(I)$], GOF=1.364. (CCDC 228769).

5.1.3. Cortisone 3. $C_{21}H_{28}O_5$, M=360.45, orthorhombic, space group $P2_12_12_1$(#19), a=7.785(2), b=10.001(3), c=23.610(6), V=1838.264(7) Å^3, Z=4, D_{calc}=1.30 g cm^{-3}, T=173.2 K, μ=0.92 cm^{-1}, (Mo K_α=0.71069 Å), R=0.032 (R_w=0.036) for 10936 observed reflections [$I>2.0\sigma(I)$], GOF=0.881. (CCDC 228770).

5.1.4. Hydrocortisone 4. $C_{21}H_{30}O_5(CH_3OH)$, M=394.51, orthorhombic, space group $P2_12_12_1$(#19), a=7.680(2), b=14.230(4), c=18.439(5), V=2015.050(8) Å^3, Z=4, D_{calc}=1.30 g cm^{-3}, T=173.2 K, μ=0.93 cm^{-1}, (Mo K_α=0.71069 Å), R=0.043 (R_w=0.056) for 12276 observed reflections [$I>2.0\sigma(I)$], GOF=1.071. (CCDC 228771).

5.1.5. Cholic acid 5. $C_{24}H_{40}O_5(CH_3OH)(H_2O)$, M=458.63, orthorhombic, space group $P2_12_12_1$(#19), a=11.283(3), b=14.434(4), c=15.383(4), V=2505.291(9) Å^3, Z=4, D_{calc}=1.22 g cm^{-3}, T=173.2 K, μ=0.87 cm^{-1}, (Mo K_α=

0.71069 Å), R=0.046 (R_w=0.054) for 14967 observed reflections [$I>2.0\sigma(I)$], GOF=1.287. (CCDC 228772).

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