# Intermolecular Hydrogen Bonding of Steroid Compounds: PFG NMR Diffusion Study, Cold-Spray Ionization (CSI)-MS and X-Ray Analysis

Kazuaki SHIKII,[a,b] Hiroko SEKI,[a,c] Shigeru SAKAMOTO,[a] Yoshihisa SEI,[d] Hiroaki UTSUMI,[e] and Kentaro YAMAGUCHI*,[c,d]

[a] *Chemical Analysis Center, Chiba University; 1–33 Yayoi-cho, Inage-ku, Chiba, Chiba 263–8522, Japan:* [b] *Nissan Chemical Industries Ltd.; 722–1 Tsuboi-cho, Funabashi, Chiba 275–0026, Japan:* [c] *CREST, Japan Science and Technology Agency (JST); 4–1–8 Honcho, Kawaguchi, Saitama 332–0012, Japan: and* [d] *Faculty of Phrmaceutical Sciences at Kagawa Campus, Tokushima Bunri University; 1314–1 Shido, Sanuki, Kagawa 769–2193, Japan: and* [e] *NMR Application Lab., JEOL Ltd.; 3–1–2 Musashino, Akishima, Tokyo 196–8558, Japan.*

Received February 16, 2005; accepted April 23, 2005; published online April 28, 2005

**An extensive analysis of hydrogen bonding of steroid compounds in diluted solution is preformed by pulsed field gradient (PFG) NMR and cold-spray ionization (CSI)-MS, in the solid state by X-ray crystallographic analysis. The formation of hydrogen bond interaction are quantified and discussed. Although X-ray analysis in the crystalline state and CSI-MS measurement in solution suggested that the observed diffusion coefficient $D_{obs}$ of the steroid compounds may vary in accordance with the number of hydrogen bonds, the actual observed $D_{obs}$ value determined from the diffusion studies diminished constantly without correlation on the decreasing numbers of hydrogen bonds. Comparison of two different calibration profiles of calculated molecular volume ($V_{cal}$) *vs.* $D_{obs}$, which are obtained from compounds possessing no hydrogen bonding and the steroid compounds, formation of a chain structure (cluster) based on intermolecular hydrogen bonding of the steroid compounds is unambiguously confirmed.**

**Key words** NMR diffusion study; standard curve; hydrogen bonding; steroid compound; cold-spray ionization (CSI)-MS; X-ray

NMR is one of the most effective analytical instrumentations to elucidate the dynamic nature of organic molecule in solution.[1,2] Other instrumental analyses such as MS and X-ray help to reveal the role of hydrogen bonding in solution which are of great importance in the field of biology and organic chemistry.

A great number of reports with respect to development of the new NMR-based techniques using nuclear Overhauser effect (NOE) and molecular diffusion measurement have been presented.[3—8] Although, many reports can be found about correlation between molecular weight and diffusion coefficient of small molecule in polymer or polymer gel,[9–11] systematic studies of diffusion coefficient of small molecule itself in solution have not been reported so far.

Recently, we reported the structure analysis, in solution, of five steroid compounds (progesterone **1**, estrone **2**, cortisone **3**, hydrocortisone **4**, cholic acid **5**) by the use of cold-spray ionization (CSI)-MS,[12] PFG NMR and X-ray analysis.[13] Narcissistic aggregation (formation of large-scale aggregated chain structure or cluster) based on the intermolecular hydrogen bonding at the hydroxyl and carboxyl functionalities was confirmed. Moreover, in this previous report, reasonable correlation between hydrogen bonding and cluster formation was obtained in the crystal as well as in solution. However, quantitative analysis based on strength of the hydrogen bonding and diffusion coefficient was not achieved by PFG NMR.

Therefore, accurate NMR diffusion experiments on the five initial steroids as well as seven additional one are presented. The formation of the hydrogen bound clusters in diluted solution quantified by diffusion coefficient measurements. The results obtained from PFG NMR are further confirmed and compared with the results obtained by CSI-MS and X-ray analysis.

## Results and Discussions

A total of twelve steroid compounds including the previous steroids (**1**—**5**,[13] androsterone **6**, norethisterone **7**, dehydroepiandrosterone acetate **8**, 11$\alpha$-hyrodoxyprogesterone **9**, corticosterone **10**, prednisolone acetate **11**, cholic acid methyl ester **12**), have been analyzed.

CSI-MS measurements are performed to analyze the nature of the steroids in solution. As previously described, the twelve compounds are classified through three hydrogen bonding types, strong (type A), weak (type B) and none (type C). The strength depends on the number of hydroxyl group(s) in the backbone chain. Compounds **6**—**12** exhibit significant correlation between the number of hydrogen bonding and the cluster formation as observed in the CSI-MS spectra. Typical pattern of the CSI-MS spectra of each type, A (**11**), B (**9**) and C (**8**) are shown in Fig. 1. $Na^+$ attached to the molecular ion peaks are undeniably observed in type A compounds, which includes **3**, **4**, **5**, and **11**. Rather weak aggregation is observed in type B spectra, compounds **2**, **6**, **7**, **9**, **10**, and **12** whereas no chain interaction is seen for compounds **1** and **8** (type C). The CSI-MS classification of the cluster type is summarized in Table 1.

The self-diffusion coefficient ($D_{obs}$), obtained by PFG NMR, of the steroid compounds are measured.[14,15] The correlations of $D_{obs}$ *vs.* molecular weight $M_{cal}$[16—20] and $D_{obs}$ *vs.* calculated volume $V_{cal}$ are obtained as shown in Figs. 2a and b, respectively. The calibration profile (lines A, C) are determined from seven reference compounds; ethyl acetate, methyl cyclohexane, xylene, methyl benzoate, pyrene, isopropyl palmitate and tri-*n*-caprylin (open circle). No hydrogen bonding for these compounds is expected because they have neither hydroxyl nor carboxyl groups. The least-squares results based on the ten steroid compounds (open triangle), excluding non interactive type compounds **1** and **8** (closed

* To whom correspondence should be addressed. e-mail: yamaguchi@kph.bunri-u.ac.jp

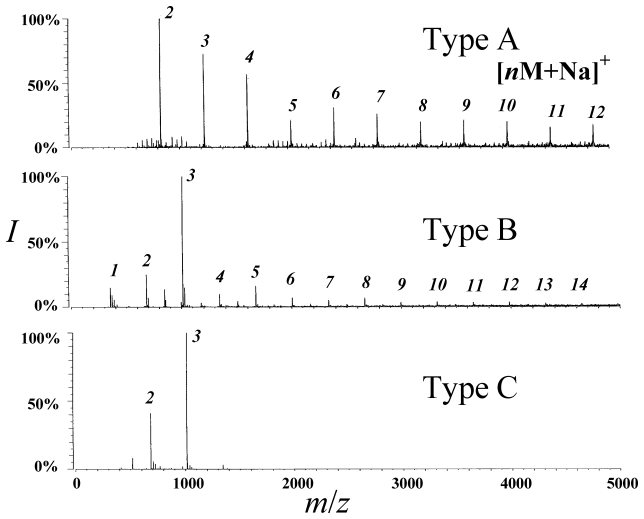


Fig. 1. Typical Three Types of Spectra, A, Strong (Compound **11**), B, Moderate (Compound **9**) and C, Weak (Compound **8)**

Table 1. Classification of the Cluster Type Observed by CSI-MS

| Steroid | Type of spectrum |
|---|---|
| **1**[a)] | C |
| **2**[a)] | B |
| **3**[a)] | A |
| **4**[a)] | A |
| **5**[a)] | A |
| **6** | B |
| **7** | B |
| **8** | C |
| **9** | B |
| **10** | B |
| **11** | A |
| **12** | B |

*a*) See ref. 13.

triangle), are represented in lines B and D. The least-squares result to determine each calibration profile is listed in Table 2. $M_{cal}$, $V_{cal}$ and $D_{obs}$ used in these experiments are shown in Table 3.

The high profile fitting, observed in line C ($R^2$=0.99) when comparing the least-squares lines A and C, suggests that the molecular volume might be critical to determine diffusion coefficient. A shift of least-squares lines in both line pair, A–B and C–D indicates a reduction of the diffusion coefficient. This strongly suggests that for these steroid compounds the formation of intermolecular hydrogen bonding in solution makes the molecules large enough to resist diffusion in solution. In the case of least-squares lines C and D ($y=ax^{-k}$), the inclination ($k$) of both lines are almost identical. The $k$ values in line C and D are $-0.65$ and $-0.63$, respectively. This is corroborated by the presence on line C (Fig. 2b) of **1** and **8**. The two compounds show no intermolecular hydrogen bonding in solution. On the other hand, the other steroid compounds, which possess hydroxyl group(s), are clearly located line D (Fig. 2b) indicating the formation of intermolecular hydrogen bonding. Although CSI-MS measurement in solution suggests that the observed diffusion coefficient $D_{obs}$ may vary on the number of hydrogen bonding, the actual observed $D_{obs}$ value determined from the diffusion studies diminished constantly without correlation on the de-

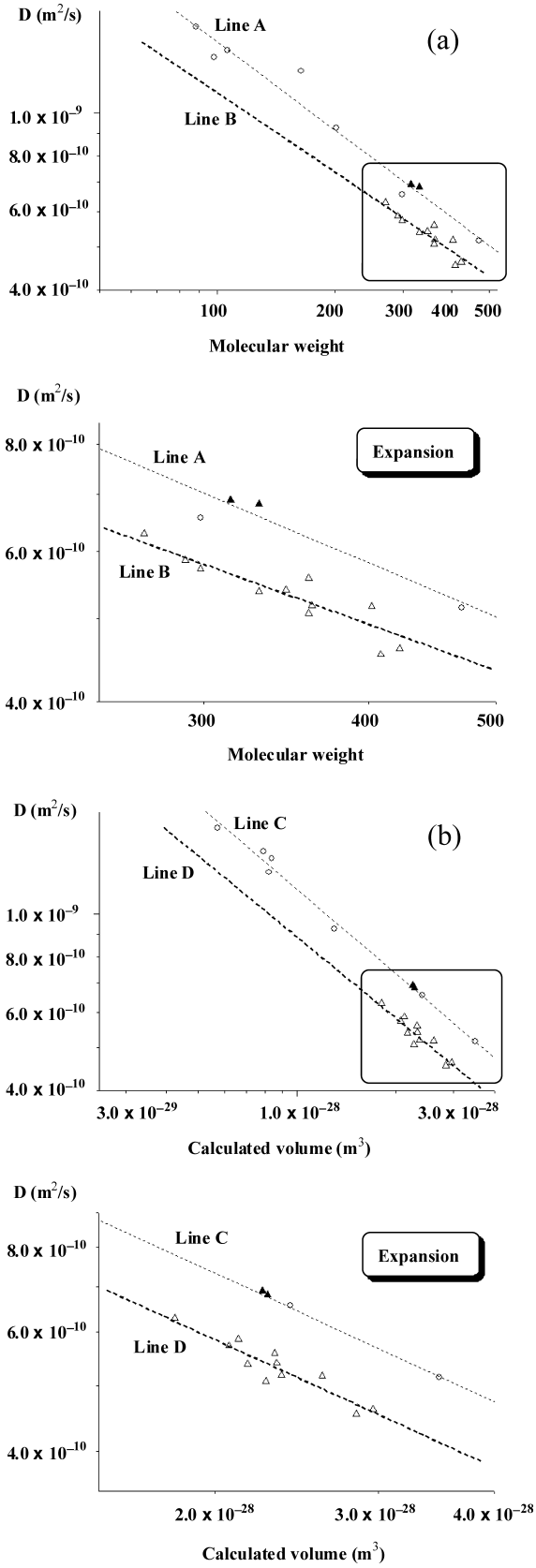


Fig. 2. (a) Correlation between Molecular Weight and $D_{obs}$, (b) Correlation between Molecular Volume and $D_{obs}$

Full (upper) and expanded (lower) range are shown. ○: reference compounds, ▲: steroid compounds, △: the steroid compounds excluding least-squares calculation.

Table 2. Least-Square Results of the Calibration Profiles

| | Reference | Steroid |
|---|---|---|
| $M_{cal}-D_{obs}$ | A: $y=2.96\times10^{-8}x^{-0.66}$ ($R^2=0.96$) | B: $y=1.93\times10^{-8}x^{-0.61}$ ($R^2=0.84$) |
| $V_{cal}-D_{obs}$ | C: $y=2.53\times10^{-27}x^{-0.63}$ ($R^2=0.99$) | D: $y=5.57\times10^{-8}x^{-0.65}$ ($R^2=0.88$) |

Table 3. Calculated Molecular Weight ($M_{cal}$), Molecular Volume ($V_{cal}$) and Observed Diffusion Coefficient ($D_{obs}$) of Steroid Compounds

| Steroid | $M_{cal}$ | $V_{cal}$ ($\times10^{-28}$ m$^3$) | $D_{obs}$ ($\times10^{-10}$ m$^2$/s) |
|---|---|---|---|
| **1**[a] | 314 | 2.25 | 6.98 |
| **2**[a] | 270 | 1.81 | 6.29 |
| **3**[a] | 360 | 2.32 | 5.58 |
| **4**[a] | 362 | 2.36 | 5.18 |
| **5**[a] | 408 | 2.84 | 4.54 |
| **6** | 290 | 2.12 | 5.85 |
| **7** | 298 | 2.07 | 5.72 |
| **8** | 330 | 2.28 | 6.81 |
| **9** | 330 | 2.28 | 5.38 |
| **10** | 346 | 2.33 | 5.40 |
| **11** | 402 | 2.61 | 5.17 |
| **12** | 422 | 2.96 | 4.61 |

*a*) See ref. 13.

creasing number of hydrogen bonds.

The crystal structures of the twelve steroid compounds have been elucidated in order to confirm the formation of intermolecular hydrogen bonding in the solid state, see Fig. 3. Hydrogen bonding in crystals are represented by dotted line. Methanol molecules are observed in the crystal packing of **4**, **5** and **12**. Strong hydrogen bonds are formed between methanol and the corresponding steroid molecule (**4**, **5**, **12**). Solvent molecule is not observed in the other crystals. **3** and **10** have two hydroxyl groups in backbone. **10** forms one-dimensional chain structure while **3** exhibit two-dimensional hydrogen bonding network. CSI-MS spectra of these two compounds are found to be quite different from each other, suggesting different cluster structure in solution. Compound **12** which formed dimeric cluster in the crystal belonged to type B classified based on CSI-MS analysis. Hydrogen bondings are observed in every hydroxyl groups of the steroid compounds. It is found that the hydroxyl group, which plays important donor roles in hydrogen bonding, might form hydrogen bonding in solution in most cases. However, no hydrogen bonding between methanol and carbonyl group is observed, methanol being a good proton acceptor.

In conclusion, formation of hydrogen bonding in steroid compounds in diluted solution is observed by using PFG NMR, CSI-MS and X-ray analysis. By NMR diffusion stud-

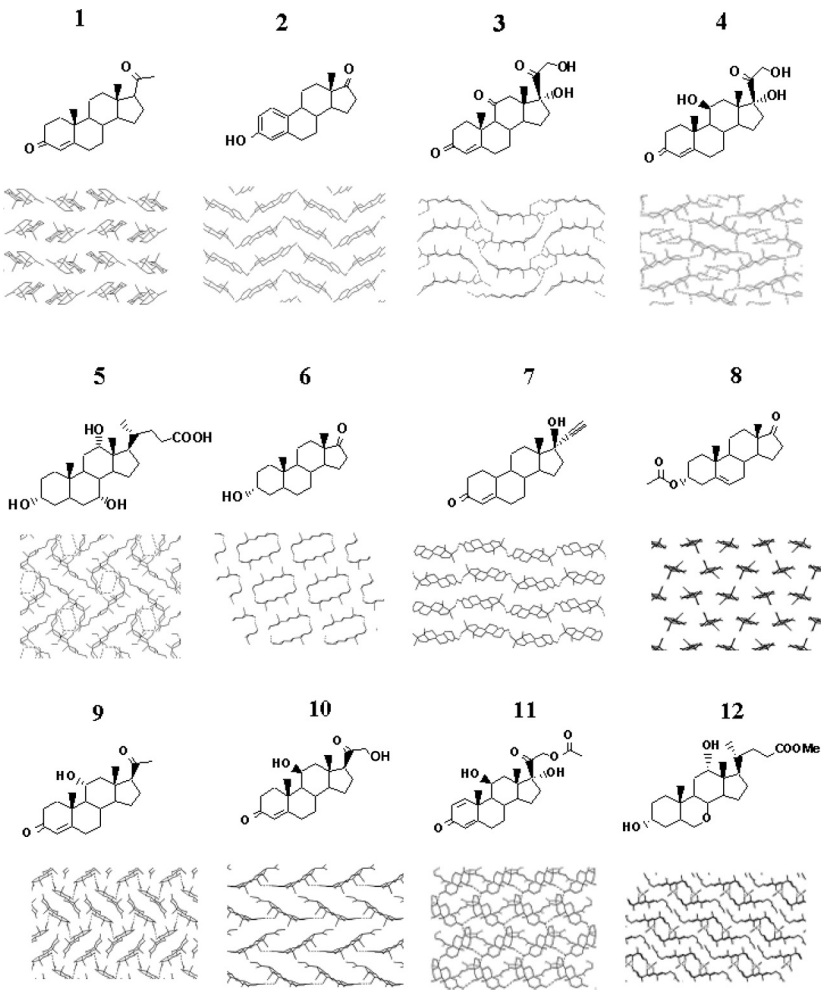


Fig. 3. The Crystal Structure of Steroid Compounds by X-Ray Analysis

The dotted lines denoted hydrogen bonding.

ies, clear least-squares lines shift of observed diffusion coefficient *vs.* molecular weight and volume comparing reference compound and steroid compound definitely prove the formation of cluster of these compounds in solution. However this result obtained from the diffusion study only characterizes the cluster formation of the steroid compound, because an averaged molecular state is observed in solution in the case of NMR measurement. Quantitative study to prove the existence in solution of weak hydrogen bond interactions is performed by CSI-MS measurement where in the solid state, X-ray structure analysis is used.

## Experimental

All the steroid compounds (**1**—**12**) were measured at the following conditions.

NMR diffusion experiments were carried out on a JEOL JNM LA-600 spectrometer. The measurement conditions were as follows: pulse sequence, bipolar-pulse-pair stimulated-echo (BPP-STE), data size 32k, spectral width 6500 Hz, gradient length 0.8—1.5 ms, diffusion time 100—110 ms, recycle delay 7.5 s, solvent $CD_3OD : D_2O = 98 : 2$, temperature 10 °C, sample concentration 10 mM. The gradient strength was varied 15 steps to 30 Gauss/cm. $D_{obs}$ were measured after 1 h at 10 °C for stabilization and by using 3 mm I.D. sample tube due to the decrease of the solution by thermal convection. The volume of the electron density isosurface was determined after optimizing the molecular geometry using MOPAC with PM3 parameters on CAChe WorkSystem Version 5.02.

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 $\mu$l/min, solvent $CH_3OH : H_2O = 98 : 2$, sample concentration 10 mM, spray temperature −20 °C, resolution (10% valley definition) 1000.

X-Ray crystallographic diffraction data were obtained on a Bruker SMART 1000 CCD diffractometer with graphite-monochromated Mo$K\alpha$ radiation. The structures were solved by direct method and refined by full-matrix least-squares method. All non-hydrogen atoms were refined anisotropically. All calculations were performed using the teXsan crystal structure solution software package. Single crystals were obtained by recrystallization from methanol solution at room temperature.

All steroid compounds were purchased from Tokyo Kasei Kogyo Co., Ltd. Reference compounds in diffusion studies were also commercially available. All reagents were used without purification. Deuterated solvents, 99.8% $CD_3OD$ and 99.9% $D_2O$, were purchased from Merck Ltd. and Cambridge Isotope Laboratories, Inc., respectively.

**Androsterone (6)** The CSI-MS spectrum is classified into type B. The diffusion coefficient is $5.85\times10^{-10}$ m$^2$/s. Crystal data: $C_{19}H_{30}O_2$, $M$=290.44, monoclinic, space group $P2_1$(#4), $a$=9.445(2), $b$=7.954(2), $c$=11.715(3), $V$=820.3(3) Å$^3$, $\alpha$=90°, $\beta$=111.251(3)°, $\gamma$=90°, $Z$=2, $D_{calc}$=1.176 g cm$^{-3}$, $T$=173.2 K, $\mu$=0.74 cm$^{-1}$, (Mo$K\alpha$=0.71069 Å), $R$=0.049, ($R_w$=0.057) for 4902 observed reflections [$I>2.0\sigma(I)$], GOF=1.620 (CCDC 261296).

**Norethisterone (7)** The CSI-MS spectrum is classified into type B. The diffusion coefficient is $5.72\times10^{-10}$ m$^2$/s. Crystal data: $C_{20}H_{26}O_2$, $M$=298.42, orthorhombic, space group $P2_12_12_1$(#19), $a$=6.514(2), $b$=12.066(3), $c$=20.748(6), $V$=1630.7(7) Å$^3$, $Z$=4, $D_{calc}$=1.215 g cm$^{-3}$, $T$=173.2 K, $\mu$=0.76 cm$^{-1}$, (Mo$K\alpha$=0.71069 Å), $R$=0.039, ($R_w$=0.052) for 9950 observed reflections [$I>2.0\sigma(I)$], GOF=1.070 (CCDC 261297).

**Dehydroepiandrosterone Acetate (8)** The CSI-MS spectrum is classified into type C. The diffusion coefficient is $6.81\times10^{-10}$ m$^2$/s. Crystal data: $C_{21}H_{30}O_3$, $M$=330.47, orthorhombic, space group $P2_12_12_1$(#19), $a$=9.008(3), $b$=12.549(4), $c$=16.567(5), $V$=1872.8(9) Å$^3$, $Z$=4, $D_{calc}$=1.172 g cm$^{-3}$, $T$=173.2 K, $\mu$=0.76 cm$^{-1}$, (Mo$K\alpha$=0.71069 Å), $R$=0.044, ($R_w$=0.056) for 11311 observed reflections [$I>2.0\sigma(I)$], GOF=1.130 (CCDC 261298).

**11$\alpha$-Hydroxyprogesterone (9)** The CSI-MS spectrum is classified into type B. The diffusion coefficient is $5.38\times10^{-10}$ m$^2$/s. Crystal data: $C_{21}H_{30}O_3$, $M$=330.47, orthorhombic, space group $P2_12_12_1$(#19), $a$=8.414(2), $b$=10.686(3), $c$=20.050(5), $V$=1802.8(6) Å$^3$, $Z$=4, $D_{calc}$=1.217 g cm$^{-3}$, $T$=173.2 K, $\mu$=0.79 cm$^{-1}$, (Mo$K\alpha$=0.71069 Å), $R$=0.034, ($R_w$=0.045) for 10953 observed reflections [$I>2.0\sigma(I)$], GOF=0.940 (CCDC 261299).

**Corticosterone (10)** The CSI-MS spectrum is classified into type B. The diffusion coefficient is $5.40\times10^{-10}$ m$^2$/s. Crystal data: $C_{21}H_{30}O_4$, $M$=346.47, monoclinic, space group $P2_1$(#4), $a$=8.308(2), $b$=12.192(4), $c$=8.953(3), $V$=906.8(4) Å$^3$, $Z$=2, $D_{calc}$=1.269 g cm$^{-3}$, $T$=173.2 K, $\mu$=0.86 cm$^{-1}$, (Mo$K\alpha$=0.71069 Å), $R$=0.043, ($R_w$=0.049) for 5346 observed reflections [$I>2.0\sigma(I)$], GOF=1.390 (CCDC 261300).

**Prednisolone Acetate (11)** The CSI-MS spectrum is classified into type A. The diffusion coefficient is $5.17\times10^{-10}$ m$^2$/s. Crystal data: $C_{23}H_{30}O_6$, $M$=402.49, monoclinic, space group $P2_1$(#4), $a$=8.519(5), $b$=13.915(8), $c$=8.985(5), $V$=1039.1(9) Å$^3$, $\alpha$=90°, $\beta$=102.688(8)°, $\gamma$=90°, $Z$=2, $D_{calc}$=1.286 g cm$^{-3}$, $T$=173.2 K, $\mu$=0.92 cm$^{-1}$, (Mo$K\alpha$=0.71069 Å), $R$=0.077, ($R_w$=0.093) for 6279 observed reflections [$I>2.0\sigma(I)$], GOF=1.790 (CCDC 261301).

**Cholic Acid Methyl Ester (12)** The CSI-MS spectrum is classified into type B. The diffusion coefficient is $4.61\times10^{-10}$ m$^2$/s. Crystal data: $C_{25}H_{42}O_5(CH_3OH)$, $M$=454.65, monoclinic, space group $C2$(#5), $a$=24.894(6), $b$=7.778(2), $c$=15.087(4), $V$=2512.7(10) Å$^3$, $\alpha$=90°, $\beta$=120.672(3)°, $\gamma$=90°, $Z$=4, $D_{calc}$=1.202 g cm$^{-3}$, $T$=173.2 K, $\mu$=0.83 cm$^{-1}$, (Mo$K\alpha$=0.71069 Å), $R$=0.046, ($R_w$=0.057) for 7644 observed reflections [$I>2.0\sigma(I)$], GOF=1.110 (CCDC 261302).

## References


1) Seki H., Nakagawa M., Hashimoto A., Hino T., *Chem. Pharm. Bull.*, **1**, 1173—1176 (1993).
2) Dell'Erba C., Gasparrini F., Grilli S., Lunazzi L., Mazzanti A., Novi M., Pierini M., Tavani C., Villani C., *J. Org. Chem.*, **67**, 1663—1668 (2002).
3) Timmerman P., Weidmann J.-L., Jolliffe A. K., Prins J. L., Reinhoudt N. D., Shinkai S., Frish L., Cohen Y., *J. Chem. Soc., Perkin Trans. 2*, **2000**, 2077—2089 (2000).
4) Cabrita E. J., Berger S., *Magn. Reson. Chem.*, **39**, S142—S148 (2001).
5) Chen A., Shapiro M. J., *J. Am. Chem. Soc.*, **120**, 10258—10259 (1998).
6) Ochiai M., Meadows P., Horiuchi H., Sakai Y., Furihata K., *Polymer J.*, **32**, 760—770 (2000).
7) Gnatyuk I., Puchkovska G., Chashechnikova I., Nozirov F., Jurga S., Peplinsla B., *J. Mol. Struct.*, **700**, 183—189 (2004).
8) Keresztes I., Williard P. G., *J. Am. Chem. Soc.*, **122**, 10228—10229 (2000).
9) Tokita M., Miyoshi T., Takegoshi K., Hikichi K., *Phys. Rev.*, **53**, 1823—1827 (1996).
10) Cukier R. I., *Macromolecules*, **17**, 252—255 (1984).
11) Peschier L. J. C., Bouwstra J. A., De Bleyser J., Junginger H. E., Leyte J. C., *J. Magn. Reson. Series B*, **110**, 150—157 (1996).
12) Yamaguchi K., *J. Mass Spectom.*, **38**, 473—490 (2003).
13) Shikii K., Sakamoto S., Seki H., Utsumi H., Yamaguchi K., *Tetrahedron*, **60**, 3487—3492 (2004).
14) Tanner J. E., *J. Magn. Reson.*, **52**, 2523—2526 (1970).
15) Wu D., Chen A., Johnson C. S., *J. Magn. Reson. Series A*, **115**, 260—264 (1995).
16) Håkansson B., Nydén M., Søderman O., *Colloid Polym. Sci.*, **278**, 399—405 (2000).
17) Raghavan R., Maver T. L., Blum F. D., *Macromolecules*, **20**, 814—818 (1987).
18) Trampel R., Schiller J., Naji L., Stallmach F., Järger J., Arnold K., *Biophys. Chem.*, **97**, 251—260 (2002).
19) Callaghan P. T., Pinder D. N., *Macromolecules*, **16**, 968—973 (1983).
20) Chen A., Wu D., Johnson C. S., Jr., *J. Am. Chem. Soc.*, **117**, 7965—7970 (1995).